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The CRAL/TRIO and GOLD Domain Protein CGR-1 Promotes Induction of Vulval Cell Fates in *Caenorhabditis elegans* and Interacts Genetically With the Ras Signaling Pathway

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ABSTRACT

Ras-mediated signaling is necessary for the induction of vulval cell fates during *Caenorhabditis elegans* development. We identified *cgr-1* by screening for suppressors of the ectopic vulval cell fates caused by a gain-of-function mutation of the *let-60 ras* gene. Analysis of two *cgr-1* loss-of-function mutations indicates that *cgr-1* positively regulates induction of vulval cell fates. *cgr-1* is likely to function at a step in the Ras signaling pathway that is downstream of *let-60*, which encodes Ras, and upstream of *lin-1*, which encodes a transcription factor, if these genes function in a linear signaling pathway. These genetic studies are also consistent with the model that *cgr-1* functions in a parallel pathway that promotes vulval cell fates. Localized expression studies suggest that *cgr-1* functions cell autonomously to affect vulval cell fates. *cgr-1* also functions early in development, since *cgr-1* is necessary for larval viability. CGR-1 contains a CRAL/TRIO domain likely to bind a small hydrophobic ligand and a GOLD domain that may mediate interactions with proteins. A bioinformatic analysis revealed that there is a conserved family of CRAL/TRIO and GOLD domain-containing proteins that includes members from vertebrates and Drosophila. The analysis of *cgr-1* identifies a novel *in vivo* function for a member of this family and a potential new regulator of Ras-mediated signaling.

RAS is a pivotal protein in signal transduction pathways that regulate important cellular processes such as proliferation, migration, and differentiation, and the *ras* gene is frequently mutated in human tumors (Barbacid 1987). The Ras signaling pathway regulates multiple cell fates during the development of the nematode *Caenorhabditis elegans*, and its role in the development of the hermaphrodite vulva has been characterized extensively (reviewed in Kornfeld 1997; Sternberg and Han 1998). The vulva is a specialized epithelial tube used for egg laying and sperm entry formed by the descendants of three hypodermal blast cells: P5.p, P6.p, and P7.p. During larval development, the anchor cell in the somatic gonad signals to P6.p by expressing the LIN-3 epidermal growth factor ligand. LIN-3 binding to the LET-23 receptor tyrosine kinase triggers activation of a conserved signaling pathway that includes the SEM-5 adapter protein, the LET-341 guanine nucleotide exchange factor, the LET-60 Ras protein, the LIN-45 Raf kinase, the MEK-2 kinase, and the MPK-1 ERK MAP kinase. MPK-1 phosphorylates multiple target proteins, including the LIN-1 ETS transcription factor. The activation of this signaling pathway causes P6.p to adopt a primary vulval cell fate and generate eight descendants. P6.p then signals to P5.p and P7.p through the LIN-12 Notch receptor, causing these cells to adopt the secondary vulval cell fate and generate seven descendants. Three additional hypodermal blast cells, P3.p, P4.p, and P8.p, are capable of adopting vulval cell fates but normally receive neither signal and thus adopt the tertiary nonvulval cell fate. Loss-of-function mutations in core signaling genes cause P5.p, P6.p, and P7.p to adopt nonvulval cell fates resulting in a vulvaless (Vul) phenotype. By contrast, gain-of-function mutations in core signaling genes such as *let-60 ras* cause P3.p, P4.p, and P8.p to inappropriately adopt vulval cell fates resulting in ectopic pseudovulvae: a phenotype designated multivulva (Muv).

The activities of the core signaling proteins are highly regulated, but the mechanisms of regulation have not been fully characterized. An advantage of the *C. elegans* system is the ability to identify regulators of the pathway by conducting sensitive screens for genes that affect Ras-mediated signaling. Others and we have described screens for mutations that suppress the Muv phenotype caused by activated *let-60 ras*. These screens identified mutations in the core signaling genes *lin-45 raf* (Hsu *et al.* 2002), *mek-2* (Kornfeld *et al.* 1995a; Wu *et al.* 1995), and *mpk-1* (Lackner *et al.* 1994; Wu and Han 1994). These screens also identified novel regulators of the

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pathway including kinase suppressor of Ras (KSR)-1, a probable adapter protein for Raf, MEK, and ERK (Kornfeld *et al.* 1995b; Sundaram and Han 1995); suppressor of activated Ras (SUR)-8, a leucine-rich repeat protein that binds Ras (Sieburth *et al.* 1998); SUR-2, a component of the mediator complex (Singh and Han 1995); SUR-6, a component of the PP2A phosphatase that may regulate Raf (Sieburth *et al.* 1999); and CDF-1 and SUR-7, proteins involved in zinc metabolism and Ras-mediated signaling (Bruinsma *et al.* 2002; Yoder *et al.* 2004). Several of these proteins have been demonstrated to have conserved functions in regulating Ras signaling in insects and vertebrates.

Here we describe the genetic and molecular characterization of *cgr-1*, a new gene identified in a screen for suppressors of activated *let-60 ras*. Genetic studies indicated that *cgr-1* positively regulates Ras-mediated signaling and functions downstream of *let-60 ras* and upstream of the *lin-1* ETS transcription factor. CGR-1 contains two conserved domains, a CRAL/TRIO domain that is likely to form a pocket that binds a small, hydrophobic ligand and a GOLD domain; both domains were necessary for the full activity of CGR-1. Bioinformatic studies showed that there is a family of proteins with both CRAL/TRIO and GOLD domains that includes members in vertebrates and insects. These proteins have not been implicated in Ras signaling, and these studies establish an *in vivo* function for a member of this protein family and identify a new regulator of Ras signaling.

MATERIALS AND METHODS

General methods and strains: *C. elegans* strains were cultured as described by Brenner (1974) and grown at 20° unless otherwise noted. The wild-type strain and parent of all mutant strains was N2. CB4856 was used for single-nucleotide polymorphism (SNP) mapping. The following mutations are described by Riddle *et al.* (1997): LGIII, *dpy-17(e164)*; LGIV, *unc-24(e138), dpy-20(e1282)*; and LGX, *unc-78(e1217), lon-2(e678).* The following mutations that affect vulval development were examined. *let-60(n1046 gf)* is a semidominant, temperature-sensitive mutation that results in a G13E substitution and a Ras protein that is constitutively active (Beitel *et al.* 1990). *let-60(ga89)* causes a gain-of-function Muv phenotype at high temperatures and a loss-of-function Vul phenotype at low temperatures and results in a L19F substitution (Eisenmann and Kim 1997). *lin-1(n383)* causes a strong loss-of-function and results in a Q298STOP substitution (Beitel *et al.* 1995). *lin-45(n2520)* partially reduces *lin-45* function and results in a S751F substitution that affects the C-terminal 14-3-3 binding site of Raf (Hsu *et al.* 2002). *sur-8(ku167)* results in a E430K substitution and causes a loss-of-function of the leucine-rich repeat protein SUR-8 (Sieburth *et al.* 1998). *lin-12(n137gf)* results in a S872F substitution and a constitutively active Notch protein (Greenwald and Seydoux 1990). *lin-31(n1053)* is an apparent null allele that results in a W57STOP substitution, causing a truncation in the middle of the DNA-binding domain of the winged-helix transcription factor LIN-31 (Miller *et al.* 2000). *lin-15(n765)* is a temperature-sensitive, partial loss-of-function mutation that affects both *lin-15A* and *lin-15B* transcripts (Clark *et al.* 1994). *mpk-1(n2521)* results in a L124F substitution that causes a partial loss-of-function of ERK MAP kinase (Lackner *et al.* 1994). *cgr-1(n2528)* and *cgr-1(am114)* are described here.

Identification of *cgr-1* alleles: We previously described a screen for suppressors of the *let-60(n1046)* Muv phenotype (Lackner *et al.* 1994; Kornfeld *et al.* 1995a,b; Jakubowski and Kornfeld 1999; Hsu *et al.* 2002). In brief, we used EMS to mutagenize *let-60(n1046)* hermaphrodites, placed 2794 F_1 progeny on separate petri dishes, and examined the F_2 self-progeny for non-Muv animals. We identified 33 independently derived mutations that reduced the penetrance of the Muv phenotype from 93% to <10%. One of these mutations was *n2528*. Seven additional mutations were isolated in a related screen (Beitel *et al.* 1990).

