

MsGC- β 3 forms active homodimers and inactive heterodimers with NO-sensitive soluble guanylyl cyclase subunits

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Summary

Soluble guanylyl cyclases are typically obligate heterodimers, composed of a single α and a single β subunit. MsGC- β 3, identified in the tobacco hornworm *Manduca sexta*, was the first example of a soluble guanylyl cyclase that exhibited enzyme activity without the need for coexpression with additional subunits. Subsequent studies have revealed that the mammalian β 2 subunit also shares this property. Using a combination of gel filtration chromatography, coprecipitation and site-directed mutagenesis we show that, as predicted, MsGC- β 3 forms active homodimers. We also demonstrate that MsGC- β 3 is capable of forming heterodimers with the nitric oxide

(NO)-sensitive guanylyl cyclase subunits MsGC- α 1 and MsGC- β 1. These heterodimers, however, show no enzyme activity and, like mammalian β 2 subunits, act in a dominant negative manner when combined with the NO-sensitive subunits to disrupt their activation by NO. In addition, we show that the unique C-terminal domain of MsGC- β 3 is not necessary for enzyme activity and might act as an auto-inhibitory domain.

Key words: cGMP, guanylyl cyclase, nitric oxide, protein dimerization, tobacco hornworm, *Manduca sexta*.

Introduction

Soluble guanylyl cyclases have classically been described as obligate heterodimers that form the principal receptor for nitric oxide (NO) (Lucas et al., 2000). Two recent reports, however, show that there is likely to be an additional class of soluble guanylyl cyclases that do not need a second subunit for activity. The first of these reports described a guanylyl cyclase subunit, named MsGC- β 3, cloned from the insect *Manduca sexta* (Nighorn et al., 1999), and the second described properties of the rat β 2 subunit (Koglin et al., 2001). Both of these subunits share several biochemical characteristics. Both yield significant guanylyl cyclase activity when expressed in heterologous cells in the absence of additional exogenous subunits and both require the presence of manganese for activity (Nighorn et al., 1999; Koglin et al., 2001). They differ principally in their response to NO: extracts of cells expressing rat β 2 subunits were weakly stimulated by NO (Koglin et al., 2001), whereas extracts containing MsGC- β 3 showed no stimulation with NO donors (Nighorn et al., 1999). Additional studies suggested that mammalian β 2 subunits could form heterodimers with the NO-sensitive α 1 subunit and that the degree of NO-stimulation of the α 1/ β 1 heterodimer was reduced when β 2 subunits were coexpressed in the same cells (Gupta et al., 1997).

Modeling of the catalytic domain of both soluble and receptor guanylyl cyclases, based on the crystal structure of adenylyl cyclases, suggests that all of these enzymes need to form dimers to generate an active site to bind the substrate,

GTP (Liu et al., 1997; Zhang et al., 1997). Thus we predict that both MsGC- β 3 and mammalian β 2 subunits should form active homodimers. In this study we demonstrate that MsGC- β 3 does form active homodimers and also show that it is capable of forming heterodimers with the NO-sensitive subunits, although these heterodimers are inactive.

Materials and methods

Animals

The rearing and staging of *Manduca sexta* (Lepidoptera: Sphingidae) has previously been described (Morton and Giunta, 1992).

Transient expression of MsGC- β 3 and guanylyl cyclase assay

The open reading frame of MsGC- β 3 in the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was used to transiently express MsGC- β 3 in COS-7 cells as described previously (Nighorn et al., 1999). When multiple guanylyl cyclases were cotransfected, the transfection efficiency was monitored by the addition of 1 μ g of a β -galactosidase expression plasmid (Invitrogen). β -Galactosidase activity was determined by incubating cell extracts with 1.3 mg ml⁻¹ *o*-nitrophenyl- β -D-galactopyranoside in a 100 mmol l⁻¹ sodium phosphate buffer, pH 7.0, containing 1.5 mmol l⁻¹ MgCl₂ and 75 mmol l⁻¹ β -mercaptoethanol at room temperature for 5–20 min. The reaction was stopped by the addition of 2 mol l⁻¹

Na_2CO_3 and the absorbance measured at 405 nm. Guanylyl cyclase activity was measured in a buffer containing 50 mmol l^{-1} Mops-KOH, pH 7.5, 60 mmol l^{-1} KCl, 8 mmol l^{-1} NaCl, 4 mmol l^{-1} MnCl_2 , $10 \mu\text{mol l}^{-1}$ each of cGMP phosphodiesterase inhibitors dipyridamole and zaprinast and 1 mmol l^{-1} GTP. The reaction was stopped with 0.2 mol l^{-1} zinc acetate and excess GTP precipitated with 0.2 mol l^{-1} Na_2CO_3 . The amount of cGMP formed was determined using a cGMP enzyme-linked immunoassay (EIA; Kingan et al., 1997). Under the conditions used, the production of cGMP was linear with respect to time for up to 30 min.

Production of C-terminal deletions of MsGC- β 3

Two deletions of MsGC- β 3 were generated that lacked the C-terminal 29 or 338 amino acids, MsGC- β 3 Δ C29 and MsGC- β 3 Δ C338, respectively. The original cDNA for MsGC- β 3 was generated by the ligation of two partial cDNAs, SGC4 and SGC25 (Nighorn et al., 1999). SGC4 coded for all but the C-terminal 29 amino acids of the open reading frame and was directly subcloned into pcDNA3.1 by excising SGC4 with *Xho*I and *Sma*I and ligating it into pcDNA3.1 at the *Xho*I and *Eco*RV sites to generate MsGC- β 3 Δ C29. MsGC- β 3 Δ C338 was generated using polymerase chain reaction (PCR). The 5' primer was designed to the 5' end of SGC4 and included an *Xho*I site: 5'GCCTCGAGGAATGTGATATTTA, and the 3' primer introduced a stop codon and a *Kpn*I site immediately following residue 602 of the open reading frame of SGC4: 5'TTGGGTACCTAGGGTCTTGATT. PCR was carried out using ElongaseTM enzyme mix (Invitrogen) for 30 cycles, according to the manufacturer's instructions, with an annealing temperature of 50°C. The PCR product was digested with *Xho*I and *Kpn*I and ligated into pcDNA3.1.

Gel filtration

Transiently transfected COS-7 cells or *Manduca* abdominal nerve cords (ANCs) were homogenized, incubated in 0.2% octyl β ,D-thioglucoopyranoside and centrifuged at $14000g$ for 30 min at 4°C. A portion (200 μ l) was applied to a gel filtration column (Bio-Sil 400-5, BioRad, Hercules, CA, USA) and eluted in 50 mmol l^{-1} Tris-HCl, pH 7.5 in the presence of 0.2% octyl β ,D-thioglucoopyranoside at 0.5 ml min^{-1} and fractions were collected every 0.2 min. The following standards were used to construct a calibration curve: thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobulin (17 kDa) (all BioRad), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) (all from Sigma, St Louis, MO, USA).

Western blots

Western blots using antisera generated to MsGC- β 3 were carried out as described previously (Nighorn et al., 1999).

