A cGMP-dependent protein kinase is implicated in wild-type motility in *C. elegans*

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Abstract

In mammals, cyclic GMP and cGMP-dependent protein kinases (cGKs) have been implicated in the regulation of many neuronal functions including long-term potentiation and long-term depression of synaptic efficacy. To develop *Caenorhabditis elegans* as a model system for studying the neuronal function of the cGKs, we cloned and characterized the *cgk-1* gene. A combination of approaches showed that *cgk-1* produces three transcripts, which differ in their first exon but are similar in length. Northern analysis of *C. elegans* RNA, performed with a probe designed to hybridize to all three transcripts, confirmed that a major 3.0 kb *cgk-1* transcript is present at all stages of development. To determine if the CGK-1C protein was a cGMP-dependent protein kinase, CGK-1C was expressed in *Sf*9 cells and purified. CGK-1C shows a K_a of 190 ± 14 nM for cGMP and 18.4 ± 2 μM for cAMP. Furthermore, CGK-1C undergoes autophosphorylation in a cGMP-dependent manner and is inhibited by the commonly used cGK inhibitor, KT5823. To determine which cells expressed CGK-1C, a 2.4-kb DNA fragment from the promoter of CGK-1C was used to drive GFP expression. The CGK-1C reporter construct is strongly expressed in the ventral nerve cord and in several other neurons as well as the marginal cells of the pharynx and intestine. Finally, RNA-mediated interference of CGK-1 resulted in movement defects in nematode larvae. These results provide the first demonstration that cGMP-dependent protein kinase is present in neurons of *C.elegans* and show that this kinase is required for normal motility.

Keywords: *C. elegans*, cGMP, cGMP-dependent protein kinase, neuronal, motility phosphorylation.

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As intracellular second messengers, the cyclic nucleotides alter cellular functions through a variety of mechanisms. cGMP is an intracellular second messenger that mediates the action of nitric oxide (NO) and natriuretic peptide hormones in the nervous system (Butt *et al.* 1993). NO activates soluble guanylyl cyclase (Arnold *et al.* 1977), while the particulate guanylyl cyclases are controlled by natriuretic peptides, guanylin and enterotoxins (Field *et al.* 1978). Within cells cGMP has several direct targets: cGMP-regulated phosphodiesterases (Francis *et al.* 1980), cGMP-gated cation channels and cGMP-dependent protein kinases (cGKs). However, the best characterized effectors of cGMP are the cGKs.

Mammals contain two different cGKs, designated type I (cGKI) and type II (cGKII). cGKIs are predominately cytoplasmic and are highly expressed in Purkinje cells (Lohmann *et al.* 1981) as well as other regions of the brain. cGKII is also expressed in the brain (Uhler 1993) but its distribution differs from that of cGKI (el-Husseini *et al.*

1995). The neuronal functions of the cGKS are not clearly defined; however, cellular regulation by cGKs outside of the nervous system is thought to be similar to that within the neurons. cGKI regulates intracellular calcium levels, particularly in smooth muscle, where cGKI mediates muscle relaxation (Lincoln 1989). cGKI is also thought to play a role in platelet aggregation (Horstrup *et al.* 1994), olfaction (Nakamura and Gold 1987), and long-term depression of neurons (Hartell 1996). cGKII has been implicated in the regulation of ion transport in

intestinal cells, where it phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Forte *et al.* 1992).

The existence of multiple intracellular cGMP receptors and the lack of well characterized physiological substrates for the cGKs have complicated attempts to define the neuronal mechanisms of cGK action. The nematode *Caenorhabditis elegans* represents a unique system in which to study the neuronal function of cGKs. *C. elegans* is a well-defined organism with 959 somatic cells, which are organized into nervous, muscular, reproductive, hypodermal, excretory and digestive systems (Hodgkin *et al.* 1998). The development of *C. elegans* has been studied in great detail and the lineage of each neuron in the worm is known (Sulston and Horvitz 1977). Furthermore, many aspects of *C. elegans* development are controlled by signalling cascades that are functionally similar to the signalling cascades present in mammals (Patterson and Padgett 2000). In addition, powerful genetic techniques simplify identification of novel genes and allow rapid characterization of a gene's function. Finally, the completion of the *C. elegans* genome sequencing project permits rapid cloning of genes of interest (Hodgkin *et al.* 1998) including potential neuronal cGK substrates.

We report here the genetic and biochemical characterization of a cGMP-dependent protein kinase from *C. elegans* which is expressed in several types of neurons. In addition, we show that RNA-mediated interference of this cGK causes a defect in motility.

Material and methods

Strains and nomenclature
The genetic nomenclature for *C. elegans* was described previously (Horvitz *et al.* 1979). We use capital letters and plain font to indicate the protein encoded by a gene. Thus, the protein produced by the *cgk-1* gene is CGK-1. All experiments were done using the Bristol strain N2 (Brenner 1974).

