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Invertebrates Yield a Plethora of Atypical Guanylyl Cyclases

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Abstract

Invertebrate model systems have a long history of generating new insights into neuronal signaling systems. This review focuses on cyclic GMP signaling and describes recent advances in understanding the properties and functions of guanylyl cyclases in invertebrates. The sequencing of three invertebrate genomes has provided a complete catalog of the guanylyl cyclases in *C. elegans*, *Drosophila*, and the mosquito *Anopheles gambiae*. Using this data and that from cloned guanylyl cyclases in *Manduca sexta*, *C. elegans*, and *Drosophila*, plus predictions and models from vertebrate guanylyl cyclases, evidence is presented that there is a much broader array of properties for these enzymes than previously realized. In addition to the classic homodimeric receptor guanylyl cyclases, *C. elegans* has at least two receptor guanylyl cyclases that are predicted to require heterodimer formation for activity. Soluble guanylyl cyclases are generally recognized as being obligate heterodimers that are activated by nitric oxide (NO). Some of the soluble guanylyl cyclases in *C. elegans* may heterodimeric, but all appear to be insensitive to NO. The β2 soluble guanylyl cyclase subunit in mammals and similar ones in *Manduca* and *Drosophila* are active in the absence of additional subunits and there is evidence that *Drosophila* and *Anopheles* also express an additional subunit that enhances this activity.

Index Entries: Cyclic GMP; guanylyl cyclase; nitric oxide; signal transduction.

Introduction

The intracellular messenger, guanosine 3′, 5′ cyclic monophosphate (cGMP) plays a central role in a wide range of physiological responses. It mediates mammalian photoreceptors' response to light, triggers smooth muscle relaxation in response to a variety of hormonal signals, and many lines of evidence implicate cGMP as an important component in forms of synaptic plasticity such as long-term potentiation and long-term depression *(1)*. Studies using invertebrates have also shown that cGMP is an important signal in the nervous system. It is known to play a significant role in ecdysis and foraging behaviors in insects *(2,3)*, olfactory detection and processing in the nematode, *C. elegans (4)* and neuronal path finding and differentiation in insects and mollusks *(5–7)*.

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A balance of two processes regulates the levels of cGMP in a cell: the synthesis of cGMP from GTP by guanylyl cyclases and the breakdown of cGMP by phosphodiesterases. Classically, guanylyl cyclases have been divided into two groups: receptor and soluble guanylyl cyclases *(1).* Receptor guanylyl cyclases are integral membrane proteins that act as homo-oligomers. They can be activated either by extracellular hormones such as the natriuretic peptides or by intracellular calcium-binding proteins, the neuronal calcium sensors (NCSs), such as the guanylyl cyclase activating proteins (GCAPS) *(1).* The soluble guanylyl cyclases, as their name suggests are generally cytoplasmic proteins. The best-studied soluble guanylyl cyclases are obligate heterodimers, consisting of an α and a β subunit that are primarily activated by the gaseous messenger, nitric oxide (NO). Examples of both receptor and NO-sensitive soluble guanylyl cyclases have been described in invertebrates.

Properties and Functions of Conventional Guanylyl Cyclases in Invertebrates

Receptor Guanylyl Cyclases

Although one of the first receptor guanylyl cyclases identified was in sea urchin sperm *(8),* less is known about invertebrate receptor guanylyl cyclases in nervous tissue compared to the NO-stimulated guanylyl cyclases. Two receptor guanylyl cyclases have been cloned from the fruit fly, *Drosophila melanogaster,* and their distribution mapped *(9–11).* One of these, named Gyc76C, is extensively expressed in the nervous system and muscle fibers *(11)* although no reports of its biochemical properties have been published. A receptor guanylyl cyclase has also been cloned from the silk moth, *Bombyx mori* that is widely expressed in many different tissues including the nervous system *(12).* Again, no biochemical characterization was made, but the interesting observation was reported that the expression of this cyclase, named BmGC-I, was regulated in flight muscle in a circadian fashion *(13).* Short PCR fragments of several predicted receptor guanylyl cyclases have also been cloned from nervous tissue of the hawkmoth, *Manduca sexta* and one of these has also been isolated as a full-length receptor guanylyl cyclase *(14).* This cyclase, named MsGC-II, is primarily expressed in the nervous system and is most similar to vertebrate retinal guanylyl cyclases, which are activated by NCSs in the presence of low levels of calcium *(1).* Transient expression of MsGC-II in COS-7 cells generated guanylyl cyclase activity, which was inhibited by high levels of calcium in the absence of exogenous calcium-binding proteins *(14).* Interestingly, addition of exogenous NCSs, either mammalian GCAP2 or frequenin, a *Drosophila* NCS, in the presence of low calcium further enhanced the activity of MsGC-II suggesting that it is activated in a similar manner to the vertebrate retinal guanylyl cyclases *(14).*

The availability of the complete genome sequence of both *Drosophila* and the mosquito, *Anopheles gambiae,* makes it possible to predict all of the likely receptor guanylyl cyclases present in these insects. In *Drosophila* there are a total of six predicted receptor guanylyl cyclases *(15)* and *Anopheles* is predicted to have an ortholog to each of them (D. B. Morton, unpublished data). Table 1 gives these predicted genes and also the orthologs in other arthropods where they are available. An important prediction is whether a given receptor guanylyl cyclase is activated by an extracellular ligand or by an intracellular NCS. A phylogenetic analysis of the *Drosophila* and vertebrate receptor guanylyl cyclases failed to make this prediction, probably because there is little sequence similarity in the extracellular domains *(15).* By contrast there is fairly good sequence similarity in the extracellular domains between the *Drosophila* and *Anopheles* orthologs and between the additional arthropod orthologs where they are available (D. B. Morton, unpublished data). The recent recognition of a family of ligand-binding domains *(16)* related to the atrial natriuretic peptide (ANP) receptor (GC-A, a ligand-activated

Table 1
Arthropod Receptor Guanylyl Cyclases

Drosophila	*Anopheles*	Other Arthropod Orthologs	Predicted ANP Binding Domain?	Closest Vertebrate Ortholog
Gyc76C	ebiP9147	*Procambarus clarkii*—PcGC-M[a] *Callinectes sapidus*—fragment[b] *Manduca*—fragment[c]	Yes	GC-A
Gyc32E	ebiP6391	*Manduca*—fragment[d]	Yes	GC-B
CG4224/ CG31183	agCP14275	*Bombyx mori*—BmGC-I *Manduca*—fragment[d]	Yes	GC-A
CG3216	agCP15265		Yes	GC-A
CG10738	ebiP9149		Yes	GC-B
CG9783	agCP14674	*Manduca*—MsGC-II	No	GC-F

The table shows the relationships between the known and predicted receptor guanylyl cyclases in arthropods. The *Drosophila* cyclases, Gyc76C and Gyc32E were identified from cDNAs *(10,11),* whereas the other *Drosophila* and *Anopheles* orthologs are predicted from the genome sequences (*15;* D. B. Morton, unpublished data). Orthologs from other arthropods are either published full-length sequences *(12,14)* or unpublished full-length sequences or fragments isolated using RT-PCR ([a] full-length sequence for a receptor GC isolated from muscle of the crayfish *Procambarus clarkii,* Liu H. -F. and Lee C. -Y., unpublished data. [b] PCR fragment from the Y organ of the blue crab, *Callinectes sapidus,* Zheng J., Liu H. -F., Lee C. -Y., and Watson R. D., unpublished data. [c] Two PCR fragments with similarities to two different regions of Gyc76C, Hazelett D. and Weeks J. C., Nighorn A., and Morton D. B. unpublished data. [d] Nighorn A. and Morton D. B., unpublished data). The presence or absence of an atrial natriuretic peptide (ANP)-binding domain and the identification of the closest vertebrate ortholog was determined by subjecting each predicted amino acid sequence to BLASTP analysis.

