Cyclic GMP-dependent protein kinase EGL-4 controls body size and lifespan in *C. elegans*

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SUMMARY

We designed an automatic system to measure body length, diameters and volume of a *C. elegans* worm. By using this system, mutants with an increased body volume exceeding 50% were isolated. Four of them are grossly normal in morphology and development, grow longer to be almost twice as big, and have weak egg-laying defects and extended lifespan. All the four mutants have a mutation in the *egl-4* gene. We show that the *egl-4* gene encodes cGMP-dependent protein kinases. *egl-4* promoter::gfp fusion genes are mainly expressed in head neurons, hypodermis, intestine and body wall muscles. Procedures to analyze

morphology and volume of major organs were developed. The results indicate that volumes of intestine, hypodermis and muscle and cell volumes in intestine and muscle are increased in the *egl-4* mutants, whereas cell numbers are not. Experiments on genetic interaction suggest that the cGMP-EGL-4 signaling pathway represses body size and lifespan through DBL-1/TGF-β and insulin pathways, respectively.

Key words: cGMP-dependent protein kinase, Body size, Lifespan, *C. elegans*, EGL-4, Big mutant

INTRODUCTION

There is great variety in the average body sizes of mature individuals among species both in animals and plants. In accordance with the body size, animals show variations in many macroscopic characteristics such as specific metabolic rate, velocity of movement (Randall et al., 1997), body structure, lifespan and ecological niche. Thus, body size is an important feature of an organism. However, the mechanisms of body size determination, especially the reasons for the differences in the average size among species, are largely unsolved.

The size of an animal is basically determined by the number and the size of its component cells. In mammals, differences in the cell number are much more important than those in individual cell size for determining inter-species differences in the mass (Conlon and Raff, 1999). In lower invertebrates, cell size variation also seems important (Böhni et al., 1999; Flemming et al., 2000). Although age and environmental factors such as food intake affect the body size of an individual, both the number and the size of cells must be determined primarily by genetic factors.

Although there is little direct evidence for a genetic factor responsible for body size difference between species, various examples are known for the change in body or organ size within a species that depends on genetic factors. Transgenic mice grew twice as big as normal through overexpression of a

growth hormone gene (Palmiter et al., 1982). Pit1 transcription factor that activates prolactin and growth hormone genes was shown to be mutated in *Snell* dwarf mutant mice (Li et al., 1990). More recently, gene disruption was performed in mice for a cyclin-dependent kinase inhibitor p27Kip1 (Nakayama et al., 1996), a cyclic GMP-dependent protein kinase (cGKII) (Pfeifer et al., 1996) and a TGFβ superfamily member GDF8 (McPherron et al., 1997). cGKII knockout mice were smaller than normal, whereas the other two were larger because of enlargement of skeletal muscle or various organs. In *Drosophila*, mutants in the components of the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway were reported to be smaller (Leevers et al., 1996; Böhni et al., 1999). In *C. elegans*, many small body size mutants have been identified. Several genes responsible for their phenotypes such as *sma-2, 3, 4* (Smad transcription factors), *daf-4* and *sma-6* (receptor) and *dbl-1/cet-1* (ligand) encode components of a TGFβ signaling pathway (Savage et al., 1996; Estevez et al., 1993; Krishna et al., 1999; Suzuki et al., 1999; Morita et al., 1999).

Of the several classes of factors that control cell size or cell growth, some are included in the results cited above. Chromosomal ploidy is a universal control factor for cell size (Brodsky and Uryvaeva, 1985; Conlon and Raff, 1999). Somatic polyploidization has been suggested to be a part of the mechanism for the evolution of body size in nematodes (Flemming et al., 2000). Cell cycle is generally an important control point determining the cell size. Protein synthesis and

transcription also seem to be important control points, which may be related to the dependence of cell size on chromosomal ploidy. Growth hormone, IGF-1, TGFβ, other growth factors and their signaling pathways could also affect cell size, at least in part, by stimulating protein synthesis or transcription. Decrease in cell size in *Drosophila* mutants for phosphoinositide-3 kinase Dp110 or IRS1-4 (Leevers et al., 1996; Böhni et al., 1999) and increase in GDF-8 mutant mice (McPherron et al., 1997) are examples of changes in cell size. Extracellular and intracellular transport of molecules may be another general determinant of the cell size (see Cossins, 1991).

The control mechanisms of cell number or cell proliferation are complex. Changes in cell numbers were noted in the mutants of insulin and TGF-β signaling components (Leevers et al., 1996; McPherron et al., 1997; Böhni et al., 1999). The size of an organ, and hence that of the whole body, in mammals is maintained by keeping the total cell mass, not the cell number, constant (Conlon and Raff, 1999). A good example of this is seen in a tetraploid mouse carrying larger and fewer cells (Henery et al., 1992). Various mechanisms keeping the organ size or cell mass constant have been proposed (Conlon and Raff, 1999; Brock and Gomer, 1999).

When searching the literature for mutations affecting body size, we noted that large mutants were much less frequently reported than small mutants. We find no literature on large body size mutants, either isolated by screening or arising spontaneously. Because cell growth and proliferation are regulated by both positive and negative regulators, one should be able to isolate large mutants carrying a defect in a negative growth regulator. To elucidate mechanisms of body size determination further, we have explored this idea by using *C. elegans*, and have succeeded in isolating many large mutants. We describe four of them and the common responsible gene, *egl-4*.

MATERIALS AND METHODS

Strains and handling of *C. elegans*

The following strains were used in addition to those isolated or prepared in this study, all of which are derivatives of a Bristol strain (N2) except for RW7000 and CB4856: wild-type strains N2, RW7000 (variety Bergerac) and CB4856, MT1072 *egl-4 (n477)* IV, MT1073 *egl-4 (n478)*IV, MT1242 *egl-4 (n612)*IV, MI162 *egl-18 (n162)* IV, FK240 *him-5 (e1490)*V, CB185 *lon-1(e185)*III, CB678 *lon-2 (e678)*X, CB4123 *lon-3 (e2175)*V, CB1370 *daf-2 (e1370)*III, DR63 *daf-4 (m63)*III, DR26 *daf-16 (m26)*I, GR1307 *daf-16 (mgDf50)*I, GR1311 *daf-3 (mgDf90)*X, CB1482 *sma-6 (e1482)*II and NU3 *dbl-1 (nk3)*V. All except for FK240 were provided by Caenorhabditis Genetics Center, Minneapolis, USA. Nematodes were handled essentially as described by Brenner (Brenner, 1974) and Sulston and Hodgkin (Sulston and Hodgkin, 1988).

Isolation of mutants

Approximately 1000 L4 hermaphrodites of wild-type N2 were mutagenized with 50 mM ethylmethane-sulfonate (EMS) for 4 hours. They were cultivated overnight on three NGM (nematode growth medium) plates (Brenner, 1974), and each young adult worm was picked and cultured separately in a plate for 4 to 6 days in a total of 50 or more plates. To obtain a synchronized population of F_2 progeny, embryos in F_1 adults from a parent were collected using alkaline hypochlorite solution (Sulston and Hodgkin, 1988), and allowed to hatch overnight in M9 buffer. Collected F_2 L1 larvae were grown in ten 9 cm NGM plates for 4 days. Worms that were longer than 1.5 mm were isolated under a dissecting microscope (Olympus SZX-12) equipped with a micrometer in the eyepiece. Isolated animals were grown and body sizes of 10 or more 4-day old adults in their progeny were measured to confirm their body size phenotype. These mutants were backcrossed with N2 four or five times to produce strains FK223 *ks60*, FK229 *ks61* and FK234 *ks62* used in this study. *ks16* mutant was also isolated with EMS, but from CE1377 *daf-6 (e1377)* in a screen for thermotaxis-defective mutants and backcrossed three times with N2 to give FK216 *ks16*. Double mutants carrying *ks61* and a mutation in *daf-16*, *daf-2*, *daf-3*, *sma-6* or *dbl-1* were made by crossing FK229 and DR26, GR1307, CB1370, CB1482 or NU3, and all the mutations in the doubles were confirmed by sequencing the respective genes.