To isolate a *cgr-1* deletion allele, we mutagenized wild-type N2 hermaphrodites on day 1 with 30 μg/ml trimethylpsoralen for 15 min, followed by a 90-sec exposure to 365 nm ultraviolet light from a UVL-21 BLAK RAY lamp (Ultra-Violet Products, Upland, CA) at 340 μW/cm^2 measured with an IL1400A ultraviolet light dose meter (International Light, Newburyport, MA). On day 2, mutagenized animals were incubated in a bleach solution (3 ml 20% $NaOCl^-$, 2 ml 4 M NaOH). Eggs were collected, plated on NGM/agar plates without bacterial lawns, and incubated overnight to obtain a population of arrested L1 larvae. On day 3, we collected larvae in M9 buffer (170 mM KH_2PO_4, 130 mM Na_2HPO_4, 86 mM NaCl, and 1 mM $MgSO_4$) and distributed ~50 larvae per plate to NGM/agar plates containing a lawn of *Escherichia coli* OP50. In total, we prepared 3840 plates with ~100 mutagenized genomes per plate. These animals were propagated until the bacteria were consumed, ~8 days at 20°. We collected the starved animals in 600 μl of M9 buffer per plate and distributed 150-μl aliquots to corresponding wells on each of two 1-ml 96-well Co-Star assay blocks (Fisher Scientific, Pittsburgh). To one 96-well plate, we added 150 μl of freezing solution (100 mM NaCl, 30 mM KH_2PO_4, 140 mM NaOH, 300 μM $MgSO_4$, and 30% glycerol) and stored these worms at −80° for future mutant recovery. To the other 96-well plate, we added 150 μl of lysis buffer (50 mM KCl, 10 mM Tris, 2.5 mM $MgCl_2$, 0.9% NP-40, 0.01% gelatin, and 60 μg/liter proteinase K), incubated it at −80° for 4–8 hr, at 60° for 12–16 hr, and at 95° for 30–60 min to prepare DNA suitable for PCR. For initial screening, DNA lysates from 10 96-well plates were pooled into 1 96-well plate. Five microliters of this pooled DNA lysate were used as a template for a nested PCR reaction, using primers to amplify a 3.1-kb fragment spanning the entire *cgr-1* genomic locus. The PCR reactions were analyzed by agarose gel electrophoresis; the *cgr-1(am114)* deletion was identified as a faster-migrating band. To recover worms with this allele, we thawed the appropriate well from the stock of frozen worms, distributed 1200 animals to individual plates, and used PCR to identify a population of animals homozygous for the deletion allele. The *cgr-1(am114)* strain was backcrossed four times to N2. The deletion endpoints were determined by DNA sequencing and are base pairs 30,198 and 32,401 on cosmid T27A10.

Genetic mapping: We used standard techniques to generate the recombinant chromosome *unc-78(e1217) cgr-1(n2528) lon-2(e678)* and hermaphrodites of the genotype *let-60(n1046); unc-78(e1217) cgr-1(n2528) lon-2(e678)*/CB4856. Unc non-Lon and Lon non-Unc self-progeny were selected, and animals homozygous for the recombinant chromosome were selected and scored for the suppression of *let-60(n1046)* Muv phenotype (Figure 1A).

To identify polymorphisms, we determined the DNA sequence of 1.2-kb fragments from predicted intergenic regions positioned between *unc-78* and *lon-2*, using DNA from CB4856 worms (as described by Jakubowski and Kornfeld 1999).

Nine polymorphisms were identified by analyzing ~10 kb of DNA. *amP59* is a 55-bp insertion at position 6269 of cosmid T14G12, *amP60* is an A-to-G substitution at position 18,282 of cosmid C02F12, *amP91* is a T-to-C substitution at position 20,029 of cosmid T27A10, *amP92* is a 1-bp deletion at position 260 of cosmid F56B6, *amP93* is a G-to-A substitution at position 3273 of cosmid R11G1, *amP94* is a T-to-C substitution at position 34,167 of cosmid T27A10, *amP95* is an A-to-G substitution at position 29,039 of cosmid T27A10, *amP96* was scored by the absence of PCR amplification of the region from 5909 to 7180 of cosmid F59D8, and *amP97* is a T-to-G substitution at position 16,482 of cosmid T27A10. Cosmids are numbered according to wormbase/NCBI.

For each of the 199 recombinant strains, the interval that contains the recombination event between N2 and CB4856 DNA was determined by using DNA sequencing or PCR amplification and gel electrophoresis to score polymorphisms (Figure 1A).

DNA cloning: All molecular biology techniques were performed as described by Sambrook *et al.* (1989), unless otherwise noted. To subclone the T27A10.7 predicted ORF, we ligated a 4785-bp *Sac*I/*Sac*II fragment of cosmid T27A10 that extends 990 bp upstream of the ATG (start) and 760 bp downstream of the TAA (stop) and does not contain sequences from adjacent predicted genes into pBluescript SK+ (Stratagene, La Jolla, CA) to create the plasmid pJG1. To engineer the G-to-A *n2528* mutation, we replaced the 1935-bp *Aat*II/*Sna*BI fragment of pJG1 with the corresponding fragment from PCR-amplified DNA derived from *cgr-1(n2528)* worms, creating pJG3. We used standard *in vitro* mutagenesis techniques to introduce a C-to-A change into pJG1 that alters codon 43 from TCG (serine) to TAG (stop), creating the plasmid pJG2. pDG125 contains the *cgr-1* genomic DNA coding region and encodes a CGR-1:GFP fusion protein; it was generated by ligating an 869-bp GFP genomic sequence from pPD95.77 (Chalfie *et al.* 1994) into pJG1 digested with *Asc*I. pDG135 and pDG141 contain *cgr-1* cDNA and encode CGR-1:GFP fusion proteins; a PCR-amplified 1104-bp fragment containing the *cgr-1* cDNA was ligated into pDG125 digested with *Age*I and *Bsp*EI. pDG141 was modified to generate pDG147, which encodes CGR-1(K230A) and pDG146, which encodes CGR-1(292Stop). To express the *cgr-1* cDNA from the *lin-31* promoter, we constructed pDG149 by ligating the 2000-bp *cgr-1* cDNA:GFP fragment from pDG141 into the 10,899-bp backbone of pJJ25 (Bruinsma *et al.* 2002). To express the *cgr-1* cDNA from the *ges-1* promoter, we constructed pDG151 by ligating the 2000-bp *cgr-1* cDNA:GFP fragment from pDG141 into the 6267-bp pJM16 plasmid (Bruinsma *et al.* 2002).

Identification and analysis of cDNA: We obtained the cDNAs, yk464g7 and yk708h11, from the *C. elegans* EST project (Kohara 1996). We isolated phage DNA according to the Lambda ZAP II *in vivo* excision protocol (Stragene, La Jolla, CA). DNA sequencing revealed a poly(A) tail attached 145 bp downstream of the STOP codon of both cDNAs. yk708h11 contained an SL1 sequence attached 2 bp upstream of the ATG start codon. The 5′ end of yk464g7 was in codon 2. To further characterize the position of the SL1 attachment site and the splicing pattern of exons 1–4, we purified total RNA from a mixed-stage population of N2 worms by Trizol extraction. RT–PCR was performed according to the Titan one-tube RT–PCR protocol (Roche Applied Science, Indianapolis), using a forward primer that is complementary to the SL1 *trans*-spliced leader and contains a *Sac*I site and a reverse primer that is complementary to exon 4 and contains a *Kpn*I site. PCR products were digested with restriction enzymes and cloned into *Sac*I/*Kpn*I-digested pBluescript SK+ (Stratagene). We isolated three of these colonies, purified the plasmid, and sequenced the cDNA. These three independently derived cDNAs had the identical sequence and contained an SL1 leader sequence attached to the *cgr-1* transcript 2 bp upstream of the start codon. We performed RT–PCR with oligonucleotide primers that span each intron and identified a single product in each case that corresponds to the predicted size on the basis of the analysis of *cgr-1* cDNAs.

We identified three differences from the coding region predicted by Genefinder: (1) Exon 4 is 5 bp shorter than predicted, (2) the 111-bp exon 5 was not predicted, and (3) exon 6 is 107 bp longer than predicted. The positions of the exons on cosmid T27A10 are as follows: exon 1, 33,043–32,943; exon 2, 32,888–32,824; exon 3, 32,777–32,584; exon 4, 31,665–31,447; exon 5, 31,391–31,281; exon 6, 31,233–31,088; exon 7, 30,556–30,487; exon 8, 30,439–30,344; and exon 9, 30,158–30,009.

Transformation rescue: Germline transformation experiments were performed as described by Mello *et al.* (1991). We co-injected *let-60(n1046)*; *cgr-1(n2528)* hermaphrodites with *C. elegans* DNA cloned in cosmids or plasmids (0.01–20 ng/μl) and with the plasmid pRF4 (80–100 ng/μl), which contains a dominant mutation *rol-6(su1006)*. Transgenic lines were established by identifying F_1 Rol animals that segregated F_2 Rol animals.

RNA-mediated interference: RNA was prepared according to the MEGAscript protocol (Ambion, Austin, TX), using the yk464g7 cDNA as template. Injections were performed as described previously (Fire *et al.* 1998). We injected RNA at 1 mg/ml into N2 or *let-60(n1046)* adult hermaphrodites raised at either 20° or 15°.

