
A regulatory cytoplasmic poly(A) polymerase in Caenorhabditis elegans

Liaoteng Wang*, Christian R. Eckmann†, Lisa C. Kadyk†‡, Marvin Wicke‡ & Judith Kimble‡†

* Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA
‡ Howard Hughes Medical Institute, 433 Babcock Drive, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA

Supplementary Information accompanies the paper on Nature's website (http://www.nature.com/nature).

Acknowledgements
We thank J. J. Casal, S. Harmer, P. Mas and F. Harmon for critical reading of the manuscript. This work was supported by an NIH grant to S.A.K. The work of M.J.Y. was initially supported by Conicet, Antorchas and the University of Buenos Aires and, more recently, by the Pew Foundation.

Competing interests statement
The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.A.K.

A regulatory cytoplasmic poly(A) polymerase in Caenorhabditis elegans

Messanger RNA regulation is a critical mode of controlling gene expression. Regulation of mRNA stability and translation is linked to controls of poly(A) tail length. Poly(A) lengthening can stabilize and translationally activate mRNAs, whereas poly(A) removal can trigger degradation and translational repression. Germline granules (for example, polar granules in flies, P granules in worms) are ribonucleoprotein particles implicated in translational control. Here we report that the Caenorhabditis elegans gene gld-2, a regulator of mitosis/meiosis decision and other germline events, encodes the catalytic moiety of a cytoplasmic poly(A) polymerase (PAP) that is associated with P granules in early embryos. Importantly, the GLD-2 protein sequence has diverged substantially from that of conventional eukaryotic PAPs, and lacks a recognizable RRM (RNA recog-
in the germ line and is developmentally regulated.

Database searches revealed that GLD-2 protein belongs to the DNA polymerase β-like superfamily of nucleotidyltransferases (NT) (Fig. 2a; refs 6, 7). Specifically, GLD-2 is a group 2 NT member, including DNA polymerase σ of Saccharomyces cerevisiae (also known as pol κ and Trf4p) and eukaryotic PAPs (Fig. 2a). GLD-2 architecture and sequence is divergent from that of canonical PAPs (Fig. 2b, d), but similar to a different cluster of NT family members (Fig. 2e). GLD-2 contains three critical carboxyamide side chains essential for catalytic activity (Fig. 2c, red) present in all DNA polymerase β superfamily members; furthermore, GLD-2 possesses putative ATP-interacting residues (Fig. 2c, green; Fig. 2d, green). Classical PAPs have a catastrophic region (Fig. 2c, gold), a ‘central’ domain (Fig. 2c, blue), and an RRM-like region (Fig. 2c, violet). By sequence comparison, GLD-2 harbours catalytic and central domains (Fig. 2b, d, colour-coded overlines), but is highly diverged from classical eukaryotic PAPs, including C. elegans PAP-1 (C. Liutjens and M.W., unpublished results) (Fig. 2d). Classical PAPs show extensive amino-acid conservation among themselves, but limited conservation with GLD-2 (Fig. 2d, black and grey boxes). Outside its catalytic and central domains, GLD-2 shares little similarity to canonical PAPs; in particular, GLD-2 has no apparent RRM-like region (Fig. 2b), which is thought to be critical for PAP RNA binding. Therefore, GLD-2 shares some key features with classical PAPs, but is divergent in motif architecture and amino acid sequence.

To examine GLD-2 protein, we generated polyclonal antibodies to the amino-terminal region (Fig. 2b) and detected a prominent protein of relative molecular mass 125,000 (M, 125K) on western blots (Fig. 3a, lanes 1, 4, 5). This protein, which corresponds in size to the predicted product of the germine glD-2 mRNA, was detected in glD-2(h292) homozygotes and glD-2(q497)/+ heterozygotes (Fig. 3a, lanes 6, 7), but not in glD-2(q497) homozygotes (Fig. 3a, lane 8). Pre-immune serum did not recognize this band, but detected others that served as a loading control (not shown). We conclude that the α-GLD-2 antibody recognizes GLD-2, that the glD-2(h292) mutant produces a nearly wild-type level of protein and that glD-2(q497) is a strong loss-of-function or null allele.