Isolation of *cgk-1* cDNA
Degenerate primers cGK5.1 (GGG AGA TCT GAG CTG GCT AT (C/T) CT (A/C/G/T) TA (C/T) AA (C/T) TG) and cGK3.1 (GGG GAA TAC CCG CA(A/G) AA(A/C) GTC CA(A/C/G/T) GT(C/T) (TT) were used in PCR reactions with pooled phage DNA from a *C. elegans* cDNA library (Stratagene, La Jolla, CA, USA) as template. The amplification resulted in a ~1.0 kb band, which was digested with *Bgl*II and *Eco*RI. The digestion products (~530 bp and ~490 bp) were cloned into pBluescript(SK) (Stratagene) and pGem3Z(+) (Promega, Madison, WI, USA), respectively. The inserts were sequenced and found to encode a protein showing homology with bovine cGK1α.

Random primer extension was used to generate radiolabeled probes for screening ~1.2 × 10^6 recombinant λZap II phage from a *C. elegans* mixed staged cDNA library (Stratagene). Inserts from three hybridizing clones were sequenced on both strands, using specific oligonucleotide primers and Sequenase DNA polymerase (Amersham, Piscataway, NJ, USA).

RT-PCR and 5′ RACE analysis
A Superscript kit (Life Technologies, Rockville, MD, USA) was used to reverse transcribe 500 nanograms of polyA + RNA with a SL1 sense primer (GGG TCT AGA TTA ATT ACC CAA GTT TGA) and a 3′ antisense primer directed against the CGK-1C cDNA sequence (CCC GTC GAC AGA GCT CCC TCA CGT GAA ACT TGC). The resulting 0.7 kb product was digested with *Sal*I and *Xba*I and ligated into pBluescript(KS) (Stratagene). Multiple independent subclones were sequenced with the Prism kit (Applied Biosystems, Foster City, CA, USA).

Briefly, 5′ RACE was performed as described (Frohman *et al.* 1988). The first PCR reaction was performed using tailed first-strand cDNA and primers Q_T (CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TT), Q_{ON} (CCA GTG AGC AGA GTG ACG AGG A), and a specific *cgk-1* internal primer (TTC TCA AAA TTC GTC GGC TCT GCT). The second PCR reaction used the product of the first reaction as template and primer Q_{1N} (CCA GTG AGC AGA GTG ACG AGG A) and a second *cgk-1* primer (GTG GAG ATG GTG ATG GTG GTT GTC). The resulting 650 bp product was cloned and sequenced.
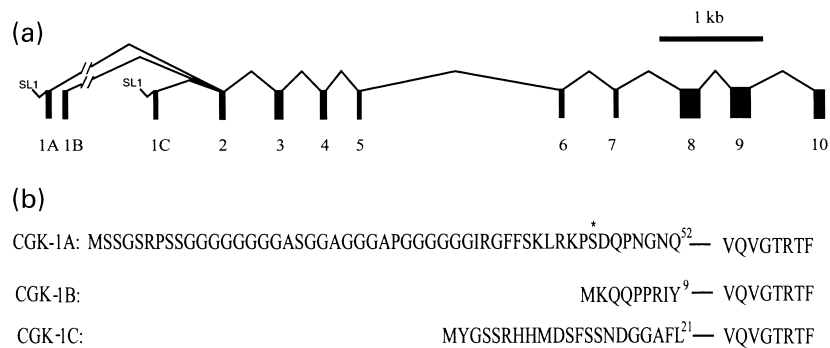
RNA isolation for northern blot
Acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987) was used to isolate total RNA from staged nematodes grown in liquid culture (Sulston and Brenner 1974). Total RNA was separated on a formaldehyde gel, transferred to a positively charged nylon membrane (MSI) and hybridized with an antisense RNA probe. Plasmid pGEMT-H6CGK-1C (see below) was used as template in a PCR reaction with primers cgk-1for (TGA TAC GTG ACG CCG TGC) and T7cgk-1rev (GGA TCC TAA TAC GAC TCA CTA TAG GGA GAA CGA AGA ATG CGT CAC CC) to amplify an ~570 bp fragment covering exon 3 to exon 7. This PCR product was used to generate a radiolabeled RNA probe for northern blotting, which should detect all three *cgk-1* isoforms. As a control for RNA loading, the same blots were hybridized with an RNA probe to the 26S RNA.

Expression and purification of CGK-1C
To aid in purification, codons for six histidine residues were appended to the amino terminal coding region of CGK-1C. pCGK-1C plasmid DNA was used as a template, and primers (GGG ATC CCA CCA TGC ACC ATC ACC ATC ATC ACC ACT ATG GGT CCA GT) and (TGG CTG GCT TAT TCT CA) were used to amplify the coding region of the cDNA. The resulting PCR fragment was subcloned into pGEMT (Promega) and designated pGEMT-H6CGK-1C. The insert containing the histidine-tagged amino terminal CGK-1C cDNA was excised from pGEMT-H6CGK-1C with enzymes *Eco*RI and *Xho*I and subcloned into

Fig. 1 The structure and organization of the *C. elegans* cgk-1 gene. (a) cgk-1 is alternatively spliced at exon 1. Exons are represented by black rectangles. Exon 1A is 257 nucleotides in length not including the SL1 spliced leader sequence. Exon 1B is at least 33 nucleotides in length (see text). Exon 1C is 102 nucleotides in length. Exons 2–10 appear common to all the cgk-1 isoforms. (b) The predicted amino acid sequences of exons 1A, 1B and 1C. Exon 1A encodes 52 amino acids and has a potential autophosphorylation site denoted by the asterick. Exon 1B encodes nine amino acids. Exon 1C encodes 21 amino acids.