Phylogeny: Homologous predicted protein sequences were identified using BLASTP with *C. elegans* CGR-1 as query. Presence of a CRAL/TRIO domain was determined using an HMM (Pfam 13.0, PF00650) (Sonnhammer *et al.* 1997), and presence of a GOLD domain was determined as described (Anantharaman and Aravind 2002). Sequences were aligned using CLUSTALX (Thompson *et al.* 1997), columns with gaps were removed, and sequences were realigned. Phylogenetic inference was performed using neighbor joining (NJ) (PHYLIP version 3.6) (Felsenstein 1989). Tree topology was tested using two methods: bootstrapping with 1000 replicates and rebuilding the phylogeny using maximum parsimony (MP). NJ and MP produced an identical branching pattern (data not shown).

RESULTS

Identification of *cgr-1(n2528)*: The gain-of-function mutation *let-60(n1046)* results in a mutant Ras protein that is constitutively active and causes a Muv phenotype that is partially penetrant and heat sensitive (Beitel *et al.* 1990). *let-60(n1046)* is similar to mutations in codon 12 of human Ras that are frequently identified in human tumors and reduce the GTPase activity of the Ras protein (Barbacid 1987). We identified the *n2528* mutation in a screen suppressors of the Muv phenotype caused by *let-60(n1046)* (see materials and methods). The strain containing *n2528* was backcrossed to wild-type animals to remove extraneous mutations. The suppression of the *let-60* Muv phenotype caused by *n2528* displayed linkage to *lon-2* on chromosome X, and three-factor mapping experiments positioned *n2528* between the visible markers *unc-78* and *lon-2* (data not shown). No other mutation isolated in this screen was positioned

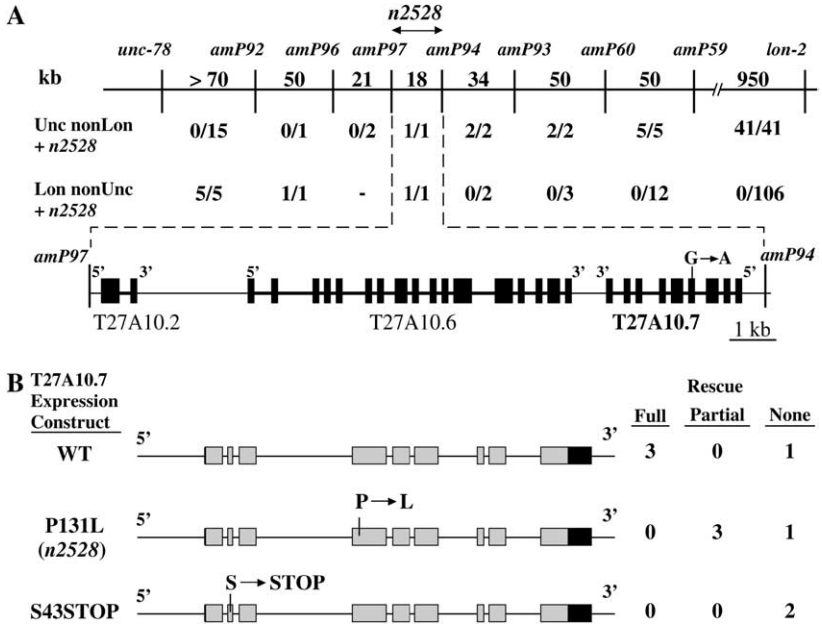

FIGURE 1.—Cloning *cgr-1*. (A) A portion of the physical map on LGX. Polymorphisms are differences between the nucleotide sequences of CB4856 and wild-type N2 and are designated *amPX*. A total of 130 Lon non-Unc and 69 Unc non-Lon progeny were selected from *let-60(n1046gf); unc-78 n2528 lon-2* / CB4856 hermaphrodites. We determined the interval that includes the recombination event by scoring polymorphisms and the presence of *n2528* by scoring suppression of the *let-60(n1046gf)* Muv phenotype. These data positioned *n2528* between *amP97* and *amP94*. This interval is expanded below; exons (boxes) and introns (thick lines) of Genefinder predicted open reading frames are shown. The G-to-A change in *n2528* animals is shown. (B) Plasmids containing wild-type (pJG1) or mutant T27A10.7 (pJG2, pJG3) were used to generate transgenic strains. The chromosomal genotype was *let-60(n1046); n2528*. Each rescue result represents an independently derived transgenic strain. We defined full rescue as >80% Muv, partial rescue as 40–80% Muv, and no rescue as <40% Muv.

in this interval, and this interval did not contain previously characterized mutations that cause a similar phenotype. These results indicated that the *n2528* mutation affects a *C. elegans* gene that has not been previously reported to be involved in Ras-mediated signaling.

***cgr-1(n2528)* is a mutation in the predicted open reading frame T27A10.7 and causes a loss of gene activity:** To identify the gene affected by the *n2528* mutation, we mapped the suppression of the *let-60(n1046)* Muv phenotype caused by *n2528* relative to SNPs. A total of 199 animals with a recombination event near *n2528* were selected using the visible markers Unc-78 and Lon-2, and a local, high-density map consisting of seven SNPs between *unc-78* and *lon-2* was used to position the *n2528* mutation to an 18-kb interval between the polymorphisms *amP97* and *amP94* (Figure 1A). We determined the DNA sequence of the three predicted open reading frames in this interval and identified 1 bp change in *n2528* mutants compared to wild-type animals. This G-to-A change results in a proline-to-leucine substitution in the predicted protein encoded by T27A10.7 (Figure 1A).

To test the hypothesis that the *n2528* mutation affects T27A10.7, we analyzed the activity of T27A10.7 in transgenic animals. A 4.8-kb fragment of genomic DNA including 1.0 kb upstream of the predicted start codon, the entire T27A10.7 coding region, and 0.8 kb downstream of the predicted stop codon was introduced into *let-60(n1046); n2528* mutant worms. Transgenic animals containing a multicopy array of this DNA displayed robust rescue of the suppression of Muv phenotype (Figure 1B), indicating that T27A10.7 is indeed the gene affected by the *n2528* mutation. To determine if the rescuing activity requires an intact open reading frame, we engineered the following two mutations into rescuing constructs: (1) a single-base change that results in a premature stop at codon 43 and (2) the G-to-A change present in the *n2528* allele. The premature stop codon eliminated the rescuing activity, whereas the *n2528* mutation reduced, but did not completely eliminate, rescuing activity (Figure 1B). These results indicate that the rescuing activity requires the protein encoded by T27A10.7 and that the *n2528* mutation partially reduces the activity or stability of this protein.

***cgr-1* encodes a protein with CRAL/TRIO and GOLD domains:** To characterize the structure of the mRNA encoded by T27A10.7, we completely sequenced two independently derived cDNAs generated by the *C. elegans* expressed sequence tags (EST) project (KOHARA 1996). In addition, T27A10.7 transcripts present in RNA isolated from mixed-stage populations of worms were analyzed by RT–PCR. These studies indicated that the transcript consists of nine exons and the predicted protein contains 383 amino acids (Figure 2, A and B). The 5′ end of the transcript contains an SL1 leader sequence that is attached two nucleotides upstream of the predicted start ATG. The 3′ end of the transcript contains a poly(A) tail. The analysis of cDNAs and RT–PCR products indicated that there is one predominant splicing pattern and did not provide evidence for alternative splicing.

A BLAST search was used to identify homologous proteins. An alignment of these proteins revealed that the predicted protein contains two conserved domains, an N-terminal CRAL/TRIO domain and a C-terminal GOLD domain (Figure 2A). The CRAL/TRIO domain was named for the *cis*-retinaldehyde binding protein and the Trio multidomain protein (BATEMAN *et al.* 2000). It forms a hydrophobic binding pocket that binds ligands such as retinaldehyde or phosphatidylinositol (SHA *et al.* 1998; STOCKER *et al.* 2002; STOCKER and BAUMANN

