The PGL Family Proteins Associate With Germ Granules and Function Redundantly in Caenorhabditis elegans Germline Development

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ABSTRACT

PGL-1 is a constitutive protein component of C. elegans germ granules, also known as P granules. Maternally supplied PGL-1 is essential for germline development but only at elevated temperature, raising the possibility that redundant factors provide sufficient function at lower temperatures. We have identified two PGL-1-related proteins, PGL-2 and PGL-3, by sequence analysis of the C. elegans genome and by a yeast two-hybrid screen for proteins that interact with PGL-1. PGL-3 is associated with P granules at all stages of development, while PGL-2 is associated with P granules only during postembryonic development. All three PGL proteins interact with each other in vitro. Furthermore, PGL-1 and PGL-3 are co-immunoprecipitated from embryo extracts, indicating that they are indeed in the same protein complex in vivo. Nevertheless, each PGL protein localizes to P granules independently of the other two. pgl-2 or pgl-3 single-mutant worms do not show obvious defects in germline development. However, pgl-1; pgl-2; pgl-3 double-mutant hermaphrodites and males show significantly enhanced sterility at all temperatures, compared to pgl-1 alone. Mutant hermaphrodites show defects in germline proliferation and in production of healthy gametes and viable embryos. Our findings demonstrate that both PGL-2 and PGL-3 are components of P granules, both interact with PGL-1, and at least PGL-3 functions redundantly with PGL-1 to ensure fertility in both sexes of C. elegans.

In multicellular organisms that undergo sexual reproduction, specialized cells, called germ cells, are solely responsible for the generation of offspring and the propagation of species. Germ cells are distinct from somatic cells in several fundamental aspects (reviewed in Marsh and Goode 1994; Saffman and Lasko 1999). Germ cells undergo the specialized cell cycle, meiosis, to reduce the ploidy from diploid to haploid and to generate gametes. The germ lineage is considered to be totipotent and immortal, since united gametes are able to generate the entire somatic body of the organism, as well as more germ cells and thus future generations. In contrast, somatic cells undergo only mitosis, show restricted developmental potential, and senescence and die with each generation. Understanding the molecular mechanisms that underlie the fundamental differences between the germ-line and soma and confer upon the germline its special characteristics remains a major issue in developmental biology.

In many organisms, primordial germ cells are set apart from the somatic lineages early in embryogenesis (Wylie 1999) and often contain distinctive, electron-dense cytoplasmic organelles, generally called “germ granules” (Eddy 1975; Saffman and Lasko 1999). The presence of germ granules in diverse organisms and the finding that ectopic granules can induce ectopic germ-cell formation in Drosophila and Xenopus (Illmensee and Mahowald 1974; Ikenishi 1987; Ephrussi and Lehmann 1992) has led to the widely accepted view that germ granules carry essential factors or “determinants” for germline development.

In the nematode Caenorhabditis elegans, germ granules, also called “P granules,” are present in germ cells throughout the life cycle (Strome and Wood 1982, 1983; Kawasaki et al. 1998). P granules are maternally contributed to the fertilized egg and partitioned to the germline blastomere (P cell) during each of four unequal divisions of the early embryo. This partitioning delivers the majority of granules to the primordial germ cell, P4. The two daughters of P4, Z2 and Z3, divide throughout

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larval development, giving rise to the ~1500 germ cells in an adult hermaphrodite. P granules are present in all descendants of P, with the exception of mature sperm. At all stages except oogenesis and early embryogenesis, P granules are closely associated with the outer surface of the nuclear envelope. This perinuclear localization is a common feature of germ granules in diverse organisms (Eddy 1975). An ultrastructural analysis of the perinuclearly localized P granules (Pitt et al. 2000) revealed their tight association with nuclear pore clusters, suggesting the possible involvement of P granules in nuclear-cytoplasmic transport of RNAs and/or proteins.

P granules are known to contain poly(A)⁺ RNAs (Seydoux and Fire 1994) and numerous proteins that are predicted to bind RNA. Two maternal mRNAs that associate with P granules in early embryos are nos-2 (Subramaniam and Seydoux 1999) and pos-1 (Schisa et al. 2001). nos-2 and the related gene nos-1 encode proteins similar to the Drosophila germ-granule component Nanos (Wang and Lehmann 1991) and function redundantly to regulate germ-cell proliferation and viability (Subramaniam and Seydoux 1999). pos-1 encodes a zinc-finger protein that functions at least in part to protect germline fate (Tabara et al. 1999). In addition, several class II maternal mRNAs (Seydoux and Fire 1994), which are degraded rapidly in somatic blastomeres but persist in germline blastomeres during early embryogenesis, have been shown to localize to P granules in adult gonads (Schisa et al. 2001). All of the identified protein components of P granules contain RNA-binding motifs and thus are predicted to associate with RNA. One class of proteins, including PIE-1 (Mello et al. 1992, 1996), GLD-1 (Jones et al. 1996), MEX-3 (Draper et al. 1996), MEX-1 (Guedes and Priess 1997), and POS-1 (Tabara et al. 1999), associates with P granules only during early embryogenesis and disappears in Z2 and Z3. Most proteins in this class function in early embryogenesis to prevent either the germline blastomeres from adopting somatic fates (pie-1, mex-1, and pos-1) or certain somatic blastomeres from adopting a germline fate (mex-3).

Proteins that are constitutively associated with P granules are likely to serve crucial roles in the assembly and functions of this organelle. In this class are PGL-1, GLH-2, GLH-3, and GLH-4 (Subramaniam et al. 1999; Kuznicki et al. 2000). GLH-1, a novel protein, is associated with P granules at all stages of development. The presence of an RNA-binding motif, an RGG box, at its C terminus predicts that PGL-1 is an RNA-binding component of P granules. pgl-1 mutants are sterile, with both a maternal and a zygotic component to the sterility. Sterility is the result of defects in germline proliferation and gametogenesis. Interestingly, the sterility caused by null alleles of pgl-1 is highly sensitive to temperature, suggesting that either PGL-1 functions as a molecular chaperone whose function is critical primarily at elevated temperature or other partially redundant proteins exist, which function with PGL-1 and are sufficient for fertility at permissive temperature. The latter possibility gained support from our identification and analysis of two pgl-1-related genes, pgl-2 and pgl-3. The proteins encoded by both genes are indeed components of P granules, although PGL-2 is notably absent from P granules in embryos. The three PGL proteins interact with each other in vitro, and at least PGL-1 and PGL-3 are associated with each other in vivo as well. Each PGL protein localizes to P granules independently of the other two. A deletion allele of pgl-3 does not result in germline defects on its own but significantly enhances the sterility of pgl-1 at low temperatures, supporting the hypothesis that PGL-1 and PGL-3 function redundantly during germline development. A deletion allele of pgl-2 does not result in germline defects on its own or significantly enhance the phenotype of pgl-1 or pgl-1: pgl-3, suggesting that PGL-2 is not a major player in germline development.