receptor guanylyl cyclase) enables better predictions to be made. All of the arthropod receptor guanylyl cyclases except MsGC-II and its orthologs have a predicted ANP receptor domain in the extracellular region (*see* Table 1). This is consistent with the finding from a BLASTP analysis that shows that the closest vertebrate receptor guanylyl cyclase to these arthropod cyclases are GC-A or GC-B, both ligand-activated receptor guanylyl cyclases whereas MsGC-II is closest to the retinal guanylyl cyclases (Table 1). This suggests that MsGC-II and its orthologs are likely to be regulated by intracellular calcium levels and the other arthropod receptor guanylyl cyclases are orphan receptors with unknown extracellular ligands. Insects do not appear to have peptides that are similar to ANP, but predictions have been made for other possible ligands for these orphan receptor guanylyl cyclases *(15).*

The best evidence in invertebrate nervous tissue for a specific ligand that activates a receptor guanylyl cyclase is found in crustaceans. A peptide hormone, crustacean hypoglycemic hormone (CHH), activates an integral membrane guanylyl cyclase in lobster muscle *(17).* Neurohemal gland extracts that contain a similar hormone in crabs elevated the cGMP levels in a specific subset of neurons in the crab stomatogastric nervous system *(18).* A separate set of neurons showed an increase in cGMP in response to NO, strongly suggesting that the peptide-containing extract acts on a receptor guanylyl cyclase in the first set of neurons. The recent report of a full-length receptor guanylyl cyclase from crayfish muscle and the fragment of a related receptor guanylyl cyclase from the blue crab Y organ (*see* Table 1) is particularly intriguing as they are most closely related to *Drosophila* Gyc76C, which is also expressed in

muscle *(11).* A fragment of a *Manduca* ortholog has also been isolated from muscle (Hazelett, D. and Weeks J. C., personal communication). As insects also have CHH-like peptides *(15),* it will be exciting to determine if the Gyc76C receptor guanylyl cyclase and its orthologs are all activated by similar peptides. The genome of *C. elegans* contains at least 32 different predicted receptor and soluble guanylyl cyclases *(19),* but as many of these appear unusual, they will be discussed in more detail below.

NO-Sensitive Guanylyl Cyclases

Both the α and the β subunits of the NO-sensitive soluble guanylyl cyclase have been cloned from *Drosophila* and *Manduca (9,20,21).* Their sequences are similar to their mammalian orthologs, although the *Drosophila* β subunit contains an insert of 118 amino acids in its regulatory region that is not seen in any other β subunit *(20).* When the subunits are transiently expressed in heterologous cells both display similar properties to the mammalian enzymes. They are obligate heterodimers, requiring co-expression of both subunits to form an active enzyme, they show higher levels of basal activity in the presence of manganese compared to magnesium and the activity is stimulated many-fold by the addition of NO donors *(20,21).* The β subunit from *Anopheles* has also been cloned *(22)* and the recent completion of the *Anopheles* genome project shows that an α subunit is also present *(23),* but no expression studies have been carried out to determine whether these subunits have similar properties to those in other species. All of the amino acid residues known to influence the biochemical properties of the NO-sensitive soluble guanylyl cyclase are conserved in the insect subunits *(15).*

The distribution of the NO-sensitive soluble guanylyl cyclases in the nervous system has been mapped in several invertebrate species using cGMP immunohistochemistry after the tissues have been stimulated with NO donors. These species include *Manduca (24,25), Drosophila (26),* the lobster *Homarus americanus (27),* and the snail *Helix pomatia (28).* The investigations all reveal that a distinct subset of both central and peripheral neurons respond to NO with an increase in cGMP in all species studied. ODQ is a specific inhibitor of NO-sensitive soluble guanylyl cyclase, which has been demonstrated to inhibit the NO-stimulated activity of the *Manduca* α and β subunits *(29).* In some mapping studies, ODQ has been used to block the NO-stimulated increase in cGMP immunoreactivity suggesting that this method does provide a reliable indication of the cells that express the α and β subunits of the NO-sensitive soluble guanylyl cyclase. The identity and function of some of these neurons has enabled predictions to be made for the function of the NO-stimulated cGMP increase, which suggests roles in feeding behavior and olfactory processing. A number of studies have also shown that the ability of NO donors to stimulate cGMP increases is transient during development and pharmacological experiments have revealed a role for NO-stimulated cGMP in neuronal outgrowth and synaptic differentiation *(5,30–33).* The functions and distribution of NO synthase (NOS) and NO-sensitive guanylyl cyclase in invertebrates have been reviewed elsewhere *(34,35)* and will not be discussed further here.

Atypical Guanylyl Cyclases in Invertebrates

Atypical Receptor and Receptor-Like Guanylyl Cyclases

The Possible Formation of Heterodimeric Receptor Guanylyl Cyclases in C. elegans

The completion of the *C. elegans* genome sequencing project revealed an astonishing number of guanylyl cyclases *(36).* Compared to the seven identified in vertebrates, there are 25 different receptor guanylyl cyclases in *C. elegans* and seven soluble guanylyl cyclase subunits compared to the four identified in vertebrates *(19).* The expression patterns of many of the receptor guanylyl cyclases have been mapped

using green fluorescent protein (GFP) coupled to the predicted promoter of each cyclase and many found to be expressed in a small number of chemosensory neurons *(37).* Mutant phenotypes have been described for two of the receptor guanylyl cyclases, ODR-1 and DAF-11, both of which affect chemosensory signaling *(4,19).* ODR-1 is expressed in the AWC chemosensory neurons and mutants of the *odr-1* gene are defective in chemotaxis to all odorants that are sensed by the AWC neurons *(4).* Chemotaxis was restored by over-expression of both full-length ODR-1 and ODR-1 that lacked the extracellular domain, whereas a point mutation equivalent to one that eliminated catalytic activity in mammalian receptor guanylyl cyclases failed to restore chemotaxis *(4).* These results suggest that cGMP generation is necessary for chemotaxis and ODR-1 is unlikely to function as the odorant receptor, but probably downstream of the receptor. *C. elegans* will adapt to high levels of individual odors by down regulation of chemotaxis to that specific odor, while retaining sensitivity to other odors *(38).* Interestingly, *odr-1* mutants that are rescued by over expression of ODR-1 lost the ability to adapt to butanone but retained the ability to adapt to benzaldehyde and isoamyl alcohol *(4).* Importantly, chemotaxis to all three odors was abolished in the *odr-1* mutants and was restored by ODR-1 over-expression demonstrating that ODR-1 is a common component in the signaling cascade of all three odors but is only a specific component of the adaptation cascade to one of the odors *(4).* Still unknown, however, is how ODR-1 is activated and how this activation differs between the different odors.

DAF-11 also codes for a receptor guanylyl cyclase in *C. elegans* and is also expressed in chemosensory neurons *(19).* DAF-11 was first identified as mutations in the *daf-11* gene that are defective in regulated dauer formation, an alternative developmental stage *(39).* Wild-type animals will only form dauers in response to certain environmental cues that include chemosensory and thermosensory cues, whereas *daf-11* mutants constitutively form dauers, even in the absence of these cues *(39).* Addition of exogenous 8-bromo-cGMP rescued this mutant phenotype, preventing dauer formation in the absence of these sensory cues, suggesting that the guanylyl cyclase catalytic activity of DAF-11 was required for normal dauer formation *(19).* DAF-11 is expressed in several different chemosensory neurons, including the AWC neurons, which also express ODR-1 *(19).* Somewhat surprisingly, *daf-11* mutants are also defective in chemotaxis to some of the same odorants that *odr-1* mutants fail to respond to *(19).* This suggests that they both act in the same pathway. One explanation for this is that together they form a heterodimeric guanylyl cyclase, which would be unusual as all other receptor guanylyl cyclases are thought to be homo-oligomers *(1).* Another possible explanation would be that both cyclases act as homodimers, but they are both required to be present to generate sufficient cGMP to enable odorant detection.