(a)

1 kb

SL1   SL1

1A 1B   1C   2   3   4   5   6   7   8   9   10

(b)

CGK-1A: MSSGSRPSSGGGGGGGGASGGAGGGAPGGGGGGGIRGFFSKLRKPSDQPNGNQ^{52}— VQVGTRTF

CGK-1B: MKQQPPRIY^{9}— VQVGTRTF

CGK-1C: MYGSSRHHMDSFSSNDGGAFL^{21}— VQVGTRTF

plasmid PZ8-CGK-1C, which contained the full length CGK-1C coding region minus the amino terminal region. The resulting plasmid was designated H$_6$PZ8-CGK-1C. The histidine tagged full length CGK-1C cDNA insert was excised from H$_6$PZ8-CGK-1C with enzymes *Spe*I and *Not*I and subcloned into the bacmid vector pFastBac (BRL) creating pFastBac-H$_6$CGK-1C. This plasmid was purified and used to transfect a culture of *Sf9* cells. Supernatant from the primary infection was used in subsequent infections to increase the viral titer.

For protein expression, 500 mL of *Sf9* culture was infected at a density of 6×10^6 cells/mL. After 72 h, cells were collected by centrifugation and stored at $-70°C$. CGK-1C protein was purified over a Ni-NTA-agarose affinity resin column (Qiagen, Valencia, CA, USA) to apparent homogeneity as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Commassie blue staining.

Kinase, autophosphorylation and dissociation assays

Kinase assays (Gamm *et al.* 1995) were performed at 30°C using the synthetic heptapeptide Arg-Lys-Arg-Ser-Arg-Ala-Glu (H$_2$Btide) (Glass and Krebs 1982) as phosphate acceptor and purified CGK-1C protein (1.5 nM final concentration) from *Sf9* cells. KT5823 inhibitor assays were similar to the kinase assay described above, except that the cyclic nucleotide concentration was held constant at 2 μM and increasing concentrations of inhibitor were used.

Autophosphorylation assays (de Jonge and Rosen 1977) also at 30°C were also similar to the kinase assay, except that bovine serum albumin (BSA) and substrate peptides were omitted from the reaction mix. The reaction mixture consisted of the kinase buffer described above and a fixed concentration (0.2 μM) of CGK-1C protein with or without 10 μM cGMP. [^{32}P] incorporation was determined by excising the CGK-1C band from the blot and scintillation counting.

[^{3}H]-cGMP dissociation assays were performed at 30°C as described (Taylor and Uhler 2000) except that purified CGK-1C was added to the reaction mixture.

Construction and analysis of *cgk-1C* promoter::GFP transgene

Two primers (AGC TTG CAT GCC TGC AGG TAT GCA AGG AGT ACT TCG ACT) and (CTG GCC AAT CCC GGG GGA TCC ATA CAT TTG GTG AGA) were used in a PCR reaction where *C. elegans* genomic DNA was the template. A 2.4-kb DNA fragment that included the start codon and upstream sequence of CGK-1C was subcloned into the TA-cloning vector (Invitrogen). The 2.4 kb fragment was released with *Bam*HI and *Pst*I and subcloned into pPD95.69 (provided by Andrew Fire, Carnegie Institution, Washington, DC, USA). The resulting plasmid, pCGK-1C::GFP was transformed into *Escherichia coli*, purified by miniprep (Qiagen), ethanol precipitated and resuspended in 1X injection buffer (Fire 1986). Finally, p*cgk-1C*::GFP (at 5, 15 or 25 ng/μL) and pRF4 [*rol-6(su1006*dm*)*] (at 100 ng/μL) were injected into young adult nematodes using the method outlined by Mello *et al.* (1991). The Rol-6 phenotype was used to select animals that carried the p*cgk-1C*::GFP construct.

The phasmids of worms expressing the pcgk-1C::GFP construct were stained with DiIC18 (St John 1991) for 30 min, washed, treated with 10 mM NaN$_3$, placed on a slide containing an agar pad and then photographed using confocal microscopy.