MATERIALS AND METHODS

**Strains and alleles:** Maintenance and genetic manipulation of *C. elegans* were carried out as described in Brenner (1974). *C. elegans* strain N2 variety Bristol was used as the wild-type strain. Mutations used in this study were LGL, glh-1(bn103), glh-4(bn2); LGIII, pgl-2(bn123); LGIV, pgl-1(ct131, bn101), him-
 Yeast two-hybrid screen: pgl-2 and pgl-3 cDNAs were isolated in a yeast two-hybrid screen (Fields and Song 1989; Durfee et al. 1993) using full-length pgl-1 cDNA (Kawasaki et al. 1998) as “bait” and an oligo(dT)-primed C. elegans cDNA library, λACt-RBl, provided by R. Burstead (Barstead and Waterston 1989) as “prey.” The positive cDNA clones were sorted by Southern hybridization analysis using some of their cDNA inserts as probes.

Northern and in situ hybridization analyses: Northern hybridization analysis was done as in HolDerMan et al. (1998). Band intensity of transcripts was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and the levels of pgl-2 and pgl-3 transcripts were normalized to the level of rpp-1 transcript, which encodes a ribosomal protein (Evans et al. 1997). cDNA clones pBS-pgl-2 and pGEM-pgl-3, which contain the longest inserts (1.7 and 2.4 kb for pgl-2 and pgl-3, respectively) among the clones isolated from the yeast two-hybrid screen, were used as probes. In situ hybridization was performed as described in Tabara et al. (1996) with some modifications. For pgl-1, an expressed sequence tag (EST) cDNA clone, yk22598, was used as probe. For pgl-2, pBS-pgl-2 described above, was used as probe. For pgl-3, EST cDNA clones, yk5186d and yk231a5, were used as probes for larval and embryonic in situ hybridizations, respectively.

Antibody production: A 1539-bp pgl-2 cDNA fragment encoding the entire PGL-2 sequence except the first 20 amino acids was cloned into the expression vector pET-30a+ (Novagen). A portion of pgl-3 cDNA (279 bp) corresponding to the PGL-3 region from Ser-448 to Ser-540 (see Figure 2), which is relatively unique to PGL-3, was PCR amplified using primers 5'-GAGGATTCCAGTGTGCGAACTCCATG-3' and 5'-TCC CAGCTTAGCTAAAAATTGACGATTG-3' and cloned into the expression vector pET-28a+ (Novagen). The 6x His-tagged fusion proteins were expressed in Escherichia coli BL21(DE3) by isopropyl thigalactoside (IPTG) induction and purified using Ni-NTA agarose columns (Qiagen, Chatsworth, CA) under denaturing conditions. The purified fusion proteins were electrophoresed on SDS-polyacrylamide gels, cut from the gels, and injected with Freund’s adjuvant into rabbits for the PGL-2 fusion protein and into rats for the PGL-3 fusion protein. Antibodies against PGL-2 and PGL-3 were purified from crude antisera by blot affinity purification (O’Millsted 1986). Monospecific antibodies bound to the 6x His fusion proteins on nitrocellulose-membrane strips were eluted with 0.2 M glycine-HCl (pH 2.8).

Immunofluorescence analysis: DNA staining of intact worms and extruded gonads was carried out as in Kawasaki et al. (1998). Immunostaining of extruded gonads and embryos was done as in Strome and Wood (1983). For immunostaining, the following primary antibodies were used: affinity-purified rabbit anti-PGL-1 diluted 1:100, affinity-purified rabbit anti-PGL-2 diluted 1:50, affinity-purified rat anti-PGL-3 diluted 1:10, affinity-purified rabbit anti-GLH-I diluted 1:100, rabbit anti-HIM-3 (a gift from M. Zetka) diluted 1:500, rabbit anti-phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1:200, mouse monoclonal antichromatin antibody PA3 (a gift from M. Monestier) diluted 1:100, mouse monoclonal anti-PGL-1 antibody K76 (Strome 1986) undiluted supernatant, and mouse monoclonal spermatogenesis-specific antibody SP56 undiluted supernatant. Secondary antibodies used were Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted 1:400 or 1:100, Alexa Fluor 488 goat anti-rat IgG (Molecular Probes) diluted 1:100, and FITC-conjugated goat anti-mouse IgG (Jackson) diluted 1:100. Samples were examined using a Zeiss Axioskop microscope equipped with Nomarski differential interference contrast and epifluorescence optics. Images were either photographed with a Tri-X pan film, scanned, and processed using Adobe Photoshop (Adobe Systems) or acquired with an AxioCam CCD camera (Zeiss) and processed using Openlab 3 (Improvement).

Glutathione S-transferase fusion protein construction, expression, and pull-down experiments: Full-length pgl-1 cDNA (2190 bp) was amplified by PCR from pBS-pgl-1 (Kawasaki et al. 1998) using primers 5'-CTCCAGATTGACGCTAACAAG CGAGAA-5' and 5'-GCCGCGCCTTAAAGACCTCCTGTC CAC-3'. The PCR product was digested with Xhol and Nofl and ligated to a glutathione-S-transferase (GST) gene fusion vector, pGEX-5X-3 (Amerham Pharmacia Biotech), prelin- carized by digestion with SalI and NotI and form pGEX-3gl-1. Full-length pgl-2 cDNA (1.7 kb) was PCR amplified from pBS- pgl-2 described in the previous section, using primers 5'-ACGA GATCTTGAAGCCCTAGTAAAGG-3' and 5'-ACTAGAAGC TTCAGGGAATCTCTCATAC-3' and cloned into BglII and HindIII sites in pGEX-2*, a derivative of pGEX-2T (Amerham Pharmacia Biotech), to form pGEX-pgl-2. Full-length pgl-3 cDNA (2.4 kb) was PCR amplified from pGEM-pgl-3, using primers 5'-CCGCTTGACACTAGTGGTAAGAGCGAACCAGG-3' and 5'-CCCGGATCTACTAAGTTGGAACCTCCAGG-3' and cloned into the EcoRI and SalI sites in pGEX-2* to form pGEX- pgl-3. E. coli BL21(DE3) was transformed with pGEX-pgl-1, pGEX-pgl-2, pGEX-pgl-3, or pGEX-2* (as control) and induced with IPTG to express GST-PGL-1, GST-PGL-2, GST-PGL-3, or GST. Purification of the GST fusion proteins was done as in Amiri et al. (2001). Radiolabeled PGL-1, PGL-3, and IFE-1 were synthesized by in vitro transcription-translation using pBS-pgl-1, pGEM-pgl-3, and pBS-ife-1, respectively, as templates with [35S]methionine in the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Binding assays were done as in Amiri et al. (2001).