Generation of tagged Manduca guanylyl cyclases

The coding regions of MsGC- α 1, MsGC- β 1 and MsGC- β 3 Δ C338 were amplified using PCR and primers that

incorporated restriction sites at the 3' and 5' ends such that when ligated into pcDNA3.1-His (Invitrogen), the coding sequence was in-frame with N-terminal hexa-histidine and ExpressTM tags. The primers used were 5'ATGGTACCAATGACGTGTCCATTCC and 5'ATGGG-CCCGTAAGAAGCTAAGTTG for MsGC- α 1, 5'ATGAA-TTCAAATGTACGGGTTTGTG and 5'ATGGGCCCTT-AGAGATTTAATGGATC for MsGC- β 1 and 5'ATG-AATTCCGATGTACGGCCTA and 5'ATGGGCCCTAGGG-TCTTGATTCCCT for MsGC- β 3 Δ C338. PCR was carried out for 3 cycles at an annealing temperature of 50°C followed by 25 cycles with an annealing temperature of 60°C, with extension times of 2 min in each case, and the products were cloned into the TOPOII vector (Invitrogen). After being sequenced, the inserts were excised with *Kpn*I and *Apa*I for MsGC- α 1 and *Eco*RI and *Apa*I for MsGC- β 1 and MsGC- β 3 Δ C338 and ligated into pcDNA3.1-His. COS-7 cells were transiently transfected with each construct and western blots probed with an anti-express antibody (Invitrogen) to confirm that each generated full-length proteins. For heterodimerization studies two 10 cm plates were transiently cotransfected with full-length, untagged MsGC- β 3 in combination with either empty vector, tagged MsGC- α 1, tagged MsGC- β 1 or tagged MsGC- β 3 Δ C338. Transfected cells from the two plates were combined, homogenized, centrifuged at $14000g$ for 30 min at 4°C and the supernatants incubated with nickel-chelated agarose discs (Pierce, Rockford, IL, USA) for 6 h at 4°C. The agarose was washed with phosphate-buffered saline (PBS) and bound proteins eluted with SDS-sample buffer and analyzed by western blot. To measure the guanylyl cyclase activity of bound proteins, the agarose was given an additional wash of 10 mmol l^{-1} imidazole in PBS and the proteins eluted with 400 mmol l^{-1} imidazole in PBS and assayed for activity as described above.

Generation of point mutations in MsGC- β 3

Point mutations were introduced into the catalytic domain of MsGC- β 3 using the methods described previously (Kunkel et al., 1987). Briefly, single-stranded uracil-containing MsGC- β 3 in pcDNA3.1 was generated using CJ236 *E. coli* and the M13 bacteriophage. This was used as a template for second-strand synthesis using a phosphorylated primer that contained a single mismatched base designed to convert E469 to lysine (5'GTCACCTATTGTCTTTCACCTTATACAC) or R537 to glutamine (5'CGAATAGACAATACTGCGGCATCTTGA). After confirmation of the sequence, the plasmid DNA was used to transfect COS-7 cells and assayed for guanylyl cyclase activity as described above.

Results

Gel filtration of MsGC- β 3 demonstrate the formation of homodimers

Previous studies showed that MsGC- β 3 was the first example of a soluble guanylyl cyclase that did not require coexpression with additional subunits for enzyme activity

Table 1. Lack of solubility of recombinant MsGC- β 3 and MsGC- β 3 Δ C338

| Treatment | Supernatant | | Pellet | | | |
|---|-----------------|-------------------------------|-----------------|-----|-------------------------------|-----|
| | MsGC- β 3 | MsGC- β 3 Δ C338 | MsGC- β 3 | (%) | MsGC- β 3 Δ C338 | (%) |
| None (50 mmol l ⁻¹ Tris-HCl) | 0.98±0.03 | 2.30±0.05 | 5.30±0.30 | 84 | 3.77±0.18 | 62 |
| 0.5 mol l ⁻¹ NaCl | 0.78±0.02 | 1.96±0.03 | 4.52±0.12 | 85 | 4.44±0.24 | 69 |
| 1% Chaps | 2.16±0.04 | 1.60±0.03 | 6.20±0.10 | 74 | 4.74±0.20 | 75 |
| 1% Digitonin | 3.21±0.04 | 3.14±0.08 | 6.27±0.15 | 66 | 4.24±0.07 | 67 |
| 1% Octyl β ,D-thioglucopyranoside | 3.80±0.04 | 5.49±0.07 | 7.12±0.44 | 65 | 2.60±0.04 | 32 |
| 1% Triton X-100 | 4.36±0.03 | 4.29±0.05 | 4.81±0.20 | 52 | 5.30±0.10 | 55 |
| 1% Tween 20 | 2.19±0.10 | 4.01±0.09 | 6.20±0.10 | 74 | 4.80±0.27 | 54 |

COS-7 cells were transiently transfected with MsGC- β 3 or MsGC- β 3 Δ C338 as described in Materials and methods and homogenized in 50 mmol l⁻¹ Tris HCl, pH 7.5. Salt or detergents were added to separate samples as shown, which were then incubated for 30 min at 4°C and centrifuged at 14000g for 30 min at 4°C. The pellet was resuspended in the same detergent that was used for solubilization and both supernatant and pellet fractions assayed for guanylyl cyclase activity.

Values are the means \pm S.E.M. of 3 measurements.

% indicates percentage of total enzyme activity in pellet fractions.

(Nighorn et al., 1999). Subsequent studies have shown that the rat β 2 subunit also shares these properties, exhibiting enzyme activity in the absence of additional subunits (Koglin et al., 2001). Other soluble guanylyl cyclases that have been investigated, whether mammalian or insect, are obligate heterodimers, requiring coexpression of an alpha and a beta subunit for activity (Lucas et al., 2000; Morton and Hudson, 2002). The other major class of guanylyl cyclases, the receptor guanylyl cyclases, exists as homodimers (Lucas et al., 2000). This suggested that both MsGC- β 3 and mammalian β 2 subunits would exist as homodimers, but direct evidence for this has not yet been provided. To determine the apparent molecular mass of MsGC- β 3 using gel filtration, MsGC- β 3 must be a soluble protein. Previous studies, however, showed that recombinant MsGC- β 3 was primarily present in the insoluble fraction of heterologous cell homogenates (Nighorn et al., 1999), presumably due to the presence of a consensus isoprenylation site at its C terminus (Nighorn et al., 1999). To solubilize sufficient quantities of MsGC- β 3 for gel filtration, homogenates of COS-7 cells that had been transfected with a plasmid coding for MsGC- β 3 were incubated in a variety of different detergents and the soluble and pellet fractions assayed for guanylyl cyclase activity (Table 1). Although several detergents did solubilize some of the MsGC- β 3 activity, significant levels remained in the pellet fraction. To overcome this problem, a version of MsGC- β 3 that lacked the C-terminal 29 residues, containing the putative isoprenylation site, was also transfected into COS-7 cells. This deletion had no effect on the distribution of guanylyl cyclase activity in the soluble and particulate fractions of transfected cells, suggesting that isoprenylation was not responsible for the protein's insolubility (data not shown). To investigate this further, another deletion mutant was generated, lacking the entire C-terminal domain. When an expression plasmid coding for MsGC- β 3 Δ C338 was transfected into COS-7 cells and assayed for guanylyl cyclase activity, similar levels of activity were again detected, compared to cells transfected with full-length MsGC- β 3, but

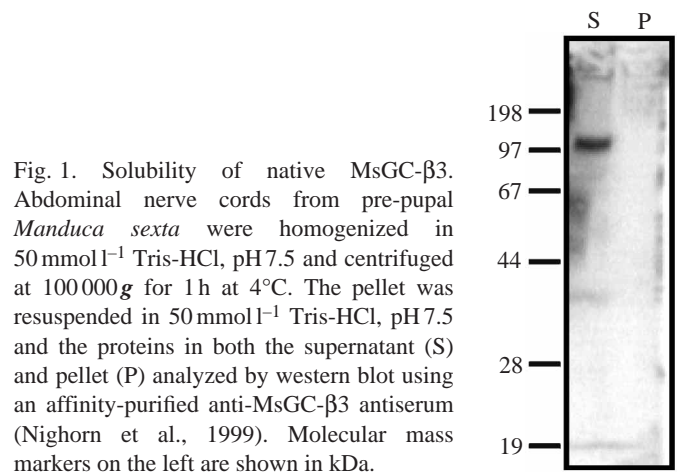


Fig. 1. Solubility of native MsGC- β 3. Abdominal nerve cords from pre-pupal *Manduca sexta* were homogenized in 50 mmol l⁻¹ Tris-HCl, pH 7.5 and centrifuged at 100 000g for 1 h at 4°C. The pellet was resuspended in 50 mmol l⁻¹ Tris-HCl, pH 7.5 and the proteins in both the supernatant (S) and pellet (P) analyzed by western blot using an affinity-purified anti-MsGC- β 3 antiserum (Nighorn et al., 1999). Molecular mass markers on the left are shown in kDa.

although more of the activity was present in the supernatant fraction, most of the activity was still in the particulate fraction (Table 1).