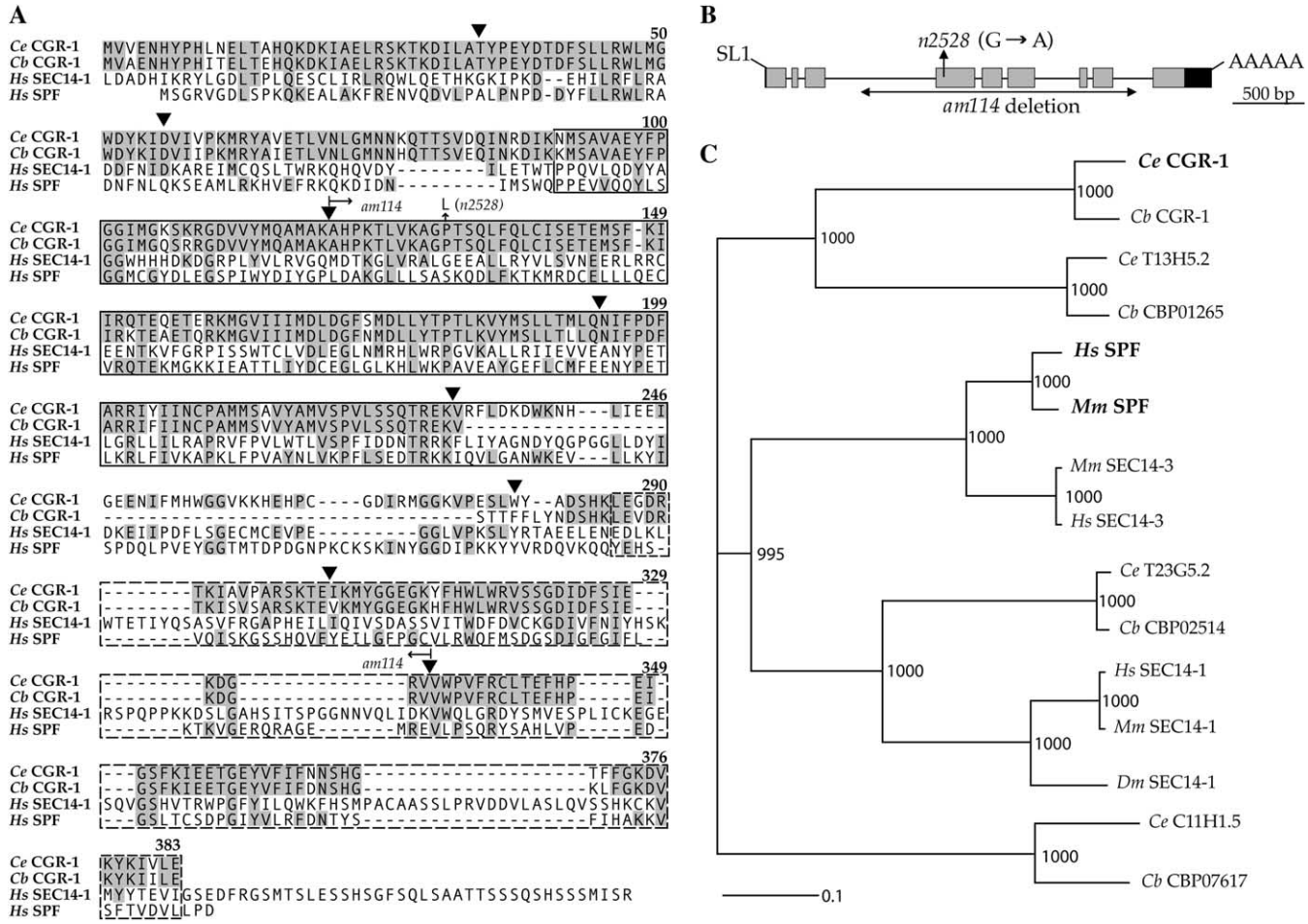

FIGURE 2.—*cgr-1* encodes a protein with CRAL/TRIO and GOLD domains. (A) Full-length *C. elegans* CGR-1 protein (Ce CGR-1) is aligned with the predicted full-length *C. briggsae* CGR-1 protein (Cb CGR-1), with amino acids 239–715 of the predicted human SEC14-1 protein (Hs SEC14-1; CHINEN *et al.* 1996), and with the full-length predicted human SPF protein (Hs SPF; SHIBATA *et al.* 2001). Amino acid numbers refer to Ce CGR-1. Shaded residues are identical to Ce CGR-1. The CRAL/TRIO domain is boxed with a solid line and the GOLD domain is boxed with a hatched line. Triangles indicate intron positions. Arrows indicate the position of the *cgr-1(n2528)* mutation (P131L) and the coding region removed by the *cgr-1(am114)* deletion (amino acids 121–334). (B) Diagram of the *cgr-1* locus. Boxes represent exons. Shaded boxes and the solid box show coding and untranslated regions, respectively. The thin line represents introns. An SL1 leader sequence is spliced 2 nucleotides upstream of the start codon. A poly(A) tail is attached 145 nucleotides downstream of the stop codon. Arrows indicate the position of the *n2528* mutation and the endpoints of the *am114* deletion. (C) Neighbor-Joining tree illustrating phylogenetic relationships of predicted CRAL/TRIO and GOLD domain-containing proteins. Boldface type indicates proteins that have been biochemically or genetically characterized. Branch lengths are proportional to divergence (scale represents 10% divergence). Numbers at each node indicate bootstrap support out of 1000 replicates. All nodes were well supported. *Mm, mus musculus*; *Dm, Drosophila melanogaster.*

2003). The GOLD domain was identified by protein alignments and it forms a β-barrel (ANANTHARAMAN and ARAVIND 2002; STOCKER *et al.* 2002). We named the T27A10.7 gene *cgr-1* for *C*RAL/TRIO and *G*OLD domain suppressor of activated ***ras***.

To characterize evolutionary conservation of this protein family, we identified human, mouse, Drosophila, and nematode predicted proteins that contain a CRAL/TRIO and a GOLD domain and constructed a neighbor-joining phylogeny (Figure 2C). There are four such proteins encoded by the genomes of *C. elegans* and the related nematode *C. briggsae*. The phylogeny shows that CGR-1 has diverged to a similar extent from the vertebrate SEC14-1 and SPF/SEC14-3 proteins. However, *C. elegans* T23G5.2 is closely related to the SEC14-1 proteins, and all five proteins in this branch contain a similar N-terminal extension. CGR-1 and the SPF/SEC14-3 proteins lack this N-terminal extension, suggesting that they may be more closely related.

Identification of a *cgr-1* deletion mutation: To obtain a null mutation of *cgr-1*, we took advantage of our molecular analysis by screening for a deletion in the *cgr-1* locus (see MATERIALS AND METHODS). We identified one allele, *cgr-1(am114)*, containing a deletion of 2.2 kb that begins in intron 3, removes exons 4–8, and ends in intron 8 (Figure 2). The deleted region encodes most of

TABLE 1

***cgr-1* interactions with Muv mutations**

Genotype	% Muv[a]	*n*[b]	Induced P*n*.p cells[c]	*n*[b]
Wild type	0	341	3.0	12
let-60(n1046)	71	362	4.3	10
let-60(n1046); cgr-1(n2528)	4	388	3.0	10
let-60(n1046); cgr-1(am114)	<1	376	3.0	10
let-60(ga89)[d]	19	308	ND[e]	—
let-60(ga89); cgr-1(n2528)[d]	2	293	ND	—
lin-15(n765)	99	150	ND	—
cgr-1(n2528) lin-15(n765)[f]	7	174	ND	—
lin-1(n383)	100	415	5.3	10
lin-1(n383); cgr-1(n2528)	99	504	5.3	10
lin-1(n383); cgr-1(am114)	100	356	5.2	10
lin-31(n1053)	73	356	ND	—
lin-31(n1053); cgr-1(n2528)	57	188	ND	—
lin-12(n137)	100	151	ND	—
lin-12(n137); cgr-1(n2528)[g]	100	153	ND	—
let-60(n1046)[h]	33	193	ND	—
let-60(n1046); amEx57[h,i]	89	220	ND	—

[a] The multivulva (Muv) phenotype, defined as one or more protrusions along the ventral cuticle that are displaced from the normal location of the vulva, was scored using a dissecting microscope.

[b] Number of hermaphrodites analyzed.

[c] Fourth larval stage (L4) hermaphrodites were examined using Nomarski optics. The P*n*.p cells, P3.p–P8.p, were classified as induced if they appeared to have produced more than two descendants.

[d] Hermaphrodites were analyzed at 25° to increase the penetrance of the *ga89* Muv phenotype.

[e] Not determined.

[f] Complete genotype: *cgr-1(n2528) lon-2(e678) lin-15(n765).*

[g] Complete genotype: *lin-12(n137); cgr-1(n2528) lon-2(e678).*

[h] Hermaphrodites were analyzed at 15° to reduce the penetrance of the *n1046* Muv phenotype.

[i] The *amEx57* extrachromosomal array consists of multiple copies of pJG1 (*Pcgr-1/cgr-1* genomic DNA) and the transformation marker pRF4. Four transgenic lines were generated; these data are from one representative line.

the CRAL/TRIO domain and approximately half of the GOLD domain, suggesting that *am114* is likely a null mutation.