RNA-mediated interference

To prepare double-stranded RNA (dsRNA), we used PCR to generate a template containing nucleotides 4–455 of the *fog-3* cDNA and separately used PCR to generate a template containing nucleotides 119–463 (exon 2 to exon 4) of the *cgk-1C* cDNA. Both DNA fragments had inward directed T7 promoters at each end. We prepared dsRNAs by *in vitro* (Megascript; Ambion, Austin, TX, USA) transcription, precipitation and resuspension in 1X injection buffer (Fire 1986), and allowed the two strands to anneal at 37°C for 1 h. The final concentration was estimated to be 1–2 mg/mL by ethidium bromide staining. Injections were carried out as described by Guo and Kemphues (1995) and Fire *et al.* (1998).
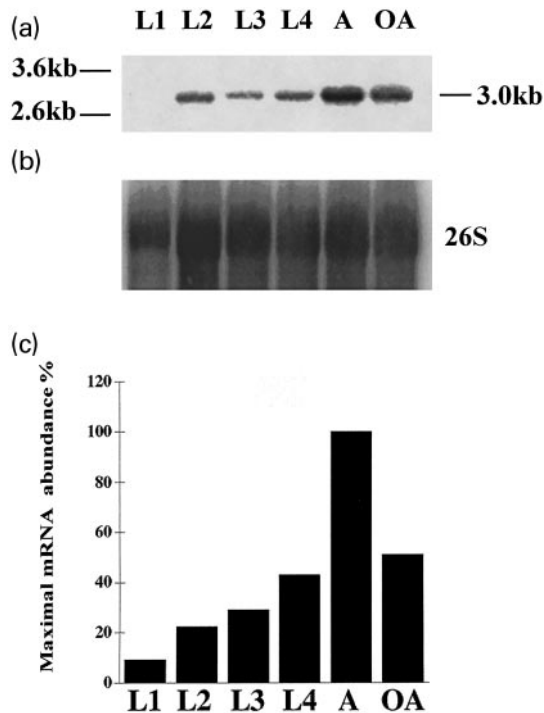
Fig. 2 Developmental northern analysis. Staged specific total RNA was hybridized to antisense *C. elegans cgk-1* probe. (a) A single 3.0 kb transcript was detected at all stages of development. The probe was generated from exons 3 to 7 and should recognize all three *cgk-1* mRNAs. (b) A 26S ribosomal RNA probe was used to test for loading errors. (c) Graph representing normalized mRNA levels following phosphoimager analysis.

Results

Isolation and characterization of *cgk-1* cDNAs

One pair of primers based on regions of homology shared by bovine, human and *Drosophila* cGK sequences successfully amplified a 1-kb fragment with high homology to other cGKs from a mixed stage *C. elegans* cDNA library (Stratagene). We used hybridization screening to isolate the full-length coding region corresponding to this fragment.

Comparison of the *cgk-1* sequence with genomic DNA (1998) showed that *cgk-1* contains 10 exons and is located on chromosome *IV*. However, a BLAST search against expressed sequence tags (ESTs) from *C. elegans* revealed the presence of additional *cgk-1* transcripts (Fig. 1a). Each of the three transcripts is predicted to be ~3000 nt long, and shares exons 2 through 10, but the translation start sites for each transcript is distinct. We independently isolated two cDNA sequences corresponding to exon 1A by 5′RACE; these transcripts were both trans-spliced with the SL1 leader sequence. Exon 1A also encodes a potential autophosphorylation site (Fig. 1b) that is not present in amino acids encoded by exons 1B and 1C. In addition, we isolated three

cDNA fragments corresponding to exon 1C from a cDNA library, following amplification with SL1 and internal primers. Finally, we have detected rare exon 1B transcripts in RT-PCR experiments (data not shown).

Cgk-1 is expressed throughout development

To determine *cgk-1* mRNA levels during development, northern blots of staged total RNA were probed using an antisense RNA probe specific for exons 3–7 of *cgk-1*. The probe detected a 3.0-kb transcript expressed at all stages of development (Fig. 2). Fig. 2(c) shows normalized mRNA levels following phosphoimager analysis. A small increase in the expression of total *cgk-1* transcripts is seen in adult stage nematodes.

The sequence of CGK-1C predicts a protein with high similarity to known cGMP-dependent protein kinases

Because the first exon of each transcript is short, the three predicted CGK-1 proteins are extremely similar (Fig. 1b). Overall CGK-1C shows 48% amino acid identity with human cGKIα (Sandberg *et al.* 1989), 43% amino acid identity with human cGKII (Orstavik *et al.* 1996), 44% amino acid identity with *Drosophila* cGKI and 43% amino acid identity with *Drosophila* cGKII (Kalderon and Rubin 1989). Homology is highest in the catalytic domain (62% identity with human cGKI, 57% identity with human cGKII, 58% identity with the *Drosophila* cGKI and 61% identity with *Drosophila* cGKII). CGK-1C has a predicted ATP binding site corresponding to residues 442–455 as well as a conserved lysine residue (K^{468}) that is found in serine/threonine protein kinases (Fig. 3).

CGK-1C has the biochemical properties of a cGMP-dependent protein kinase

To determine if CGK-1C is preferentially activated by cGMP over cAMP, we performed radiometric kinase assays with increasing concentrations of cGMP or cAMP. The synthetic peptide RKRSRAE was used as substrate in these assays because it has been shown to be a good substrate for mammalian cGKs (Glass and Krebs 1982). Activation constants for both cGMP and cAMP were determined by Eadie/Hofstee plots (Eadie 1942; Hofstee 1942). CGK-1C has a K_a of 190 ± 14 nM for cGMP and 18.4 ± 2 μM for cAMP (Fig. 4a). CGK-1C activation constants for cGMP and cAMP are very similar to activation constants determined for other members of the cGMP-dependent protein kinase family. For example, the mammalian cGKIα has a K_a of 190 ± 14 nM for cGMP and 18.4 ± 2 μM for cAMP (Gamm *et al.* 1995). Furthermore, CGK-1C has a V_{max} of 1.47 μmole/min/mg of protein for cAMP and 2.1 μmole/min/mg of protein for cGMP which is comparable with the V_{max} values obtained for bovine cGK Iα (4.4 μmole/min/mg with cGMP) and mouse cGK II (1.2 μmole/min/mg with cGMP) (Gamm *et al.* 1995).