To determine whether the insolubility was the result of expression in a heterologous cell line, western blots were used to detect native MsGC- β 3 in pellet and supernatant fractions of nervous tissue homogenates. Surprisingly, native MsGC- β 3 was present exclusively in the soluble fraction of nervous tissue homogenates (Fig. 1), suggesting that MsGC- β 3 is not isoprenylated *in vivo*. In combination with the solubility data described above, these data suggest that the recombinant protein forms aggregates that are largely insoluble when expressed in COS-7 cells.

To determine the native molecular mass of the soluble fraction of recombinant MsGC- β 3, COS-7 cells were transfected with either MsGC- β 3 or MsGC- β 3 Δ C338 and the proteins solubilized by incubation with octyl β ,D-thioglucopyranoside. This detergent was chosen because, in addition to being one of the more effective in solubilizing both forms of MsGC- β 3, it also has a relatively high critical micelle concentration of 9 mmol l⁻¹. After centrifugation, soluble

Fig. 2. Gel filtration of recombinant and native MsGC-β3. Supernatants from extracts of COS-7 cells that had been transiently transfected with either MsGC-β3 (solid line), MsGC-β3ΔC338 (broken line) or nerve cords (CNS; dotted line) were homogenized in 50 mmol l⁻¹ Tris HCl, pH 7.5 and separated by gel filtration in the presence of 0.2% octyl β,D-thioglucopyranoside. Fractions from COS-7 cell extracts were assayed for guanylyl cyclase activity and fractions from nerve cord homogenates were assayed by western blot. Molecular mass estimates were generated by comparing with standard proteins run in parallel.

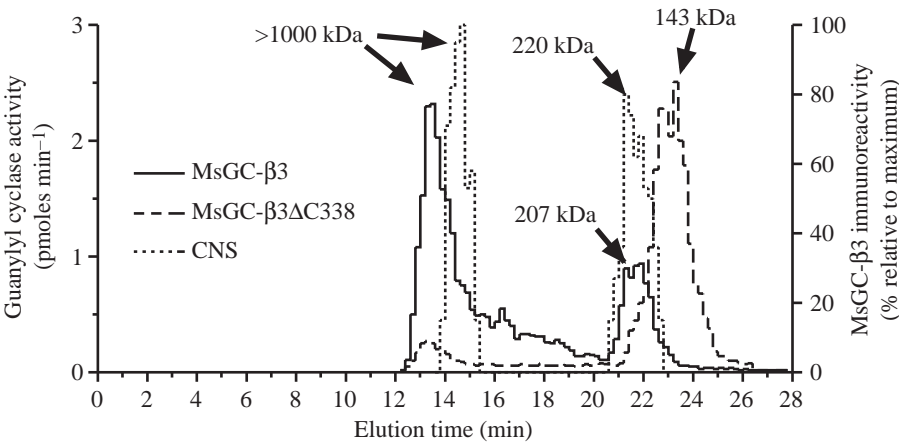


Table 2. Coexpression of MsGC-β3 with MsGC-α1 and MsGC-β1 reduces NO-stimulated guanylyl cyclase activity

| Plasmids transfected | Basal activity | Activity with | |
|---|----------------|------------------------------|-------------|
| | | 125 μmol l ⁻¹ SNP | Stimulation |
| 10 μg MsGC-α1+10 μg MsGC-β1 | 7.72±0.72 | 43.10±0.96 | 5.6-fold |
| 10 μg MsGC-α1+10 μg MsGC-β1+10 μg MsGC-β3 | 4.63±0.73 | 16.10±1.69 | 3.5-fold |
| 10 μg MsGC-α1+10 μg MsGC-β1+20 μg MsGC-β3 | 7.72±0.24 | 13.80±1.27 | 1.8-fold |
| 10 μg MsGC-β3 | 11.10±1.00 | 9.63±0.43 | 0.9-fold |
| 20 μg MsGC-β3 | 24.4±0.10 | 22.1±1.10 | 0.9-fold |

COS-7 cells were transfected with the plasmids as shown plus a plasmid coding for β-galactosidase and assayed for guanylyl cyclase activity in the presence or absence of 125 μmol l⁻¹ sodium nitroprusside (SNP) as described in Materials and methods, except that the MnCl₂ was replaced by 4 mmol l⁻¹ MgCl₂.

Extracts were also assayed for β-galactosidase activity and the guanylyl cyclase activity shown for each plasmid combination was normalized to the β-galactosidase activity present in the vector-transfected sample.

Activity levels are expressed as pmoles cGMP formed min⁻¹ mg⁻¹ protein.

Values are means ± S.E.M. of 3 determinations.

proteins were separated by gel filtration and each fraction assayed for enzyme activity (Fig. 2). Gel filtration of full-length MsGC-β3 confirmed that a large proportion of even the solubilized protein existed as large aggregates (>1000 kDa) that eluted in the void volume of the column. The calculated mass of monomeric MsGC-β3 is 106 kDa (Nighorn et al., 1999) and the elution profile of full-length MsGC-β3 from the gel filtration column showed a second peak of guanylyl cyclase activity at 207 kDa, consistent with the formation of homodimers. When extracts of COS-7 cells transfected with MsGC-β3ΔC338 (calculated monomeric mass of 69 kDa) were separated on the gel filtration column, a single peak of activity was detected with an apparent molecular mass of 143 kDa, again consistent with the formation of homodimers (Fig. 2).

To test whether native MsGC-β3 also formed homodimers, we separated the soluble fraction of nerve cord homogenates by gel filtration using the same conditions as for recombinant MsGC-β3 (Fig. 2). Each fraction was assayed by western blot for MsGC-β3 immunoreactivity (IR). The profile of MsGC-β3-IR showed two peaks, the first eluted with the void volume, presumably reflecting the formation of large aggregates, and the second eluted with an apparent mass of about 220 kDa, consistent with the formation of homodimers. Thus, in the

presence of detergent, both native and recombinant MsGC-β3 elute at a position consistent with the formation of homodimers, and in addition appear to form large aggregates.