Measurement of body size

To synchronize adult worms, young adults were picked up and grown on NGM plates. To measure 4-day old adults, worms were transferred once to remove the progeny. Alternatively, parent adults were allowed to lay eggs for 4-6 hours and removed, and the remaining eggs were cultured for five days to obtain a synchronous population (2.5-3 day adults). Animals were put in APF medium (del Castillo and Morales, 1967) containing 50 mM NaN3 and then put on a sectioned counting chamber (Erma Burker-Turk 1/10mm deep) after one hour. Their images were taken with Zeiss Axiophot 2 microscope and analyzed using an image-analyzing device 'Senchu-gazou-kaiseki-souchi SVK-3A' (Showa Denki Co. Ltd, Fukuoka, Japan). In this system, an outline and a centerline of an indicated animal are drawn, and the length of the centerline of any curve is measured as the body length, automatically. Then an indicated number (such as 50 or 100) of lines perpendicular to, and equally spaced along, the centerline are drawn to the outline. The lengths of these perpendiculars are measured automatically to give average and maximum diameters of the animal. The volume of the animal is estimated as the sum of the volumes of all the truncated cones obtained by rotating the divided images around the centerline, assuming that orthogonal sections of a worm are circular.

Measurement of lifespan

A population of worms synchronized by allowing to hatch for four hours was grown on a 6 cm plate. At the fifth day, well-grown adults were picked with a platinum picker onto fresh 3.5 cm NGM plates (five worms per plate, 50 or more per experiment) and grown in a wet chamber at 20°C. Thereafter, animals were occasionally transferred to fresh plates to remove the progeny, and the plates were examined at various times for live, dead and missing individuals. Missing animals were not included for the calculation of lifespan. Worms were scored as dead when they were unresponsive to prodding with a picker.

Complementation test and mapping of an *egl-4* mutation

For complementation tests between *ks16* and each of *ks60*, *ks61* and *ks62*, males of *ks16*; *him-5 (e1490)* carrying a green fluorescent protein (GFP) marker *flr-1::gfp/nls* were crossed with hermaphrodites of *ks60*, *ks61 or ks62*. F1 young adult hermaphrodites carrying the GFP marker were cultured, and four days later, their volumes were measured and compared with those of the single mutants, N2 and *ks16;him-5 Ex[flr-1::gfp/nls]* (*n*=20 or more). Complementation between *ks16* and *n477* or *egl-18* (*n162*) was similarly examined but without a GFP marker.

For mapping of an *egl-4* mutation based on single nucleotide polymorphisms (SNPs), FK216 *ks16* was crossed with CB4856 and large F2 worms were isolated. Their progeny were assayed for the volume, and approximately 100 candidate *ks16* homozygous recombinant lines were established. SNPs of these lines were analyzed either by digestion with a restriction enzyme (RFLP) or by sequencing
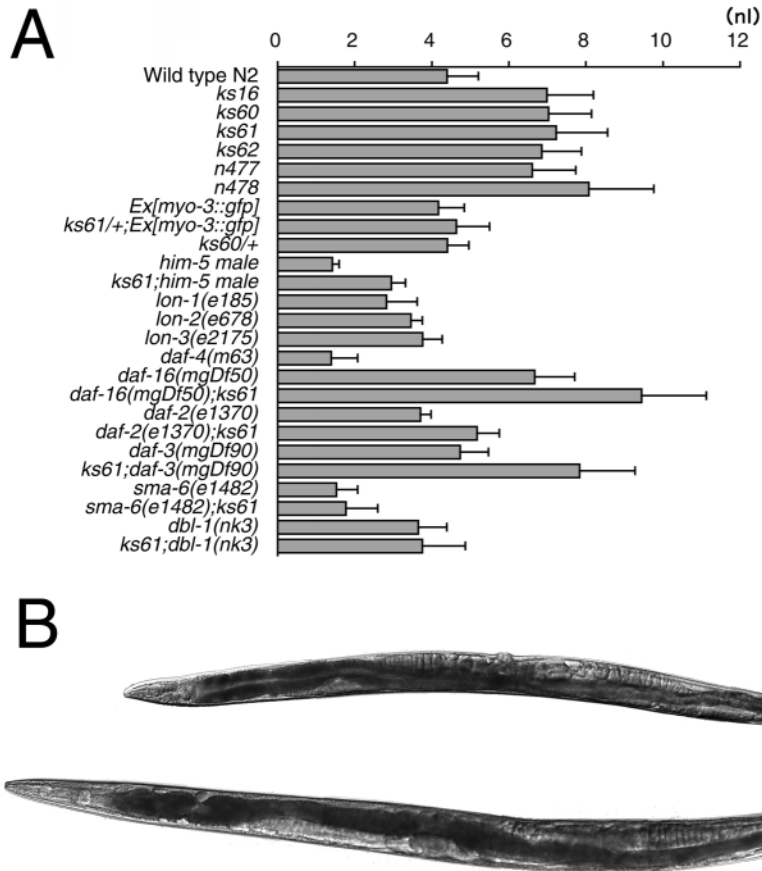
Fig. 1. Isolation of larger body size mutants. (A) Body volumes of 4-day old adults. The numbers of examined animals are as follows: N2 476, *ks16* 86, *ks60* 211, *ks61* 168, *ks62* 80, *n477* 53, *n478* 17, *Ex[myo-3::gfp]* 42, *ks61/+;Ex[myo-3::gfp]* 41, *ks60/+* 19, *him-5 (e1490)* male 10, *ks61;him-5* male 15, *lon-1(e185)* 10, *lon-2(e678)* 43, *lon-3(e2175)* 43, *daf-4(m63)* 13, *daf-16(mgDf50)* 32, *daf-16(mgDf50);ks61* 37 (results with two lines), *daf-2 (e1370)* 67, *daf-2 (e1370);ks61* 157 (results with three lines), *daf-3 (mgDf90)* 46, *ks61;daf-3* (results with three lines) 74, *sma-6 (e1482)* 29, *sma-6;ks61* 52, *dbl-1 (nk3)* 86 and *ks61;dbl-1* 32. Error bars indicate standard deviation (s.d.) (B) DIC microphotographs of a 4-day old adult of N2 (top) and a larger body size mutant (*ks60*, bottom). The pictures were taken in 5-8 parts per animal using an AxioCam CCD camera mounted on Zeiss Axiophot 2 with a 20×/0.5 lens and assembled. Scale bar: 200 µm.

PCR products as described by Wicks et al. (Wicks et al., 2001). Information of SNPs on LGIV was also obtained by sequencing and comparing N2 and CB4856 genomes.

Germline transformation

Germline transformation was done as described by Mello et al. (Mello et al., 1991). Circular plasmid DNAs were injected at 50 or 100 µg/ml and the total DNA concentration was adjusted to 100 µg/ml with the addition of pBluescript plasmid DNA. For rescue experiments, a *kin-8::gfp* plasmid (Koga et al., 1999) was co-injected as a marker.

Molecular biology

Mutation sites of *egl-4* (*ks16*, *ks61*, *ks62*, *n477*, *n478 and n612*) were identified in cDNA synthesized by RT-PCR from each mutant and that of *ks60* was identified in PCR products obtained from genomic DNA. The mutation sites for *ks16*, *ks61* and *n477* were confirmed in the genomic DNA.

To obtain *egl-4* promoter::gfp fusion genes, 5.5kb and 1.4kb sequences upstream of the initiation codons for PKGa and PKGb, respectively, were amplified by PCR and subcloned between *Pst*I and *Bam*HI sites of pPD95.69 vector carrying a gene for GFP (S65C) and SV40 NLS to produce Ppkga and Ppkgb plasmids. For expression of PKGa2 and PKGb1 cDNA, cDNA was amplified from yk341c9 and yk87g12 clones respectively, obtained from Y. Kohara, and then inserted at a *Kpn*I site downstream of a *Pst*I-*Bam*HI fragment carrying promoter a or promoter b in pPD49.26 vector to produce Ppkga-PKGa and Ppkgb-PKGb. pPD95.69 and pPD49.26 vectors were provided by A. Fire.