Fig. 3 Homology of *C. elegans* CGK-1C amino acid sequence to known cGMP-dependent protein kinase amino acid sequences. The predicted amino acid sequence of the *C. elegans* CGK-1C (top) is shown compared with human cGK I (Hs cGKI)and II (Hs cGKII) and the *Drosophila* cGK I (Dm cGKI) and II (Dm cGKII). Positions where two or more sequences show identity are indicated by black shading. The amino acid numbers of the various sequences are indicated at the right of the diagram.

KT5823 is a ATP analog inhibitor of mammalian cGKs (Kase *et al.* 1987) and is used widely for *in vivo* applications in mammalian systems (Grider 1993; Fujii *et al.* 1995). If CGK-1C is functionally similar to other cGMP-dependent protein kinases, it should be inhibited by KT5823. To assess CGK-1C sensitivity to KT5823 *in vitro*, kinase assays were performed in the presence of varying KT5823 concentrations (see Materials and methods). KT5823 inhibited CGK-1C with an IC_{50} of 3.8 μM (Fig. 4b).

To determine if purified CGK-1C undergoes autophosphorylation after activation by cGMP, recombinant CGK-1C was incubated with $[\gamma\text{-}^{32}P]$-ATP in the presence or absence of 10 μM cGMP. The reactions were terminated by addition of SDS loading buffer, and the samples analyzed by
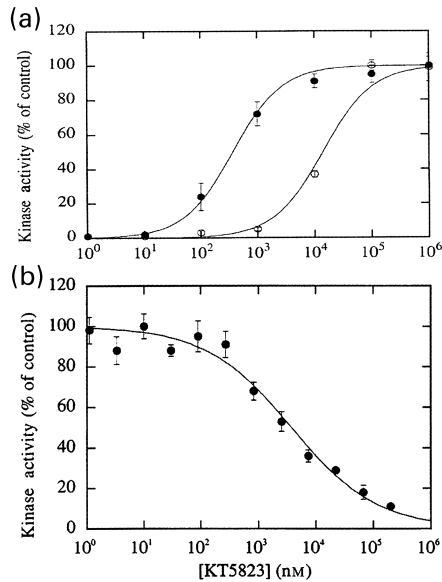
Fig. 4 Kinase activities of CGK-1C. (a) The kinase activity of CGK-1C protein (1.5 nM) was measured in the presence of increasing cyclic nucleotide concentrations. Filled circles indicate activation by cGMP and open circles indicate activation by cAMP. Activity was determined and expressed as the percentage of the highest CGK-1C activity obtained for cGMP and cAMP, respectively. These experiments were performed six times for each cyclic nucleotide concentration. (b) Increasing concentrations of cGMP-dependent protein kinase inhibitor KT5823 inhibits CGK-1C activity with an IC_{50} of 3.8 μM. Activity in these experiments was expressed as a percentage of CGK-1 activity in the absence of inhibitor. These experiments were performed three times with similar results.
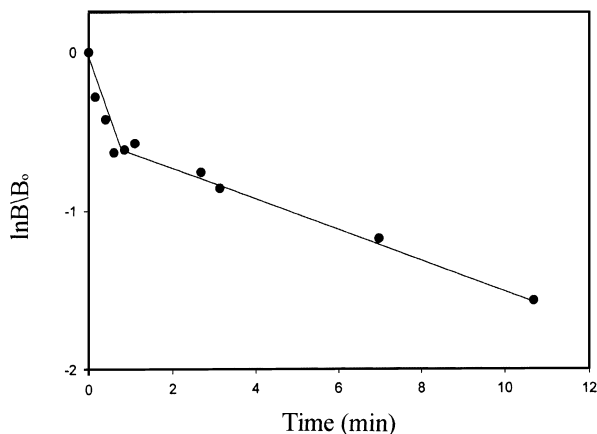

Fig. 5 CGK-1C has a biphasic dissociation for [³H]-cGMP. Briefly, 450 nM of CGK-1C was incubated in KPEH buffer, with 1 mM cGMP and 3.2 μM [³H]-cGMP, for 30 min. To measure [³H]-cGMP dissociation kinetics from the two cGMP binding sites, 100X cold cGMP was added to the reaction mix and vortexed, following which samples were collected and analyzed (see Materials and methods). The $t_{1/2}$ for dissociation from the fast dissociating site in CGK-1C was ~0.15 min and the $t_{1/2}$ from the slow dissociating site was ~8 min.