Coexpression of MsGC-β3 with MsGC-α1 and MsGC-β1 reduces NO-stimulated activity

Previous studies demonstrated that MsGC-β3 did not form NO-sensitive heterodimers with either of the *Manduca* NO-sensitive guanylyl cyclase subunits, MsGC-α1 or MsGC-β1 (Nighorn et al., 1999). These data did not, however, indicate whether heterodimers were formed or had any basal enzyme activity. The mammalian β2 subunit acts in a dominant negative manner, forming heterodimers with the mammalian α1 subunit that are less sensitive to NO than the α1/β1 combination (Gupta et al., 1997). To determine whether MsGC-β3 behaved in a similar manner, we cotransfected COS-7 cells with all three subunits: MsGC-α1, MsGC-β1 and MsGC-β3 (Table 2). Coexpression with MsGC-β3 substantially reduced the NO activation of MsGC-α1/MsGC-β1. When the amount of MsGC-β3 was doubled, the activation by NO was further reduced. These data suggested that MsGC-β3 was capable of forming heterodimers with either MsGC-α1 or MsGC-β1 and that these heterodimers were NO-

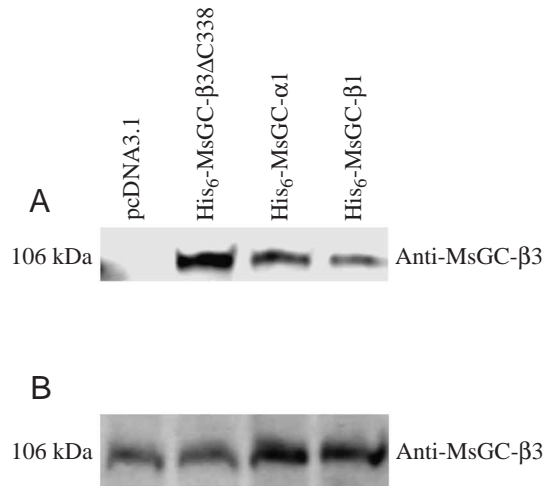


Fig. 3. Heterodimerization of MsGC-β3 with MsGC-α1 and MsGC-β1. Extracts of COS-7 cells that were transiently cotransfected with MsGC-β3 and either empty vector (pcDNA3.1), or hexa-histidine (His₆)-tagged MsGC-β3ΔC338, MsGC-α1 or MsGC-β1, were incubated with nickel-chelated agarose and the bound proteins analyzed by western blot. (A) Blot probed with MsGC-β3 antiserum shows that MsGC-β3 is pelleted when cotransfected with either tagged MsGC-β3ΔC338, tagged MsGC-α1 or tagged MsGC-β1, but the intensity of the immunoreactivity is highest when cotransfected with tagged MsGC-β3ΔC338. (B) Western blot of input extract stained with anti-MsGC-β3.

insensitive and were either catalytically inactive or exhibited substantially reduced levels of activity.

MsGC-β3 forms inactive dimers with tagged MsGC-α1 and tagged MsGC-β1

To determine directly whether MsGC-β3 was capable of forming heterodimers with either MsGC-α1 or MsGC-β1 we generated hexa-histidine tagged versions of all three guanylyl cyclases. We used MsGC-β3ΔC338 rather than a tagged full-length version of MsGC-β3, because the MsGC-β3 antisera recognized an epitope in the C-terminal domain (D. B. Morton, unpublished data) and hence could be used to distinguish between the tagged and untagged versions. COS-7 cells were cotransfected with each of the tagged guanylyl cyclases in combination with full-length untagged MsGC-β3, the extracts incubated with nickel-chelated agarose and the proteins bound to the beads analyzed by western blot. Probing the blot with

MsGC-β3 antisera (Fig. 3A) showed that each of the tagged guanylyl cyclases was capable of binding to and pulling down MsGC-β3 from solution. Somewhat lower levels of MsGC-β3 were detected with either tagged MsGC-α1 or MsGC-β1 compared to tagged MsGC-β3, although the levels of MsGC-β3 in each extract were similar (Fig. 3B). It is not known, however, whether the lower levels of MsGC-β3 pelleted with either MsGC-α1 or MsGC-β1 reflect a difference in affinity of MsGC-β3 for forming homodimers compared to heterodimers.

These data show that both MsGC-α1 and MsGC-β1 are capable of forming heterodimers with MsGC-β3. To determine if these heterodimers exhibited any guanylyl cyclase activity, we eluted the bound proteins from the beads with 400 mmol l⁻¹ imidazole and assayed the eluate for guanylyl cyclase activity. Very low levels of enzyme activity were measured (less than 1% of the input levels) and subsequent experiments showed that 400 mmol l⁻¹ imidazole inhibited the guanylyl cyclase activity by 70–80% (data not shown).

Site-directed mutagenesis confirms that the heterodimers are inactive

An alternative approach to determine whether heterodimers form active enzymes is to use site-directed mutagenesis to eliminate the activity generated by MsGC-β3 homodimers so that any activity present when MsGC-β3 is coexpressed with either MsGC-α1 or MsGC-β1 will be from heterodimers. Solving the crystal structure of the catalytic domain of adenylyl cyclase followed by homology modeling of the catalytic domain of guanylyl cyclases enabled the critical residues that bind GTP to be predicted (Liu et al., 1997; Zhang et al., 1997). This modeling predicts that homodimeric receptor guanylyl cyclases have two GTP-binding sites, whereas heterodimeric α1/β1 guanylyl cyclases have a single GTP-binding site, with the α subunit providing some of the critical residues and the remainder provided by the β subunit (Liu et al., 1997). MsGC-β3, like the receptor guanylyl cyclases, has all of the critical residues necessary for binding GTP (Morton and Hudson, 2002; Fig. 4) and hence MsGC-β3 homodimers should form two GTP-binding sites. When MsGC-β3 forms a heterodimer with MsGC-α1 it is possible that a single active GTP-binding site is formed with MsGC-α1 acting as the α chain and MsGC-β3 acting as the β chain. Conversely, MsGC-β3 could act as the α chain in heterodimers formed with MsGC-β1, again potentially forming a single active GTP-binding site. When MsGC-β3 is

Fig. 4. Alignment of regions of the catalytic domain of selected guanylyl cyclases showing residues predicted to contact Mg-GTP. Homology modeling has been used to predict the residues (shaded) of soluble and receptor guanylyl cyclases that bind Mg-GTP (Liu et al., 1997). Sequences shown are the rat soluble guanylyl cyclase subunits α1, β1 and β2, the *Manduca* soluble subunits MsGC-α1, MsGC-β1 and MsGC-β3, and the rat receptor guanylyl cyclase, GC-A. Light shading shows the residues from the α chain and dark shading those from the β chain. Note that the homodimeric guanylyl cyclases, rat β2, GC-A and MsGC-β3, possess all the residues from both α and β chains. *Residues mutated in MsGC-β3E469K and MsGC-β3R573Q.

| | | | | | | | | | |
|----------|-----|-------------|---------|-----|--------|------------|-----|--------------|-------------|
| Rat α1 | 481 | MLFSDIVGFT | TAICSQ | 520 | DVYKVE | TIGDAYCVAG | 588 | VKMPRYCLFGNN | VTLANKFESCS |
| MsGC-α1 | 483 | MLFSDIVGFTS | ICAT | 522 | DVYKVE | TIGDAYCVAS | 590 | KTMLKYCLFGHN | VTLANKFESGS |
| Rat GC-A | 873 | IYFSDIVGFT | ALSAE | 912 | DVYKVE | TIGDAYMVVS | 983 | LKMPRYCLFGDT | VNTASRMESNG |
| Rat β2 | 469 | ILFSDVVTFT | NICAA | 508 | DVYKVE | TIGDAYMVVG | 577 | DKMPRYCLFGDT | VNTASRMESHG |
| MsGC-β3 | 425 | ILFSDVVTFT | TEICSR | 464 | RVYKVE | TIGDAYMVVS | 533 | LKMPRYCLFGDS | VNTASRMESTS |
| MsGC-β1 | 422 | LLFSGIVGF | FANYCAR | 459 | NVYKVE | TVGDKYMAVS | 526 | HRMPRYCLFGNT | VNLTSCRETTG |
| Rat β1 | 431 | ILFSGIVGF | NAFCSK | 468 | FVYKVE | TVGDKYMTVS | 535 | QRMPRYCLFGNT | VNLTSCRETTG |

coexpressed with either MsGC- α 1 or MsGC- β 1 it is difficult to determine the contribution of the heterodimers to the total guanylyl cyclase activity because they are dominated by MsGC- β 3 homodimers. This is especially true if MsGC- β 3 has a lower affinity for MsGC- α 1 and MsGC- β 1 as was suggested by the data shown in Fig. 3A. A series of deletion mutants have been made in mammalian α 1 and β 1 subunits, which demonstrate that particularly critical residues are R592 in the α 1 subunit and E473 in the β 1 subunit (Beuve, 1999). α 1R592Q/ β 1 and α 1/ β 1E473K were both inactive (Beuve, 1999). If these predictions extend to MsGC- β 3, then single mutations at each site will yield inactive homodimers but active heterodimers when expressed with wild-type MsGC- β 3. To test this, we generated two mutants: MsGC- β 3E469K and MsGC- β 3R537Q, which have the equivalent mutations to β 1E473K and α 1R592Q respectively (Fig. 4).