DNA sequencing was done in an ABI Prism Genetic Analyzer 310

or 3100. Isolation and analysis of DNA and RNA were done basically as described by Sambrook et al. (Sambrook et al., 1989).

Antibody preparation

The cDNA fragment of PKGa corresponding to amino acids 35-138 was subcloned into pGEX4T3 (Amersham Pharmacia Biotech) and pMALc-2 (New England BioLabs) to generate glutathione S-transferase (GST) and maltose-binding protein (MBP)-fusion proteins, respectively. GST and MBP-fusion proteins were expressed in *E. coli* DH5α and purified by affinity chromatography with glutathione sepharose and amylose resin, respectively. To produce anti-PKG antibody, rabbits were immunized with the GST fusion protein, and the PKG-specific antibody was affinity purified with an Affigel 10 (Bio-Rad) column coupled with the MBP fusion protein.

Immunoprecipitation and kinase assay

Wild-type N2 or *egl-4* (*ks16*) mutant worms were washed twice and disrupted by sonication in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1% Triton X-100 and one tablet of Complete Mini Protease Inhibitor Cocktail (Roche)/6 ml. After centrifugation at 15,000 *g* for 30 minutes, protein concentration of a lysate was determined by BCA assay kit (Pierce). The lysate containing 1 mg protein was incubated with 2 µl of purified anti-EGL-4 antibody and the immunocomplexes were collected with 20 µl of a 50% suspension of protein A Sepharose (Amersham). After washing three times with lysis buffer by centrifugation for 1 minute at 2000 rpm, immunocomplexes were examined by kinase assay and western blotting probed with anti-PKG antibody (western blot data not shown). The kinase assay was performed in 50 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 1 mM
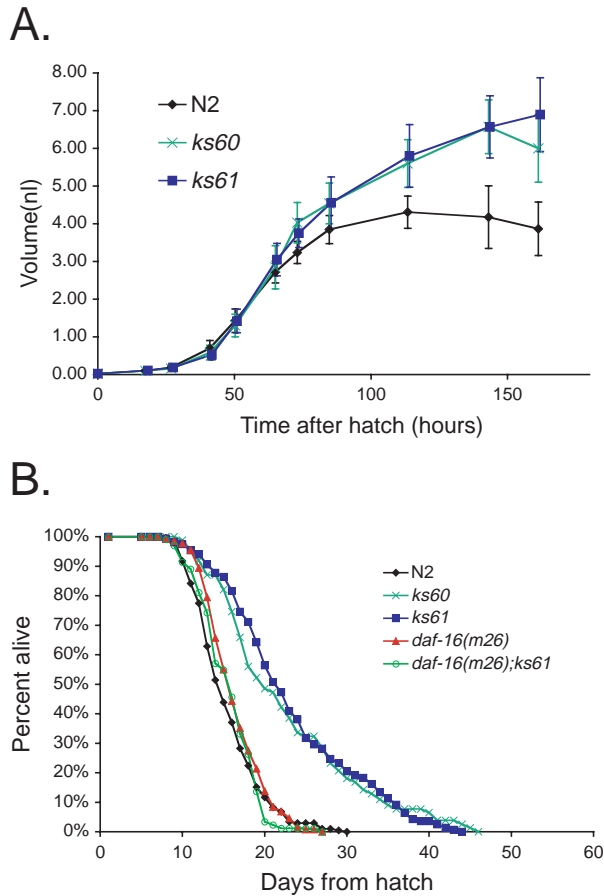
A.

B.

Fig. 2. Growth and lifespan of the mutants and wild type.
(A) Growth curves. The worms were synchronized by allowing them
to hatch for 4 hours and then grown at 20°C. Most worms became
adults before 51 hours. Each point represents results on an
independent culture plate. Average numbers of the examined animals
at a point are as follows: N2, 32; *ks60*, 31; *ks61*, 25. Error bar
indicates s.d. (B) Lifespan. The first time points represent the day
when synchronized L1 larvae were put on NGM plates. Mean
lifespan, standard deviation, number of examined animals excluding
the missing ones/total number of examined animals, and number of
tests performed independently are as follows: N2, 14.5±4.1 days,
207/300(6); *ks60*, 21.5±8.7 days, 80/150(2); *ks61,* 22.4±8.2 days,
143/200(4); *daf-16(m26),* 15.5±3.6 days, 131/150(3); *daf-16(m26);ks61,* 14.8±3.5 days, 88/150(3).

ditiothreitol, an indicated concentration of cGMP, 5 μCi [γ-^{32}P]ATP
and 100 mM KEMPTIDE (LRRASLG, Sigma) as substrate at 30°C
for 10 minutes. Reaction mixtures were absorbed on P81
phosphocellulose papers (Whatman) and they were washed four times
with 75 mM H$_3$PO$_4$. The papers were dried and the radioactivities
were measured by Cherenkov scintillation counting.

Morphological analysis of organs and cells

For anatomy of intestine, hypodermis or muscle, *dss-1p::gfp*, *col-19p::gfp* or *myo-3p::gfp* reporter plasmid was injected at 100 or 50
μg/ml into wild type, FK216 *egl-4* (*ks16*) and FK229 *egl-4* (*ks61*)
strains to obtain fluorescent transgenic lines. The *C. elegans dss-1*
gene in YAC Y119D3 on LGIII was cloned as a homolog of human
SHFM1/DSS-1 gene (Crackower et al., 1996) by K. Ishii and T. Tani
in our laboratory (unpublished). A 3.0 kb genomic fragment including
1.4 kb sequence upstream of the initiation codon was cloned into

*Pst*I/*Bam*HI sites of pPD95.75 vector plasmid (A. Fire) to produce a
dss-1p::gfp promoter fusion gene. This reporter is expressed
exclusively in the entire intestine. *col-19p::gfp* reporter plasmid
carrying 845 bp sequence upstream of the initiation codon of the *col-19* gene was constructed by inserting a PCR product from plasmid
pJA1 into *Pst*I/*Bam*HI sites of pPD95.77. The promoter drives
expression of GFP specifically in adult hypodermis as described for
lacZ expression (Liu et al., 1995). A *myo-3p::gfp* reporter carrying
approximately 2 kb upstream sequence of *myo-3* gene was made by
T. Ishihara by exchanging the *lacZ* gene in pPD18.49 (Fire et al.,
1990) for a GFP (S65C) gene. 4-day old fluorescent adults were
picked from agar plates and put directly into 50 mM sodium azide for
analysis.

For anatomy of gonad, adult worms were dissected and fixed
essentially as described by Francis et al. (Francis et al., 1995). The
fixed worms were stained with 5 μg/ml FITC and 50 mM NH$_4$Cl
in 1 ml PBS at 4°C overnight, transferred into a hole slide glass
(Toshinriko Co. Ltd., Japan) and extruded gonads were cut from uteri
to obtain half gonads for observation.

Anesthetized worms expressing GFP, and gonads stained with
FITC, were analyzed under a Zeiss LSM-410 confocal laser-scanning
microscope. An entire series of 1 μm optical sections were obtained
usually at the resolution of 512×512 (1024×1024 for Fig. 6A-D) with
20×/0.75 objective lens and pinhole of 12 (20 for gonads). For entire
intestine, hypodermis or muscle of a worm, two or three separate
sectionings were needed. A fluorescent muscle cell was examined
within a single section using a 40×/0.75 lens. Contrast and brightness
parameters for intestine, hypodermis or muscle were selected as those
that gave correct volumes of FITC-stained L3 larvae, the volumes of
which were also obtained by the automatic volume measurement
system described before. Most typical combinations of contrast and
brightness were 250/9000 and 250 or 225/9500. A series of optical
sections obtained as above were analyzed in a Zeiss KS 3D-Lite three-dimensional (3D) image processing system equipped with 3D for
LSM software, to reconstruct a 3D image and to calculate the volume
of an object. Segment (threshold) parameters for 3D image
construction and volume measurement were selected to give correct
volumes and normal morphology: low thresholds were 36 or 20 for
GFP and 55 for gonad, and the high threshold was 255. The precision
of the volume calculation by this KS 3D-Lite was confirmed in model
experiments using fluorescent Polybead Microparticles YG90
(Polyscience, Inc.; 87.9±9.14 μm in diameter) or an optical segment
of an FITC-stained worm of a specified volume.