SDS-PAGE. These experiments suggest that CGK-1C is autophosphorylated rapidly with maximal autophosphorylation occurring within 5 min (data not shown). Quantitation of phosphate incorporation demonstrated that only 0.02 mol of phosphate were incorporated per mol of enzyme in 5 min assays in the absence of cGMP; however, 1.75 mol of phosphate were incorporated in the presence of cGMP. This value is similar to that of cGKIß which incorporates ~2 mol of phosphate per mol of enzyme in autophosphorylation assays (Chu *et al.* 1998).

Mammalian cGKs have a characteristic biphasic dissociation curve for cGMP. The biphasic curve is the result of two distinguishable cyclic nucleotide binding sites: one high affinity and one low affinity site per cGK monomer (Corbin and Doskeland 1983; Wolfe *et al.* 1989a). To assess CGK-1C binding properties, we measured $t_{1/2}$-values for its cyclic nucleotide binding sites by studying the time course of [³H]-cGMP dissociation. Our results demonstrate that *C. elegans* CGK-1C exhibits a biphasic pattern (Fig. 5). The $t_{1/2}$ for dissociation from the rapidly dissociating site in CGK-1C was ~0.15 min and $t_{1/2}$ from the slowly dissociating site was ~8 min. These dissociation values are similar to dissociation values described for mammalian cGKs. For example, cGKIα has a $t_{1/2}$ for the fast and slow sites of 0.5 min and 25 min, respectively (Gamm *et al.* 1995). The stoichiometry of cGMP binding (0.7–0.8 mol of cGMP per mole of enzyme) was significantly lower than the expected value of 2 mol of cGMP per mole of enzyme. We believe this is due in part to occupancy of the cGMP-binding sites by cyclic nucleotides derived for the *Sf9* cells during protein production.

The *cgk-1C* promoter drives GFP expression in neurons as well as other cell types

To elucidate where CGK-1C is expressed in *C. elegans* we used its promoter to drive expression of a GFP transgene which served as a reporter (see Materials and methods). The 2.4 kb region immediately upstream of *cgk-1C* was cloned into a promoterless GFP expression vector and injected with *rol-6(su1006*dm*)* into young adult nematodes. The *rol-6(su1006*dm*)* gene produces a rolling phenotype when expressed (Mello *et al.* 1991) and allowed rapid selection of animals that had formed extrachromosomal arrays that might express the p*cgk-1C*::GFP construct. Six transgenic lines were isolated and examined for GFP expression. All lines had similar expression patterns. We observed fluorescence in a subset of neurons in the head, nerve ring, and ventral nerve cord including some motor neurons (Figs 6a and b). Furthermore, p*cgk-1C*::GFP expression was found in several neurons in the tail. One of these neurons was PQR, which was identified by its axon morphology and its position relative to the phasmid neurons (Fig. 6c); PQR has been
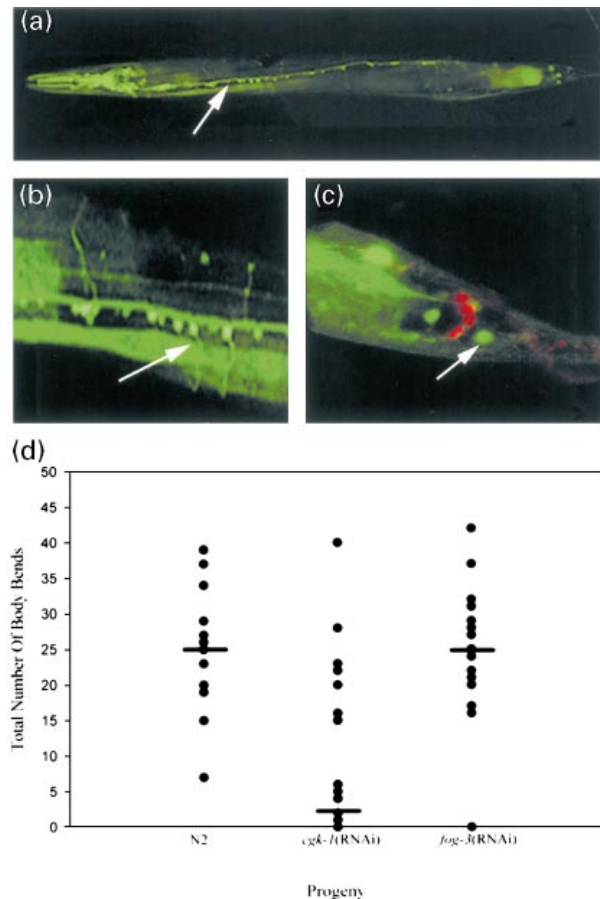
Fig. 6 Promoter *cgk-1C*::GFP is expressed in some neurons in *C. elegans*. Transgenic animals with a GFP construct under the control of the 2.4 kb promoter of *cgk-1C* were imaged using confocal microscopy. (a) L2 animal expressing GFP in the pharyngeal marginal cells, neurons in the head, and ventral nerve cord (arrow). (b) Enlargement of a segment of the ventral nerve cord. Arrow indicates a motor neuron, which was identified by location and axonal morphology. (c) Image of the tail region in an adult animal. Arrow indicates GFP expression in neuron PQR, which was identified by location and axonal morphology. DiIC18-filled phasmids were detected by the red fluorescence. (d) dsRNA was generated from the cDNA of *cgk-1C*, injected into young adult animals and XX-self-progeny were observed. To quantitate the motility defect, blind tests were performed on F1 progeny from animals injected with *cgk-1* dsRNA, *fog-3* dsRNA or N2 animals (see text) and the number of complete body flexes (bends) per minute was determined. Eighteen of 25 *cgk-1(RNAi)* animals made fewer than 10 bends/min and had median movement value of 2 bends/min. Progeny for N2 animals and *fog-3* had a median value of 25 and 24 bends/min, respectively. Similar experiments were performed two additional times, but with additional controls.

