Previously characterized soluble guanylyl cyclases form α-β heterodimers that can be activated by the gaseous messenger, nitric oxide. In mammals, four subunits have been cloned, named α1, α2, β1, and β2. We have identified a novel soluble guanylyl cyclase isofrom from the nervous system of the insect Manduca sexta that we have named M. sexta guanylyl cyclase β3 (MsGC-β3). It is most closely related to the mammalian β subunits but has several features that distinguish it from previously identified soluble cyclases. Most importantly, MsGC-β3 does not need to form heterodimers to form an active enzyme because guanylyl cyclase activity can be measured when it is expressed alone in COS-7 cells. Moreover, this activity is only weakly enhanced in the presence of the nitric oxide donor, sodium nitroprusside. Several of the amino acids in rat β1 subunits, previously identified as being important in heme binding or necessary for nitric oxide activation, are substituted with nonsimilar amino acids in MsGC-β3. There are also an additional 315 amino acids C-terminal to the catalytic domain of MsGC-β3 that have no sequence similarity to any known protein. Northern blot analysis shows that MsGC-β3 is primarily expressed in the nervous system of Manduca.

The intracellular messenger cGMP mediates a wide variety of cellular and physiological processes. It functions as the primary messenger in visual transduction (1, 2), is an important regulator in vascular smooth muscle and kidney function (3, 4), and has been implicated in a number of forms of neuronal plasticity (5–7).

The enzymes that regulate the synthesis of cGMP fall into two major classes: the cytoplasmically localized, soluble guanylyl cyclases and the membrane-associated, receptor guanylyl cyclases (4). Soluble guanylyl cyclases are obligate heterodimers composed of an α subunit and a β subunit. Two α (α1 and α2) and two β (β1 and β2) subunits have been cloned from mammalian tissues (8–13). MsGC-β3 and a C-terminal extension of the α1 and β1 subunits, named MsGC-α1 and MsGC-β1 (19). Here we describe the cloning and preliminary characterization of a novel β subunit, MsGC-β3. This new subunit binds significant basal activity when expressed alone and shows modest stimulation in the presence of NO.

**MATERIALS AND METHODS**

**Animals**—The rearing and staging of M. sexta (Lepidoptera: Sphingidae) has been described previously (17).

**RNA Isolation and Degenerate Oligonucleotide RT-PCR—**Poly(A)+ RNA was isolated from prepupal M. sexta abdominal central nervous system using Trizol reagent (Life Technologies, Inc.) and oligo(dT)+cellulose columns (Life Technologies, Inc.). First strand cDNA was generated from 5 μg of poly(A)+ RNA using oligo(dT) primers and Superscript II RT (Life Technologies, Inc.) and resuspended in 40 μl of water. Degenerate oligonucleotide primers were designed against the amino acid sequences DYYKVETI (CCRAIIARCARTAICKNGCAT) and MPRYCLPG (GAYGTITAAYAARTGWICNACAT). These sequences are in the catalytic domain (Fig. 1) and are highly conserved in both receptor and soluble guanylyl cyclases. PCR was performed in a 20-μl reaction containing 1 μl of cDNA, 200 pmol of each degenerate primer, 2 mm MgCl2, 1 × PCR buffer II (Perkin-Elmer), all four deoxynucleotides at 200 μM, 12.5 μCi of [35S]dATP, and 2 units of AmpliTaq (Perkin-Elmer). Thirty cycles of 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 30 s were performed. The resulting PCR products were analyzed on an 8% polyacrylamide sequencing gel, and bands below 235 base pairs, the expected size of receptor cyclases, were cut out, eluted, reamplified, T/A cloned into pCRII (Invitrogen), and manually sequenced.

cDNA Library Construction and Screening—cDNA libraries were constructed from 5 μg of poly(A)+ RNA isolated from prepupal abdomen by the gaseous messenger, nitric oxide (NO)1 (14). The soluble cyclases are found in the cytosol of cells, although the mammalian β2 subunit has a consensus isoprenylation site, suggesting that it might be associated with membranes. The receptor guanylyl cyclases, by contrast, are integral membrane proteins with transmembrane and extracellular domains (4).