Immuno precipitation and Western blot analyses: Embryo extract was prepared as in Xu et al. (2001). A total of 90 μl of N2 embryo extract was incubated with 3 μl of either rabbit anti-PGL-1 (Kawasaki et al. 1998) or rat anti-PGL-3 antiserum at 4°C for 1 hr after the extract was preabsorbed with 3 μl of either normal rabbit serum or normal rat serum (both from Jackson), respectively. The mixture was then incubated with 20 μl of protein A/G PLUS-agarose (Santa Cruz) at 4°C for 2 hr. The immuno-complex was precipitated by centrifugation, washed four times with 500 μl of RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) at 4°C for 10 min, resuspended in SDS sample buffer, boiled, and analyzed by SDS-PAGE. Western blot analysis was carried out as in Kawasaki et al. (1998) with some modifications. For detecting PGL-1 and PGL-3, affinity-purified rabbit anti-PGL-1 diluted 1:100 and affinity-purified rat anti-PGL-3 diluted 1:40, respectively, were used as primary antibodies, and as secondary antibodies, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated donkey anti-rat IgG (both from Jackson), respectively, were used (both at 1:10,000 dilution). Bound antibodies were visualized using enhanced chemiluminescence Western blot detection reagents (Amerham Pharmacia Biotech).

Isolation of pgl-2 and pgl-3 deletion mutants: Worm libraries mutagenized with trimethylpsoralen and UV irradiation (Yan- dell et al. 1994) were screened for a deletion mutation in either the pgl-2 locus (B0523.3) or the pgl-3 locus (C18G1.4) according to the protocol of G. Molder and R. Barstead (http://pmc1.fsl.ohiou.edu/Knockout/) with some modifications as described in Karashima et al. (2000). PCR primers used to screen for a pgl-2 deletion mutation were as follows: external primer set, pgl-2 367F, 5’-CGTTTTCTGTATACAGG-
RESULTS

Identification of the pgl-2 and pgl-3 genes: In a previous study (KAWASAKI et al. 1998), we showed that PGL-1, a constitutive component of P granules, is essential for C. elegans germline development, but only at elevated temperatures. We reasoned that this temperature sensitivity might be explained by the presence of other proteins that function redundantly with PGL-1. Indeed, our searches of the C. elegans genome (C. ELEGANS SEQUENCING CONSORTIUM 1998) revealed two open reading frames (B0523.3 and C18G1.4) whose predicted protein products show significant sequence similarity to PGL-1. The encoded proteins were also identified in a yeast two-hybrid screen for C. elegans proteins that interact with PGL-1 (Figure 1). In this screen, full-length PGL-1 protein was fused to the GAL4 DNA-binding domain (GAL4DB-PGL-1) and used as bait. Proteins encoded by the library cDNAs were fused to the GAL4 activation domain. Among 2 × 10^6 independent cDNA transformants screened, 270 clones were found to activate both HIS3 and lacZ reporter genes only in the presence of GAL4DB-PGL-1. Most (260) of the positive clones were found to correspond to one of four genes: ife-1, which encodes one of the five C. elegans isoforms of eukaryotic translation initiation factor 4E (eIF4E; AMIRI et al. 2001); pgl-1 itself, suggesting that PGL-1 protein can form multimers; and the two pgl-1-like genes, B0523.3 and C18G1.4, termed pgl-2 and pgl-3, respectively (Figure 1).

The sequences of the longest cDNA inserts for both pgl-2 and pgl-3 were determined, and their splicing patterns were deduced by comparison with their corresponding genomic sequences (C. ELEGANS SEQUENCING CONSORTIUM 1998). Their 5' ends were determined by sequencing RT-PCR products generated using a genespecific downstream primer and an upstream primer corresponding to the transspliced leader SL1 or SL2 (SPIETH et al. 1993). The pgl-2 gene contains seven exons (and six introns including a very long fifth intron of 2208 bp). pgl-2 mRNA is transspliced to SL1 and encodes a predicted protein of 532 amino acids. PGL-2 protein has 34% identity (140/414) and 67% similarity (279/414) with PGL-1 in its N-terminal 414 amino acids (Figure 2). Its C-terminal portion is not very similar to PGL-1. The pgl-3 gene contains eight exons. pgl-3 mRNA is transspliced to both SL1 and SL2 and encodes a predicted protein of 693 amino acids. PGL-3 protein has 62% identity (431/693) and 77% similarity (535/693) with PGL-1 throughout its length (Figure 2). Also, PGL-5 contains the same RNA-binding motif, an RGG box, as PGL-1 at its C terminus (Figure 2). An RGG box consists of multiple repeats of Arg-Gly-Gly, often interspersed with aromatic amino acids (Kiledjian and Dreyfuss 1992). The RGG box of PGL-3 consists of 59 amino acids and contains six Arg-Gly-Gly repeats. As the RGG-box motif is known to act as an RNA-binding domain in some RNA-binding proteins (see BURD and DREYFUS 1994 for review), PGL-3, like PGL-1, is predicted to be an RNA-binding protein.

pgl-2 and pgl-3 transcripts are enriched in the germ-line: Densitometric quantification of Northern hybridization blots (Figure 3) revealed that wild-type adult hermaphrodites contain six times more pgl-2 mRNA and 14 times more pgl-3 mRNA than glp-4(bn2ts) adult hermaphrodites do, which have a severely underproliferated germline at restrictive temperature (BEANAN and STROME 1992). These results indicate that both pgl-2 and pgl-3 transcripts are enriched in the germline.