shown to express a guanylyl cyclase (Yu *et al.* 1997). GFP was also expressed in the pharyngeal marginal cells, body muscle, intestine, vulval muscles, and spermatheca (data not shown).

RNA-mediated interference against *cgk-1* causes impaired movement

To examine potential physiological roles of CGK-1 in *C. elegans*, RNA-mediated interference experiments were performed (Fire *et al.* 1998). Double-stranded RNA was generated from nucleotides 119–463 of the coding region of *cgk-1C* and injected into the germline of young adult animals at ~1 mg/mL. We observed the XX self-progeny of these animals and all of the F1 animals developed with normal size and morphology. However, many of these animals moved slowly and irregularly.

To study this phenotype further, blind tests were conducted using wild type (N2) animals, F1 animals from hermaphrodites injected with *fog-3* dsRNA (Chen *et al.* 2000) as a control and F1 animals from hermaphrodites injected with *cgk-1* dsRNA. The animals were placed on foodless plates to encourage foraging and the number of complete body flexes per minute was counted. We observed serious defects in movement among most of the *cgk-1* animals, but not among the controls (Fig. 6d). The small variation in phenotype of the *cgk-1(RNAi)* worms might results from varying concentrations of dsRNA found in the young animals.

Discussion

CGK-1C is a cGMP-dependent protein kinase

The *cgk-1* gene of *C. elegans* produces three transcripts, which encode proteins that differ at their amino termini but are otherwise identical. In order to eludicate the function of these proteins, we selected one, CGK-1C for biochemical characterization. Two biochemical tests indicate that this protein is indeed a cGMP-dependent protein kinase. First, we measured the ability of CGK-1C to phosphorylate a synthetic peptide, and found that CGK-1C was preferentially activated by cGMP over cAMP. In fact, we observed that the K_a for cGMP was 97-fold lower than for cAMP. Second, we showed that the ability of CGK-1C to phosphorylate the peptide substrate was inhibited by KT5823, a compound shown previously to inhibit cGMP-dependent protein kinases in mammals. CGK1-C undergoes autophosphorylation in the presence of cGMP, like other cGMP-dependent protein kinases. Additionally, CGK-1C showed a biphasic dissociation curve for cGMP that resembles those of other cGMP-dependent protein kinases. Taken together, these results suggest that the sequence similarity between CGK-1C and other cGMP-dependent protein kinases reflects common mechanisms of activation and regulation.

What about the two other products of the *cgk-1* gene? We have not tested their functions biochemically, but their considerable sequence identities suggest that their activities are similar to those of CGK-1C. The mammalian cGKIα

and β also only differ in their amino terminal sequences (Lincoln *et al.* 1988; Wernet *et al.* 1989; Wolfe *et al.* 1989b) and these differences are thought to regulate protein–protein interactions. For example, the amino terminal sequences of cGKIα are required for binding to the myosin-binding subunit (MBS) of myosin phosphatase (Surks *et al.* 1999). By contrast, cGKIß and cGKII do not bind MBS. Similarly, cGKIß has been show to specifically associate with the IP$_3$R-associated cGMP kinase substrate (IRAG) (Schlossmann *et al.* 2000) whereas cGKIα and cGKII have not been shown to associate with IRAG. We speculate that the different amino terminal sequences present in the CGK-1 isoforms might regulate protein–protein interactions or localization.

Why does *cgk-1* produce three similar transcripts?

cgk-1 is by no means unique. The *C. elegans kin-1* gene, which encodes a cAMP-dependent protein kinase catalytic subunit, has five alternative first exons (Tabish *et al.* 1999). One possibility is that the crucial factor is not the difference between the alternative first exons, but instead between the promoter regions attached to these exons. Since these promoter regions are large for *C. elegans*, it is not unreasonable to suspect that each drives the expression of *cgk-1* in a complex and distinct set of cells. Alternatively, the different first exons might themselves play an important role in the regulation of each protein's activity, as we speculated above. In this regard, some versions of KIN-1 can be myristoylated, but others cannot (Tabish *et al.* 1999).