***cgr-1* positively regulates Ras signaling and is necessary for ectopic vulval cell fates induced by *let-60* and *lin-15* but not by *lin-1*, *lin-31*, or *lin-12*:** *cgr-1 (n2528)* reduced the penetrance of the Muv phenotype caused by *let-60(n1046)* from 71 to 4% (Table 1, rows 2 and 3). To determine if the suppression of the Muv phenotype is caused by a change of cell fates, we used Nomarski optics to analyze the number of P*n*.p cells that adopt vulval fates. Wild-type hermaphrodites displayed an average of 3.0 induced P*n*.p cells, since P5.p, P6.p, and P7.p adopt vulval cell fates whereas P3.p, P4.p, and P8.p adopt nonvulval cell fates (Table 1, row 1). *let-60(n1046)* increased the number of induced P*n*.p cells to 4.3 (Table 1, line 2). *let-60(n1046); cgr-1(n2528)* hermaphrodites displayed an average of 3.0 induced P*n*.p cells (Table 1, line 3). The *cgr-1(am114)* deletion allele exhibited similar interactions with *let-60*. *let-60(n1046); cgr-1(am114)* hermaphrodites displayed a Muv penetrance of <1% and an average of 3.0 induced P*n*.p cells (Table 1, row 4). These results indicated that *cgr-1* is necessary for activated *let-60 ras* to induce ectopic vulval cell fates.

To determine if *cgr-1* mutations have allele-specific interactions with *let-60(n1046)*, we analyzed a different mutation, *let-60(ga89)*, that results in a L19F substitution. The *ga89* missense mutation causes a gain-of-function at high temperatures and a loss-of-function at low temperatures (Eisenmann and Kim 1997). *cgr-1(n2528)* strongly suppressed the gain-of-function Muv phenotype caused by *let-60(ga89)* at 25° (Table 1, rows 5 and 6). This indicates that the *cgr-1* phenotype is independent of the specific mutation that activates *let-60 ras*.

To test if *cgr-1* interacts with other genes that control induction of vulval cell fates, we examined the interaction of *cgr-1* with *lin-15*, *lin-1*, *lin-31*, and *lin-12*. *lin-15* is a complex locus that encodes two novel proteins that function in the synthetic Multivulva pathway (Clark *et al.* 1994; Huang *et al.* 1994). Loss-of-function mutations in *lin-15* cause a Muv phenotype, and genetic epistasis experiments indicate that *lin-15* acts downstream of the *lin-3* epidermal growth factor ligand and upstream of the *let-23* receptor tyrosine kinase and *let-60 ras* (Sternberg *et al.* 1992). *cgr-1(n2528)* suppressed the *lin-15(n765)* Muv phenotype from 99 to 7% at 20° (Table 1, rows 7 and 8). Thus, *cgr-1* is necessary for the ectopic vulval cell fates induced by a loss of *lin-15*.

TABLE 2

***cgr-1* interactions with Vul mutations**

Genotype	% egg-laying defective[a]	n^b	P5.p–P7.p descendants (range)[c]	% animals with 22 P5p–P7.p descendants	n^b
Wild type	1	142	22 (22)	100	12
cgr-1(n2528)[d]	4	156	22 (22)	100	10
cgr-1(am114)[e]	4	306	22 (22)	100	12
lin-45(n2520)[f]	0	188	22 (22)	100	11
lin-45(n2520); cgr-1(am114)[g]	31	130	21 (18-22)	64	11
sur-8(ku167)[h]	2	170	22 (22)	100	10
sur-8(ku167); cgr-1(am114)[i]	28	125	20 (16-22)	20	10

[a] Eggs were placed on individual petri dishes, and worms were observed every 1–2 days. Worms were scored as egg-laying defective if they laid fewer than five eggs and produced viable progeny that hatched internally.

[b] Number of hermaphrodites analyzed.

[c] L4 stage hermaphrodites were examined using Nomarski optics, and the number of descendants of P5.p–P7.p was inferred from their position and morphology. The range represents the lowest and highest number of descendants counted in an individual animal.

[d] Hermaphrodites were raised at 20° to analyze egg laying and at 15° to analyze P5.p–P7.p.

[e] Complete genotype of hermaphrodites used to analyze P5.p–P7.p: *cgr-1(am114) lon-2(e678).*

[f] Complete genotype of hermaphrodites used to analyze P5.p–P7.p: *lin-45(n2520) unc-24(e138).*

[g] Complete genotype of hermaphrodites used to analyze P5.p–P7.p: *lin-45(n2520) unc-24(e138); cgr-1(am114) lon-2(e678).*

[h] Complete genotype of hermaphrodites used to analyze P5.p–P7.p: *sur-8(ku167) dpy-20(e1282).*

[i] Complete genotype of hermaphrodites used to analyze P5.p–P7.p: *sur-8(ku167) dpy-20(e1282); cgr-1(am114) lon-2(e678).*

lin-1 encodes an ETS-domain transcription factor that inhibits the primary vulval cell fate and functions downstream of *mpk-1* (Beitel *et al.* 1995). The strong loss-of-function *lin-1(n383)* mutation causes a highly penetrant Muv phenotype with an average of 5.3 induced P*n*.p cells (Table 1, row 9). The *cgr-1(n2528)* and *cgr-1(am114)* mutations did not significantly affect the Muv phenotype or the ectopic induction of vulval cell fates caused by *lin-1(n383)* (Table 1, rows 10 and 11). The *lin-31* gene encodes a predicted transcription factor that contains an HNF-3 forkhead domain and appears to act at the level of *lin-1* to regulate vulval cell fates (Miller *et al.* 1993). The strong loss-of-function *lin-31(n1053)* mutation causes a Muv phenotype with a penetrance of 73% (Table 1, row 12). The *cgr-1(n2528)* mutation only slightly reduced the Muv phenotype caused by *lin-31(n1053)* (Table 1, row 13). Thus, *cgr-1* is not necessary for the ectopic vulval cell fates caused by a loss of *lin-1* and *lin-31.*

The activity of the *lin-12* Notch gene causes P5.p and P7.p to adopt 2° vulval cell fates (Yochem *et al.* 1988). The *lin-12(n137)* gain-of-function mutation causes a Muv phenotype because all six P*n*.p cells adopt secondary vulval cell fates (Greenwald and Seydoux 1990). *cgr-1(n2528)* did not affect the Muv phenotype caused by *lin-12(n137)* (Table 1, rows 14 and 15). Thus, *cgr-1* is not necessary for the ectopic 2° vulval cell fate induced by activated *lin-12.*

The result that *cgr-1* loss-of-function mutations suppress the Muv phenotype caused by mutations of *let-60 ras* and *lin-15* indicates that *cgr-1* activity promotes Ras signaling. If this is the case, then overexpression of *cgr-1* might enhance Ras signaling. To test this prediction, we generated transgenic animals that contain multicopy arrays of the wild-type *cgr-1* genomic DNA locus. In a wild-type genetic background overexpression of *cgr-1* did not significantly affect vulval development. In *let-60(n1046gf)* hermaphrodites, overexpression of *cgr-1* increased the penetrance of Muv from 33 to 89% at 15° (Table 1, rows 16 and 17). This result demonstrates that *cgr-1* is sufficient to promote Ras-mediated signaling in a sensitive genetic background and provides additional evidence that *cgr-1* positively regulates Ras signaling.

***cgr-1* promotes vulval cell fates in P5.p, P6.p, and P7.p:** In an otherwise wild-type background, *cgr-1* mutations caused an egg-laying defect that was 4% penetrant, indicating that most mutant hermaphrodites formed a functional vulval passageway (Table 2, rows 2 and 3). These mutants displayed 22 descendants of P5.p, P6.p, and P7.p, indicating that the vulval cell lineages are frequently normal. To determine if *cgr-1* mutations affect the fates of P5.p–P7.p in sensitized genetic backgrounds, we constructed double mutants containing *cgr-1(am114)* and a mutation that partially reduces Ras-mediated signaling. The *lin-45 raf* gene is necessary for all Ras-mediated signaling, since strong loss-of-function mutations in *lin-45* cause a highly penetrant vulvaless phenotype (Han *et al.* 1993; Hsu *et al.* 2002). We previously identified the partial loss-of-function allele *lin-45 (n2520)* as a suppressor of *let-60 ras* (Hsu *et al.* 2002). In an otherwise wild-type genetic background, *lin-45(n2520)* does not cause an egg-laying defect or affect the lineages of P5.p–P7.p (Table 2, row 4). The *lin-45(n2520); cgr-1(am114)* double-mutant strain displayed an egg-laying defect that was 31% penetrant and a reduction in the average number of P5.p–P7.p descendants (Table 2, row 5). The gene *sur-8*

TABLE 3

***cgr-1* mutations cause larval lethality**

Genotype	% larval lethal[a] at 20°	*n*[b]	% larval lethal[a] at 15°	*n*[b]
Wild type	1	142	1	275
cgr-1(n2528)	8	57	49	162
cgr-1(am114)	12	206	100	248
cgr-1(RNAi)[c]	1	842	26[d]	983
mpk-1(n2521)	11	137	—	—
mpk-1(n2521); cgr-1(am114)[e]	100	44	—	—
let-60(n1046)	ND[f]	—	5	129
let-60(n1046); cgr-1(n2528)	9	47	15	153
let-60(n1046); cgr-1(am114)	ND	—	73	145
lin-1(n383)	ND	—	8	117
lin-1(n383); cgr-1(am114)	ND	—	100	171

[a] Eggs were placed on individual petri plates, and worms were examined every 1–2 days. A worm was scored as larval lethal if it died during the L1–L4 stages or persisted in these larval stages for >14 days.