Numbers of intestinal nuclei were determined after staining with
diamidino-phenylindole (DAPI) (Sulston and Hodgkin, 1988). Body
wall muscle cells were counted using polarized light microscopy
(Waterston et al., 1980).

RESULTS

Isolation of mutants with an increased body size

As a basis to study body size in *C. elegans*, we designed a
system to measure body length, diameters and volume of a
worm with an automatic image analysis (see Materials and
Methods). We screened progeny of the wild-type strain N2 that
had been mutagenized with EMS for mutants with increased
body volumes by using this system. To date, we have obtained
more than 20 independent mutants larger than the wild type in
adult volume by 50% to more than 100%. Three independent
mutants obtained in a screen (*ks60*, *ks61*, *ks62*), and another
mutant, *ks16*, which had been isolated by I. Mori in our
laboratory in a screen for thermotaxis-defective mutants, were
almost twice as large four days after reaching adult stage (Fig.
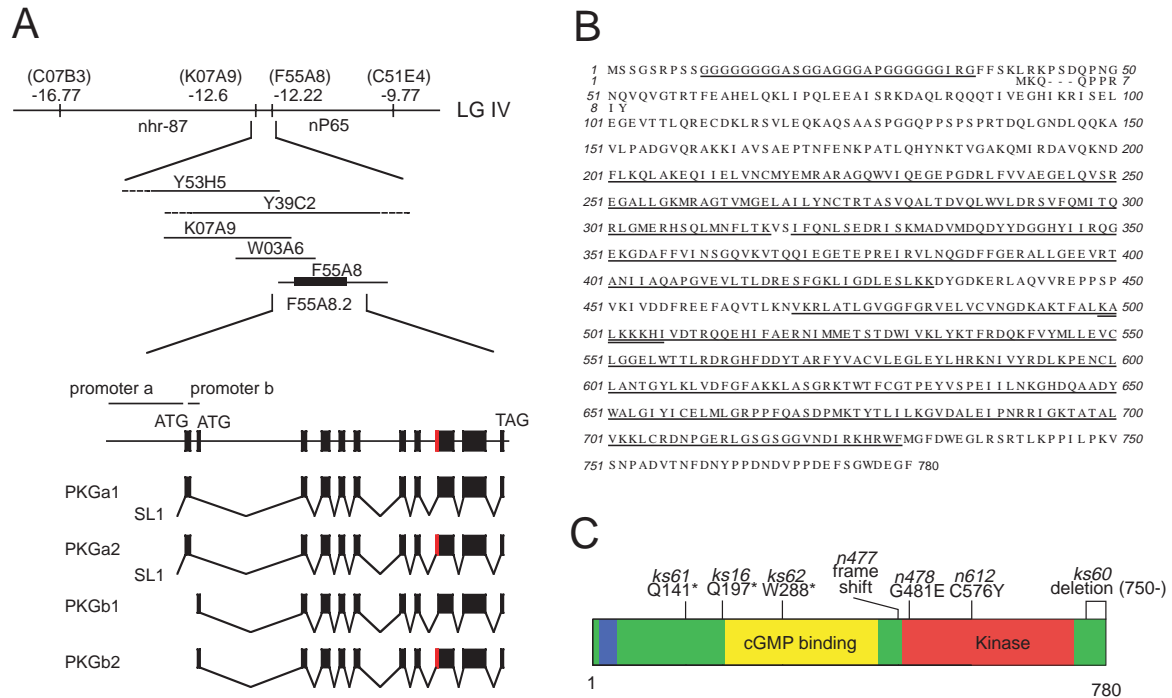1A,B), and were analyzed in this work.

A

(C07B3) −16.77   (K07A9) −12.6   (F55A8) −12.22   (C51E4) −9.77

LG IV

nhr-87   nP65

Y53H5

Y39C2

K07A9

W03A6

F55A8

F55A8.2

promoter a   promoter b

ATG ATG   TAG

PKGa1   SL1

PKGa2   SL1

PKGb1

PKGb2

B

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  1 MSSGSRPSSGGGGGGGGGASGGAGGGAPGGGGGGIRGFFSKLRKPSDQPNG 50
  1                                   MKQ---QPPR 7
 51 NQVQVGTRTFEAHELQKLIPQLEEAISRKDAQLRQQQTIVEGHIKRISEL 100
  8 IY
101 EGEVTTLQRECDKLRSVLEQKAQSAASPGGQPPSPSPRTDQLGNDLQQKA 150
151 VLPADGVQRAKKIAVSAEPTNFENKPATLQHYNKTVGAKQMIRDAVQKND 200
201 FLKQLAKEQIIELVNCMYEMRARAGQWVIQEGEGPGDRLFVVAEGELQVSR 250
251 EGALLGKMRAGTVMGELAILYNCTRTASVQALTDVQLWVLDRSVFQMITQ 300
301 RLGMERHSQLMNFLTKVSIFQNLSEDRISKMADVMDQDYYDGGHYIIRQG 350
351 EKGDAFFVINSGQVKVTQQIEGETEPREIRVLNQGDFFGERALLGEEVRT 400
401 ANIIAQAPGVEVLTLDRESFGKLIGDLESLKKDYGDKERLAQVVREPPSP 450
451 VKIVDDFREEFAQVTLKNVKRLATLGVGGFGRVELVCVNGDKAKTFALKA 500
501 LKKKHIVDTRQQEHIFAERNIMMETSTDWIVKLYKTFRDQKFVYMLLEVC 550
551 LGGELWTTLRDRGHFDDYTARFYVACVLEGLEYLHRKNIVYRDLKPENCL 600
601 LANTGYLKLVDFGFAKKLASGRKTWTFCGTPEYVSPEIILNKGHDQAADY 650
651 WALGIYICELMLGRPPFQASDPMKTYTLILKGVDALEIPNRRIGKTATAL 700
701 VKKLCRDNPGERLGSGSGGVNDIRKHRWFMGFDWEGLRSRTLKPPILPKV 750
751 SNPADVTNFDNYPPDNDVPPDEFSGWDEGF 780
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C

ks61 Q141*   ks16 Q197*   ks62 W288*   n477 frame shift   n478 G481E   n612 C576Y   ks60 deletion (750-)

cGMP binding   Kinase

1   780

Fig. 3. Identification of the *egl-4* gene. (A) Physical map of the *egl-4* region, related genomic clones, exon-intron organization of F55A8.2 and structure of mRNAs. Black boxes indicate protein-coding regions. Red boxes represent 5′ extensions of exon 8 in mRNAs for a2 and b2. (B) Amino acid sequences of PKGa1 and PKGb1 predicted by the cDNAs. They differ only in the N termini (the sequence of a1 is the upper line). Underlines denote a glycine-rich domain, two cGMP binding domains and a kinase domain. Double underlines denote putative NLS (see Discussion). (C) Schematic of the primary structure of the EGL-4(PKGa1) protein and the mutation sites. Blue box represents the glycine-rich domain.

The average size of the mutants in 4-day old adults (4 days after becoming adults) is approximately 1.7 mm long, 90-100 μm in maximum diameter and 7-8 nl in volume in contrast to 1.4 mm, 80 μm and 4.5 nl, respectively, in the wild type. The mutations are recessive because heterozygotes (−/+) have a normal body size. *ks61;him-5* males are also bigger than *him-5* males (Fig. 1A). *lon-1*, *lon-2* and *lon-3* mutants are not larger, or are smaller, than the wild type (Fig. 1A), although longer. Known small body size mutants *daf-4*, *sma-6*, *sma-2*, *sma-3* and *sma-4* are really smaller (Fig. 1A and data not shown).