Nematodes have two cGKs

Humans, mice and fruit flies each have two distinct cGK genes, which encode a type-I and a type-II cGMP-dependent protein kinase. In mammals, evidence suggests that the two types of cGKs serve different physiological functions (Lohmann *et al.* 1997). We used BLAST searches to screen the completed *C. elegans* genome (1998) and identified a second potential cGK gene, which we are currently characterizing. Thus, nematodes, like other animals, are likely to contain two types of cGMP-dependent protein kinases. These two forms of the protein may have arisen early in evolution from a common ancestor and have been preserved since that time, or might instead have arisen independently in each lineage. Our sequence analysis of CGK-1 suggests that it is most likely to have arisen from the same ancestral gene that gave rise to mammalian cGKII and *Drosophila* cGKI (Jarchau *et al.* 1994) (unpublished results).

What functions does CGK-1 regulate in *C. elegans*?

We observed movement defects following RNA-mediated interference of *cgk-1,* which suggest that *cgk-1* functions in the neuro-musculature system; however, we do not rule the possibility that movement defects could be due to inhibition of *cgk-1* in non-neuronal cells. CGK-1 might also control behaviors that were not affected by RNAi, because RNAi is not equally effective in all tissues, or because a second cGMP-dependent protein kinase might be functionally redundant to CGK-1 in some cells. The isolation of *cgk-1* deficient mutants should help complete our analysis of the role of CGK-1 in *C. elegans*.

In *Drosophila*, higher activity of the *foraging* gene (*for*) (also known as *dg2*) which encodes a neuronal cGMP-dependent protein kinase causes a more active foraging behavior than sitter strains which have lower *for* activity (Osborne *et al.* 1997). Similar roles for cGKs in mouse behavior have not been reported; however, mice lacking cGKI show erectile dysfunction and impaired smooth muscle relaxation (Pfeifer *et al.* 1998; Hedlund *et al.* 2000) while cGKII deficient mice exhibit dwarfism and intestinal secretory defects (Pfeifer *et al.* 1996).

Mammalian cGKs are known to regulate intracellular Ca^{2+} levels in smooth muscle cells (Lincoln 1989). High calcium/calmodulin levels in muscle activate the myosin light chain kinase which phosphorylates myosin light chain, the first step in muscle contraction (Somlyo and Somlyo 1994). By lowering Ca^{2+} levels, cGKs indirectly inactivate myosin kinase, causing muscle relaxation. cGKI can regulate intracellular Ca^{2+} levels by inhibiting phospholipase C activation (Barnett *et al.* 1995) and the generation of inositol 1,4,5-trisphosphate (IP$_3$). cGK also phosphorylates the IP$_3$ receptor (Komalavilas and Lincoln 1994) directly, which attenuates Ca^{2+} release. Additionally, cGK activates Ca^{2+}-ATPases in the plasma membrane of the sarcoplasmic reticulum (Cornwell *et al.* 1991), which lower intracellular cytosolic Ca^{2+} levels (Gonzalez *et al.* 1996).

Within Purkinje cell neurons, the phosphatase inhibitor G-substrate in a well characterized substrate for cGKI and G-substrate phosphorylation can cause activation of genes containing cAMP-responsive elements (Hall *et al.* 1999). Finally, voltage-operated Ca^{2+} channels and L-type calcium channels (Chik *et al.* 1995) can be directly phosphorylated and inhibited by cGK and may be important neuronal substrates for mammalian cGKs. By analogy, the interference of *cgk-1* in *C. elegans* might affect the regulation of calcium channels or phosphatase regulation in neurons, thereby impairing neurosecretion and movement.

Future studies

C. elegans is an excellent system for identifying genes that are important to function of the nervous system. UNC-4 is a neuronally expressed homeodomain transcription factor which specifies presynaptic input in the ventral nerve cord in *C. elegans*. *unc-4* mutants have impaired backward motility which is caused by miswiring of VA motor neurons (Miller and Niemeyer 1995). Like *unc-4*, *cgk-1C* is expressed in the ventral nerve cord and RNAi experiments result in motility defects. Another gene mutation which phenocopies the *cgk-1* RNAi is *unc-29*. *unc-29* encodes a non-α subunit of the nicotinic acetylcholine receptor

(Fleming *et al.* 1997). Mutant *unc-29* animals exhibit motility defects during early larva stages (Fleming *et al.* 1997) and expression studies have shown that *unc-29* is expressed in the ventral nerve cord.

The *C. elegans* genome sequence contains 29 putative guanylyl cyclases (Bargmann 1998, 1998). The majority of these cyclases are predicted to be particulate, and only 6 are thought to be soluble (Yu *et al.* 1997). Some of the particulate cyclases are known to be specifically expressed in neurons (Yu *et al.* 1997). For example, the guanylyl cyclase ORD-1 is neuronal and has been reported to modulate olfaction (L'Etoile and Bargmann 2000). Also, the guanylyl cyclase DAF-11 has been shown to modulate chemosensory transduction and dauer formation in *C. elegans* (Vowels and Thomas 1992). The functional role of PQR is not known, but the expression of a guanylyl cyclase and *cgk-1C* in PQR suggests that a cGMP signalling pathway functions in this neuron.

We are now searching for *cgk-1* alleles to use in genetic mosaic analysis to resolve the question of which tissues must express CGK-1 for normal movement. The fact that we can screen for mutations that suppress the motility defect makes it possible to carry out a genetic dissection of pathways in which CGK-1 is involved and identify important physiological CGK-1 substrates.

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