[b] Number of hermaphrodites analyzed.

[c] Wild-type N2 hermaphrodites were injected with *cgr-1* double-stranded RNA. To analyze progeny, we placed each hermaphrodite on a separate petri dish, transferred it to a fresh plate daily, and counted the number of eggs laid. Eggs that did not yield L4 stage larvae by day 4 at 20° or day 6 at 15° were scored as larval lethal.

[d] The average of 11 independently injected hermaphrodites is shown. Individual results varied from 4 to 96%.

[e] We analyzed self-progeny of *mpk-1(n2521) dpy-17(e164); cgr-1(am114)/unc-78(e1217) lon-2(e678)* hermaphrodites. Forty-four of 158 (28%) of the progeny did not develop to adulthood, and we inferred that these animals had the genotype *cgr-1/cgr-1*. Forty of 158 (25%) of the progeny were Unc Lon, indicating that they had the genotype *unc-78 lon-2/unc-78 lon-2*. Fifty-five of 158 (35%) of the progeny segregated Unc Lon self-progeny, indicating that they had the genotype *cgr-1/unc-78 lon-2*. Nineteen of 158 (12%) of the progeny were non-Lon Unc, sterile adults. On the basis of the expected segregation ration of 1:2:1, we inferred that these animals had the genotype *cgr-1/unc-78 lon-2*, although it is possible that some of these had the genotype *cgr-1/cgr-1*.

[f] Not determined.

encodes a leucine-rich repeat protein that binds Ras and promotes Ras-mediated signaling (Sieburth *et al.* 1998). The *sur-8(ku167)* partial reduction-of-function mutation causes an egg-laying defect that is 2% penetrant and does not affect the cell lineages of P5.p–P7.p in an otherwise wild-type genetic background (Table 2, row 6). *sur-8(ku167); cgr-1(am114)* double-mutant hermaphrodites displayed an egg-laying defect that was 28% penetrant and a reduction in the average number of P5.p–P7.p descendants that was 80% penetrant (Table 2, row 7). Thus, in these sensitized genetic backgrounds *cgr-1* is necessary for P5.p–P7.p to generate normal vulval cell lineages.

***cgr-1* is required for larval development:** In addition to vulval defects, *cgr-1* mutants exhibited other abnormalities including a slightly dumpy appearance, a small increase in the amplitude of body bends, a propensity to spend time away from the lawn of *E. coli* on the petri dish, delays in larval development, and larval lethality. To characterize the abnormalities during larval development, we placed one egg on a petri dish and monitored larval development using a dissecting microscope. *cgr-1(n2528)* mutants displayed a larval lethal phenotype that was cold sensitive: 8% of these mutants died at 20° whereas 49% died at 15° (Table 3, row 2). For the *cgr-1(n2528)* mutants that developed into fertile adults, ~90% experienced some developmental delay during the larval stages, and we observed mutants that were L1 or L2 larvae for 8–10 days before developing into fertile adults. *cgr-1(am114)* mutants displayed a higher-penetrance larval lethal phenotype that was also cold sensitive: 12% of these mutants died at 20° whereas 100% died at 15° (Table 3, row 3). The time until larval death was highly variable; ~10% of *cgr-1(am114)* mutants died as L1 larvae within 3 days and 75% persisted as L1 or L2 larvae for >10 days.

The function of many *C. elegans* genes can be reduced transiently by RNA interference (RNAi), using double-stranded RNA (Fire *et al.* 1998). To analyze *cgr-1*, we injected double-stranded *cgr-1* RNA into the gonad of wild-type hermaphrodites and examined their progeny. *cgr-1(RNAi)* caused a cold-sensitive larval lethal phenotype with a penetrance of 1% at 20° and 26% at 15° (Table 3, row 4). *cgr-1(RNAi)* larvae displayed developmental delays and arrests that were similar to the defects caused by the *cgr-1* mutations. These results support the model that *cgr-1* promotes larval viability and provide additional evidence that *n2528* and *am114* cause a reduction of *cgr-1* gene activity.

The core genes of the Ras-signaling pathway including *lin-3*, *let-23*, *sem-5*, *let-341*, *let-60*, *lin-45*, *mek-2*, and *mpk-1* are necessary for the development of the excretory duct cell (Yochem *et al.* 1997). Strong loss-of-function mutations of these genes cause a larval lethal phenotype characterized by a rigid, rod-like morphology. By contrast, the *cgr-1(lf)* mutants do not display this

rigid, rod-like morphology, but instead die as thin and scrawny larvae or persist as larvae for many days. To determine if *cgr-1* function during larval development involves Ras-mediated signaling in the excretory duct cell and/or another cell, we analyzed interactions with Ras pathway genes. The *let-60(n1046)* allele partially rescued the larval lethality caused by *cgr-1*, reducing the *cgr-1(am114)* larval lethal phenotype from 100 to 73% and the *cgr-1(n2528)* larval lethal phenotype from 49 to 15% (Table 3, rows 8 and 9). *mpk-1(n2521)* is a partial loss-of-function mutation that causes only 11% larval lethality (Table 3, row 5) (Lackner *et al.* 1994). *mpk-1(n2521)* enhanced the penetrance of the *cgr-1(am114)* larval lethal phenotype from 12 to 100% at 20°; most double mutants displayed the arrested larval development characteristic of *cgr-1* mutants and not the rigid, rod-like morphology characteristic of mutants with a defective excretory duct cell (Table 3, row 6). The LIN-1 ETS transcription factor is an important target of Ras signaling during the development of the excretory duct cell, and a loss-of-function mutation of *lin-1* suppresses the larval lethal phenotype caused by a loss-of-function mutation in a core signaling gene, such as *mek-2* (Kornfeld *et al.* 1995a). If *cgr-1(lf)* mutations cause larval lethality by reducing Ras signaling in the excretory duct cell, then the *cgr-1* lethal phenotype might be suppressed by a loss-of-function mutation in *lin-1*. However, *lin-1(n383)* did not suppress the larval lethality caused by *cgr-1(am114)*, since these double mutants displayed a larval lethal phenotype that was 100% penetrant (Table 3, row 11). The results that the *cgr-1* larval lethal phenotype displayed interactions with *let-60* and *mpk-1* but not with *lin-1* suggest that *cgr-1* promotes larval viability by functioning in a signaling pathway that is distinct from the pathway that specifies the excretory duct cell. These results do not exclude the possibility that *cgr-1* functions in the excretory duct cell and that part of the *cgr-1* phenotype is caused by a defect in this cell.

CGR-1 expression pattern: To investigate the expression pattern of CGR-1, we generated a plasmid that encodes a CGR-1:GFP fusion protein. To mimic the endogenous CGR-1 expression pattern, we used a 4.8-kb genomic DNA fragment that includes 1 kb of the *cgr-1* promoter region and the complete *cgr-1* coding region; the GFP coding region was inserted at the *cgr-1* stop codon. Transgenic animals containing this plasmid displayed rescue of the *cgr-1(n2528)* suppression of Muv phenotype, indicating that the fusion protein is functional and that it is expressed in the cells that are necessary for *cgr-1* function. CGR-1:GFP fluorescence was visible in the intestinal cells during all stages of development; it was not visible in P*n*.p cells (Figure 3). The fluorescence signal in intestinal cells appears to be diffuse and cytosolic; it was largely excluded from the nucleus and was not concentrated at the periphery of the cell.

***cgr-1* functions in P*n*.p cells to mediate vulval induction:** The observation that CGR-1:GFP was not visible in P*n*.p cells suggests that CGR-1 may be expressed in P*n*.p cells at a level that is below the detection threshold of this assay or that CGR-1 may affect the fate of P*n*.p cells by functioning in another cell type. To investigate the CGR-1 site of action, we used two tissue-specific promoters to express CGR-1: the *ges-1* promoter that is expressed specifically in intestinal cells (Aamodt *et al.* 1991) and the *lin-31* promoter that is expressed specifically in P*n*.p cells (Tan *et al.* 1998). *cgr-1* expression directed by the *ges-1* promoter did not rescue the *cgr-1(n2528)* suppression of *let-60(n1046)* Muv phenotype (Table 4, rows 19 and 20). By contrast, *cgr-1* expression directed by the *lin-31* promoter partially rescued the *cgr-1(n2528)* phenotype (Table 4, rows 16–18). These results indicate that CGR-1 activity in P*n*.p cells is sufficient to rescue the *cgr-1* mutant defects in vulval development and suggest that CGR-1 normally functions cell autonomously in the P*n*.p cells to promote induction of the primary vulval cell fate.