Morphology of the mutants is grossly normal as noted by normal ratios of body length to maximum diameter (around 18) and by Nomarsky photomicrographs (Fig. 1B), but the intestine is darker (Din phenotype).

Growth of the mutants and the wild type was monitored by their volumes (Fig. 2A; results on *ks16* and *ks62* are similar and not shown). The mutant embryos and larvae are not significantly larger than those of the wild type. The mutant body size markedly increases in the adult stage. Although growth of the wild type ceases approximately 2 days after the adult stage is reached, the mutants continue to grow for 4 or 5 days.

The four mutants have weak egg-laying (Egl) phenotypes (Trent et al., 1983). Namely, egg-laying is slightly delayed on the average, resulting in more and irregularly arranged embryos in the uterus in 2-day old adults (data not shown), and the brood size is slightly decreased (number of larvae born per parent = 316±48 for N2, 200±37 for *ks60*, 229±36 for *ks61* and 227±25 for *egl-4 (n477)*, *n*=10). We found that all four mutants

had an extended lifespan: the mean and maximum lifespans were increased by approximately 50% over those of the wild type N2 (Fig. 2B and data not shown).

The four mutants are *egl-4* mutants

Because the four mutants showed similar phenotypes, complementation tests were done. *ks16* did not complement with each of the other three in the body sizes as described under Materials and Methods. We initially mapped *ks16* with Tc1 polymorphisms (Williams et al., 1992), and further with SNPs on the left arm of chromosome IV. In this region, there are two known egg-laying defective genes *egl-4* and *egl-18*. *ks16* did not complement with *egl-4* (*n477*), and did complement with *egl-18* (*n162*) in body size and Egl phenotypes. *egl-4* mutants were reported to have an increased body length (Daniels et al., 2000). *egl-4* mutants *n477* and *n478* had an increased body volume (Fig. 1A). These results, together with identification of mutation sites in the mutants, indicate that all the four big mutants independently isolated are *egl-4* mutants.

Identification of the *egl-4* gene

To identify the *egl-4* gene, we mapped *egl-4* (*ks16*) using SNPs to a region between cosmids K07A9 and F55A8 (Fig. 3A). DNA from yeast cells carrying YAC Y39C2 located in this region, but not DNA for another YAC Y53H5, completely rescued body size, Egl and Din phenotypes of *ks16* mutation, thus supporting the notion that *egl-4* is located in this region. This region spans approximately 50 kb and contains only two probable candidate genes F55A8.2a and F55A8.2b. These
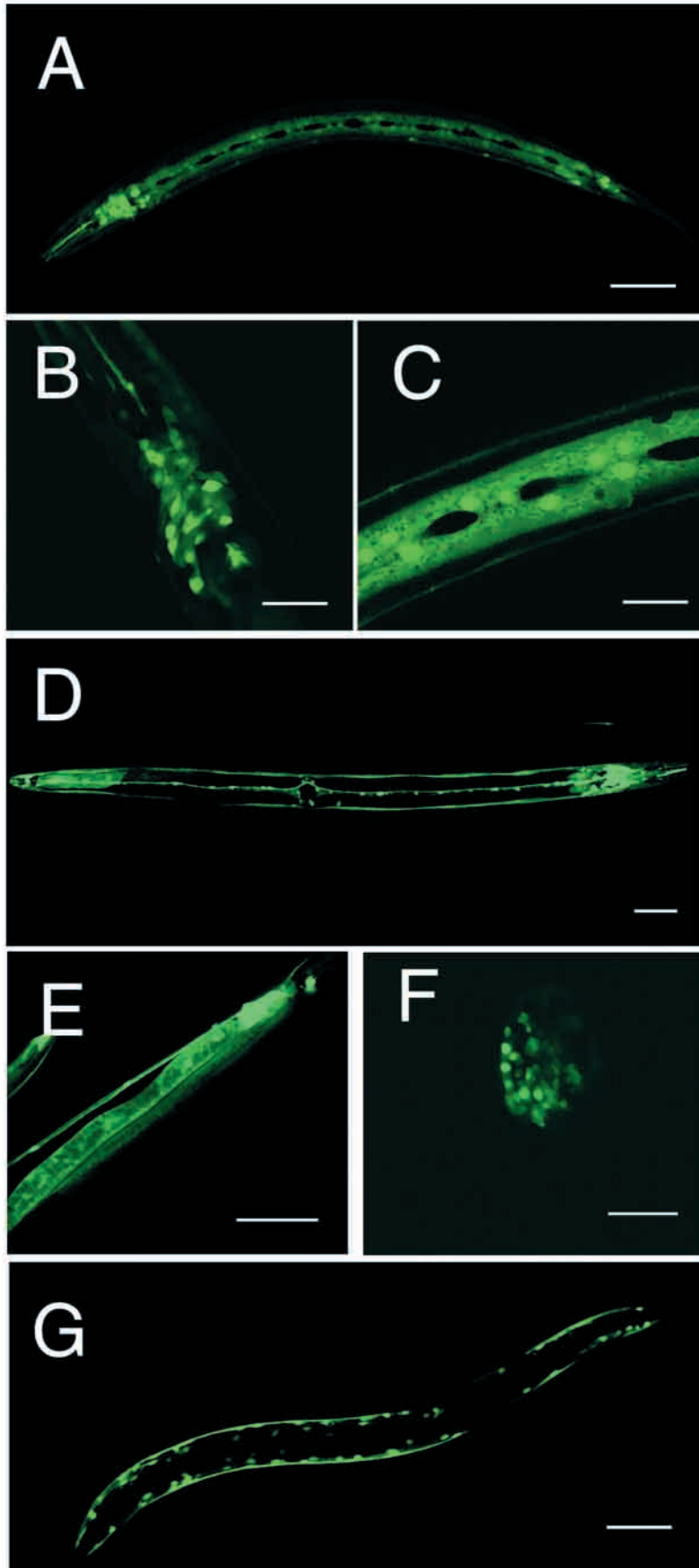
Fig. 4. Expression patterns of *egl-4* promoter::*gfp/nls* fusions. Larvae (A-E,G) and an embryo (F) expressing green fluorescent protein (GFP) under promoter a (A-F) or promoter b (G). B and C are higher magnification images of head neurons (B) and a part of hypodermis (C) of the worm shown in A. Stages are L3 (A-C), L4 (G), young adult (D,E) and embryo (F). A-C, E and G are lateral views, and D is a ventral view. The images were obtained with a Zeiss LSM-410 (A-C,G) or LSM-510 (D-F) confocal microscope. Scale bars: 50 μm (A,D,E,G) and 20 μm (B,F).

genes encode homologues of a mammalian cyclic GMP-dependent protein kinase (PKG) on the basis of the genome sequence databases. We sequenced cDNAs obtained by RT-PCR and cDNA clones obtained from Y. Kohara, to identify four alternative forms of the cDNA. F55A8.2a and F55A8.2b encoding PKGa and PKGb differ in the promoters and the first exons. Use of an alternative splice donor site at the edge of exon 8 results in three additional amino acids in PKGa2 and PKGb2 (Fig. 3A). PKGa1 is predicted to consist of 780 amino acids with a glycine-rich domain, two tandem cyclic nucleotide-binding domains and a kinase domain, whereas PKGb1 consists of 735 amino acids with the glycine-rich domain deleted (Fig. 3B,C). These PKGs are highly conserved in other species such as man and *Drosophila*. *C. elegans* PKGa1 has 48.3% and 41.6% amino acid identity over the entire protein, and 63.8% and 58.7% identity in the kinase domain, with the human cGKIa and cGKII (Accession numbers D45864, D70899), respectively.