Fig. 5 shows that these predictions are correct. COS-7 cells transfected only with MsGC- β 3E469K had no guanylyl cyclase activity, whereas cells cotransfected with wild-type MsGC- β 3 and MsGC- β 3E469K had a similar or greater level of activity than cells transfected only with wild-type MsGC- β 3 (Fig. 5A). In these experiments the total amount of plasmid was kept constant and densitometry of western blots showed that there was no significant difference in the total amount of MsGC- β 3-IR when MsGC- β 3E469K was cotransfected with MsGC- β 3 (Fig. 5C). The other point mutation gave slightly different results (Fig. 5). Firstly, and similarly, COS-7 cells expressing only MsGC- β 3R537Q had no guanylyl cyclase activity, demonstrating that R537 is critical for enzyme activity. The level of activity when MsGC- β 3R537Q and wild-type MsGC- β 3 were cotransfected was always less than cells that only expressed wild-type MsGC- β 3. Averaging three separate experiments the level of activity from cells expressing both MsGC- β 3R537Q and wild-type MsGC- β 3 was $78 \pm 12\%$ of the activity from cells expressing only wild-type MsGC- β 3. To demonstrate that the level of activity was proportional to the amount of plasmid transfected, we also transfected cells with 5 μ g of MsGC- β 3 plasmid and 5 μ g of empty pcDNA3.1 vector. In these experiments, the level of activity was approximately half ($44.3 \pm 2.9\%$) that seen when 10 μ g of MsGC- β 3 was used.

Because MsGC- β 3 can potentially act as both the α and β strands, we predicted that if MsGC- β 3 forms active heterodimers with either MsGC- α 1 or MsGC- β 1 then a single functional GTP-binding site should be formed between MsGC- β 3E469K (eliminating the β strand function) and MsGC- β 1, but not with MsGC- α 1. Similarly, a single GTP-binding site will be formed in heterodimers between MsGC- β 3R537Q (eliminating the α strand function) and MsGC- α 1 but not with MsGC- β 1. Because both mutants form inactive enzymes when expressed alone it follows that when either is coexpressed with either MsGC- α 1 or MsGC- β 1 (which also are inactive when expressed alone), any activity that is detected will come from the heterodimers. The results from these experiments are also shown in Fig. 5A. No activity is seen with any of these combinations, demonstrating that

neither mutant forms active heterodimers with either MsGC- α 1 or MsGC- β 1.

Two additional controls were performed to confirm that each mutant was capable of forming dimers. The first control tested that each mutant only affected one of the GTP binding sites. This was accomplished by cotransfecting each of the mutants together. Both mutants should each affect the same GTP binding site, leaving the other unaffected, and hence generate an active enzyme. The results of this experiment show that the MsGC- β 3E469K/MsGC- β 3R537Q heterodimer was active (Fig. 5B). Interestingly, when the two mutants were expressed together, there was substantially more enzyme activity than when an equivalent amount of wild-type MsGC- β 3 plasmid was transfected alone. The second control was to test that each mutant was capable of forming heterodimers with the NO-sensitive subunits. The lack of activity when the mutants were coexpressed with either MsGC- α 1 or MsGC- β 1 could have been because the mutated residues were critical for dimer formation. If inactive heterodimers were formed we predicted that each mutant would act like a dominant negative when coexpressed with both NO-sensitive subunits together. This experiment is shown in Fig. 5D and demonstrates that each mutant reduced both the basal and the NO-stimulated guanylyl cyclase activity, confirming that inactive heterodimers were formed.

Enzyme properties of MsGC- β 3 Δ C338 compared to MsGC- β 3

The results from the C-terminal domain deletion experiments demonstrated that this region of MsGC- β 3 was not necessary for enzyme activity (Table 1). Studies on the mammalian β 2 subunit, however, have shown that residues within the C-terminal domain affect the enzymatic properties of the β 2 subunit, rendering it active in the presence of magnesium ions, whereas the wild-type enzyme was only active in the presence of manganese ions (Koglin et al., 2001). To determine whether the C-terminal domain of MsGC- β 3 also affected the relative sensitivities of the enzyme to magnesium and manganese, we measured the activity of MsGC- β 3 and MsGC- β 3 Δ C338 in the presence of both of these cations and in the presence of different concentrations of GTP. These results are shown in Fig. 6. In the first report describing the properties of MsGC- β 3 (Nighorn et al., 1999), we used a radio-enzyme assay for guanylyl cyclase activity and failed to detect any activity for MsGC- β 3 in the presence of magnesium, whereas Fig. 6A shows that using a more sensitive EIA detection method we could clearly detect guanylyl cyclase activity in the presence of 4 mmol l⁻¹ MgCl₂. Interestingly, for a variety of GTP concentrations, we consistently measured higher levels of activity for MsGC- β 3 Δ C338 in the presence of magnesium, whereas no difference was detected in the presence of manganese. Using this data we calculated the values for K_m and V_{max} , shown in Table 3. In the presence of manganese the activities of MsGC- β 3 and MsGC- β 3 Δ C338 were indistinguishable and yielded similar values for K_m and V_{max} . By contrast, in the presence of magnesium, MsGC- β 3 Δ C338 yielded a significantly lower