We have been using the insect Manduca sexta as a model system to study cGMP regulation and have previously shown that a neuropeptide, eclosion hormone, is a potent stimulator of cGMP levels in the central nervous system (15). Biochemical characterization of the eclosion hormone-stimulated cGMP increase suggested that eclosion hormone might activate a NO-insensitive, soluble guanylyl cyclase (15–18). In an attempt to identify the pathway for eclosion hormone-stimulated cGMP levels, we used reverse transcription-polymerase chain reaction (RT-PCR) to identify guanylyl cyclases from Manduca nervous tissue. This approach has yielded a number of different guanylyl cyclases. We have previously described the cloning and expression characteristics of the Manduca homologues of the α1 and β1 subunits, named MsGC-α1 and MsGC-β1 (19). Here we describe the cloning and preliminary characterization of a novel β subunit, MsGC-β3. This new subunit binds significant basal activity when expressed alone and shows modest stimulation in the presence of NO.

1 The abbreviations used are: NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one; RT, reverse transcriptase; PCR, polymerase chain reaction; SNP, sodium nitroprusside; MsGC-β3, M. sexta guanylyl cyclase β3; kb, kilobase; GST, glutathione S-transferase.
Novel Guanylyl Cyclase Subunit

inal central nervous systems. Oligo(dT)-primed, double-stranded cDNA was generated using a Superscript Choice cDNA construction kit (Life Technologies, Inc.) according to the manufacturer’s instructions, except that the reverse transcription reaction was performed in a thermocycler at 37 °C for 15 min followed by a slow cool down to 50 °C over 30 min. The cDNA was then ligated into EcoRI-cut and ZAPII (Stratagene) and packaged using Gigapack Gold III (Stratagene) packaging extract. The library was screened using nitrocellulose filters (Schleicher & Schuell), hybridized, and washed according to the manufacturer’s instructions.

DNA Sequencing and Sequence Analysis—Manual DNA sequencing was performed using Sequenase kit, Version 2.0 (Amersham Pharmacia Biotech). Most sequencing was carried out at an automated sequencing facility running an ABI model 377 sequencer. Sequence analysis was performed using GeneWorks (Intelegentic) DNA analysis software, and protein sequence alignments used the ClustalW program through the Baylor College of Medicine search learner.

Cell Expression and Guanylyl Cyclase and cGMP Assays—The open reading frame of MsGC-β3 was cloned into pcDNA3.1 (Invitrogen), and an 8-μg aliquot was transfected into a 10-cm dish of COS-7 cells using LipofectAMINE (Life Technologies, Inc.). Three days after transfection, the cells were harvested and homogenized in 1 ml of 25 mM Tris-HCl, pH 7.4, containing 100 μM phenylmethylsulfonyl fluoride. Cell extracts were assayed for guanylyl cyclase activity by monitoring the conversion of [γ-32P]ATP to cGMP as described previously (20). Under these conditions used, the production of cGMP was linear with respect to time. To assay cGMP levels in intact COS-7 cells, cells were plated onto 24-well plates, and each well was transfected with 0.2 ml of each plasmid. After 3 days, the cells were incubated in saline (composition: NaCl (120 mM), KCl (5.4 mM), CaCl2 (1.8 mM), Tris-HCl (25 mM) and glucose (15 mM), pH 7.4) for 30 min at 37 °C, followed by a further 5 min in the presence of 6 mM sodium nitroprusside (SNP). The saline was then removed, and the cells were lysed with ethylendiamine tetra-acetic acid (EDTA) (100 mM). Following centrifugation, the supernatant was lyophilized, redissolved in 50 mM sodium acetate, pH 6.2, and assayed for cGMP content with a commercial radioimmunoassay kit (NEN Life Science Products).

Northern Blot Analysis—Ten μg of total RNA was separated on a formaldehyde-agarose (1%) gel and blotted onto Zetaprobe membrane (Bio-Rad). The blot was UV cross-linked, dried, and hybridized overnight at 42 °C with a hybridization solution consisting of 50% formamide, 5× saline/sodium phosphate/EDTA, 5× Denhardt’s solution, 1% SDS, 10% dextran sulfate, 100 μg/ml sonicated salmon sperm DNA, and 32P-labeled probe at 105 cpm/ml. Probes were generated by random priming of a gel-purified fragment, containing the entire open reading frame, as a template.