Analysis of the catalytic domains of ODR-1 and DAF-11 provides circumstantial evidence that the first of these possibilities may be correct: that ODR-1 and DAF-11 form active heterodimers but inactive homodimers. Based on the crystal structure of adenylyl cyclase, a model for the catalytic site of guanylyl cyclases has been proposed that predicts two subunits arranged in an anti-parallel manner form the catalytic domain *(40).* There are 17 residues predicted to interact with the Mg-GTP substrate that are arranged along the interface between the two subunits with each subunit contributing a subset of residues to the catalytic site. Receptor guanylyl cyclases, such as the retinal guanylyl cyclase RetGC-1, form homodimers, and are predicted to form two catalytic centers whereas heterodimeric cyclases such as the α and β subunits of the NO-sensitive soluble guanylyl cyclase form a single catalytic site with each subunit contributing complementary residues *(40).* A multiple sequence alignment of ODR-1, DAF-11 and RetGC-1 reveals that neither ODR-1 nor DAF-11 have all of the necessary residues, but together these two cyclases can form a single active catalytic site (*see* Fig. 1 and Table 2). The residues that are substituted in one or other of

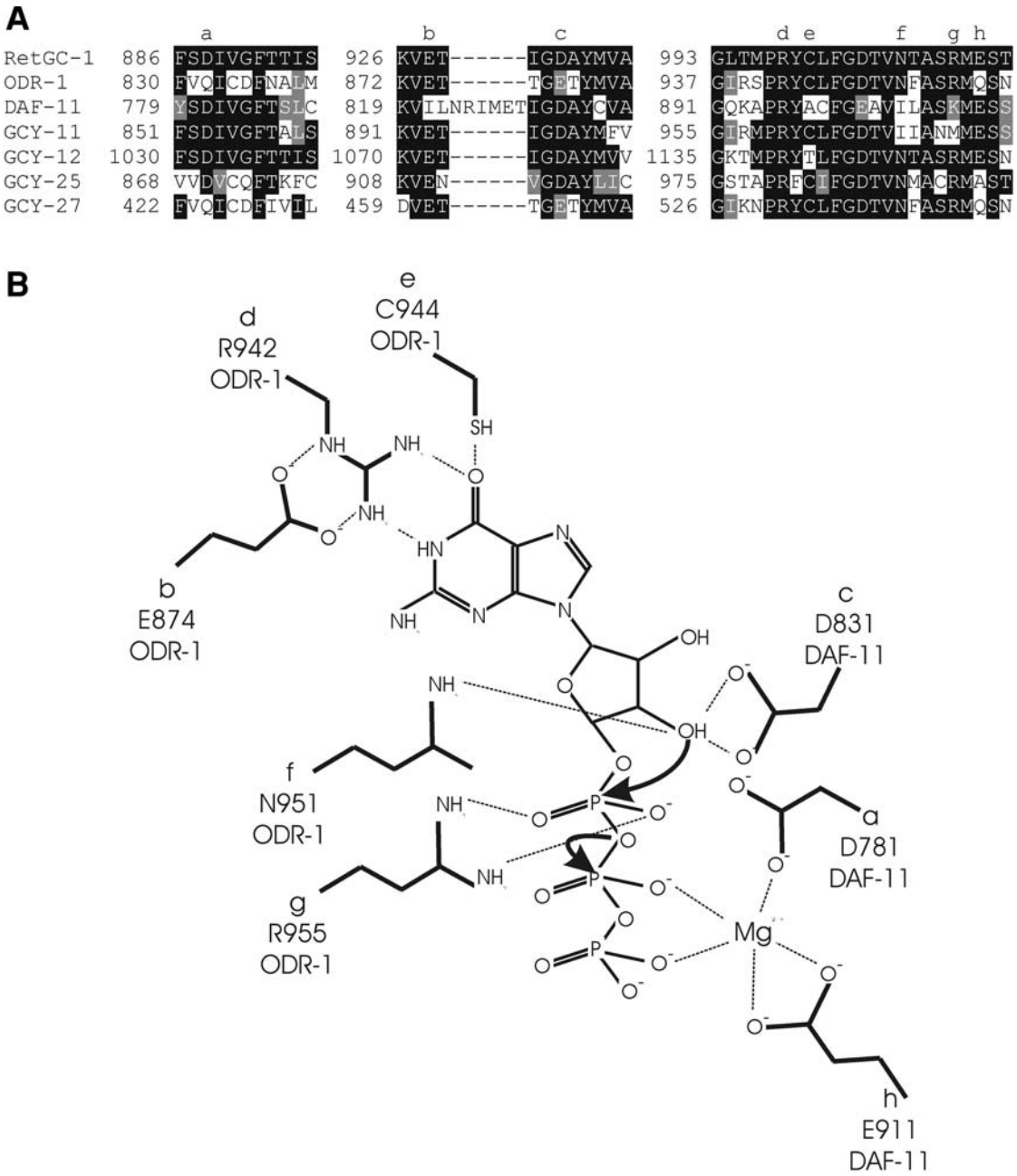

Fig. 1. **(A)** Multiple sequence alignment of portions of selected receptor guanylyl cyclases from *C. elegans* compared to the human retinal guanylyl cyclase RetGC-1. The residues marked with lower case letters are shown in **B** and are known to be critical for enzyme activity. **(B)** Model of the GTP-binding site in the proposed ODR-1/DAF-11 heterodimeric receptor guanylyl cyclase. Specificity for binding the guanine group is provided by E874, R942, and C944 of ODR-1. Residues equivalent to E874 and C944 are substituted in DAF-11. N951 and R955 in ODR-1 and D831 in DAF-11 catalyze the condensation reaction of the 3′ hydroxyl of the ribose to the α phosphate group. N951 is substituted in DAF-11. D781 and E911 in DAF-11 are required for stabilizing the β,γ bisphosphate moiety and the Mg^{2+} ion and are substituted in ODR-1. E911, C944, and N951 are also substituted in GCY-25, GCY-12, and GCY-11 respectively, which might also need to form heterodimers for activity. The model and figure are modified from refs. *1,40,* and *41.*

Table 2
Conserved and Substituted Residues in the Predicted GTP-Binding Sites of Selected Invertebrate Guanylyl Cyclases

Known Heterodimeric Guanylyl Cyclases

Residues contributed by the α subunit							Residues contributed by the β subunit								
MsGC-α1	**D**	T	I	**D**	R	**E**	MsGC-β1	F	**E**	M	**R**	**C**	**N**	S	**R**
DGC-α1	–	–	–	–	–	–	DGC-β1	–	–	–	–	–	–	–	–
MsGC-β1	**G**	A	V	–	T	–	MsGC-α1	–	–	C	–	–	**T**	N	**K**
DGC-β1	**G**	G	V	–	T	–	DGC-α1	–	–	C	**K**	–	**T**	N	**K**