To analyze the temporal and spatial distribution of the PGL-1 and PGL-3 proteins, we prepared antisera that recognize both species of PGL-1 and PGL-3. In C. elegans, PGL-1 is expressed in the hermaphrodite germline (Figure 4A), and PGL-3 is expressed in both the gonadal and intestinal germlines of the hermaphrodite (Figure 4B).
these transcripts, we performed in situ hybridization of 

\( pgl-2 \) and \( pgl-3 \) probes to whole-mount wild-type embryos and worms at different developmental stages (Tabara et al. 1996) and compared their patterns with that of \( pgl-1 \). Zygotic \( pgl-1 \) transcript first becomes detectable during the twofold stage of embryogenesis in the primordial germ cells, Z2 and Z3 (Figure 4G). \( pgl-1 \) transcript is highly enriched in the germline throughout larval development and in adults (Figure 5, left). \( pgl-1 \) mRNA is maternally loaded into embryos, as the signal is detectable in 1-cell embryos (Figure 4A). Signal appears evenly distributed until the 4-cell stage (Figure 4, B and C), after which it diminishes in somatic blastomeres. Signal persists primarily in P3 and E at the 8-cell stage (Figure 4D), in P3 at the 15-cell stage (Figure 4E), and in P4 at and after the 24-cell stage (Figure 4F). \( pgl-1 \) signal disappears from P4 before it divides to Z2 and Z3 around the 90-cell stage (data not shown).

Zygotic \( pgl-3 \) transcript first becomes detectable in the germline of L3 larvae (Figure 5, right), much later than the appearance of \( pgl-1 \) transcript. The signal becomes stronger in later stages of larval development and in adults and is highly concentrated in the germline, especially in the pachytene region. Like \( pgl-1 \) mRNA, \( pgl-3 \) mRNA is maternally loaded into embryos (Figure 4O) and is selectively retained by the germline blastomeres (Figure 4, Q–S). \( pgl-3 \) signal becomes undetectable before \( P_4 \) is generated at the 24-cell stage (Figure 4T). No signal is detectable throughout the rest of embryogenesis (Figure 4U) or in early larval stages (Figure 5, right).

The significance of the persistence of both \( pgl-1 \) and \( pgl-3 \) maternal mRNAs primarily in the P cells of early embryos is not known, but this pattern is seen for many other class II maternal mRNAs (Seydoux and Fire 1994). Interestingly, the 3'-untranslated region sequences of \( pgl-1 \) and \( pgl-3 \) mRNAs are very similar (data not shown), suggesting that the localization and/or stability of these two mRNAs may be controlled by a similar post-transcriptional mechanism(s).
Zygotic pgl-2 transcript becomes detectable in L1 larvae but is not significantly enriched in the germline (Figure 5, middle). The signal becomes more intense in the germline, with weak signal in somatic tissues, at later stages of larval development and in adults. In embryos, pgl-2 mRNA shows a uniform distribution in all cells through the early cleavage stages until the 100- to 200-cell stage (Figure 4, H–M), after which it gradually disappears (Figure 4N).

In summary, our in situ hybridization results demonstrate the following. First, pgl-1, pgl-2, and pgl-3 transcripts accumulate primarily in the germline of L3 and later-stage hermaphrodites and are maternally loaded into embryos. Second, the levels of pgl-1 and pgl-3 transcripts in the germline are much higher than the level of pgl-2. Third, transcripts of pgl-1 and pgl-3, but not of pgl-2, show the class II maternal mRNA behavior of persisting in the germline blastomeres and disappearing from the somatic blastomeres of early embryos. Fourth, pgl-1 joins nos-1 in being transcribed in the primordial germ cells, Z2 and Z3, of embryos (Subramaniam and Seydoux 1999).

PGL-2 and PGL-3 are components of P granules: Antibodies were raised against PGL-2 and a central region

Figure 4.—In situ hybridization analysis of pgl-1, pgl-2, and pgl-3 transcripts in whole-mount wild-type embryos at different developmental stages. Anterior is left, ventral is down, for all but the last row of embryos. Wild-type embryos were hybridized with cDNA probes for pgl-1 (A–G), pgl-2 (H–N), and pgl-3 (O–U), respectively. Developmental stages of embryos are indicated. Bar, 10 μm.
of PGL-3 and then purified by blot affinity purification (Olms ted 1986; see materials and methods). Specificity of the affinity-purified antibodies was demonstrated by immunostaining analysis of the pgl-2 and pgl-3 deletion mutants (Figure 6). Affinity-purified anti-PGL-2 antibody stained granules in wild-type N2 and pgl-3(bn104) mutants but not in pgl-2(bn123) mutants, and affinity-purified anti-PGL-3 antibody stained granules in wild-type N2 and pgl-2(bn123) mutants but not in pgl-3(bn104) mutants.

The affinity-purified antibodies against PGL-2 and PGL-3 stained the same granules as were stained by antibodies to PGL-1 (Figures 7 and 8), demonstrating that both PGL-2 and PGL-3 proteins are components of P granules. However, the distributions of the three PGL proteins show some temporal and spatial differences. In wild-type worms, PGL-1 appears to be associated with P granules evenly in all germ cells in both sexes throughout the life cycle, with one exception: PGL-1 disappears from P granules during spermatogenesis, perhaps to release IFE-1 from P granules (Amiri et al. 2001). Similar to PGL-1, PGL-3 is associated with P granules in both sexes at all stages of development except in spermatogenesis. But unlike PGL-1, PGL-3 is present in a distinct gradient in the adult germline: progressively more enriched in the pachytene and diplon tene meiotic regions than in the distal mitotic region (compare Figure 7, E and F). Compared to pgl-1 mRNA, pgl-3 mRNA also seems more enriched in the meiotic region of the gonad than in the mitotic region (Figure 5, compare left and right columns).

Notably, PGL-2 is associated with P granules only during postembryonic development (Figure 7B); it is not detectable in embryos (Figure 8, B, D, and F). PGL-2 is first detected in L1 larvae, and like its transcript, it is not significantly enriched in the germ line in newly hatched L1’s (data not shown). Concomitant with mitotic proliferation of germ cells, PGL-2 appears to accumulate in P granules, with weak signal still remaining in somatic tissues (data not shown). It remains associated with P granules in adult gonads in both sexes until it disappears during spermatogenesis and during the final stages of oogenesis (data not shown).