By immunocytochemistry, GLD-2 was found to be predominantly cytoplasmic in both germ line (Fig. 3b) and early embryo (Fig. 3c). Within the germ line, GLD-2 was detectable in the mitotic region and became abundant during pachytene and oogenesis (Fig. 3b). GLD-2 decreased during spermatogenesis in both sexes, and was undetectable in mature sperm (not shown). In early embryos, GLD-2 was diffuse in the cytoplasm of early P0 embryos, co-localized with P granules in late P0 embryos and remained associated with P granules in germline blastomeres (Fig. 3c, not shown). P granules are essential for germline development. In ~100-cell embryos, GLD-2 was undetectable.

Given its presence in oocytes and early embryos, we tested whether GLD-2 was required for embryogenesis. To deplete both maternal and zygotic glD-2 mRNAs, wild-type adult hermaphrodites were treated with double-stranded RNA corresponding to either the glD-2(5′-5′) or its common region (exons 16–18) to produce glD-2(RNAi) embryos (see Methods). In both cases, most glD-2(RNAi) embryos failed to hatch (99%, n > 500 in 26–36 h period after treatment). To visualize chromosomes in glD-2(RNAi) embryos, we used a strain carrying a histone-GFP transgene (AZ212). Whereas mock-treated AZ212 embryos cleaved normally (Fig. 3d), glD-2(RNAi) AZ212 embryos did not cleave and possessed malformed nuclei in clusters (Fig. 3e). We conclude that glD-2 activity is required for embryogenesis, and that GLD-2 protein co-localizes with P granules.

A specific interaction between GLD-2 and another germline regulator, GLD-3 (ref. 5), was discovered in yeast two-hybrid screens. Specifically, using GLD-2 as ‘bait’, 2,000,000 transformants
were screened and 30 gld-3 cDNAs (T07F8.3) found; using GLD-3 as bait, 1,500,000 transformants were screened and 94 gld-2 cDNAs recovered. To identify the region of GLD-2 critical for GLD-3 binding, GLD-2 variants were assayed for GLD-3 interaction. A GLD-2 fragment comprising both catalytic and central domains was essential (amino acids 544–924) (Fig. 4a). A GLD-2-E875K mutant, designed after gld-2(h292)4, interacted poorly with GLD-3 (Fig. 4a, E875K and Δ7). Indeed, β-galactosidase activity was reduced 7- to 16-fold by GLD-2(h292)-E875K (Fig. 4a, compare for example Δ2 to Δ7), but GLD-2 levels were equivalent (Fig. 4b). Importantly, GLD-2-E875K was present at normal levels in C. elegans (Fig. 3a, lane 6), even though it disrupts gld-2 function. We conclude that GLD-2 binds specifically to GLD-3, and that GLD-2-E875K is defective in GLD-3 binding. Therefore, the GLD-2/GLD-3 interaction appears to be important for development.

Given its sequence similarity to nucleotidyltransferases and its cytoplasmic location, we considered that GLD-2 might be a cytoplasmic PAP, even though its architecture and sequence diverged substantially from classical PAPs. To test this idea, we initially assayed incorporation of radiolabelled ATP into an RNA substrate. Specifically, GLD-2 was translated in vitro, either on its own or together with GLD-3. The in vitro translation mixture was incubated with [32P]-ATP and an unlabelled poly(A) substrate, and incorporation of label into acid-insoluble material was measured (see Methods). GLD-2 on its own had low activity, whereas GLD-3 had none; however, GLD-2 and GLD-3 together gave a robust response (Fig. 4c). We also measured incorporation in three control reactions (no protein and two GLD-2 mutants together with GLD-3). GLD-2-D608A was designed to abolish the catalytic site GLD-2–GLD-3 binding (Fig. 4a). The GLD-2-E875K level was abolished by the E875K mutation (Fig. 4d, lane 6), which disrupts GLD-2/GLD-3 binding (Fig. 4a). The GLD-2-E875K level was equivalent to wild-type GLD-2 (Fig. 4d, compare lanes 4 and 6). Third, GLD-2-dependent incorporation is substrate dependent and requires ATP (not shown). Thus, replacement of ATP with GTP, CTP or UTP did not yield incorporation onto the substrate. Finally, products produced by GLD-2 plus GLD-3 were selectively retained on oligo(dT) cellulose, suggesting they were polyadenylated (not shown).