Predicted heterodimeric guanylyl cyclases

DAF-11	**781**	786	829	**831**	877	**911**	ODR-1	830	**874**	881	**942**	**944**	**951**	954	**955**
ODR-1	**Q**	N	T	**E**	–	**Q**	DAF-11	Y	**I**	C	–	**A**	**I**	–	**K**
GCY-6	–	–	–	–	–	–	GCY-6	–	–	F	–	–	–	–	–
GCY-11	–	–	–	–	–	–	GCY-11	–	–	–	–	–	**I**	N	**M**
GCY-12	–	–	–	–	–	–	GCY-12	–	–	–	–	**T**	–	–	–
GCY-15	–	V	–	–	–	–	GCY-15	–	–	–	–	–	–	–	–
GCY-21	–	V	–	–	–	–	GCY-21	–	–	–	–	–	–	–	–
GCY-27	**Q**	I	T	**E**	–	**Q**	GCY-27	–	–	–	–	–	–	–	–
GCY-31	–	–	–	–	–	–	GCY-31	–	–	–	–	–	**Y**	N	**K**
GCY-35	–	–	–	–	–	–	GCY-35	–	–	–	–	–	–	N	**K**
GCY-33	**R**	S	–	**E**	K	–	GCY-33	–	–	–	–	–	–	–	–
GCY-36	–	Q	V	–	–	–	GCY-36	–	–	–	–	–	–	–	–
CG14885	**E**	Y	V	**M**	–	–	CG14885	–	–	–	–	–	–	–	–
CG14886	**E**	Y	V	**M**	–	–	CG14886	–	–	–	–	–	–	–	–
ebiP3998	**E**	I	V	**K**	–	–	ebiP3998	–	–	–	–	–	–	–	–
GCY-25	–	–	V	–	K	**A**	GCY-25	V	–	–	–	–	–	C	–
GCY-32	–	Q	V	–	–	–	GCY-32	–	–	–	**K**	–	**T**	–	**Q**
GCY-34	–	Q	V	–	–	–	GCY-34	–	–	–	–	–	**T**	–	**Q**
GCY-37	–	F	L	–	–	**C**	GCY-37	–	**L**	L	–	–	–	K	**S**

Homodimeric guanylyl cyclases

MsGC-β3	–	–	–	–	–	–	MsGC-β3	–	–	–	–	–	–	–	–
CG4154	–	–	–	–	–	–	CG4154	–	–	–	–	–	–	–	–
agCP12881	–	–	–	–	–	–	agCP12881	–	–	–	–	–	–	–	–

The first part of the table shows the residues that are predicted to contact the Mg-GTP substrate in known insect heterodimeric soluble guanylyl cyclase subunits. The α subunits from both *Manduca* and *Drosophila* have identical residues in the equivalent positions to the mammalian α subunits predicted in models for the catalytic sites *(40,41).* Similarly, the β subunits also have 100% conserved residues. By contrast the α subunits have substitutions in some of the equivalent positions in β subunits and the β subunits have substitutions in the corresponding positions of α subunits as previously described *(15).* A dash represents the presence of an identical residue and residues marked in bold have been shown to be critical for catalytic activity *(40,41),* and are shown in Fig. 1. The second part of the table shows predicted heterodimeric guanylyl cyclases. DAF-11 has all the conserved residues contributed by α subunits with some substitutions in residues provided by β subunits, whereas ODR-1 has all the residues contributed by β subunits and some substitutions in residues contributed by α subunits. The numbers represent the positions of the residues in DAF-11 for the α subunits and ODR-1 for the β subunits; also shown in Fig. 1. Similarly, GCY-6, -11 and -12, soluble guanylyl cyclases GCY-31 and -36 have all the residues contributed by the α subunit conserved, but substitutions in positions for β subunits. Conversely, the residues contributed by the β subunits are all conserved in GCY-15, -21, and -27, soluble guanylyl cyclases GCY-33 and -35 and the *Drosophila* and *Anopheles* subunits CG14885, CG14886, and ebiP3998. The *C. elegans* receptor guanylyl cyclase GCY-25 and soluble guanylyl cyclases GCY-32, -34, and -37 have substitutions in residues that are predicted to be contributed by both subunits and so might not make active enzymes with any other subunit. The final part of the table shows that MsGC-β3 and its orthologs have all the residues contributed by both α and β subunits and are predicted to form active homodimers.

the *C. elegans* guanylyl cyclases are shown and those that are known to be necessary for activity in other guanylyl or adenylyl cyclases in other species are highlighted in Table 2. The aspartic acid at the equivalent position to D781 in DAF-11 and the glutamic acid at the equivalent position to E911 in DAF-11 are replaced by glutamines in ODR-1. These residues are both part of a triad of acidic residues that are important for binding the β,γ bisphosphate moiety and the Mg^{2+} ion. Mutations to either of these residues reduced the level of activity in adenylyl cyclase to less than 10% of wild type *(40).* The asparagine at the equivalent position to N951 in ODR-1 appears to be critical in the condensation reaction of the 3′ hydroxyl of the ribose to the α phosphate group *(40)* and is substituted with an isoleucine in DAF-11. The other important residues are E874 and C944 in ODR-1 that are substituted for isoleucine and alanine respectively in DAF-11 and are known to be important for substrate specificity *(41).* Transforming *odr-1* mutants with ODR-1 where E874 was converted to an alanine failed to rescue the mutant *(4).* Mutating the equivalent residues to E874 and C944 in ODR-1 to lysine and aspartic acid respectively, which are the residues present in adenylyl cyclases, changes the RetGC-1 from a guanylyl cyclase to an adenylyl cyclase *(41).* Thus, neither ODR-1 nor DAF-11 should form an active homodimeric guanylyl cyclase, but together they should form an active guanylyl cyclase with a single GTP-binding site. If this prediction is correct, and the similar phenotypes of *odr-1* and *daf-11* mutants strongly support this, it will be the first example of an obligate heterodimeric receptor guanylyl cyclase. Importantly, residues equivalent to D781 and N951 are also substituted in β1 and α1 subunits respectively of the heterodimeric soluble guanylyl cyclases (Table 2), demonstrating that they do not act as dominant negative mutations, but only affect a single catalytic center.

A similar analysis of the other 23 receptor guanylyl cyclases showed that most have all 17 of the residues that are predicted to interact with Mg-GTP or have conservative substitutions and so would be expected to form active homodimers (D. B. Morton, unpublished data). GCY-6, GCY-15 and GCY-21 have substitutions in residues that have not been tested for their effects on guanylyl cyclase activity and so might also be capable of forming active homodimers (Table 2). However, GCY-11, GCY-12, GCY-25 and GCY-27 have substitutions in residues that have been shown to be necessary for activity and hence might form obligate heterodimers (Fig. 1; Table 2). GCY-12 substitutes a threonine for the cysteine at the equivalent position C944 in ODR-1 (Fig. 1) although it is possible that the hydroxyl of the threonine could substitute for the sulfhydryl of the cysteine to bind to the O6 of the guanine ring *(41)* and form an active homodimer. GCY-25 and GCY-27 have substitutions (Table 2) for the acidic residues (D781 and E911 in DAF-11) that bind to the β,γ bisphosphate moiety and the Mg^{2+} ion and should, like ODR-1, be inactive as a homodimer. GCY-11 has substitutions in two of the residues that are critical for the formation of the cyclic phosphate-ribose bond. In addition to an isoleucine in place of the asparagine in the equivalent position to N951 in ODR-1, GCY-11 has a methionine in place of the arginine at the equivalent position of R955 of ODR-1 (Fig. 1; Table 2). As the cellular localization for GCY-11, GCY-25 and GCY-27 have not been reported; predictions of which guanylyl cyclase will form their partners cannot be made. GCY-11 has also been reported to lack a predicted transmembrane domain, but whether this is due to an inaccurate prediction of its intron/exon boundaries or whether it is a pseudogene is not known *(19).*

MsGC-I: A Receptor-Like Guanylyl Cyclase Lacking Extracellular and Transmembrane Domains