RT-PCR—Total RNA was isolated using Trizol reagent, and 5-μg aliquots were treated with DNAsel (Life Technologies, Inc.) for 30 min at 37 °C. cDNA was synthesized using oligo(dT) primers and Superscript RT in 20 μl for 1 h at 37 °C. The cDNA was diluted to 40 μl with water, and PCR was performed in 20 μl containing 1 μl of cDNA, 200 pmol each primer, 2 mM MgCl2, PCR buffer II (Perkin-Elmer), all four deoxynucleotides at 200 μM each, and 2 units of Amplitaq (Perkin-Elmer). Thirty cycles of 94 °C for 20 s, 66 °C for 20 s, and 72 °C for 30 s were performed. Products were analyzed on a 1% agarose gel and stained with ethidium bromide. The primers for MsGC-β3 and MsGC-α1 were designed and checked for false hybridization to other guanylyl cyclase cDNAs, using Oligo 4.0 (Molecular Biology Insights). The primers for MsGC-β3 were TAATAGTTCTCAGAATGCCTC (forward) and TGAAGATTGTCACCGTGT (reverse) (Table 1). The primers for MsGC-α1 were CTTTAACTATGCACTGATGCGT (forward) and CGTTTCTGTTTCTGTT (reverse) (Table 1). The resulting GST-MsGC-β3 fusion protein was purified using glutathione-coupled Sepharose (Amersham Pharmacia Biotech) and affinity purified using GST-mouse β2-subunit cDNA (NH2-activated Sepharose (Amersham Pharmacia Biotech). COS-7 cells or nervous tissue was homogenized in phosphate-buffered saline containing protease inhibitors (Complete Mixture, Boehringer Mannheim) and SDS sample buffer added (final concentrations: 20 mM Tris-HCl, pH 6.8, 1.6% SDS, 4% β-mercaptoethanol, 4% glycerol and 20 μg/ml bromophenol blue). The proteins were separated on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Primary antisera were used at 1:100 for whole sera or 1:10 for affinity purified antisera and detected using 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit antibodies (The Jackson Laboratory) and visualized with chemiluminescence and a digital imaging system (Chemilumager, Alpha Innotech).

RESULTS

Cloning of MsGC-β3—Using RT-PCR with degenerate oligonucleotide primers, we isolated cDNA fragments of a variety of different guanylyl cyclases from the abdominal central nervous system of M. sexta. Two of these have been described previously (19), appear to be the Manduca homologues of the mammalian soluble guanylyl cyclase subunits, α1 and β1, and have been named MsGC-α1 and MsGC-β1, respectively. In the course of this earlier study, we isolated an additional cDNA fragment that also had high sequence similarity to mammalian β subunits. Using this fragment, we screened a cDNA library and isolated five overlapping cDNA clones. These clones, when sequenced and compared, could be combined to form a cDNA construct of 5271 base pairs (Fig. 1A). The size of this cDNA construct approximately matched the 5.1-kb band seen on Northern blots (see Fig. 4A). This composite clone has an open reading frame of 940 amino acids, beginning with a consensus ribosome binding site. There are multiple stop codons in all frames both 5′ and 3′ to this open reading frame. The translated N terminus of this clone also shows a significant level of identity to other cloned β subunits of guanylyl cyclases suggesting that this construct represents the full-length sequence of MsGC-β3. Using a unique BamHI site, a composite clone was created from clones SGC4 and SGC25, which contained the entire open reading frame. Both strands of this composite clone were analyzed and the resulting sequence placed into GenBankTM under accession number AF064514. This clone was then used for further expression studies.

BLAST analysis of the protein encoded by the open reading frame showed that it was most similar to β subunits of soluble guanylyl cyclases. The most closely related sequence was a Caenorhabditis elegans clone (GenBankTM accession number 1109803, Ref. 20). Of previously identified and characterized guanylyl cyclases, it had the most similarity to vertebrate β1 subunits, with a 57% overall sequence identity to the rat β1 subunit compared with 29% sequence identity to the rat β2 subunit (Table 1). This is, however, much lower than the 59% identity seen between MsGC-β1 and rat β1 (Ref. 19 and Table 1). In addition, the newly identified Manduca sequence has a 315 amino acid region C-terminal to its catalytic domain that is not present in any other guanylyl cyclase of any species cloned to date. The C. elegans clone also has a C-terminal extension but has very low similarity to the Manduca sequence (Table 1). BLAST analysis of the 315-amino acid sequence from the Manduca sequence did not reveal any significant similarity to any other sequence in the data base. The clone does not appear to be the Manduca homologue of a β2 subunit, and as it has novel features not seen in previously cloned and characterized guanylyl cyclases, we propose to name this clone MsGC-β3. MsGC-β3 shows an alignment of the amino acid sequences of MsGC-β3 with the C. elegans sequence, MsGC-β1, and the rat β1 subunit.