We identified mutation sites for the seven *egl-4* alleles (Fig. 3C). The alleles *ks61*, *ks16* and *ks62* carry a nonsense mutation near the N terminus, and are probably null. *ks60* has a 958 bp deletion including the entire last exon. The allele *n477* has an eight-base pair insertion between V464 and T465, which causes a frameshift and a stop codon and leads to loss of the kinase domain. *n478* and *n612* have a missense mutation in the kinase domain. The glycine 481 and cysteine 576 for which *n478* and *n612*, respectively, have a substitution are conserved among species. This cysteine 576 might form a disulfide bond essential for structural integrity of the protein. As to the body size, *n478* is the biggest among those examined, *n612* is the smallest (a weak allele) and the other five have intermediate and similar body sizes (Fig. 1A and data not shown for *n612*).

egl-4 cDNA a2 and b1 were expressed under promoters a and b, respectively, in *ks16*. Expression under promoter a completely rescued body size, Egl and Din phenotypes, but expression under promoter b failed to rescue (volumes were 5.0±0.39 nl for N2, 7.1±0.66 nl for *ks16*, 4.3±0.73 nl for *ks16;Ex[Ppkga/PKGa]* and 6.9±0.57 nl for *ks16;Ex[Ppkgb/PKGb]* 120 hours after hatch, n=16-33).
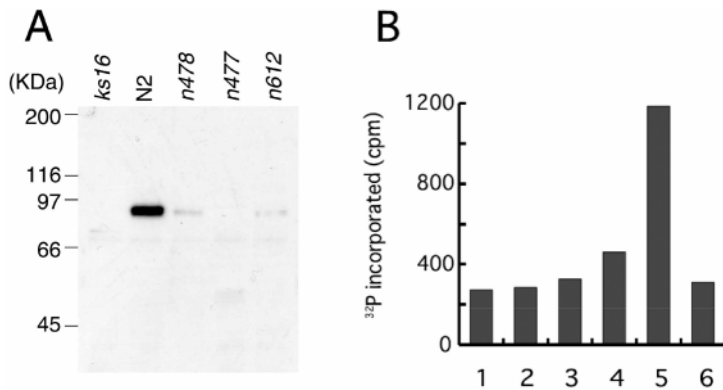
Fig. 5. Characterization of endogenous EGL-4 proteins. (A) Extracts of wild-type N2 and indicated mutants (10 μg protein each) were separated in a 7.5% polyacrylamide-SDS gel, subjected to western blotting and probed with purified anti-EGL-4 antibodies. (B) Protein kinase activities in extracts from *egl-4* (*ks16*) (lane 1) or N2 (lanes 2-6) were assayed in the absence (lane 2) and presence of 0.1 μM (lane 3), 1 μM (lane 4) or 10 μM (lanes 1, 5, 6) cGMP. For lane 6, the antibodies were preincubated with the antigen GST-EGL-4.

Expression patterns

To examine expression patterns of the *egl-4* gene, we prepared constructs to express a GFP fusion protein with a nuclear localization signal (NLS) under the control of promoter a (5.5 kb) or promoter b (1.4 kb). Under promoter a, GFP was predominantly expressed in head neurons (Fig. 4A,B), a few cells in the tail (Fig. 4A) and hypodermis except for seam cells (Fig. 4A,C). A total of 30-50 fluorescent neurons of varying intensities were observed in the head. Stronger or more consistent expression was observed in RMDV, RMD, SMDV, RIB, ASK and probably RMDD and SMDD neurons. Expression in AWC, ASE, AVJ and ASH was sometimes seen. In the tail, DVB and DVC neurons were probably expressing. GFP was also expressed in ventral cord neurons (Fig. 4D) and intestine, typically near the tail (Fig. 4D,E). L2 and L3 larvae show extensive expression most notably in head neurons and hypodermis, and the expression is weaker in L4 and adults. Expression is also seen in later embryos (Fig. 4F). Under promoter b, GFP was expressed in body wall muscles (Fig. 4G).

EGL-4 proteins have protein kinase activity

We developed polyclonal antibodies to a common N-terminal part (35-138 in PKGa1) of the EGL-4 proteins. The purified antibodies detected a single protein band of approximately 80 kDa in a wild-type extract (Fig. 5A), the size of which is consistent with the molecular masses of the predicted products of the *egl-4* gene (735 and 780 amino acids). The protein band was not detected in *ks16* nonsense and *n477* frameshift (and nonsense) mutants, and the amount of the protein was significantly decreased in *n478* and *n612* missense mutants. Instability of mutant products might be a major reason for decrease of the 80 kDa protein band in *n478* and *n612* mutants and absence of a protein band in *n477* in Fig. 5A. These results showed specificity of the antibodies used.

EGL-4 proteins immunoprecipitated with the antibodies were assayed for kinase activity on KEMPTIDE peptide carrying a serine residue, in the presence of various concentrations of cGMP (Fig. 5B). An extract of the wild-type N2 showed little or no kinase activity in the absence of cGMP (lane 2) as compared to the activity of a *ks16* mutant extract

Table 1. Volume of an organ or cell in wild type and *egl-4* mutants

Object	Strain	Genotype	Volume (nl) (mean±s.d.)	Relative value of the mean (%)	Average body length (mm)	Number examined
Intestine of 4-day-old adults	FK290	*Ex[dss-1p::gfp]*	0.89±0.24	100	1.30	21
	FK296	*egl-4 (ks16);Ex[dss-1p::gfp]*	1.43±0.48	161	1.79	20
	FK299	*egl-4 (ks61);Ex[dss-1p::gfp]*	1.59±0.32	178	1.67	20
Hypodermis of 4-day-old adults	FK321	*Ex[col-19p::gfp]*	1.08±0.43	100	1.26	7
	FK322	*egl-4 (ks16);Ex[col-19p::gfp]*	1.99±0.91	184	1.70	13
	FK323	*egl-4 (ks61);Ex[col-19p::gfp]*	1.90±0.62	176	1.69	17
Muscle of 4-day-old adults	FK254	*Ex[myo-3p::gfp]*	0.73±0.16	100	1.37	11
	FK257	*egl-4 (ks16);Ex[myo-3p::gfp]*	1.04±0.24	142	1.66	9
	FK271	*egl-4 (ks61);Ex[myo-3p::gfp]*	0.95±0.21	130	1.73	9
Single body wall muscle cell of 4-day-old adults	FK254	*Ex [myo-3p::gfp]*	$7.1±3.4×10^3$*	100	1.29	20
	FK257	*egl-4 (ks16);Ex[myo-3p::gfp]*	8.8±3.4*	124	1.54	20
	FK271	*egl-4 (ks61);Ex[myo-3p::gfp]*	9.6±3.5*	136	1.55	20
Dissected half gonad of 2-day-old adults	N2		0.59±0.11	100		20
	FK216	*egl-4 (ks16)*	0.62±0.10	105		21
	FK223	*egl-4 (ks60)*	0.60±0.14	102		21
Dissected half gonad of 4-day-old adults	N2		0.70±0.16	100		20
	FK216	*egl-4 (ks16)*	0.52±0.14	74		23
	FK223	*egl-4 (ks60)*	0.51±0.13	73		22