The CRAL/TRIO and GOLD domains are important for CGR-1 function: The CRAL/TRIO domain of yeast SEC14 binds phosphatidylinositol (PI), and purified SEC14 displays PI transfer activity (Phillips *et al.* 1999). A lysine residue that is necessary for this activity is conserved in CGR-1 and corresponds to lysine 230 (Figure 2). To test the importance of CGR-1 lysine 230, we generated a plasmid that encodes mutant CGR-1 with a lysine 230-to-alanine substitution. The CGR-1(K230A) mutant partially rescued the *cgr-1(n2528)* suppression of Muv phenotype (Figure 4 and Table 4, rows 10 and 11). The *cgr-1(n2528)* mutation results in a proline 131-to-leucine substitution in the CRAL/TRIO domain. Our genetic studies indicated that this mutation partially reduces *cgr-1* activity. In addition, expression of CGR-1(P131L) partially rescued the *cgr-1* suppression of Muv phenotype (Figure 4 and Table 4, rows 6–9). The analysis of CGR-1(K230A) and CGR-1(P131L) mutants indicates that the CRAL/TRIO domain is necessary for the full function of CGR-1, although it is also possible that these mutations reduce protein stability. The residual activity of these proteins might result from residual function of the CRAL/TRIO domain or from CGR-1 functions that are independent of the CRAL/TRIO domain. To address this issue, we generated a plasmid-encoding CGR-1 protein with a deletion of the entire CRAL/TRIO domain. However, this construct could not be maintained in transgenic animals, perhaps because expression of the GOLD domain alone causes toxicity.

To investigate the role of the GOLD domain, we constructed a plasmid that encodes mutant CGR-1 protein with a deletion of the entire GOLD domain. Expression of CGR-1(K292 Stop) partially rescued the *cgr-1* suppression of Muv phenotype (Figure 4 and Table 4, rows 12–15). This result suggests that the GOLD

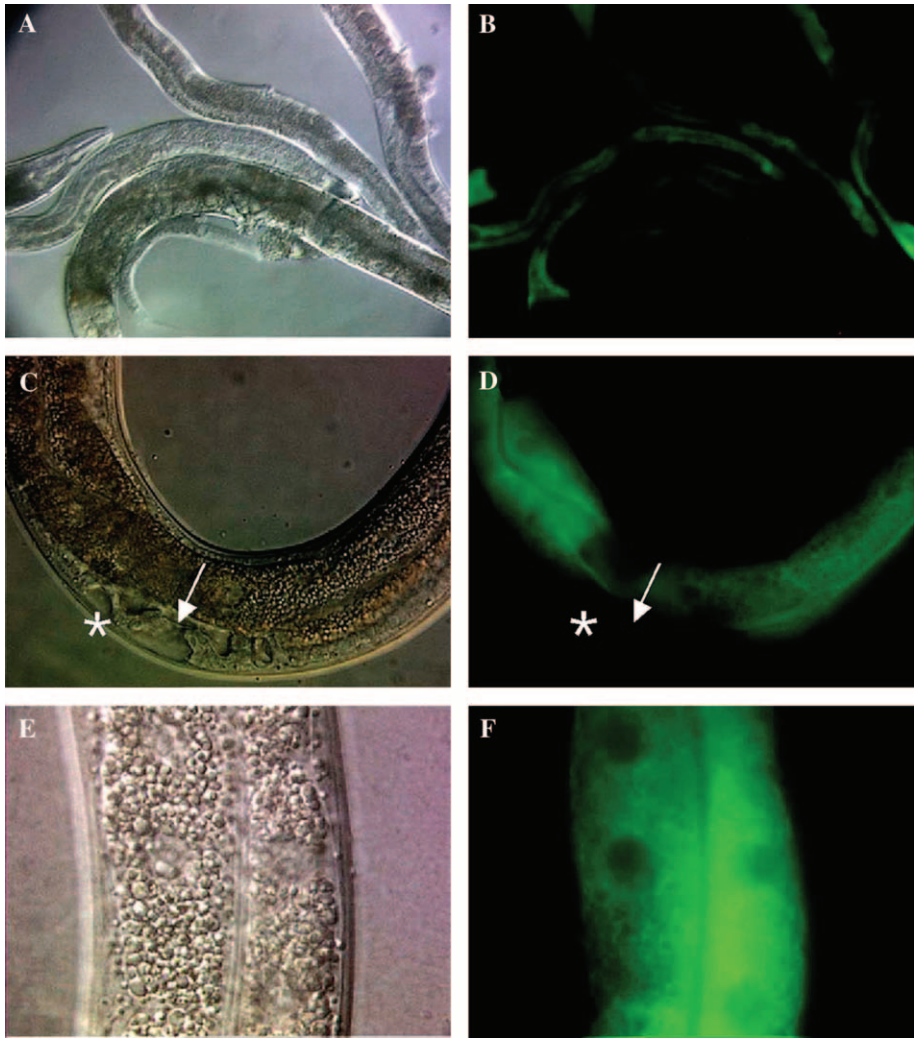

FIGURE 3.—Expression pattern of CGR-1. The genotype of all hermaphrodites was *let-60(n1046); cgr-1(n2528); amEx82*. The *amEx82* array contains pDG125 (*Pcgr-1*:*cgr-1* genomic:GFP) and pRF4. (A and B) Nomarski and fluorescent images (100×) of adult hermaphrodites. CGR-1:GFP expression is visible in intestinal cells. (C and D) Nomarski and fluorescent images (400×) of an L4 hermaphrodite. The arrow marks the vulva. The asterisk marks an ectopic vulval invagination. CGR-1:GFP expression is visible in intestinal cells but not in vulval cells. (E and F) Nomarski and fluorescent images (630×) of adult intestinal cells. CGR-1:GFP exhibits cytoplasmic localization and appears to be largely excluded from nuclei.

domain is necessary for the full activity of CGR-1, although it is also possible that this mutation reduces protein stability. In addition, it indicates that some CGR-1 functions are independent of the GOLD domain.

DISCUSSION

The *cgr-1(n2528)* mutation was identified by using random chemical mutagenesis and a forward genetic screen. This strategy was chosen because it is a sensitive and unbiased approach to identify novel genes that mediate Ras signaling. Indeed, *C. elegans cgr-1* and similar genes in other species have not been implicated previously in Ras signaling. Here we present genetic and molecular experiments demonstrating that CGR-1 promotes Ras-mediated signal transduction. These results identify a new protein that contributes to Ras-mediated signaling and demonstrate an *in vivo* function for a member of the evolutionarily conserved family of CRAL/TRIO and GOLD domain proteins.

***cgr-1* positively regulates Ras-mediated signaling:** Several techniques were used to manipulate *cgr-1* activity, and these studies indicate that *cgr-1* positively regulates Ras signaling. Two *cgr-1* chromosomal mutations were analyzed: the *cgr-1(n2528)* missense mutation and the *cgr-1(am114)* deletion mutation. Both *cgr-1* mutations caused larval lethal and suppression of Muv phenotypes. They can be arranged in an allelic series, since *am114* caused a higher penetrance of both phenotypes. The *cgr-1* vulval phenotype was rescued by overexpression of wild-type *cgr-1*, and rescuing activity was diminished by introducing the *n2528* mutation. These results suggest that *n2528* causes a partial reduction of *cgr-1* function and that *am114* causes a larger and perhaps complete reduction of *cgr-1* function. Consistent with this conclusion, double-stranded RNA interference, a technique that reduces gene function, phenocopied the *cgr-1* larval lethal phenotype. In addition, overexpression of wild-type *cgr-1* enhanced Ras-mediated signaling. These findings indicate that *cgr-1* is necessary for the full activity of the Ras-mediated signaling pathway and that *cgr-1* can be sufficient to promote Ras-mediated signaling. Our analysis also showed that P*n*.p cells usually adopt a normal pattern

TABLE 4

CGR-1 expression in transgenic animals

Genotype[a]	*cgr-1* expression construct	% Muv[b]	*N*[c]
Wild type	None	0	341
let-60(n1046)	None	71	362
let-60(n1046); cgr-1(n2528)	None	4	388
let-60(n1046); cgr-1(n2528); amEx84	*Pcgr-1*/CGR-1:GFP	92	90
		91	118
let-60(n1046); cgr-1(n2528); amEx62	*Pcgr-1*/CGR-1(P131L)	68	117
		47	100
		47	74
		26	86
let-60(n1046); cgr-1(n2528); amEx89	*Pcgr-1*/CGR-1 (K230A):GFP	79	250
		46	370
let-60(n1046); cgr-1(n2528); amEx88	*Pcgr-1*/CGR-1 (ΔGOLD):GFP	63	295
		52	367
		48	446
		38	397
let-60(n1046); cgr-1(n2528); amEx90	*Plin-31*/CGR-1:GFP	49	222
		40	264
		21	213
let-60(n1046); cgr-1(n2528); amEx91	*Pges-1*/CGR-1:GFP	10	118
		9	206

[a] Extrachromosomal arrays were obtained by coinjecting pRF4 and the following CGR-1 expression plasmids: *amEx84*, containing pDG135; *amEx62*, containing pJG3; a*mEx89*, containing pDG147; *amEx88*, containing pDG146; *amEx90*, containing pDG149; and *amEx91*, containing pDG151 (described in MATERIALS AND METHODS).