Several different functional domains have been identified in mammalian soluble guanylyl cyclases (21–23). These consist of the N-terminal putative heme-binding domain, a putative dimerization domain (based on analogy with receptor guanylyl cyclases), and the C-terminal catalytic domain. Table I shows the percentage of amino acid identities in these domains when MsGC-β3 is compared with other β subunits. The level of

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sequence identity in the catalytic domain is very similar (≈40%) whether MsGC-β3 is compared with the C. elegans gene, rat β1, rat β2, or MsGC-β1. MsGC-β3 is most similar, however, to the C. elegans gene and the rat β1 subunit in its heme-binding and dimerization domains. Although MsGC-β3 is similar to rat β1, the degree of similarity is considerably lower (< 40%) compared with the sequence identities seen when MsGC-β1 is compared with rat β1 or when MsGC-α1 is compared with rat α1 (50–72%). These comparisons again suggest that MsGC-β3 is a novel β subunit and not a Manduca homologue of an identified vertebrate guanylyl cyclase.

Further analysis of the predicted amino acid sequence for MsGC-β3 reveals a number of other novel features. Several studies of mammalian guanylyl cyclases, using sequence comparison and mutational analysis, have identified 23 amino acids proposed to be involved in heme binding, NO activation, and/or dimerization (23–25). These residues are marked in Fig. 1B, and the sequence alignment shows that six of these residues are conserved between MsGC-β3 and any of the other sequences. In the regulatory region, those residues conserved between all guanylyl cyclases are shaded in black, and those conserved in all guanylyl cyclases except MsGC-β3 are marked with an asterisk. In the C-terminal domain, unique to MsGC-β3 and C. elegans, the residues marked with a double underline show potential phosphorylation sites, and those marked with a single underline show the potential isoprenylation site. The sequences used for designing primers used in the RT-PCR for cloning MsGC-β3 are marked P1 and P2. The nucleic acid sequence of the SGC4/SGC25 composite clone including the entire open reading frame of MsGC-β3 has been deposited with GenBank™, accession number AF064514.
and glutamic acid, respectively, in MsGC-β3. Friebe et al. (23) have shown that mutating these cysteines to serines in the rat β1 subunit produces a guanylyl cyclase that, when co-expressed with a wild-type α1 subunit, has detectable (but low) basal activity in the presence of manganese but cannot be activated by NO. Interestingly, histidine-105 (position based on rat β1 sequence), which has been suggested to be the axial ligand for heme binding and also shown to be required for NO-stimulated guanylyl cyclase activity (24), is present in MsGC-β3 and the C. elegans gene. The high degree of similarity between MsGC-β3 and the C. elegans gene suggests that it is also a novel β subunit and could also be considered a β3 subunit.

MsGC-β3 has a C-terminal 315-amino acid domain that is not present in any other guanylyl cyclase. This region shows no significant similarity to any other protein in the data base. A search for protein motifs revealed that there are a number of possible protein phosphorylation sites within this C-terminal domain, and a potential C-terminal isoprenylation site was also found. Interestingly, the rat β2 subunit also has a potential C-terminal isoprenylation site, although the modification has not yet been demonstrated in vivo (4). None of the phosphorylation sites are conserved in the C. elegans gene, nor is the isoprenylation site.