The three PGL proteins associate with each other in vitro and in vivo: To verify that the interactions between PGL-1 and itself, PGL-2, PGL-3, and IFE-1 detected by
Figure 6.—Immunofluorescence analysis of antibody specificity. Adult or L4 hermaphrodite gonads from wild-type N2 (A and B), pgl-2(bn123) mutants (C and D), or pgl-3(bn104) mutants (E and F) were stained with either affinity-purified rabbit anti-PGL-2 antibody (A, C, and E) or affinity-purified rat anti-PGL-3 antibody (B, D, and F). Anti-PGL-2 stained N2 (A) and pgl-3 (E) gonads but failed to stain pgl-2 (C) gonads. Anti-PGL-3 stained N2 (B) and pgl-2 (D) gonads but failed to stain pgl-3 (F) gonads. Bar, 100 μm.

Figure 7.—Immunofluorescence analysis of PGL protein distributions in adult hermaphrodite germlines. The distal end of each gonad arm is top left. Adult hermaphrodite gonads from wild-type N2 (A, B, E, and F) or pgl-1(bn101) mutants (C, D, G, and H) were costained either with mouse monoclonal antibody K76, which recognizes an epitope on PGL-1 (A and B), 35S-PGL-1 (~100 kD) bound to both GST-PGL-1 and GST-PGL-2, but not to GST alone (Figure 9A, lanes 1–4). Similarly, 35S-PGL-3 (~90 kD) bound to both GST-PGL-1 and GST-PGL-2, but not to GST alone (Figure 9A, lanes 5–8). 35S-IFE-1 (~25 kD) bound to GST-PGL-1, but not to GST-PGL-2 or GST-PGL-3 (Figure 9B). These results indicate that PGL-1, PGL-2, and PGL-3 are all able to bind directly to each other and that at least PGL-1 can bind to itself in vitro in the absence of other C. elegans proteins. Although the three PGL proteins share significant sequence similarity, only PGL-1 can bind to IFE-1 in vitro. In the absence of other C. elegans proteins.

To investigate whether the three PGL proteins are associated with each other in vivo, we performed co-immunoprecipitation experiments on embryo extracts (see MATERIALS AND METHODS). After preabsorption with normal serum, wild-type embryo extract was incubated with either rabbit anti-PGL-1 or rat anti-PGL-3 antiserum, followed by precipitation using protein A/G agarose and Western blot analysis of the immunoprecipitated complex (Figure 9C). PGL-1 was co-immunoprecipitated by anti-PGL-3 antiserum (lane 3), and PGL-3 was co-immunoprecipitated by anti-PGL-1 antiserum (lane 7), whereas no PGL protein was immunoprecipitated by normal sera (lanes 4 and 8). PGL-2 was not detected in any of the embryo extract fractions (data not shown), as expected from the immunostaining results described above (see Figure 8). Our results indicate that PGL-1 and PGL-3 are associated with each other in embryos. We expect that PGL-2 is associated with PGL-1 and PGL-3 during postembryonic stages.

PGL proteins localize to P granules independently of each other: GLH proteins, another family of constitutive P-granule components, localize to P granules independently of each other (Kuznicki et al. 2000). To test whether there is a hierarchy among PGL proteins in their recruitment to P granules, the localization of each PGL protein in various pgl mutant backgrounds was
examined. In pgl-1 mutant worms, both PGL-2 and PGL-3 localize to P granules (Figure 7, D and H); this is observed even in the defective mutant gonads of pgl-1 mutant worms raised at the restrictive temperature. Similarly, in pgl-2 and pgl-3 mutant worms, the remaining PGL proteins localize to P granules at all temperatures (not shown). Moreover, in pgl-2; pgl-1, pgl-2; pgl-3, and pgl-1; pgl-3 double-mutant worms, the single remaining PGL protein localizes to P granules at all temperatures (not shown). These results indicate that, although the PGL proteins associate with each other in vitro and in vivo, this association is not required for any of them to localize to P granules. All three PGL proteins depend on GLH-1 for proper localization to P granules (KAWASAKI et al. 1998; KUZNICKI et al. 2000; N. MEYER, A. ORSBORN, K. BENNETT and S. STROME, unpublished results), suggesting that GLH-1 is upstream of all three PGL proteins in a P-granule assembly pathway. Consistent with this, the localization of GLH-1 to P granules in the adult germline does not require the presence of any of the three PGL proteins (Figure 10F). A normal pattern of GLH-1 in embryos may require the PGLs, a possibility that is being investigated (N. MEYER and S. STROME, unpublished results). Regardless of the order of assembly, we have found no evidence from in vitro binding tests for a direct interaction between PGL-1 and GLH-1 (data not shown).

PGL-3, but not PGL-2, has a redundant function with PGL-1 in hermaphrodite germline development: To examine the roles of PGL-2 and PGL-3 in C. elegans development and to assess their functional redundancy with PGL-1, we isolated deletion alleles of pgl-2 and pgl-3 (see MATERIALS AND METHODS). The pgl-2 allele, bn123, has a 472-bp deletion (corresponding to nucleotides 7839–8310 in cosmid B0523) accompanied by a 16-bp insertion of unknown sequence. The deletion/insertion is predicted to cause premature termination after the first 188 amino acids of PGL-2 (asterisk in Figure 2). The pgl-3 allele, bn104, has a 2456-bp deletion (corresponding to nucleotides 12,925–15,380 in cosmid C18G1). The deletion starts 167 bp upstream of the first ATG and removes the N-terminal 654 amino acids of the 693 amino acids of the predicted PGL-3 protein (Figure 2). Thus, both bn123 and bn104 are predicted to be either null or strong loss-of-function alleles. Indeed, as described above, pgl-2(bn123) and pgl-3(bn104) mutant worms fail to stain with anti-PGL-2 (Figure 6C) and anti-PGL-3 antibody (Figure 6F), respectively. Using a null allele of pgl-1 (bn101 or ct131), pgl-2(bn123), and pgl-3(bn104), we constructed all combinations of double and triple mutants and analyzed their phenotypes as described below.

pgl-1 mutants develop into sterile worms at elevated temperature (KAWASAKI et al. 1998). We initially compared pgl-2, pgl-3, and double and triple mutants with pgl-1 by shifting homozygous mutant mothers (P0’s) from 20° to 16°, 20°, and 26° as L4’s and assessing the fertility/sterility of their F1 hermaphrodite progeny (Table 1). When grown at permissive temperatures (16° and 20°), 10–14% of pgl-1 single-mutant hermaphrodites developed into sterile adults that lacked healthy looking embryos in their uterus (their uterus either contained a mass of unfertilized gametes/regenerating embryos or was empty). Growth at restrictive temperature (26°) resulted in 99% sterile adult hermaphrodites. pgl-2 single mutants did not display significant sterility at any temperature and did not enhance the sterility of pgl-1 at permissive temperatures. pgl-3 single mutants also did not display significant sterility at any temperature, but did enhance the sterility of pgl-1 at permissive temperatures: 71 and 80% of F1 hermaphrodite progeny from pgl-1; pgl-3 double-mutant mothers were sterile (38 and 33% of them possessed an empty uterus) at 16° and 20°, respectively. Triple mutants resembled pgl-1; pgl-3 double mutants. These results suggest that PGL-1 and PGL-3, but not PGL-2, function redundantly and that the low-temperature fertility of most pgl-1 mutants depends on worms having functional PGL-3 protein.