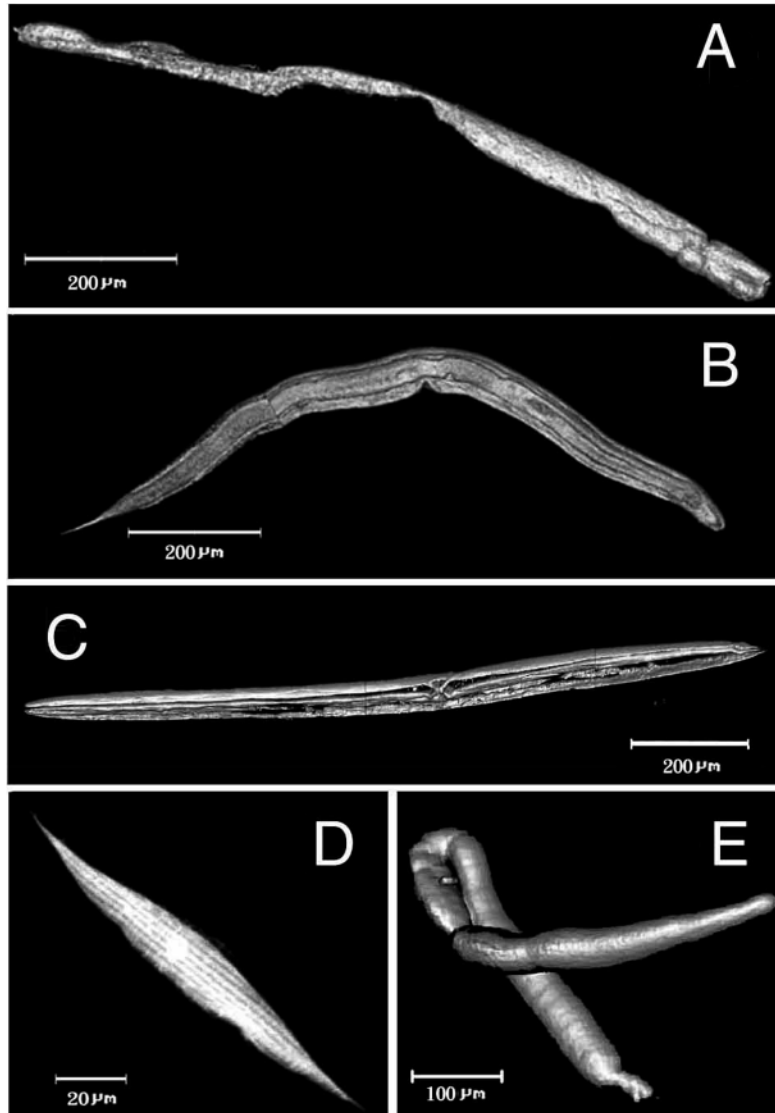*Volume in $μ^3$, all other volumes are in nl.

Fig. 6. Three-dimensional (3D) images of organs and a cell in the wild type. 3D reconstructed images of intestine (A), hypodermis (B), muscles expressing *myo-3p::gfp* (C), a body wall muscle cell (D) and a dissected half gonad (E) are shown. The stages are 4-day old (A-D) or 2-day old (E) adults.

sometimes partly bloated and more fragile (data not shown).

Results of volume measurements are presented in Table 1. The volume of whole intestine is increased by 60-80% in the 4-day adult mutants. The average number of intestinal nuclei did not change in the mutants even in the 4-day adults (33.3 and 33.6 for *ks16* and wild type, *n*=21 and 20), indicating that the number of intestinal cells (20) did not change in the mutants. Therefore, intestinal cells in the mutants must be 60-80% larger on average. The volume of hypodermis is increased by approximately 80% in the mutants. In the muscles expressing *myo-3p::gfp* (body wall muscles, vulval, uterine and intestine-associated muscles) (Okkema et al., 1993), a great majority of which consists of body wall muscles, the volume increased by 42% or 30% in the mutants. For body wall muscles, direct analysis of a single, fluorescently isolated cell was possible because of occasional loss of the extrachromosomal GFP fusion gene (Fig. 6D). Body wall muscle cell volumes in the mutants increased by 24% or 36%. The number of body wall muscle cells was not increased in the mutants (48 or 47 in adjacent two quadrants in *ks16* as was in N2). Volume of a dissected half gonad excluding the uterus in the mutants of 2-day old adults was similar to that of the wild type, and it was rather decreased in 4-day adults. This finding and somewhat abnormal morphology suggest a defect in the mutant gonad. The uterus containing embryos was not precisely analyzed, but it may be somewhat larger in the mutants because of the Egl phenotype.

(lane 1), and the activity was stimulated by cGMP dependent on the concentration (lanes 3, 4 and 5). These results indicate that endogenous EGL-4 proteins have protein kinase activity.

Some organs and cells in the mutants have an increased volume

Because *egl-4* mutants have a markedly increased body size in 4-day old adults, the number or size of some types of cells is probably increased. We analyzed morphology of four major organs. To do this, a whole animal expressing GFP specifically in intestine, hypodermis or muscle, or dissected and stained gonad, was examined using a confocal laser-scanning microscope. Based on a series of sectional fluorescent images, a 3D image was reconstructed and its volume was calculated with an image processing system as described in Materials and Methods. Fig. 6 shows examples of 3D images of these organs in the wild type. The morphology of intestine, hypodermis and muscle in the *egl-4* mutants in the 4-day adults looked normal as compared to those of the wild type. The morphology of a dissected gonad was somewhat abnormal: a mutant gonad was more irregular,

Genetic interaction of the *egl-4* gene

Loss of function or reduced function mutations in several genes such as *daf-2* (insulin receptor homolog), *age-1* (PI-3 kinase) and *pdk-1* (Akt/PKB kinase) in the insulin-like branch of the pathway controlling dauer larva formation in *C. elegans* result in an increased lifespan (Kenyon et al., 1993; Paradis et al., 1999). Because extension of the lifespan in these mutants is suppressed by a mutation in a downstream gene *daf-16* encoding a putative transcription factor (Kenyon et al., 1993; Ogg et al., 1997; Paradis et al., 1999), we asked whether a *daf-16* mutation also suppresses extension of the lifespan in an *egl-4* mutant. Lifespan of a *daf-16* (*m26*); *egl-4* (*ks61*) double mutant was indistinguishable from that of *daf-16* (*m26*) single mutant and was similar to that of the wild type, indicating that the lifespan extension was suppressed by a *daf-16* mutation (Fig. 2B). This result was confirmed for *daf-16* (*mgDf50*) null (complete deletion) mutation in a separate experiment: the lifespan for wild type, *daf-16*, *egl-4(ks61);daf-16*, and *egl-4* was 14.2±2.6, 11.6±2.0, 13.7±2.4 and 18.8±5.7 days, respectively.

Interaction with respect to body size of an *egl-4* mutation

with *daf-16* (*m26*) was not clear because three independent double mutants gave different results (data not shown). We prepared two double mutant lines carrying *egl-4* (*ks61*) and a null mutation *mgDf50* in *daf-16*. Interestingly, the *daf-16* (*mgDf50*) mutant showed an increased body volume as did *egl-4* mutants, and both the double mutant lines were bigger than either single mutant (Fig. 1A; see also Discussion). We also prepared three double mutant lines carrying a reference allele (*e1370*) of *daf-2* and *egl-4* (*ks61*). The *daf-2* mutant was slightly smaller than N2 and body sizes of the double mutants were in-between those of *daf-2* and *egl-4* (Fig. 1A).

Several phenotypes of *egl-4* mutants such as Egl and partially constitutive dauer formation (Daf-c) were reported to be suppressed by *daf-3* (*e1376*) mutation and partially by *daf-5* (*e1385*) mutation (Trent et al., 1983; Daniels et al., 2000). We made three double mutant lines carrying *egl-4* (*ks61*) and a null mutation *daf-3* (*mgDf90*). The *daf-3* (*mgDf90*) single mutant showed normal body size, and this *daf-3* mutation did not suppress the increased body volume (Fig. 1A), nor the body length (data not shown) of the *egl-4* mutant.

We also tested interaction of *egl-4* with Sma TGFβ pathway (*dbl-1-sma-6/daf-4-sma-2, 3* and *4*) by constructing a double mutant with *sma-6* (*e1482*) or *dbl-1* (*nk3*) null mutation. Both mutations clearly suppressed *egl-4* (*ks61*) mutation in body size (Fig. 1A).

DISCUSSION

Isolation of large body size mutants

We developed a system for rapid and accurate measurement of the body sizes of *C. elegans* using an automatic image analysis. By taking advantage of this system, we succeeded for the first time in a systematic isolation of mutants with volumes 50% larger than those of the wild type by 50% or more. To date, approximately 20 such large mutants have been independently isolated, the largest of which is over double the size. Synchronization of animals and less dense culture conditions helped identification of a mutant. Another key was to measure volumes of 4-day old adults in the progeny of an isolate to confirm that it was really large on the average. The four mutants that were isolated earlier and which were nearly twice as big were found to be mutants of the same *egl-4* gene. However, because some of more recent isolates are not allelic with *egl-4* or show phenotypes differing from those of the *egl-4* mutants, it is certain that various types of mutants have been obtained. Similar careful experiments may allow isolation of large mutants in other organisms as well.