**Expression of MsGC-β3 in COS-7 Cells**—To examine the enzyme properties of MsGC-β3, we subcloned its open reading frame into the mammalian expression vector pcDNA3.1. We then transiently transfected this construct into COS-7 cells, either alone or in combination with plasmids containing the other Manduca soluble guanylyl cyclase subunits. Cell extracts were then assayed for guanylyl cyclase activity in the presence or absence of SNP as a NO donor (Fig. 2). As guanylyl cyclases have been shown to vary in their activity depending on whether magnesium or manganese is present (26), we tested each cell extract in the presence of each metal ion. In the presence of magnesium, only cells co-transfected with both MsGC-α1 and MsGC-β3 showed guanylyl cyclase activity above that seen for cells transfected with control plasmid. As reported previously, this activity could be stimulated by SNP (19). MsGC-β3 showed no guanylyl cyclase activity when expressed alone, and it did not appear to form an active heterodimer when co-expressed with either MsGC-α1 or MsGC-β1. In the presence of manganese, cells co-transfected with MsGC-α1 and MsGC-β1 showed higher basal and lower SNP-stimulated activity when compared with control extracts assayed in the presence of magnesium. This is a similar result to the enzymatic activity of mammalian soluble guanylyl cyclases (23). In cells transfected with only MsGC-β3 and assayed in the presence of manganese, we detected significant basal guanylyl cyclase activity above that seen in cells transfected with control plasmid. We did not, however, detect any stimulation of activity in the presence of SNP. This suggests that MsGC-β3 does not need to form heterodimers to form an active guanylyl cyclase and that this active enzyme is NO-insensitive. When MsGC-β3 was co-expressed with either MsGC-α1 or MsGC-β1, significant basal guanylyl cyclase activity above that seen for cells transfected with control plasmid was detected. As reported previously, this activity could be stimulated by SNP (19). MsGC-β3 showed no guanylyl cyclase activity when expressed alone, and it did not appear to form an active heterodimer when co-expressed with either MsGC-α1 or MsGC-β1. In the presence of manganese, cells co-transfected with MsGC-α1 and MsGC-β1 showed higher basal and lower SNP-stimulated activity when compared with control extracts assayed in the presence of magnesium. This is a similar result to the enzymatic activity of mammalian soluble guanylyl cyclases (23). In cells transfected with only MsGC-β3 and assayed in the presence of manganese, we detected significant basal guanylyl cyclase activity above that seen in cells transfected with control plasmid. We did not, however, detect any stimulation of activity in the presence of SNP. This suggests that MsGC-β3 does not need to form heterodimers to form an active guanylyl cyclase and that this active enzyme is NO-insensitive. When MsGC-β3 was co-expressed with either MsGC-α1 or MsGC-β1, significant basal guanylyl cyclase activity above that seen for cells transfected with control plasmid was detected.

![Graph](image_url)

**Fig. 2. Guanylyl cyclase activity in COS-7 cells transfected with Manduca-soluble guanylyl cyclase subunits.** COS-7 cells were transiently transfected with pcDNA3.1 vectors containing the open reading frame of the Manduca guanylyl cyclase subunits. Cell extracts were then assayed for guanylyl cyclase activity in the presence of 4 mM MgCl₂ (A) or 4 mM MnCl₂ (B) and in the absence (open columns) or presence (closed columns) of 250 μM SNP. Each assay was carried out in triplicate, and the columns show the means ± S.E. of three separate experiments.

**Table II**

| Transfection | No addition | 100 μM SNP | 5 μM hematin/100 μM DTT |
|--------------|-------------|------------|-------------------------|
|              | Control     | 100 μM SNP | Control                 |
| pcDNA3.1     | ND          | 2.8 ± 1.90 | —                       |
| MsGC-β3      | 0.64 ± 0.13 | 2.11 ± 0.08| 0.51 ± 0.11             |
| MsGC-β3 + MsGC-α1 | 0.50 ± 0.26 | 1.40 ± 0.16| ND                      |
| MsGC-α1 + MsGC-β3 | 1.11 ± 0.15 | 23.4 ± 0.28| 1.05 ± 0.11             |

* ND, not detectable.

* Not determined.
pressed with either MsGC-α1 or MsGC-β1, the total guanylyl cyclase activity present in the cell extracts was less than when MsGC-β3 was expressed alone and a small but significant increase in activity was detected in the presence of SNP. It is not clear whether this increase was due to the formation of low levels of NO-sensitive heterodimers. COS-7 cells transfected with control plasmid alone also showed a small increase in guanylyl cyclase activity in the presence of SNP, presumably due to the expression of low levels of endogenous soluble guanylyl cyclase.