The GLD-2/GLD-3 enzyme represents a new type of poly(A) polymerase (Fig. 5). Canonical PAPs, which include nuclear and cytoplasmic enzymes, are all closely related12–15; they are monomeric and possess three key domains (Fig. 5, left)8,9. By contrast, GLD-2 appears to function as a heterodimer (Fig. 5, right). GLD-2 harbours the catalytic and central domains; GLD-3 has five consecutive K homology (KH)-related motifs9 which may, at least in

Figure 3 The GLD-2 protein. Polyclonal anti-GLD-2 antibodies were affinity purified.

a. Western blot of proteins from wild-type embryos (E), larvae (L1–L4), and adults (A) (lanes 1–5), and adults of genotype gld-2(h292)/gld-2(h292) (lane 6), gld-2(q497)/gld-2(+/+) (lane 7), and gld-2(q497)/gld-2(q497) (lane 8). b. GLD-2 protein is in germline cytoplasm. Extruded WT adult hermaphrodite germ line; GLD-2 is abundant in pachytene region and oocytes. Magnified view shows lack of GLD-2 in nuclei (arrowheads) and presence of GLD-2 in granular form (arrows). A control gld-2(q497) extruded germ line showed no anti-GLD-2 staining (not shown). c. GLD-2 protein is associated with P granules in early embryos. Embryos stained with antibody to P granule marker, PGL-1, to GLD-2, and to nuclear pore antigen. Top, late P0 embryo, GLD-2 colocalizes with P granules; second panel down, 28-cell embryo, P4, white arrowhead; third panel, ~100-cell embryo, germline precursor cells, Z2 and Z3, arrows; bottom, magnified view of P2 blastomere to show PGL-1 and GLD-2 co-localization (arrows). d. e. Transgenic strain AZ212. Left, Nomarski image; right, nuclei visualized by histone::GFP marker. Both control and gld-2(RNAi) embryos are of approximately same age. d. Mock injected control. e. gld-2(RNAi).
GLD-2 and GLD-3 are likely to function together during nematode development. First, GLD-2 and GLD-3 have similar, albeit not identical, functions in germline development and embryogenesis (refs 4, 5, and this work). Second, both are cytoplasmic and associated with P granules (ref. 5, this work), large complexes of RNA and protein that are critical for germline development19,20. GLD-2 and GLD-3 may polyadenylate mRNAs associated with P granules (for example, nos-2; ref. 16) or may be stored there for segregation to germline blastomeres. GLD-2 may be targeted to specific mRNAs by GLD-3, which is a Bic-C family KH protein. Other KH proteins (EMR8, NOVA, hnrNPK) bind RNAs through sequence-specific interactions17-20. GLD-2 may also be targeted to specific mRNAs indirectly via the interaction of GLD-3 with FBP3. FBP is a sequence-specific RNA-binding protein and member of the PUF family21. PUF proteins appear to repress mRNAs by promoting poly(A) removal22, GLD-3 antagonizes FBP3, and works with GLD-2 to promote poly(A) addition (this work). Therefore, GLD-2/GLD-3 may switch FBP from a repressive to an activating mode.

Regulatory cytoplasmic PAPs of the GLD-2/GLD-3 class may be common. Within the large superfamily of DNA polymerase β-like nucleotidyltransferases, several are closely related to GLD-2 (Fig. 2e). To date, most have no assigned function, but Schizosaccharomyces pombe Cid13 and Cid1 appear to be rcPAPs22,23. The similarity between GLD-2 and Cid1 is particularly striking, as both are involved in cell cycle control. GLD-2 promotes entry into meiosis at the expense of mitosis9, and Cid1 inhibits mitosis25. We suggest that GLD-2 and Cid1 may in fact be components of an ancient regulatory circuit controlling the cell cycle, and that other GLD-2 relatives may similarly be regulatory cytoplasmic PAPs.