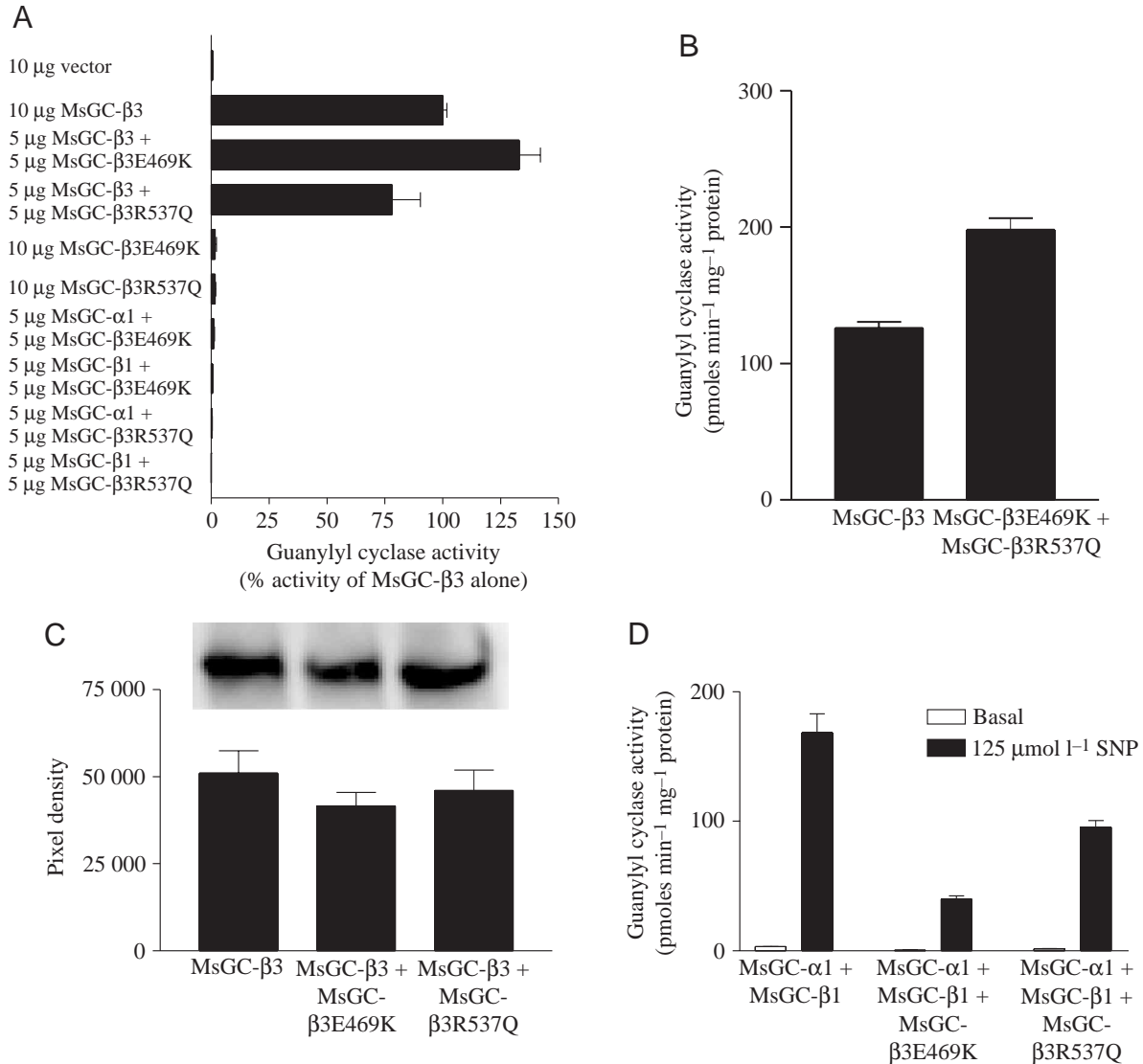


Fig. 5. Point mutations in MsGC-β3 demonstrate that MsGC-β3/MsGC-α1 and MsGC-β3/MsGC-β1 heterodimers are inactive. Two separate point mutations were generated that converted glutamate 469 to lysine (MsGC-β3E469K) and arginine 537 to glutamine (MsGC-β3R537Q). These two constructs were transfected into COS-7 cells as shown and cell extracts were assayed for guanylyl cyclase activity. (A) Each point mutation is inactive when transfected individually or in combination with either MsGC-α1 or MsGC-β1. When each is cotransfected with wild-type MsGC-β3, the level of guanylyl cyclase activity measured was similar or greater than the level of activity measured when wild-type MsGC-β3 was transfected alone. The results shown are the sum of six separate transfection experiments and because the absolute level of GC activity varied between experiments, all the data are expressed as % activity compared to the activity measured when wild-type MsGC-β3 was transfected alone. Analysis of variance (ANOVA) showed that the activity measured when either mutant was transfected alone or in combination with MsGC-α1 or MsGC-β1 was not significantly different ($P > 0.05$) from the activity measured in COS-7 cells transfected with vector alone. (B) Coexpression of the two mutants generates an active enzyme. MsGC-β3E469K and MsGC-β3R537Q were transiently coexpressed in COS-7 cells (5 μg of each plasmid) and the guanylyl cyclase activity measured and compared to COS-7 cells that had been transfected with 10 μg of wild-type MsGC-β3. Values are means \pm S.E.M. of three determinations. (C) Western blot of representative transfections showing that each mutant generates an equivalent level of MsGC-β3 immunoreactivity. COS-7 cells were transfected with 10 μg MsGC-β3, 5 μg MsGC-β3 + 5 μg MsGC-β3E469K or 5 μg MsGC-β3 + 5 μg MsGC-β3R537Q and cell extracts analyzed by western blot. Each sample was run on six separate lanes and the pixel density in each band quantified. A representative band is shown above each histogram. There was no significant difference (ANOVA; $P > 0.05$) between the pixel density of MsGC-β3 immunoreactivity for each transfection. (D) Each mutant acts as a dominant negative when coexpressed with the NO-sensitive guanylyl cyclase subunits. COS-7 cells were transiently transfected with the plasmids shown and assayed for guanylyl cyclase activity in the presence of 4 mmol l⁻¹ MgCl₂ \pm 125 μmol l⁻¹ sodium nitroprusside (SNP). The activity levels were normalized for transfection efficiency and show that both mutants reduce the levels of basal and NO-stimulated guanylyl cyclase activity. Both the basal and NO-stimulated activity was significantly lower (ANOVA; $P < 0.01$) when the mutants were cotransfected than in their absence. Values are means \pm S.E.M. of three determinations.

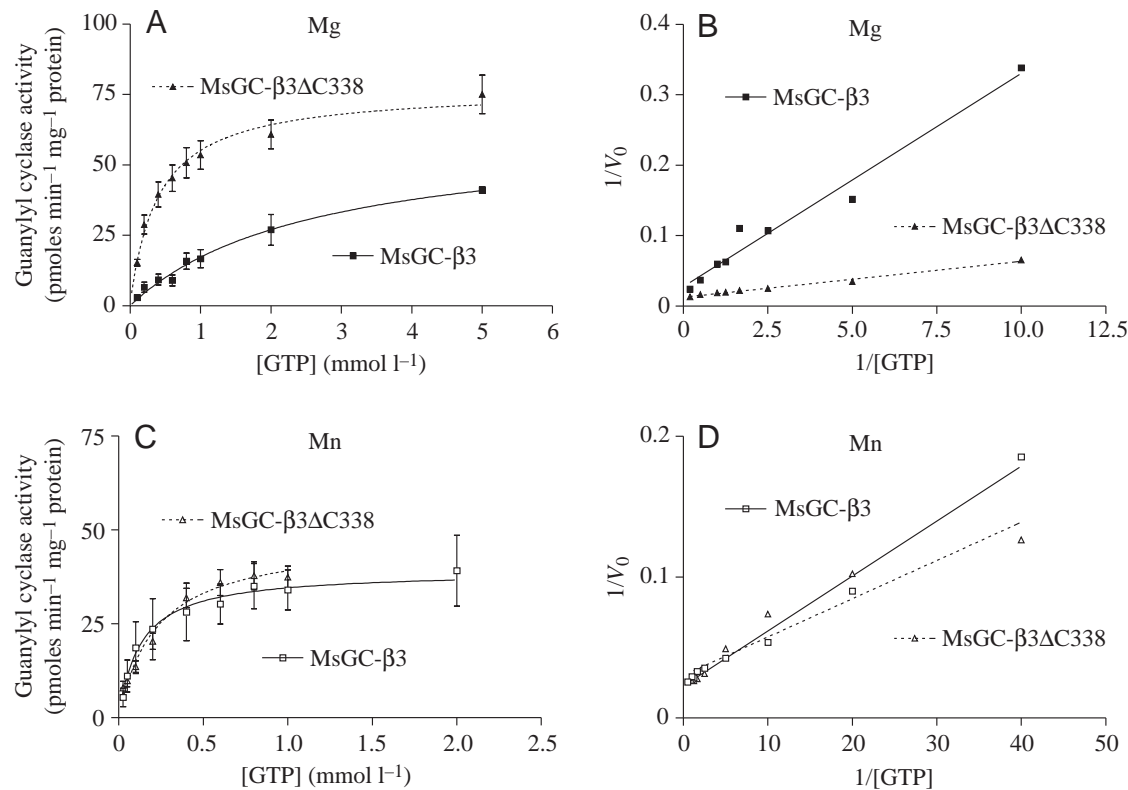


Fig. 6. Enzymatic properties of MsGC-β3 compared to MsGC-β3ΔC338. COS-7 cells were transiently transfected with the plasmids indicated and assayed for guanylyl cyclase activity with GTP concentrations of 0.1 mmol l⁻¹ to 5 mmol l⁻¹ in the presence of 4 mmol l⁻¹ MgCl₂ (A,B) or 4 mmol l⁻¹ MnCl₂ (C,D). For each concentration the reaction was allowed to proceed for 5, 15 and 30 min and linear regression of these values used to calculate a rate of reaction. The values were also plotted as double reciprocal plots (B,D), which demonstrate that both MsGC-β3 and MsGC-β3ΔC338 yield Hill coefficients of 1.0. Values for the V_{\max} and K_m were calculated from the untransformed data using the following equation: $\text{Rate} = (V_{\max} \times [\text{GTP}] / (K_m + [\text{GTP}]))$ using GraphPad Prism 3.0 and are shown in Table 3.