Friebe et al. (23) report that mutant β subunits, missing either cysteine-78 or cysteine-214, show a substantial reduction in their ability to form NO-sensitive heterodimers. NO sensitivity was restored, however, by reconstitution with heme. Table II shows that this does not occur with MsGC-β3. We transfected COS-7 cells with either MsGC-β3 alone or MsGC-β3 together with MsGC-α1. We then assayed the cell extracts for guanylyl cyclase activity after incubation with 5 μM hematin and 100 μM dithiothreitol. In neither case did we see an increase in NO-stimulated activity. By contrast, when cells transfected with MsGC-α1 together with MsGC-β1 were treated in a similar fashion, heme reconstitution did increase the NO-stimulated activity, presumably because some endogenous heme was lost during cell homogenization.

These experiments suggest that MsGC-β3 either does not bind heme, or binds it in such a manner that renders the cyclase NO-insensitive. Recently, a compound, 1H-1,2,4oxadiazo[4,3-α]quinazolin-1-one (ODQ), has been identified as a potent inhibitor of mammalian guanylyl cyclases (27). It appears to act as a heme site inhibitor, inhibiting activity by irreversible oxidation of the heme group (28). We tested whether ODQ could inhibit MsGC-β3 by assaying guanylyl cyclase activity of transfected COS-7 cell extracts in the presence of different concentrations of ODQ (Fig. 3). ODQ has an IC_{50} of 0.72 μM for inhibiting mammalian soluble guanylyl cyclase (28), yet 100 μM had no inhibitory effect on MsGC-β3.

As a control for the effectiveness of ODQ, we also assessed its effects on Manduca NO-sensitive soluble guanylyl cyclase (Fig. 3). These results showed that 100 μM ODQ inhibited Manduca NO-sensitive activity by 95% with an IC_{50} of 2.9 μM. Interestingly, basal activity of the Manduca NO-sensitive cyclase showed no sensitivity to ODQ. This is in contrast to the mammalian enzyme, which showed 80% inhibition of basal activity in the presence of 30 μM ODQ (28).

The C terminus of MsGC-β3 contains a potential isoprenylation site (Fig. 1B). To determine whether MsGC-β3 is membrane-associated, we separated transfected COS-7 cell homogenates into particulate and cytoplasmic fractions and assayed each separately for guanylyl cyclase activity. We found that the majority of MsGC-β3 activity was pelleted by ultracentrifugation (Table III), suggesting that the enzyme was associated with membranes. Whether this is a result of isoprenylation or not will require further study.

In addition to assessing the enzyme activity of MsGC-β3 in homogenates of transfected COS-7 cells, we also measured the cGMP content of intact COS-7 cells after they had been transiently transfected with MsGC-β3. We found that in unstimulated COS-7 cells, MsGC-β3 forms an active guanylyl cyclase capable of causing a much larger accumulation of cGMP than when COS-7 cells are either co-transfected with MsGC-α1 and MsGC-β1, or transfected with vector alone (Table IV). Surprisingly, when the COS-7 cells were stimulated with SNP, all of the transfected cells showed a significant increase in cGMP content. Presumably, the increase in cGMP in response to SNP in COS-7 cells transfected with vector was due to the presence of endogenous soluble guanylyl cyclase. When this background value was subtracted from the cGMP levels in cells transfected with Manduca guanylyl cyclases, over a 100-fold stimulation in cGMP content was observed with cells co-transfected with MsGC-α1 and MsGC-β1, whereas cells transfected with MsGC-β3 showed less than a 3-fold stimulation.