[b] The Muv phenotype was scored as described in Table 1. Each value represents an independently derived transgenic strain. All adult hermaphrodites that displayed the Rol phenotype were scored on several petri dishes.

[c] Number of hermaphrodites analyzed.

of cell fates when a *cgr-1* mutation is in an otherwise wild-type background. One possibility is that *cgr-1* provides a function that is essential for Ras signaling but genes that are homologous to *cgr-1* are partially redundant. Our analysis revealed three other *C. elegans* genes in the *cgr-1* family; these genes have not been characterized, and they may be partially redundant with *cgr-1*. A second possibility is that *cgr-1* function is important but not essential; the Ras signaling pathway may be robust enough to specify the vulval cell fates in many cases despite the absence of this positive regulator.

The genetic studies define a portion of the Ras signal transduction pathway that is likely to be the site of *cgr-1* action. *cgr-1(lf)* mutations strongly suppressed the Muv phenotype caused by *lin-15(lf)* or *let-60(gf)* mutations. The *lin-15(lf)* phenotype is not efficiently suppressed by loss-of-function mutations in upstream signaling genes such as *lin-3*, and the *let-60(gf)* Muv phenotype is not efficiently suppressed by loss-of-function mutations in upstream signaling genes such as *sem-5* (FERGUSON *et al.* 1987; CLARK *et al.* 1992; STERNBERG *et al.* 1992). Thus, the suppression of these Muv phenotypes by *cgr-1(lf)* mutations indicates that *cgr-1* functions downstream of *lin-15* and *let-60* if these genes function in a linear signaling pathway. The *cgr-1(lf)* mutations did not suppress the Muv phenotypes caused by *lin-1(lf)*, *lin-31(lf)*, or *lin-12(gf)* mutations. These results indicate that *cgr-1* does not simply prevent execution of vulval cell fates

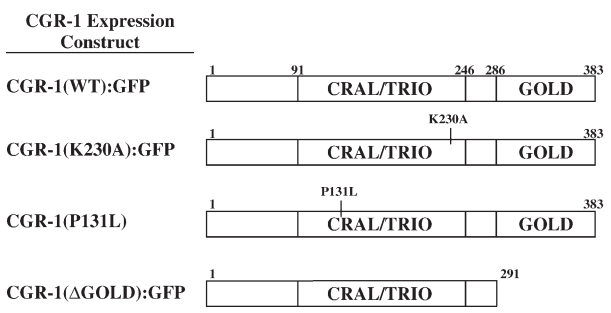

FIGURE 4.—Structure/function analysis of CGR-1. Transgenic strains that expressed the diagrammed CGR-1 protein and had the chromosomal genotype *let-60(n1046); cgr-1(n2528)* were assayed for rescue of the *cgr-1* suppression of Muv phenotype. Extrachromosomal arrays are described in Table 4 and criteria for rescue are described in Figure 1.

and that *cgr-1* functions upstream of *lin-1*, *lin-31*, and *lin-12* if these genes function in a linear signaling pathway. The signal transduction pathway downstream of *let-60 ras* and upstream of *lin-1* includes the core signaling genes *lin-45* Raf, *mek-2* MEK, and *mpk-1* ERK and the positive modifiers *ksr-1* and *cdf-1*. Our data do not establish the order of action of *cgr-1* relative to these genes. These results are also consistent with the model that *cgr-1* functions in parallel to the genes in the Ras pathway. For example, the induction of vulval cell fates is positively regulated by a Wnt signaling pathway that includes the positive regulator *bar-1* and the negative regulator *pry-1* (Eisenmann *et al.* 1998; Hoier *et al.* 2000; Gleason *et al.* 2002). The activity of this Wnt pathway positively regulates transcription of the *lin-39* homeotic gene, which promotes vulval cell fates. If *cgr-1* functions in parallel to the Ras signaling pathway, then it might promote Wnt signaling.

The suppression of the Muv phenotype caused by *cgr-1(lf)* mutations demonstrates that *cgr-1* affects the cell fates of P3.p, P4.p, and P8.p. In two sensitized genetic backgrounds, *cgr-1(lf)* mutations caused a Vul phenotype characterized by a reduced number of descendants of P5.p, P6.p, and P7.p. Thus, *cgr-1* affects the fates of all six P*n*.p cells. To address whether *cgr-1* acts cell autonomously in these P*n*.p cells, we expressed *cgr-1* in specific tissues. CGR-1 is highly expressed in intestinal cells, but specific expression in these cells did not affect vulval development. Expression in intestinal cells may mediate a different function of CGR-1. By contrast, CGR-1 expression in P*n*.p cells was sufficient to promote vulval cell fates, indicating that *cgr-1* acts cell autonomously in P*n*.p cells. These findings are consistent with the model that *cgr-1* functions in P*n*.p cells to affect the signaling pathway at a step downstream of *let-60 ras* and upstream of the LIN-1 transcription factor.

An *in vivo* function for a CRAL/TRIO and GOLD domain protein: A large number of proteins contain a CRAL/TRIO or a GOLD domain, often in combination with other conserved motifs. Here we focus on proteins that contain both motifs, since these are likely to share an ancestral gene and may have retained conserved functions. We used bioinformatic searches to identify proteins with both domains in vertebrates, insects, and nematodes, and we constructed a phylogeny for this protein family. These studies demonstrate that this protein family has been conserved during the evolution of multicellular animals. Only two of these proteins have been functionally characterized: supernatant protein factor (SPF) has been analyzed biochemically, and CGR-1 is described here. The other proteins are predicted on the basis of genomic DNA sequences and have not yet been characterized.

SPF was discovered because it stimulates conversion of squalene to lanosterol by liver microsomes (Tchen and Bloch 1957). Purified SPF can stimulates two enzymes, squalene epoxidase and squalene-2-3-oxide lanosterol cyclase (Ferguson and Bloch 1977; Caras and Bloch 1979). The mechanism of SPF has been investigated extensively but has not yet been fully defined. Purified SPF can facilitate squalene transfer between membranes, and it appears to enhance the availability of substrates to cholesterol biosynthetic enzymes. However, it does not bind squalene but rather binds anionic phospholipids (Caras *et al.* 1980).

The gene encoding SPF was recently cloned (Shibata *et al.* 2001), and the crystal structure was recently reported (Stocker *et al.* 2002). The structure of the CRAL/TRIO domain of SPF is similar to that of SEC14, and both form hydrophobic binding pockets that mediate binding to hydrophobic ligands (Sha *et al.* 1998). The GOLD domain forms an unusual eight-stranded jelly-roll barrel structure characteristic of viral proteins that bind to the outer surface of cell membranes (Stocker *et al.* 2002). The function of the GOLD domain is not well characterized. One possible function of the GOLD domain is suggested by an analysis of GCP-60. The GOLD domain of GCP-60 is necessary for binding to the Golgi resident protein, Giantin (Sohda *et al.* 2001), suggesting that the GOLD domain may mediate protein–protein interactions. SPF was independently identified on the basis of its ability to bind α-tocopherol and called tocopherol-associated protein (TAP) (Zimmer *et al.* 2000). However, the physiological significance of tocopherol binding is unclear. These enzymatic and structural studies of SPF complement the genetic and *in vivo* studies of CGR-1 and suggest intriguing possibilities for the biochemical mechanism of CGR-1.

CGR-1 is not likely to regulate cholesterol synthesis, like SPF, since *C. elegans* does not synthesize cholesterol and appears to lack the enzymes that are stimulated by SPF. However, the CRAL/TRIO and GOLD domains of CGR-1 are highly conserved and likely to adopt structures that are similar to SPF. Thus, CGR-1 is likely to contain a hydrophobic binding pocket and to interact with a hydrophobic ligand. Both Ras and Raf interact with lipids, and we speculate that CGR-1 might regulate signaling by affecting these interactions. Ras is membrane tethered by a lipid modification, and CGR-1 might affect the localization or membrane environment of Ras. Raf is recruited to the plasma membrane where it becomes activated by mechanisms that have yet to be fully defined. Raf can bind phospholipids such as phosphatidyl serine and phosphatidic acid, and these interactions may promote Raf activation (Ghosh *et al.* 1996; McPherson *et al.* 1999). CGR-1 might promote the activation of Raf by facilitating access to such a hydrophobic ligand. Although more studies are required to elucidate the mechanism of action of CGR-1, our results document that CGR-1 plays an important role in Ras-mediated signaling. This function may be conserved, and other members of the CGR-1 family may also regulate signal transduction.

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