Sterility in pgl-1 mutants at 26° has both a maternal and a zygotic component (KAWASAKI et al. 1998). The
Figure 10.—Analysis of germlines in pgl double and triple mutants. Gonad arms were extruded from wild-type N2 (A, C, and E), pgl-1(bn101); pgl-3(bn104) sterile (B), or pgl-2(bn123); pgl-1(bn101); pgl-3(bn104) fertile (D and F) adult hermaphrodites raised at 20°. For A and B, gonads were fixed and stained with DAPI to visualize nuclei; the distal end of each gonad is indicated with an asterisk. For C–F, gonads were fixed and double stained with PA3 mouse monoclonal antibody to chromatin (C and D) and rabbit anti-GLH-1 (E and F); an optical section in the pachytene region of the distal gonad is shown. (A) Gonad arm from a wild-type hermaphrodite. The germline is well proliferated. Arrowheads indicate diakinesis-stage oocytes. Arrows indicate sperm. (B) Gonad arm from a pgl-1; pgl-3 sterile mutant. The germline lacks detectable PGL-1 and PGL-3 (not shown). It is underproliferated, lacks oocytes, and contains a few sperm. Mutant germlines tend to degenerate and shrink, especially in the proximal region, as worms age. (C and E) Gonad from a wild-type hermaphrodite. (D and F) Gonad from a pgl-2; pgl-1; pgl-3 fertile hermaphrodite. In both samples, anti-GLH-1 stains perinuclear P granules (E and F). Bars, 10 μm.

zygotic component is seen in the F1 pgl-1/pgl-1 progeny from pgl-1/+ mothers. These F1 homozygous progeny inherit maternal pgl-1(+) product but do not synthesize zygotic product, and presumably as a result, some of the worms are sterile at 26° (KAWASAKI et al. 1998). To examine the zygotic component of pgl-3 enhancement of pgl-1 sterility, we allowed pgl-1 unc-24/+; pgl-3 dpy-11/+ + mothers (n = 8) to produce self-cross progeny at permissive temperature and compared the sterility of pgl-1 unc-24; pgl-3 dpy-11 (Unc-24; Dpy-11) progeny and pgl-1 unc-24 (Unc-24) progeny. Scoring only the “empty uterus” class of sterile worms, we observed higher sterility in pgl-1; pgl-3 double-mutant progeny (14%, n = 83).
TABLE 1
Percentage of sterile hermaphrodite progeny produced by *pgl* mutant mothers at different temperatures

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% sterile F1's at 16°C</th>
<th>% sterile F1's at 20°C</th>
<th>% sterile F1's at 26°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>unc-24 (IV); dpy-11 (V)</em></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.5 (0.5)</td>
</tr>
<tr>
<td><em>pgl-1 unc-24 (IV)</em></td>
<td>14 (3)</td>
<td>10 (1)</td>
<td>99 (99)</td>
</tr>
<tr>
<td><em>pgl-2 (III)</em></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td><em>pgl-3 dpy-11 (V)</em></td>
<td>0.5 (0.2)</td>
<td>0.1 (0.1)</td>
<td>2.2 (0.6)</td>
</tr>
<tr>
<td>*pgl-2 (III); <em>pgl-1 unc-24 (IV)</em></td>
<td>5 (2)</td>
<td>9.2 (1)</td>
<td>99 (99)</td>
</tr>
<tr>
<td>*pgl-2 (III); <em>pgl-3 dpy-11 (V)</em></td>
<td>0.4 (0)</td>
<td>0.5 (0.1)</td>
<td>0.6 (0.6)</td>
</tr>
<tr>
<td>*pgl-1 unc-24 (IV); <em>pgl-3 dpy-11 (V)</em></td>
<td>71 (38)</td>
<td>80 (33)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>*pgl-2 (III); *pgl-1 unc-24 (IV); <em>pgl-3 dpy-11 (V)</em></td>
<td>65 (42)</td>
<td>77 (32)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

P0 mothers were transferred to the indicated temperatures as L4's. Their F1 hermaphrodite progeny were scored as adults on a dissecting microscope for sterility vs. fertility. The percentage of sterile F1's represents the percentage of F1 hermaphrodite progeny that lacked healthy appearing embryos in their uteruses. Numbers in parentheses indicate the percentage of F1 hermaphrodite progeny that had an empty uterus. More than 500 F1 worms were scored for each genotype/temperature, except that 96 *pgl* triple-mutant worms were scored at 26°C. The *bn101* allele of *pgl-1* was used.

PGl Proteins and Germline Development

than in *pgl-1* single-mutant progeny (2%, *n* = 248). Thus, *pgl-3* enhancement of *pgl-1* sterility has a zygotic component. Also, because *pgl-1/+; pgl-3/+* out-cross progeny from *pgl-1; pgl-3* double-mutant mothers show higher "empty uterus" class sterility (17%, *n* = 465) than do *pgl-1/+* out-cross progeny (3%, *n* = 645) from *pgl-1* single-mutant mothers at permissive temperature, *pgl-3* enhancement also has a maternal component.

*pgl-1; pgl-3* hermaphrodites show more severe germline defects than *pgl-1* hermaphrodites do: To characterize how germline development is compromised in *pgl* multiple mutants, individual gonads of mutant hermaphrodites raised at restrictive temperature were examined and classified (Table 2). In wild-type adult hermaphrodites, each of the two gonad arms contains >300 germ nuclei at 26°C (*unc-24; dpy-11* in Table 2). Each gonad arm exhibits a distal-to-proximal pattern of development (Schedl 1997): a distal region in which germ nuclei divide mitotically (this region contains some phospho-histone H3-positive M-phase nuclei); a transition zone in which germ nuclei exit from the mitotic cell cycle and enter meiotic prophase I (indicated by the appearance of HIM-3, a meiotic chromosome core component; Zetka et al. 1999); and a pachytene zone in which germ nuclei exhibit a characteristic thread-like chromatin morphology [seen by 4′,6-diamidino-2-phenylindole (DAPI) staining]. In the proximal region of each arm, oocytes develop and arrest at diakinesis of meiotic prophase I (Figure 10A). Sperm made during the L4 larval stage (recognized by the spermatogenesis-specific antibody SP56) are stored in the spermatheca through which oocytes pass and become fertilized.