**Table III**

MsGC-β3 activity is primarily present in the pellet fraction of COS-7 cell homogenates

| Cell fraction     | Guanylyl cyclase activity |
|-------------------|---------------------------|
|                   | pmol/min/mg of protein    |
| Homogenate        | 9.4 ± 0.2                 |
| 100,000 × g supernatant | 8.2 ± 0.5           |
| 100,000 × g pellet | 22.7 ± 0.5               |

**Table IV**

Activity of MsGC-β3 in intact COS-7 cells

| Transfection          | cGMP content | cGMP content (minus control transfection) | Fold stimulation |
|-----------------------|--------------|------------------------------------------|------------------|
|                       | Basal | 1 μM SNP | Basal | 1 μM SNP |                                      |
| pcDNA3.1              | 5.9 ± 0.9 | 199 ± 18 | 245 ± 15 | 657 ± 36 | 2.7                                  |
| MsGC-β3               | 251 ± 15 | 857 ± 36 | 3.3 ± 1.7 | 452 ± 68 | 137.0                               |
| MsGC-α1 + MsGC-β1     | 9.2 ± 1.7 | 652 ± 68 |                                      |                 |                                      |
analysis. We found a single 5.1-kb transcript present at high levels in the abdominal central nervous system. The same transcript was found at lower levels in brain and trachea (Fig. 4A). A signal was also detected in the heart, although it could not be resolved into discrete bands. No signal was detectable in fat body, muscle or gut. We also examined the expression of MsGC-β3 in several tissues of adult Manduca, just prior to ecdysis. No message could be detected in any tissues (brain, abdominal central nervous system, antennae, or muscle) at that stage (data not shown) suggesting that MsGC-β3 is developmentally regulated. Because MsGC-β3 codes for a guanylyl cyclase that is relatively insensitive to NO, we were especially interested in determining whether its transcript could be detected in tissues that are known to increase cGMP in response to eclosion hormone. Previous studies have shown that the eclosion hormone-stimulated cGMP increases are not mimicked by NO (16, 29). These targets include the epitracheal glands (30) and the transverse nerve (16). To determine whether MsGC-β3 could be detected in these tissues, we used RT-PCR. We were able to detect a strong, RT-dependent band in epitracheal glands and transverse nerve samples, as well as the abdominal central nervous system, muscle, and trachea (Fig. 4B). Because MsGC-β3 is expressed in trachea, and it is possible that tracheal epithelial cells could have contaminated both the epitracheal gland and transverse nerve samples, we examined the expression of MsGC-α1 as a control. We found that although both MsGC-β3 and MsGC-α1 were detectable in trachea, only MsGC-β3 could be detected in the epitracheal gland and transverse nerve samples.

To demonstrate that the MsGC-β3 protein is indeed expressed in Manduca nervous tissue, we constructed a GST fusion protein containing amino acids 178–940 of MsGC-β3 and immunized rabbits with the resulting protein. MsGC-β3 has a predicted molecular mass of 106 kDa, and Fig. 4D shows that antisera that had been affinity purified against the MsGC-β3 fusion protein recognized a protein of approximately 106 kDa in COS-7 cells that had been transfected with the MsGC-β3 expression plasmid (lane d). COS-7 cells transfected with control plasmid (lane a) or plasmids containing MsGC-α1 (lane b) or MsGC-β1 (lane c) did not show the presence of any bands. Preimmune serum did not show the presence of this 106 kDa band in COS-7 cells expressing MsGC-β3 or any of the other plasmids (Fig. 4C). When abdominal central nervous tissue was blotted and probed with the affinity purified antisera, a single band was detected with a slightly higher molecular mass compared with the band in COS-7 cells. This demonstrates that a protein of the predicted size of MsGC-β3 is expressed in Manduca central nervous system. It also suggests that some processing takes place in vivo, resulting in a protein with an apparently larger molecular mass compared with MsGC-β3 expressed in COS-7 cells. The primary sequence of MsGC-β3 does not give any clues as to the nature of this processing.