A more direct demonstration of the existence of atypical guanylyl cyclases has come from a series of studies that used RT-PCR and degenerate oligonucleotide primers to clone guanylyl cyclases from the nervous system of *Manduca.* In addition to identifying MsGC-II *(14)* and the subunits for the NO-sensitive

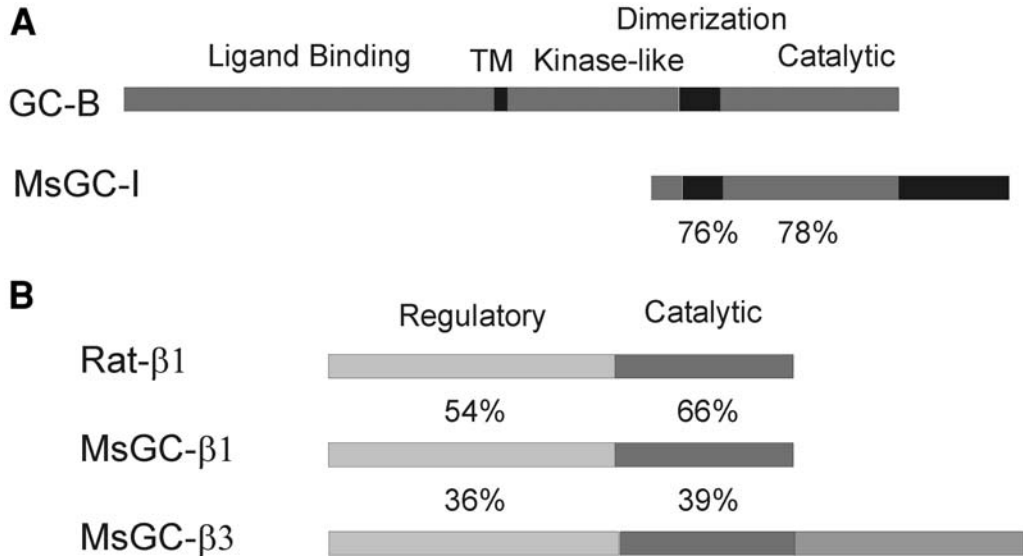

Fig. 2. Schematic representation of the atypical guanylyl cyclases from *Manduca.* **(A)** MsGC-I is closely related to the mammalian receptor guanylyl cyclase GC-B. MsGC-I has conserved dimerization and catalytic domains but is lacking the ligand-binding transmembrane (TM) and most of the kinase-like domains that are characteristic of receptor guanylyl cyclases. **(B)** MsGC-β3 is most closely related to mammalian β1 subunits although the *Manduca* MsGC-β1 subunit is more closely related to the mammalian β1 subunits. MsGC-β3 also has a unique C-terminal domain. The percentages given are the amino acid similarities between the indicated domains.

guanylyl cyclases *(21),* two novel guanylyl cyclases named MsGC-I *(42)* and MsGC-β3 *(29)* were also isolated. The predicted primary amino acid sequence of MsGC-I showed that it was most closely related to the receptor guanylyl cyclases, but lacked the extracellular and transmembrane domains, while the catalytic and dimerization domains showed greater than 75% similarity to a mammalian receptor guanylyl cyclase GC-B *(42)* (*see* Fig. 2A). Northern and western blots confirmed the predicted size of the transcript and protein demonstrating that MsGC-I was not a cloning artifact *(42).* Transient transfection of MsGC-I into COS-7 cells demonstrated that MsGC-I had guanylyl cyclase activity, which was higher in the presence of manganese compared to magnesium and that MsGC-I was primarily present in the soluble fraction of COS-7 cells *(42).* Gel filtration of the soluble fraction of transfected COS cells confirmed that MsGC-I formed active homodimers as was expected for a receptor-like guanylyl cyclase *(42).* An immunocytochemical study of the distribution of MsGC-I showed that it was widely expressed in a number of brain regions including a number of higher order synaptic and sensory neuropils *(43).* One particularly intriguing characteristic of MsGC-I was that although it was present in the soluble fraction of transfected COS-7 cells, it was located in the particulate fraction of CNS homogenates *(42).* This suggested that MsGC-I was bound to another protein in vivo that might regulate its activity *(42).* One possibility for this is the receptor guanylyl cyclase, MsGC-II, as it is expressed in some of the same regions of the brain and co-transfection studies suggested that they might heterodimerize *(14).* The calcium regulation of MsGC-II described above also provided some circumstantial evidence that MsGC-I and MsGC-II interact in vivo. The body wall of larval

Manduca is innervated by a population of dendritic arborization neurons *(44),* most of which respond to NO donors with an increase in cGMP. One specific neuron, named ddaB, responded poorly to this stimulus, but showed a large increase in cGMP in response to incubation with the calcium chelator EGTA, which was assumed to reduce the intracellular calcium concentration in ddaB *(44).* Further studies showed that MsGC-I was also expressed in ddaB, suggesting that it was activated by low calcium in a manner similar to the vertebrate retinal guanylyl cyclases *(45).* Although, this could not be mimicked in vitro even with the addition of exogenous NCSs *(45),* it is possible that only the MsGC-I/MsGC-II heterodimer could bind these proteins.

The identification of MsGC-I as a guanylyl cyclase with a novel structure raises the question of whether it is unique to *Manduca.* There is a report of a rat guanylyl cyclase, named ksGC, which like MsGC-I lacks extracellular and transmembrane domains *(46).* Subsequent reports suggested that this was a cloning artifact and was a truncated version of the full-length receptor guanylyl cyclase GC-G *(47).* There are, however, several lines of evidence that suggest ksGC could represent an alternatively spliced transcript of GC-G. The predicted transcript size of GC-G is at least 3.4 kb whereas the cDNA of ksGC is 2.9 kb *(46,47).* Northern blots in both studies showed that skeletal muscle, lung, kidney and intestine expressed multiple transcripts including one large enough to code for GC-G, but also transcripts that were too small to code for full-length GC-G but could code for ksGC *(46,47).* In addition, a recent search of Genebank identified two mouse cDNAs from hippocampus and ES cells that appear to code for mouse orthologs of ksGC (accession numbers AK049940 and AK010727) and also lack the extracellular and transmembrane domains. Thus, it appears that mammals also express guanylyl cyclases with a similar structure to MsGC-I and these represent an additional class of guanylyl cyclases. Searches of the annotated genome of *C. elegans*, *Drosophila* and *Anopheles* also identified predicted guanylyl cyclases that lacked extracellular and transmembrane domains (*15,* D. B. Morton, unpublished data) but whether these represent incorrectly predicted coding regions or whether they are also members of this new class of guanylyl cyclase remains to be determined.