**Methods**

**Molecular cloning of gld-2**

Three-factor mapping places gld-2 0.05 map unit to the right of bli-4. Cosmids in this region were injected into strain JK1716 [hli-4(e957) gld-2(m6697) dpy-5(e61)] or strain JK1732 [hli-4(e937) gld-2(k692) dpy-5(e61)] amc-13(e51)]. Cosmid ZC308 gave transformants resistant to amc-13(e51). A stretch of 22 As was found at the end of the 3′ untranslated region (UTR). To determine the gld-2 3′ end, semi-nested PCR was performed using λE. coli, C. elegans mixed-stage oligo(dT) primed complementary DNA library (gift from A. Puoti). One PCR product was confirmed and sequenced. A stretch of 22 As was found at the end of the 3′ untranslated region (UTR). To determine the gld-2 5′ ends, reverse transcriptions (RT) were performed using SuperScript II Reverse Transcriptase (Gibco BRL) and poly(A)+ RNA from either wild-type mixed-stage worms or gfp-1(q224) mutants raised at 25°C, which have no germ line. The resultant cDNAs were then used as templates for semi-nested PCR with SL1 (a trans-spliced leader in C. elegans) as the constant 5′ primer. All PCR products were cloned into pSTBlue-1 and sequenced. The 4.7-kb mRNA is SL1 trans-spliced, comprises 19 exons including an 86-nucleotide 5′ UTR and 1,105-nucleotide 3′ UTR.

**Transcript analyses**

Northern blots were performed as described44. Templates for making RNA probes (gld-2 5′, middle, 3′; efh-3) were made by polymerase chain reactions (PCRs) from pJK830, pJK831, pJK832 and pBluescript-efh-3 (gift from P. Anderson). To determine the gld-2 2′ end, semi-nested PCR was performed using λE. coli, C. elegans mixed-stage oligo(dT) primed complementary DNA library (gift from A. Puoti). One PCR product was confirmed and sequenced. A stretch of 22 As was found at the end of the 3′ untranslated region (UTR). To determine the gld-2 5′ ends, reverse transcriptions (RT) were performed using SuperScript II Reverse Transcriptase (Gibco BRL) and poly(A)+ RNA from either wild-type mixed-stage worms or glp-1(q224) mutants raised at 25°C, which have no germ line. The resultant cDNAs were then used as templates for semi-nested PCR with SL1 (a trans-spliced leader in C. elegans) as the constant 5′ primer. All PCR products were cloned into pSTBlue-1 and sequenced. The 4.7-kb mRNA is SL1 trans-spliced, comprises 19 exons including an 86-nucleotide 5′ UTR and 1,105-nucleotide 3′ UTR.

**Antibody production, western blot and immunocytochemistry**

Polyclonal antibodies were generated from rabbits using a keyhole limpet haemocyanin (KLH)-conjugated peptide corresponding to GLD-2 amino acids 108–127 (Genemed Synthesis) or from rats using a GST–GLD-2 fusion protein carrying amino acids 13–330 of GLD-2. Rabbit anti-PGL-1 antibody was a gift from S. Strome. Monoclonal antibody 414, the anti-nuclear pore monoclonal, was purchased from BABCO. Western blots were performed using the GLD-2 peptide antibody as described44. Immunocytochemistry followed published procedures45 using the GST–GLD-2 fusion-protein antibody, which was specific for GLD-2 as demonstrated on gld-2(q497) extruded germ lines and gld-2(RNAi) embryos.
Poly(A) polymerase assay

Proteins were in vitro translated using the TNT coupled transcription-translation system (Promega), and assayed using buffer conditions essentially as described17. For scintillation counting, poly(A) (Roche) was used as substrate. For gel assays, we used RNA oligo, C20A60 (DHaracon) a 45-nucleotide and supplemental 1 mM MgCl2. Products were analysed on 12% sequencing gels.

Received 8 May; accepted 16 July 2002; doi:10.1038/nature01039.