*pgl-1* hermaphrodites raised at 26°C are almost 100% sterile (Table 1) and contain a moderately underproliferated germline (Table 2). Each gonad arm contains approximately 150 germ nuclei on average, and ~25% of the gonad arms lack phospho-histone H3 staining and therefore M-phase nuclei. However, the degree of underproliferation is highly variable among *pgl-1* gonad arms (Kawasaki et al. 1998). Seventy percent of the gonad arms contain relatively few germ nuclei, generally lack SP56 sperm staining (~80% of this subpopulation), and do not exhibit the proximal region of the L4 larval stage (recognized by the spermatogenesis-specific antibody SP56).

### TABLE 2
Analysis of germline development in *pgl* mutant hermaphrodites raised at 26°C

<table>
<thead>
<tr>
<th>Hermaphrodite genotype</th>
<th>Average no. of germ nuclei per gonad arm</th>
<th>% gonad arms lacking phospho-histone H3 positive germ nuclei</th>
<th>% gonad arms lacking oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>unc-24 (IV); dpy-11 (V)</em></td>
<td>310 ± 52</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>pgl-3 dpy-11 (V)</em></td>
<td>327 ± 68</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>pgl-1 unc-24 (IV)</em></td>
<td>148 ± 185</td>
<td>23</td>
<td>70</td>
</tr>
<tr>
<td>*pgl-1 unc-24 (IV); <em>pgl-3 dpy-11 (V)</em></td>
<td>68 ± 50</td>
<td>48</td>
<td>97</td>
</tr>
<tr>
<td>*pgl-2 (III); *pgl-1 unc-24 (IV); <em>pgl-3 dpy-11 (V)</em></td>
<td>66 ± 42</td>
<td>50</td>
<td>95</td>
</tr>
</tbody>
</table>

P0 mothers were transferred to 26°C as L4’s, and the germlines of their young adult F1 hermaphrodite progeny were analyzed. The *bn101* allele of *pgl-1* was used.

* Average number ± standard deviation. A total of 40–92 gonad arms were scored for each genotype.

* On average, five phospho-histone H3-positive germ nuclei per gonad arm were observed.
not contain any oocytes (Table 2). The remaining 30% of the gonad arms contain more germ nuclei and contain oocytes, which are abnormal in appearance and are defective.

*pgl-3* hermaphrodites raised at 26°C are fertile (Table 1) and contain a well-proliferated and fully differentiated germline (Table 2). However, *pgl-1; pgl-3* double-mutant hermaphrodites raised at 26°C display more severe germline defects than do *pgl-1* single-mutant hermaphrodites (Table 2). *pgl-1; pgl-3* gonad arms contain ~70 germ nuclei on average, and ~50% of the gonad arms lack phospho-histone H3 staining. Approximately 70% of the gonad arms lack SP56 sperm staining and 97% of them do not contain any oocytes. Although many of them contain transition zone (HIM-3 positive) nuclei, pachytene nuclei are rarely observed (as judged by nuclear morphology in DAPI-stained samples). *pgl-1; pgl-3* gonads tend to degenerate as adults age, leading to progressively more abnormal morphologies.

At lower temperatures, *pgl-1; pgl-3* double-mutant hermaphrodite germlines are also more underproliferated and more undifferentiated (Figure 10B) than *pgl-1* single-mutant hermaphrodite germlines: *pgl-1; pgl-3* double-mutant hermaphrodites and *pgl-1* single-mutant hermaphrodites contain ~150 and ~500 germ nuclei per gonad arm on average, and ~30 and ~2% of their gonad arms lack oocytes, respectively.

In summary, absence of PGL-3 enhances the Pgl-1 mutant phenotype in hermaphrodite germlines in several respects: increased percentage of animals that are sterile at lower temperatures and more pronounced defects in germ cell proliferation and in production of sperm and oocytes at all temperatures. Absence of PGL-2 does not further enhance any of these phenotypes.

The ability of *pgl-1; pgl-3* males to produce out-cross progeny is greatly reduced: To test whether *pgl* mutants are also defective in male germline development, *pgl* mutations were combined with a *him-3* mutation, *ct131* (ZH2001), which leads to increased production of self-cross male progeny. Mutant *P₀* mothers were shifted to either 20°C or 25°C, single *F₁* self-cross male progeny were mated with single *unc-24; dpy-11* hermaphrodites and analyzed for production of out-cross progeny. The *ct131* allele of *pgl-1* was used.

### TABLE 3

Production of out-cross progeny by *pgl* mutant males at different temperatures

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Average no. of out-cross progeny per male^a^ (% out-cross progeny)^b^ at 20°C</th>
<th>Average no. of out-cross progeny per male^a^ (% out-cross progeny)^b^ at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>him-3</em> (IV)</td>
<td>149 ± 74 (82)</td>
<td>57 ± 71 (45)</td>
</tr>
<tr>
<td><em>pgl-1</em> <em>him-3</em> (IV)</td>
<td>61 ± 83 (35)</td>
<td>1 ± 3 (1)</td>
</tr>
<tr>
<td><em>pgl-2</em> (III); <em>him-3</em> (IV)</td>
<td>120 ± 71 (85)</td>
<td>43 ± 71 (33)</td>
</tr>
<tr>
<td><em>him-3</em> (IV); <em>pgl-3</em> (V)</td>
<td>120 ± 55 (89)</td>
<td>33 ± 55 (27)</td>
</tr>
<tr>
<td><em>pgl-2</em> (III); <em>pgl-1</em> <em>him-3</em> (IV)</td>
<td>65 ± 87 (35)</td>
<td>4 ± 18 (4)</td>
</tr>
<tr>
<td><em>pgl-1</em> <em>him-3</em> (IV); <em>pgl-3</em> (V)</td>
<td>22 ± 41 (14)</td>
<td>0 ± 0 (0)</td>
</tr>
</tbody>
</table>

*P₀* mothers were transferred to 20°C or 25°C as L4’s. Their male *F₁* progeny were mated to *unc-24; dpy-11* hermaphrodites and analyzed for production of out-cross progeny. The *ct131* allele of *pgl-1* was used.