Atypical Soluble Guanylyl Cyclases

MsGC-β3: An Homodimeric Soluble Guanylyl Cyclase Similar to Mammalian β2 Subunits

In addition to MsGC-I, our search for guanylyl cyclases expressed in *Manduca* CNS identified another novel cyclase that was most closely related to the β subunits of NO-sensitive soluble guanylyl cyclases *(29).* Sequence analysis of the cDNA revealed two structural elements that had not been seen previously in any other guanylyl cyclase and as it was not obviously an ortholog of either the mammalian β1 or β2 subunits, we named it MsGC-β3 *(29).* Firstly, the open reading frame extended the C-terminal portion of the protein 315 residues beyond the predicted catalytic domain forming an additional domain with no similarity to other proteins (Fig. 2B). In addition, two cysteine residues in the regulatory domain that are conserved in all other conventional β subunits are replaced in MsGC-β3 *(29).* Site-directed mutagenesis of these two cysteines in the rat β1 subunit generated a NO-insensitive guanylyl cyclase when it was co-expressed with a wild-type α1 subunit *(48).*

Transient transfection of COS-7 cells with a plasmid coding for MsGC-β3 revealed two novel properties for this enzyme. In contrast to conventional soluble guanylyl cyclases that are obligate heterodimers, MsGC-β3 formed an active enzyme in the absence of additional subunits and this activity was insensitive to stimulation by NO donors *(29).* Co-expression with the *Manduca* α subunits or incubation with protoporphyrin failed to generate an enzyme that was activated by NO and MsGC-β3 was also unaffected by the soluble guanylyl cyclase inhibitor, ODQ *(29).* Subsequent experiments have shown that MsGC-β3 is also insensitive to

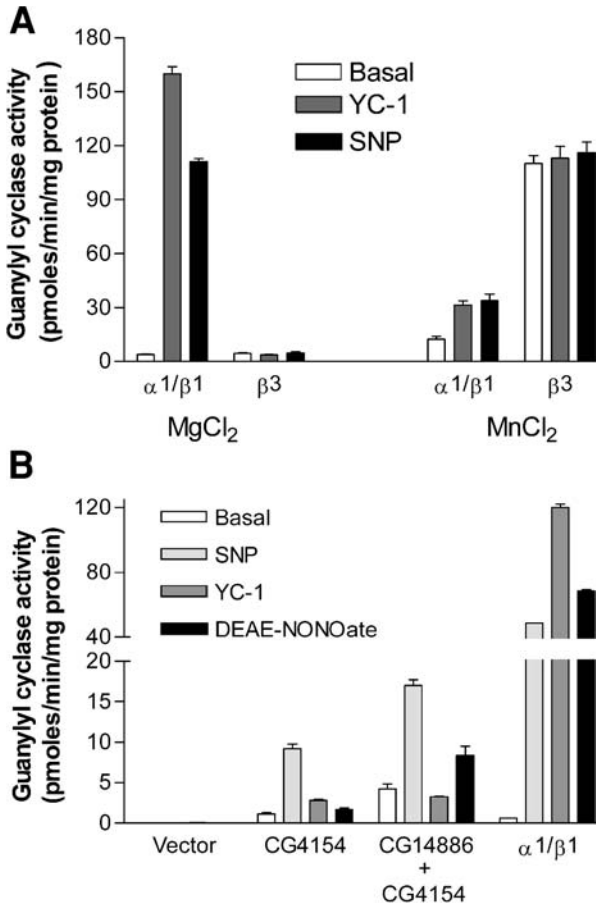

Fig. 3. Guanylyl cyclase activity of soluble guanylyl cyclases of *Manduca* and *Drosophila* transiently expressed in COS-7 cells. **(A)** *Manduca* subunits. MsGC-α1 and MsGC-β1 are only active when expressed together (α1/β1), whereas MsGC-β3 (β3) is active when expressed in the absence of additional subunits. NO donors such as sodium nitroprusside (SNP) or the NO-independent activator YC-1 are potent stimulators of MsGC-α1/β1 heterodimers in the presence of Mg. In the presence of Mn, the basal activity is increased and the stimulated activity is reduced. MsGC-β3 is insensitive to either SNP or YC-1 and shows a large increase in basal activity in the presence of Mn. **(B)** *Drosophila* subunits. CG4154 is active in the absence of additional subunits, whereas CG14886 is only active when co-expressed with CG4154. Both CG4154 and CG4154 + CG14886 are slightly stimulated by SNP, are unaffected by YC-1, and are slightly stimulated by another NO donor, DEA-NONOate. By contrast, the *Manduca* subunits MsGC-α1 + MsGC-β1 (α1/β1) are potently stimulated by all three activators *(70).* Details of the methods used are detailed in refs. *21* and *49.*

an NO-independent soluble guanylyl cyclase activator, YC-1 (*see* Fig. 3A).

Although MsGC-β3 does not require co-expression with additional subunits for activity, its similarity to MsGC-β1 suggested that it might nevertheless form heterodimers with MsGC-α1. To test this we used hexa-histidine tagged versions of all three subunits in pull-down experiments and showed that MsGC-β3 was capable of forming heterodimers with both MsGC-α1 and MsGC-β1 *(49).* In addition, co-expressing all three subunits together resulted in lower NO-stimulated activity than just co-expressing MsGC-α1 with MsGC-β1, showing that MsGC-β3 acted as a dominant negative subunit and suggested that the heterodimers were inactive *(49).* Although it was clear that the MsGC-α1/MsGC-β3 or MsGC-β1/MsGC-β3 heterodimers were not activated by NO, it was not possible to determine directly whether they had any basal activity because it couldn't be distinguished from the MsGC-β3 homodimer activity *(49).* To overcome this problem we designed and made point mutations in MsGC-β3 that formed inactive homodimers but active heterodimers with wild-type MsGC-β3. These experiments showed that the heterodimers formed with either MsGC-α1 or MsGC-β1 were inactive *(49).* These findings, together with the report that mammalian α1 and β1 subunits formed inactive homodimers *(50)* suggest that there is little specificity in the formation of dimers, although specificity could be conferred by cell-specific expression patterns or by different relative affinities between the subunits.

One of the unusual structural features of MsGC-β3 is the C-terminal extension *(29).* To determine whether this feature contributed to its unusual biochemical properties, we made a version that lacked the terminal 338 amino acids and studied its properties in transiently transfected COS-7 cells *(49).* This deletion mutant, MsGC-β3ΔC338 showed no major differences in activity compared to the wild-type MsGC-β3. It was active as a homodimer and was insensitive to NO stimulation *(49).* A more detailed analysis however, showed that in the presence of Mg ions MsGC-β3ΔC338 had a

lower K_m for GTP than MsGC-β3, although both versions had a similar K_m for GTP in the presence of Mn ions and also similar estimates for the value of V_{max} in both Mg and Mn *(49).* As the in vivo substrate for guanylyl cyclases is Mg-GTP, this data suggested that the C-terminal domain might act as an auto-inhibitory domain. The kinetic analysis also revealed another unexpected property of MsGC-β3. Homodimeric guanylyl cyclases such as the receptor guanylyl cyclases are thought to contain two GTP-binding sites and exhibit kinetics with positive cooperativity *(1).* As MsGC-β3 also forms homodimers it would also be expected to contain two GTP-binding sites, but it exhibited Michaelis-Menten kinetics with a Hill Coefficient of 1.0 suggesting either that it had a single GTP binding site or that the two sites acted independently *(49).*

One of the most important characterizations of MsGC-β3 is to determine how it is activated. Circumstantial evidence relating to this was provided by showing that it is expressed in cells that are known to be targets for the neuropeptide eclosion hormone (EH). EH is released at the end of each molt and functions as part of an endocrine cascade that triggers a stereotyped behavior known as ecdysis, enabling the insect to escape from the cuticle of the previous developmental stage *(51,52).* The mechanism of action of EH has been studied in detail and it has been known for over 20 yr that it is mediated by an increase in cGMP *(53,54).*

There are three known populations of cells that respond to EH with an increase in cGMP. Within the CNS of *Manduca* there is a network of about 50 peptidergic neurons that show an increase in cGMP shortly after being exposed to EH *(55).* EH also acts in the periphery on a series of glands, known as epitracheal glands, where it acts on the Inka cell in the epitracheal gland stimulating an increase in cGMP *(56,57).* The third known EH target is a group of cells, known as the subtransverse nerve region (STNR) cells *(58).* The STNR cells are located along the posterior margin of the transverse nerve of the abdominal CNS and have been used for several studies aimed at understanding the mechanism of the EH-stimulated increase in cGMP *(54).* A series of biochemical and immunocytochemical studies have shown that EH increases the levels of cGMP by activating an NO-insensitive soluble guanylyl cyclase (reviewed in ref. *54*). Additional immunocytochemical studies have shown that MsGC-β3, which has the biochemical properties expected for the EH-activated guanylyl cyclase, is expressed in both the Inka cells in the epitracheal glands and the STNR cells of the transverse nerve *(59).* This suggested that EH stimulates an increase in cGMP in these cells by activating MsGC-β3. A model for this activation has been proposed suggesting that this activation is via protein phosphorylation *(54).* Direct evidence for this model, however, has not yet been provided.