DISCUSSION

This paper describes the cloning and initial characterization of a novel β isoform of soluble guanylyl cyclases from M. sexta that we have named MsGC-β3. Previously described guanylyl cyclases can be classified as either receptor guanylyl cyclases, which are integral membrane proteins, or soluble guanylyl cyclases, which are generally cytoplasmically localized (4). Soluble guanylyl cyclases are thought to exist as heterodimers of an α and a β subunit, and two α and two β isoforms have been identified in mammals (8–13). MsGC-β3 has a high degree of similarity to the rat β1 subunit throughout all three of its domains, demonstrating that MsGC-β3 clearly belongs to the soluble guanylyl cyclase family rather than the receptor guanylyl cyclase family. The principal difference between the α and β subunits is that the hemebinding domains of α subunits are longer: 372 amino acids for rat α1 as compared with 311 for rat β1 (9, 11). The equivalent region of MsGC-β3 also contains 311 amino acids, which identifies it as a β subunit rather than an α subunit. Although sequence analysis shows that MsGC-β3 is most similar to mammalian β1 subunits, there are a number of
features that suggest that it is a novel \(\beta\) subunit rather than the *Manduca* homologue of \(\beta 1\) or \(\beta 2\). First, we have previously cloned and characterized another *Manduca* subunit that has a higher degree of similarity to rat \(\beta 1\) than does MsGC-3 (19). Second, MsGC-3 has a C-terminal extension of 315 amino acids not seen in any other guanylyl cyclase cloned to date. Finally, MsGC-3 lacks several amino acids that are conserved in all other soluble cyclases, some of which have also been shown to be necessary for NO activation (23). A search of the database also reveals a gene from *C. elegans* that appears to be homologous to MsGC-3. The *C. elegans* gene is lacking five of the 6 highly conserved amino acid residues absent in MsGC-3 and also has an extended C-terminal tail.

In addition to the sequence information, expression data also show that MsGC-3 has novel features when compared with other guanylyl cyclases. Previous studies have shown that soluble guanylyl cyclases are obligate heterodimers (11). We have demonstrated that the *Manduca* homologues of \(\alpha 1\) and \(\beta 1\) subunits also conform to this pattern (19) (Fig. 2). MsGC-3, by contrast, shows significant guanylyl cyclase activity when expressed alone in COS-7 cells. This is true both when the cells are homogenized and enzyme activity measured *in vitro* and when guanylyl cyclase activity is assessed in intact cells by measuring cGMP accumulation. Like other guanylyl cyclases, the activity measured in homogenates is higher when Mn-GTP is used as a substrate compared with when Mg-GTP is used. Unlike heterodimeric guanylyl cyclases, however, MsGC-3 responds very poorly to NO. When measured in COS-7 cell homogenates, in the presence of either manganese or magnesium, the guanylyl cyclase activity of MsGC-3 shows no stimulation with SNP. When measured in intact COS-7 cells, MsGC-3 did show significantly higher accumulations of cGMP when treated with SNP. This increase was substantially less, however, than that found in COS-7 cells that were co-transfected with MsGC-\(\alpha 1\) and MsGC-\(\beta 1\). It will be very interesting to determine whether the *C. elegans* homologue is also active as a homomer and whether it is also NO-insensitive.

An important question raised by these findings is whether or not MsGC-3 is capable of binding heme. Cysteine-78 and cysteine-214 have been shown to be important in NO sensitivity in the rat \(\beta 1\) subunit (23). When these cysteines were point-mutated to serines and the recombinant subunit co-expressed with wild-type \(\alpha 1\) subunits, the resulting guanylyl cyclase was NO-insensitive, although NO-sensitivity could be restored by incubation with heme (23). This suggests that loss of these two cysteines results in a guanylyl cyclase capable of binding heme, but with a much lower affinity. MsGC-3 lacks both of these cysteines and is NO-insensitive. However, NO-sensitivity cannot be restored with heme reconstitution, whether the activity is measured in extracts of COS-7 cells transfected with MsGC-3 alone or co-transfected with MsGC-\(\alpha 1\). The soluble guanylyl cyclase inhibitor, ODQ, is thought to act by irreversible oxidation of the heme group resulting in a NO-insensitive enzyme (28). ODQ has no effect on the activity of MsGC-3, suggesting either that MsGC-3 does not bind heme or that it binds heme in an oxidized state and hence is unaffected by either NO or ODQ. The fact that a slight stimulation by SNP is seen in intact COS-cells suggests that MsGC-3 does bind heme in *vivo*, but that it is rapidly lost or irreversibly oxidized during the homogenization process. It will also be interesting to determine whether MsGC-3 forms homodimers, or if it is capable of forming heterodimers with other soluble guanylyl cyclase subunits. The dimerization domain of soluble guanylyl cyclases has not been investigated in detail. It is defined primarily by comparison to receptor guanylyl cyclases (22) as the region on the immediate N-termi-
Identification and Characterization of a Novel β Subunit of Soluble Guanylyl Cyclase That Is Active in the Absence of a Second Subunit and Is Relatively Insensitive to Nitric Oxide

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