Table 3. Kinetic parameters of MsGC-β3 and MsGC-β3ΔC338

| | V_{\max} (pmoles min ⁻¹ mg ⁻¹ protein) | | K_m (mmol l ⁻¹) | | Hill coefficient | |
|--------------|---|-------------------|-------------------------------|-------------------|-------------------|-------------------|
| | MgCl ₂ | MnCl ₂ | MgCl ₂ | MnCl ₂ | MgCl ₂ | MnCl ₂ |
| MsGC-β3 | 62.3±11.2 | 39.0±5.0 | 2.62±0.81 | 0.13±0.07 | 0.90±0.06 | 1.01±0.06 |
| MsGC-β3ΔC338 | 77.0±4.7 | 47.9±3.7 | 0.40±0.08 | 0.22±0.05 | 1.16±0.13 | 0.89±0.06 |
| ANOVA | NS | NS | $P < 0.001$ | NS | NS | NS |
| MsGC-β3ΔC29 | 26.8±0.9 | ND | 0.27±0.03 | ND | 1.03±0.07 | ND |

COS-7 cells were transiently transfected with MsGC-β3, MsGC-β3ΔC338 or MsGC-β3ΔC29 as described in Materials and methods. Cell extracts were assayed for guanylyl cyclase activity in the presence of different concentrations of GTP and 4 mmol l⁻¹ MgCl₂ or 4 mmol l⁻¹ MnCl₂. For each GTP concentration, samples were incubated for 5, 15 and 30 min to ensure linearity of the rate of cGMP formation.

Values for V_{\max} and K_m for MsGC-β3 and MsGC-β3ΔC338 were calculated from the data shown in Fig. 6A,B. The Hill coefficient was calculated as the slope of the line produced by plotting $\log[\text{rate}/(V_{\max} - \text{rate})]$ versus $\log[\text{GTP}]$. A similar set of experiments was carried out using MsGC-β3ΔC29 but the data is not included in the graphs in Fig. 6. NS, not significant; ND, not determined.

K_m although the calculated value for V_{\max} was statistically indistinguishable. The linearity of double-reciprocal plots (Fig. 6B,D) and calculations of the Hill coefficients (Table 3) demonstrated that neither enzyme showed cooperativity with respect to GTP. In a separate experiment we also analyzed the

influence of GTP concentration on the activity of MsGC-β3ΔC29 in the presence of magnesium. The resulting values of K_m and V_{\max} are also shown in Table 3 and showed that deletion of the C-terminal 29 residues was sufficient to reduce the K_m for GTP.

Discussion

When MsGC- β 3 was first cloned and partially characterized, it exhibited several properties that were unique among soluble guanylyl cyclases (Nighorn et al., 1999). When MsGC- β 3 was expressed in COS-7 cells it was active in the absence of additional subunits and was insensitive to NO, whereas all previously described soluble guanylyl cyclases were heterodimers that were stimulated by NO (Morton and Hudson, 2002). The primary sequence of MsGC- β 3 revealed a potential structural basis for these two properties. The lack of two critical cysteine residues in the regulatory domain provided a rationale for its lack of NO sensitivity (Nighorn et al., 1999). The catalytic domain of both soluble and receptor guanylyl cyclases has been modeled based on the crystal structure of adenylyl cyclases and has been used to predict which residues bind the GTP substrate (Liu et al., 1997). This analysis showed that soluble, heterodimeric guanylyl cyclases contain a single GTP binding site and homodimeric receptor guanylyl cyclases contain two (Liu et al., 1997). By comparing the residues predicted to bind to GTP in MsGC- β 3 we predicted that it should form active homodimers with two GTP binding sites (Morton and Hudson, 2002; Fig. 4). The results described in the present study confirm that MsGC- β 3 does form active homodimers. Firstly, in the presence of detergent both recombinant and native MsGC- β 3 run on a gel filtration column as homodimers. Although both recombinant and native MsGC- β 3 also appear to form large aggregates in the presence of detergents, it is not known whether this is due to the presence of detergents or whether MsGC- β 3 also forms aggregates *in vivo*. Secondly, the use of point mutations confirms that dimers are necessary for enzyme activity. These point mutations, MsGC- β 3E469K and MsGC- β 3R537Q, were inactive when expressed alone, but when coexpressed formed an active enzyme. The simplest explanation of this data is that each point mutation targets the same single GTP binding site. When expressed on their own, homodimers are formed with both GTP-binding sites disrupted, but when coexpressed, heterodimers are formed that contain one disrupted and one intact GTP binding site, enabling the enzyme to form cGMP.

MsGC- β 3 was the first soluble guanylyl cyclase identified that was active in the absence of additional subunits. A recent report has shown that the rat β 2 subunit is similarly active in the absence of additional subunits (Koglin et al., 2001). Analysis of the residues predicted to form the active site show that, like MsGC- β 3 and receptor guanylyl cyclases, mammalian β 2 subunits have all the residues necessary to form the catalytic site without the need for additional subunits (Fig. 4) and are all predicted to have two GTP binding sites. Initial reports on the activity of mammalian β 2 subunits suggested that they only formed active heterodimers with mammalian α subunits (Gupta et al., 1997), although subsequent studies failed to reproduce this data (e.g. Denninger and Marletta, 1999). A more recent study (Koglin et al., 2001), in combination with data from MsGC- β 3, appear to place both guanylyl cyclases in a new group of homodimeric soluble guanylyl cyclases (Morton and Hudson, 2002). A major

difference, however, between MsGC- β 3 and the mammalian β 2 subunits is that the insect guanylyl cyclase is NO-insensitive whereas rat β 2 is weakly stimulated by NO (Koglin et al., 2001).

Another similarity between the *Manduca* and rat homodimeric guanylyl cyclases is their low level of activity in the presence of magnesium compared to manganese. Although our initial report describing MsGC- β 3 failed to detect any activity in the presence of magnesium, the present study, using a more sensitive assay system, clearly showed that the enzyme is active in the presence of magnesium. Furthermore, kinetic analysis showed that the K_m for GTP in the presence of magnesium is 20-fold higher than in the presence of manganese. Wild-type rat β 2 subunits showed no detectable enzyme activity in the presence of magnesium. Mutating a single cysteine residue in the C-terminal isoprenylation sequence (CVVL), however, yielded an enzyme that did show significant activity in the presence of magnesium (Koglin et al., 2001). Interestingly, our data also revealed that alterations in the C terminus affected the K_m in the presence of magnesium. Removal of either the C-terminal 338 or 29 residues significantly reduced the calculated value of the K_m . MsGC- β 3 also terminates in a consensus isoprenylation sequence (CRLI), although our data showed that the native protein was found in the soluble fraction of nerve cord extracts, suggesting that it is not modified *in vivo*. It would be interesting to determine whether mutating the equivalent cysteine residue also affects the K_m of MsGC- β 3. It is also interesting to note the similarities in the values of K_m for the insect and rat enzymes. In the presence of 4 mmol l⁻¹ manganese, rat β 2 has a K_m of 0.375 mmol l⁻¹ when unstimulated and 0.136 mmol l⁻¹ when stimulated with NO (Koglin et al., 2001), which is very similar to the value we obtain for MsGC- β 3 of 0.13 mmol l⁻¹.