The first reports describing the cloning and properties of the mammalian β2 subunit suggested that, like the β1 subunit, it formed heterodimers with an α subunit *(60,61).* Subsequent studies, however, demonstrated that the β2 subunit was active in the absence of additional subunits, making it more similar to the *Manduca* MsGC-β3 subunit *(62).* Additional experiments revealed further similarities. A kinetic analysis of the β2 subunit showed that, like MsGC-β3, it exhibited linear Michaelis-Menten kinetics *(62).* The β2 subunit has a shorter C-terminal extension than MsGC-β3, but a point mutation within this region of β2 generated an enzyme that was active in the presence of Mg, whereas the activity of the wild-type enzyme was undetectable in the presence of Mg *(62).* Both the wild-type and point mutation showed similar levels of activity in the presence of Mn *(62).* These properties parallel the effect of removing the entire C-terminal tail of MsGC-β3 *(49).* These biochemical similarities between the mammalian β2 subunits and MsGC-β3 suggest that they form a separate family of homodimeric soluble guanylyl cyclases *(62).*

Possible Functions and Properties of Soluble Guanylyl Cyclases in C. elegans

Analysis of the *C. elegans* genome yielded seven soluble guanylyl cyclase subunits that

were all most similar to β subunits from other organisms *(19,63).* Comparison of these cyclases with other β subunits, including *Manduca* MsGC-β3, showed that all the *C. elegans* soluble subunits had substitutions in the same two cysteine residues that are missing in MsGC-β3 and are predicted to contribute to the lack of NO stimulation of MsGC-β3 *(63).* This comparison, together with the observation that *C. elegans* lacks an identifiable NO synthase gene *(36),* led to the prediction that all of the *C. elegans* soluble guanylyl cyclases would also be insensitive to NO stimulation *(63).* Unfortunately, it has not been possible to test this prediction directly as none of the subunits yields an active enzyme when transiently expressed in COS-7 cells, either alone or in any pair-wise combination (*64;* M. L. Hudson, D. S. Karow, M. A. Marletta, and D. B. Morton, unpublished data). By transforming animals with promoter::GFP constructs the expression patterns of all seven subunits have been mapped and shown to fall into two groups. Two of the subunits, GCY-31 and GCY-33, are expressed in a single pair of sensory neurons, the BAG neurons, while the remaining five subunits, GCY-32, GCY-34, GCY-35, GCY-36 and GCY-37 are all expressed in two pairs of cells, the sensory neurons AQR and PQR and the pair of URX sensory neurons (*37,64;* M. L. Hudson, D. S. Karow, M. A. Marletta, and D. B. Morton, unpublished data).

An analysis of the predicted GTP binding sites of these subunits in a similar manner to that used on the receptor guanylyl cyclases described above can be used to predict whether heterodimers or homodimers should be formed (Table 2). GCY-31 retains all of the residues found in α subunits whereas GCY-33 has all those found in β subunits, predicting that GCY-31/GCY-33 heterodimers will be active (Table 2). The asparagine residue in the equivalent position of N951 in ODR-1 (Fig. 1) that participates in the condensation reaction to form cGMP is replaced with a tyrosine residue in GCY-31 (Table 2), which is likely to render GCY-31 homodimers inactive. In addition, the aspartic acid residue in the equivalent position of D781 in DAF-11 (Fig. 1) is replaced with an arginine in GCY-33 (Table 2); again suggesting homodimers will be inactive. As both GCY-31 and GCY-33 are expressed in the same cells it makes it more likely that they form functional heterodimers. The situation with the remaining *C. elegans* soluble guanylyl cyclase subunits is not as clear cut, partially because there are five different subunits that are expressed in the same cells. None of these subunits have conserved all 17 of the residues that are predicted to interact with the Mg-GTP (Table 2). Some of the substitutions, however, have not been tested to determine whether they are critical for activity or not. GCY-35 has all the residues that are supplied by the α subunit but has two substitutions to residues supplied by the β subunit. One of these is a conservative substitution of a lysine for an arginine (R955 in ODR-1) and the other; an asparagine for a serine (S954 in ODR-1), has not been shown to be critical for activity. Hence GCY-35 might form active homodimers. GCY-36 has all those that are predicted to be supplied by the β subunit (Table 2) but has two substitutions in residues (T786 and I829 in DAF-11) that are supplied by β subunits. However, these residues have also not been shown to be critical for activity and hence GCY-36 might also form active homodimers. GCY-32, GCY-34 and GCY-37 all have substitutions for the arginine equivalent to R955 in ODR-1 and the asparagine equivalent N951 in ODR-1, residues predicted to be supplied by the β subunits and hence might be expected to act as α subunits (Table 2). However, they also have substitutions in residues supplied by α subunits, although these residues have not been tested for their necessity. This apparent redundancy makes it difficult to predict which subunits form active guanylyl cyclases in the AQR, PQR, and URX neurons.

Although there are deletion mutants for both the *gcy-31* and *gcy-33* genes, we have not at this time been able to determine a phenotype that is associated with the loss of these guanylyl cyclases (M. L. Hudson, D. S. Karow, M. A. Marletta and D. B. Morton, unpublished

data). It is possible, however, to predict a possible function for whichever of the remaining five subunits form the active guanylyl cyclase in the AQR, PQR, and URX neurons. A behavioral polymorphism exists in natural populations of *C. elegans* such that individual worms either feed in groups or feed in isolation *(65).* These two behavioral states are associated with a single amino acid change in a neuropeptide Y-like receptor, NPR-1 *(65).* (This is unrelated to the natriuretic peptide receptor 1 of the same name.) Animals with deletions of the *npr-1* gene feed in groups, termed social feeding, indicating that activation of the NPR-1 receptor inhibits social feeding. The NPR-1 receptor is expressed in a wide variety of both central and peripheral neurons *(66)* and it is possible to identify which subsets of neurons require NPR-1 expression to suppress social feeding by using a variety of heterologous promoters coupled to the *npr-1* gene in an *npr-1* loss of function background *(66).* Using the GCY-32 promoter to express NPR-1 in AQR, PQR and URX, a subset of cells that normally express NPR-1, strongly suppressed social feeding *(66).* Loss of function mutations in the cGMP-gated ion channel, TAX-2 and TAX-4, also suppressed social feeding in *npr-1* loss of function mutants, suggesting that expression of a cGMP-gated ion channel is necessary to promote social feeding *(66).* The most likely source of the cGMP that gates the TAX-2/TAX-4 channel is some combination of GCY-32, GCY-34, GCY-35, GCY-36 and/or GCY-37. Social feeding can be triggered by a variety of aversive stimuli, signaling through specific sensory neurons *(67).* Signals from these cells could activate the guanylyl cyclases in the AQR, PQR and URX neurons leading to activation of the TAX-2/TAX-4 channels and the generation of social feeding. Conversely, activation of the NPR-1 receptor, presumably by an endogenous neuropeptide leads to a down-regulation of this pathway by inhibiting the level of cGMP generated and leading to a reduction in aggregation.