Structure and function of EGL-4 proteins

Before *egl-4* was cloned, cGMP-dependent protein kinases had been isolated and characterized in *Drosophila* and mammals. Their structure, conservation, expression and functions were reviewed (Lohmann et al., 1997), although much remains unknown as to the roles and related mechanisms.

We found four types of *egl-4* cDNA PKGa1, a2, b1 and b2 (Fig. 3A), and Stansberry et al. (Stansberry et al., 2001) found three types, CGK-1A, 1B and 1C, without assignment to EGL-4. In a comparison of the expected products, our PKGa1 (780 amino acids) and PKGb1 (735 amino acids) probably correspond to CGK-1A and CGK-1B, respectively. We did not find PKG-1C (744 amino acids) and they did not find our PKGa2 and b2. However, all these expected products differ only in the N terminus or three amino acids in exon 8, and therefore we presume that their functions are similar. More important differences among the genes may be their promoters and expression patterns (see below).

EGL-4 proteins isolated with the purified antibodies from an extract of the wild-type *C. elegans* showed cGMP-dependent protein kinase activity (Fig. 5). The antibodies we used were prepared against a common part for all the expected products, suggesting that the proteins we examined were a mixture of endogenous products. Stansberry et al. (Stansberry et al., 2001) expressed CGK-1C in cultured cells and examined the kinase activity in vitro. These kinase activities are probably essential for the function of the EGL-4 proteins because three mutations *n477*, *n478* and *n612* either lead to deletion of, or are found in, the kinase domain (Fig. 3C). Mammalian cGKI has a functional NLS of eight amino acids, which resembles that of interleukin-1α, in the kinase domain (Gudi et al., 1997). A homologous sequence (KALKKKHI) is found in PKGa1 at a corresponding position (Fig. 3B).

Knockout (KO) mice for a cGMP-dependent protein kinase were reported previously. cGKII KO mice were slightly dwarf (14% less heavy and 16% shorter at 8-10 weeks) because of impaired bone growth, and showed intestinal secretary defects (Pfeifer et al., 1996). In contrast, cGKI KO mice showed gross intestinal distention or hypertrophy and showed defects in NO/cGMP-dependent smooth muscle relaxation (Pfeifer et al., 1998). The body size phenotype of *egl-4* mutants seems to be opposite to that of cGKII-deficient mice, and similar to that of cGKI KOs with respect to volume increase in intestine, although it is not clear if cGKI KO mice were larger than the wild type.

C. elegans has a gene (C09G4.2) potentially encoding another PKG. The expected product of this gene has 37.7% and 54.7% amino acid identity with EGL-4a1 overall and in the kinase domain, respectively. It has a higher homology to human cGKIa than to cGKII (41.8% compared with 35.5%) as does EGL-4, but the homologies are lower than those between EGL-4 and mammalian counterparts.

Functional foci of the *egl-4* gene

Expression of the GFP reporter was observed predominantly in head neurons and hypodermis under *egl-4* promoter a and in body wall muscles under promoter b (Fig. 4). Functional foci of the *egl-4* gene were suggested to reside in head neurons or hypodermis by rescue of body size, Egl and Din phenotypes of the *ks16* mutant with expression of a cDNA under promoter a but not with promoter b (see Results). Fujiwara et al. (Fujiwara et al., 2002) showed that expression of an *egl-4* cDNA under *tax-4* promoter rescued all the phenotypes of an *egl-4* mutant, including that of body size. L'Etoile et al. (L'Etoile et al., 2002) rescued the defect in olfactory adaptation of *n479* mutant by expression of a cDNA mainly in AWC sensory neurons in the head. These results suggest that expression of the *egl-4* gene is required only in some neurons in the head for various functions including control of body size. Thus, the action of EGL-4 on the size of intestine, hypodermis and muscle may be mediated by molecules secreted by those *egl-4* expressing neurons. Alternatively, EGL-4 could also function cell-autonomously in

these major organs for their size control as well as non cell-autonomously in neurons.

Genetic pathways related to *egl-4*

We have shown lifespan extension and increased body volume as the novel phenotypes of *egl-4* mutants. Demonstration of volume increase, not only of increase in body length (Daniels et al., 2000), is critical in discussing body size because *lon* mutants are longer but their volumes are not increased (Fig. 1A). Increase in both body size and lifespan resulting from a single mutation could be interesting in the sense that body size and lifespan are roughly correlated in vertebrates, although these are genetically separable in *egl-4*, as discussed below.

Lifespan extension in *egl-4* (*ks61*) mutant was suppressed by a *daf-16* mutation (Fig. 2B and text), suggesting that *egl-4* controls lifespan through the insulin-like signaling pathway. However, *daf-16;egl-4* double mutants were larger than either single mutant, and *daf-2;egl-4* doubles had a size in-between those of the single mutants (Fig. 1A). Both these results are interpreted to mean that *egl-4* functions to control body size independently of the insulin-like signal pathway that includes *daf-16* and *daf-2*.

As to the genetic interaction of *egl-4* in the body size control, large body size of an *egl-4* mutant was not suppressed by a *daf-3* mutation either suggesting that the pathway in which *egl-4* functions to control body size is different from the TGFβ branch of the dauer control pathway to which *daf-3* belongs. However, suppression of body size of an *egl-4* mutant by a *sma-6* or *dbl-1* mutation (Fig. 1A) suggests that EGL-4 functions upstream of DBL-1 and SMA-6 that act in another TGFβ pathway as a ligand and a receptor, respectively (Krishna et al., 1998; Suzuki et al., 1999; Morita et al., 1999). This result seems reasonable because this pathway is known to control body size (Patterson and Padgett, 2000), and is also important to elucidate mechanisms for the control of body size by *egl-4*. Thus, functions of *egl-4* are related to at least three signaling pathways. These multiple functions of *egl-4* in multiple pathways can be explained if EGL-4 kinase phosphorylates multiple substrates. Because EGL-4 requires cGMP for effective kinase activity [Fig. 5B and Stansberry et al. (Stansberry et al., 2001)], a guanylyl cyclase must be a component upstream of EGL-4.

Hsin and Kenyon (Hsin and Kenyon, 1999) and Patel et al. (Patel et al., 2002) reported that germline ablation induced extension of lifespan, and both lifespan extension and body size increase by approximately 50%, respectively, suggesting that a germline signal represses lifespan and growth. Extension of lifespan required DAF-16 but gigantism did not. Although *egl-4* mutants probably do not lack this hypothetical germline signal, EGL-4 could possibly be a component related to this signal.

Mechanisms for the control of body size by *egl-4*

We found that the volume of intestine or hypodermis was much increased in the *egl-4* mutants (Table 1). Volumes of muscles expressing *myo-3* and body wall muscle cells also increased. Although volume of uterus may be increased in adults of *egl-4* mutants, volume of gonad excluding uterus was not increased in the mutants (Table 1E). This is important because *egl* mutants in general could be regarded to be bigger because of bloating of the gonad. To our knowledge, this is the first paper reporting volume of an organ in *C. elegans,* and we established the methods used for the volume measurement. In contrast, cell numbers in intestine and body wall muscle were not increased. We suggest that volume increase in the cells of those major organs contribute to most of the body size increase in the mutants. In mammals, organ size seems to be kept constant even when cell size is increased (see Introduction). The difference between mammals and *egl-4* mutants and *Drosophila* mutants such as *chico* (Böhni et al., 1999) in organ size control may suggest that invertebrates generally have a different mechanism. Alternatively, similar cases of increase in organ size may also be found in mammals. Mechanisms for the control of cell size by *egl-4* should be elucidated in the future.

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