The finding that removal of the C-terminal domain of MsGC- β 3 reduced the K_m for GTP in the presence of magnesium but not in the presence of manganese suggests a role for this novel domain. Receptor guanylyl cyclases and NO-sensitive guanylyl cyclases show basal levels of activity in the presence of magnesium that are fully sensitive to their respective activators (peptide ligands, GCAPs or NO) (Lucas et al., 2000). In the presence of manganese, however, these guanylyl cyclases all exhibit maximal levels of catalytic activity that are insensitive to further stimulation (Lucas et al., 2000). Full-length MsGC- β 3 has a higher K_m in the presence of magnesium compared to manganese, whereas the value of K_m of MsGC- β 3 Δ C338 is similar in the presence of either cation. This suggests that the C-terminal domain acts as an auto-inhibitory domain in the presence of magnesium, but in the presence of manganese it has no effect. It is not known how MsGC- β 3 is activated *in vivo*, but an intriguing possibility is that removal of the C-terminal domain mimics this activation process, yielding a fully active guanylyl cyclase that is also produced when MsGC- β 3 functions in the presence of manganese.

Both MsGC- β 3 and rat β 2 also appear to interact with the NO-sensitive heterodimeric subunits. Coexpression of all three

rat subunits ($\alpha 1$, $\beta 1$ and $\beta 2$) showed that with increasing amounts of the $\beta 2$ subunit the NO stimulation of the resulting mixture was reduced (Gupta et al., 1997). This suggested that heterodimers between the $\beta 2$ and either $\alpha 1$ or $\beta 1$ were formed and that these were less sensitive to NO than the $\alpha 1/\beta 1$ heterodimers. MsGC- $\beta 3$ behaved in the same manner: increasing the amount of MsGC- $\beta 3$ coexpressed with MsGC- $\alpha 1$ and MsGC- $\beta 1$ reduced the NO stimulation (Table 2). By using tagged subunits we were also able to directly demonstrate that MsGC- $\beta 3$ formed heterodimers with both MsGC- $\alpha 1$ and MsGC- $\beta 1$.

In addition, coexpression of MsGC- $\alpha 1$ and MsGC- $\beta 1$ with each of the two point mutations of MsGC- $\beta 3$ demonstrated that the heterodimers formed between MsGC- $\beta 3$ and MsGC- $\alpha 1$ and MsGC- $\beta 1$ were inactive. The design of this experiment assumed that MsGC- $\beta 3$ formed two GTP-binding sites homologous to the two sites predicted for vertebrate homodimeric receptor guanylyl cyclases (Liu et al., 1997). The generation of an active enzyme when the two mutants were coexpressed appears to confirm that there are two GTP-binding sites. Neither of the mutants formed an active enzyme when coexpressed with either MsGC- $\alpha 1$ or MsGC- $\beta 1$. The conclusion that heterodimers between MsGC- $\beta 3$ and MsGC- $\alpha 1$ or MsGC- $\beta 1$ are inactive depends on demonstrating that each mutant is capable of forming an active heterodimer with wild-type MsGC- $\beta 3$. The MsGC- $\beta 3$ E469K mutant was clearly active as a heterodimer with wild-type MsGC- $\beta 3$, as the level of activity when both plasmids were cotransfected was similar or greater than the level of activity when wild-type MsGC- $\beta 3$ was transfected alone, even though only half the amount of wild-type MsGC- $\beta 3$ plasmid was used. By contrast, coexpression of MsGC- $\beta 3$ R537Q with wild-type MsGC- $\beta 3$ always yielded lower levels of activity (78%) compared to the activity measured when wild-type MsGC- $\beta 3$ was expressed alone. A 1:1 ratio of wild-type:mutant should yield a mixture of wild-type homodimers, heterodimers and mutant homodimers in a ratio of 1:2:1. If the heterodimers have half the specific activity of wild-type homodimers (because they have a single active site compared to two), the total level of guanylyl cyclase activity would be half that measured when wild-type MsGC- $\beta 3$ was expressed alone, if the total amount of plasmid was equal. By contrast, if the heterodimers were inactive, then the activity of the mixture should be 25% of the activity when only wild-type MsGC- $\beta 3$ is expressed. Thus, the MsGC- $\beta 3$ R537Q mutant also appears to form active heterodimers with wild-type MsGC- $\beta 3$.

An alternative model is that although there are two potential GTP-binding sites only one can be filled at a time – possibly because they are too close to each other to allow the simultaneous binding of two GTP molecules. If this were the case, then mutating one of the binding sites would have no effect on the specific activity of the heterodimer (or only a minimal effect), as it would still bind a single GTP molecule. A 1:1 mixture of mutant:wild-type MsGC- $\beta 3$ would then be about 75% as active as wild-type MsGC- $\beta 3$ alone. This is

closer to the situation seen with the two point mutations. Our kinetic analysis of MsGC- $\beta 3$ reveals a Hill coefficient of 1.0, i.e. that there is no cooperativity with respect to GTP, which is consistent with a single GTP molecule binding per dimer. The rat $\beta 2$ subunit also shows linear Michaelis–Menten kinetics in the presence of manganese (Koglin et al., 2001). These findings contrast the situation with the homodimeric receptor GC, GC-A, which shows positive cooperativity with respect to GTP in the presence of manganese (Wong et al., 1995). Thus, although the data strongly support our hypothesis that both mutant/wild-type MsGC- $\beta 3$ heterodimers are active, our results suggest a possible different model for the catalytic site of this homodimeric guanylyl cyclase. Interestingly, when the two mutant MsGC- $\beta 3$ subunits were expressed together, substantially higher levels of guanylyl cyclase activity were measured compared to an equivalent amount of wild-type MsGC- $\beta 3$. This suggests that eliminating one of the GTP binding sites removes a constraint of GTP binding, yielding a more active enzyme. Although these equivalent mutations have been made in the homodimeric receptor guanylyl cyclase, RetGC-1 (Tucker et al., 1998), each mutant was only expressed individually (each was inactive). It would be interesting to determine the total level of guanylyl cyclase activity if these mutations were coexpressed together or with wild-type RetGC-1, and compare the results with those we have obtained for MsGC- $\beta 3$.

Overall, our data demonstrate that although MsGC- $\beta 3$ can form heterodimers with both MsGC- $\alpha 1$ and MsGC- $\beta 1$, these heterodimers are catalytically inactive. These results are somewhat surprising, as both heterodimers should have all the residues necessary to form at least one GTP-binding site. This suggests that although heterodimers are formed, they do not fold together correctly to form an active catalytic site. A complementary series of studies showed that human $\alpha 1$ and $\beta 1$ subunits were each capable of forming homodimers and that they were inactive (Zabel et al., 1999). When the $\alpha 1$ and $\beta 1$ subunits were coexpressed, homodimers were still formed, but not as readily as heterodimers (Zabel et al., 1999). We do not know the relative affinities of MsGC- $\beta 3$ for the formation of homodimers compared to heterodimers with MsGC- $\alpha 1$ or MsGC- $\beta 1$. Thus these studies seem to suggest a general property of all soluble guanylyl cyclase subunits; they can dimerize with any other subunit but are only active as dimers with their appropriate subunit. Specificity could be generated either by different relative affinities or by cell-specific expression patterns. It is not known whether MsGC- $\beta 3$ is ever coexpressed with either of the NO-sensitive subunits, but it has been suggested that in the kidney, mammalian $\alpha 1$, $\beta 1$ and $\beta 2$ are coexpressed and their relative levels can contribute to alterations in renal NO sensitivity (Gupta et al., 1997).

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