A particularly interesting aspect of this model is the parallel that exists with the regulation of feeding behavior in *Drosophila (68).* Individual *Drosophila* larvae from naturally occurring populations also exhibit two different behavioral strategies when food is present; either remaining in the patch of food or continuing to search for additional food sources *(69).* This behavioral polymorphism is associated with different levels of cGMP-dependent protein kinase (PKG) *(3).* Although it is not known which guanylyl cyclases generate the cGMP to activate the PKG it is intriguing that in both *C. elegans* and *Drosophila* cGMP appears to be central to regulating feeding strategies *(68).*

The Drosophila and Anopheles Genomes Predict Additional Atypical Soluble Guanylyl Cyclases

A search of the *Drosophila* and *Anopheles* genomes identified three types of soluble guanylyl cyclase subunits (*15;* D. B. Morton, unpublished data). Both insects have the α1 and β1 subunits of the NO-sensitive heterodimeric soluble guanylyl cyclases (*see* above), which have been cloned and expressed in *Drosophila* and are similar to vertebrate α1 and β1 subunits in their sequence and properties *(9,19).* Both insects also have orthologs to the *Manduca* MsGC-β3 subunit that forms an active guanylyl cyclase without the need for additional subunits and is insensitive to stimulation by NO *(28).* The gene that codes for this subunit in *Drosophila* is CG4154 *(15)* and in *Anopheles* is agCP12881 (D. B. Morton, unpublished data). In both cases, the predicted genes code for proteins that have substitutions for the two cysteine residues that are critical for NO sensitivity *(15,48).* In addition, both of these subunits, like MsGC-β3, have all 17 of the residues that have been modeled to interact with the Mg-GTP substrate, suggesting that both should be able to form active homodimers (*15;* Table 2).

The third class of subunits predicted from the *Drosophila* and *Anopheles* genomes are encoded by CG14885 and CG14886 in *Drosophila* and ebiP3998 in *Anopheles* (*15;* D. B. Morton, unpublished data). They are all more closely related to β subunits than α subunits

and are all missing the same two cysteine residues as MsGC-β3 and its orthologs (*15;* D. B. Morton, unpublished data). Comparing the residues present in the catalytic domains, these subunits are predicted to require an additional subunit to form an active enzyme (Table 2). The most critical residues that are substituted are one of the acidic residues supplied by the α subunit that bind to the Mg^{2+} and bisphosphate group (D781 in DAF-11) and the aspartic acid residues equivalent to D831 in DAF-11 that participates in the condensation reaction that forms cGMP (Fig. 1; Table 2). These residues can be supplied by either the α subunit or the MsGC-β3 orthologs. We have recently cloned and transiently expressed some of these subunits from *Drosophila* and have begun to test these predictions *(70).* An example of some of this preliminary data is shown in Fig. 3B. As predicted, CG4154 forms an active guanylyl cyclase when transiently expressed in COS-7 cells in the absence of additional subunits and CG14886 is inactive when expressed on its own. Surprisingly, the activity of CG4154 is slightly stimulated by incubation with the NO donor sodium nitroprusside (SNP), although this stimulation is much lower than seen for the α1/β1 heterodimers. An alternative NO donor, DEA-NONOate, and an NO-independent activator, YC-1, are ineffective at stimulating CG4154, whereas both are potent activators of the α1/β1 enzyme. The reason that SNP stimulates CG4154 but DEA-NONOate does not is unclear. Whether this represents stimulation by alternative SNP breakdown products such as cyanide is unknown at this time. Also, as predicted, CG14886 appears to form an active enzyme when co-expressed with CG4154, as both the basal and SNP-stimulated activities are higher than when CG4154 is expressed alone. It is not yet known whether these subunits can form an active enzyme when co-expressed with the α1 subunit. Preliminary data from in situ hybridization experiments reveals that both CG4154 and CG14886 are expressed in many neurons throughout the central and peripheral nervous system *(70).* Some neurons appear to co-express both subunits, while others only seem to express one or the other *(70).*

The other unusual structural feature of MsGC-β3 is its extended C-terminal domain. The sequence of CG4154 predicted from the genome does not include a C-terminal domain, but the sequence of the cDNA includes a C-terminal domain of 268 residues *(70).* The predicted sequence of the *Anopheles* ortholog also contains a C-terminal extension of 143 residues. Multiple sequence alignments of these three sequences shows that although there is greater than 80% similarity in the catalytic and N-terminal regulatory domains between the three species there is very little overall similarity in the C-terminal domains. There is however a short region of 22 residues that is almost 100% conserved across all three species *(70).* This suggests that this region might have important functional properties and is especially intriguing in light of the evidence from MsGC-β3 that the C-terminal domain might act as an auto-inhibitory domain *(49).* The predicted size of CG14885, CG14886 and ebiP3998 are similar to that of typical β1 subunits, lacking a C-terminal extension (D. B. Morton, unpublished data).

Summary

Prior to the identification and characterization of MsGC-I and MsGC-β3 from the insect *Manduca sexta* all guanylyl cyclases could easily be classified into one of two different families—the soluble or receptor guanylyl cyclases. The unusual sequence and biochemical characteristics of these two *Manduca* enzymes has shown that there is much more diversity in the structural and regulatory properties of guanylyl cyclases than had previously been appreciated. Using comparisons with the *Manduca* enzymes plus predictions based on models of the structures of guanylyl cyclases, analysis is presented that makes predictions of the properties of all the guanylyl cyclases that have been identified from the complete genome

sequences of three invertebrates. Experimental data are also described that either directly or indirectly support several of these predictions.

Typical receptor guanylyl cyclases are homodimeric integral membrane proteins *(1).* MsGC-I is closely related to these enzymes but is likely to be cytoplasmically located as it lacks extracellular and transmembrane domains. Similar guanylyl cyclases are predicted to be present in rats, mice, *C. elegans* and other insects, but direct experimental evidence for this is still lacking at this time. *C. elegans* also expresses a large number of full-length receptor guanylyl cyclases, most of which appear to be homodimers. Several of these receptor guanylyl cyclases, however, are predicted to be active only as heterodimers. Genetic evidence in support of this is provided by the finding that loss of function mutants in two receptor guanylyl cyclases that are expressed in the same cells as each other have the same phenotype. MsGC-β3 was the first homodimeric soluble guanylyl cyclase to be identified. Subsequent data showed that similar cyclases are present in mammals and *Drosophila* and are predicted to exist in *Anopheles.* MsGC-β3 is insensitive to NO stimulation, whereas the *Drosophila* and mammalian orthologs are slightly sensitive to NO. Other variations include the soluble guanylyl cyclases in *C. elegans* that appear to be NO insensitive, some of which are also predicted to require heterodimerization for activity. In addition, *Drosophila* and *Anopheles* appear to express a subunit that requires heterodimerization either with the ortholog of the β2/β3 subunit or with the typical α1 subunit of the NO-sensitive soluble guanylyl cyclase. Taken together, this evidence highlights the unexpected diversity of mechanisms that are used to generate cGMP in the nervous system.

This diversity raises many unanswered questions concerning cGMP regulation. A particularly important piece of evidence will be to determine whether the subunits that are predicted to heterodimerize are expressed in the same cells. Another critical piece of information will be to identify the mechanism of activation of the NO-insensitive soluble guanylyl cyclases and to determine whether similar activation mechanisms are used by the guanylyl cyclases that show low sensitivity to NO or whether this represents a mechanism for reducing the sensitivity of a system to NO. Although several of the predictions made described in this review are supported by experimental evidence, some of this evidence is only circumstantial. It remains a high priority to design experiments that will directly test these predictions, especially whether heterodimerization of some of these atypical guanylyl cyclases occurs in vivo. This information will provide further insights into structure–function relationships of all guanylyl cyclases.

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