^a^Average number ± standard deviation. A total of 18–30 *F₁* males were analyzed for each genotype/temperature.

^b^Percentage of out-cross progeny among total (both self-cross and out-cross) *F₂* progeny scored. 

Absence of PGL-1 and PGL-3 causes embryonic lethality in addition to germline defects: In addition to their germline defects, *pgl* mutants produce dead embryos, arrested larvae, and more males than usual. To examine those defects, we scored developmental fates of all embryos laid by *pgl* mutant mothers that had been transferred to 26°C or 20°C as L4’s (Table 4, A and B). At 26°C, *pgl-2; pgl-1; pgl-3* triple-mutant, *pgl-1; pgl-3* double-mutant, and even *pgl-1* single-mutant mothers laid fewer fertilized embryos (26–50 embryos/mother) than con-
**TABLE 4**

Developmental fates of *pgl* mutant progeny at different temperatures

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average brood size</th>
<th>% embryonic lethal</th>
<th>% larval lethal</th>
<th>% male</th>
<th>% sterile hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. At 26°</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>unc-24; dpy-11</em></td>
<td>118 ± 13</td>
<td>2 [1–3] (940)</td>
<td>0 (940)</td>
<td>0.2 (922)</td>
<td>0 (920)</td>
</tr>
<tr>
<td><em>pgl-1 unc-24</em></td>
<td>50 ± 22</td>
<td>25 [20–43] (397)</td>
<td>9 (397)</td>
<td>5 (262)</td>
<td>100 (250)</td>
</tr>
<tr>
<td><em>pgl-1 unc-24; pgl-3 dpy-11</em></td>
<td>31 ± 17</td>
<td>37 [14–86] (247)</td>
<td>9 (247)</td>
<td>2 (134)</td>
<td>100 (131)</td>
</tr>
<tr>
<td><em>pgl-2; pgl-1 unc-24; pgl-3 dpy-11</em></td>
<td>26 ± 5</td>
<td>58 [33–82] (207)</td>
<td>5 (207)</td>
<td>4 (75)</td>
<td>100 (72)</td>
</tr>
<tr>
<td><strong>B. At 20°</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>unc-24; dpy-11</em></td>
<td>190 ± 14</td>
<td>0.4 [0–1] (1518)</td>
<td>0 (1518)</td>
<td>0.1 (1512)</td>
<td>0 (1511)</td>
</tr>
<tr>
<td><em>pgl-1 unc-24</em></td>
<td>177 ± 12</td>
<td>5 [1–17] (1412)</td>
<td>0 (1412)</td>
<td>0.8 (1399)</td>
<td>3 (1328)</td>
</tr>
<tr>
<td><em>pgl-1 unc-24; pgl-3 dpy-11</em></td>
<td>114 ± 39</td>
<td>14 [11–16] (916)</td>
<td>0.5 (916)</td>
<td>7 (778)</td>
<td>30 (720)</td>
</tr>
<tr>
<td><em>pgl-2; pgl-1 unc-24; pgl-3 dpy-11</em></td>
<td>156 ± 20</td>
<td>21 [15–29] (1245)</td>
<td>1 (1245)</td>
<td>3 (968)</td>
<td>29 (943)</td>
</tr>
</tbody>
</table>

P₀ mothers were transferred to 26°C (A) or 20°C (B) as L4’s. The broods and fates of F₁ offspring from eight P₀ mothers were analyzed. The *bn101* allele of *pgl-1* was used.

* Average number of total embryos laid per mother ± standard deviation.
* Percentage of dead embryos. Numbers in brackets indicate ranges of percentage of dead embryos among the eight mothers.
* Numbers in parentheses indicate total embryos laid.
* Percentage of arrested larvae. Numbers in parentheses indicate total embryos laid.
* Percentage of male progeny. Numbers in parentheses indicate total adult progeny.
* Percentage of sterile hermaphrodite progeny of the “empty uterus” class. Numbers in parentheses indicate total adult hermaphrodite progeny.

P₀ mothers (~120 embryos/mother) did, indicating reduced numbers or reduced competence of oocytes and/or sperm (Table 4A). Furthermore, a significant fraction of their progeny arrested as late embryos (ranging from 14 to 86% among the mothers) or larvae (5–9% of embryos on average). As a result, they produced very few viable adult progeny: averages of 9, 17, and 33 for the triple, double, and single mutants, respectively, compared to 115 for control worms. Additionally, among the surviving adult progeny, the frequency of males was higher in *pgl-1*-containing mutants (2–5%) than in controls (0.2%). Finally, as discussed above, all of the surviving adult hermaphrodite progeny were sterile at 20°C, although *pgl-1* mutant mothers laid similar numbers of fertilized embryos as control mothers. *pgl-1; pgl-3* double-mutant and *pgl-2; pgl-1* triple-mutant mothers produced a significant proportion of dead embryos (ranging from 11 to 29% among the mothers) and males (up to 7% on average; Table 4B). As described above, ~30% of the surviving adult hermaphrodite progeny were sterile with an empty uterus.

On the basis of Nomarski observation and DAPI staining, arrest of *pgl-1; pgl-3* double-mutant and *pgl-2; pgl-1; pgl-3* triple-mutant embryos appeared to occur during morphogenesis (ranging from comma to threefold) stages. Because many embryos were fragile and prone to explode, we hypothesized that they had defects in formation of the eggshell/vitelline membrane and in osmoregulation. To test this possibility, *pgl-1; pgl-3* double-mutant embryos and wild-type embryos were released from the uterus of mothers into water containing DAPI. All of the wild-type embryos excluded DAPI and developed well in water. In contrast, many *pgl-1; pgl-3* double-mutant embryos were stained with DAPI, swelled, took on an abnormal appearance, and arrested (data not shown). Thus, at least some *pgl-1; pgl-3* double-mutant embryos appear to be defective in formation of the eggshell/vitelline membrane and in osmoregulation. This phenotype suggests defects during oogenesis or during egg activation upon sperm entry. Such defects probably lead, at least in part, to the high degree of embryonic lethality displayed by *pgl* mutants at 26°C (Table 4A). As discussed below, we hypothesize that *PGL-1* and *PGL-3* are involved in the regulation of many mRNAs in the maternal germline and that the ultimate cause of defects in oogenesis and/or egg activation in *pgl-1; pgl-3* mutants is abnormal localization and/or expression of maternal mRNAs.

**DISCUSSION**

The PGL and GLH families of P-granule proteins:

Two families of proteins, the PGLs and the GLHs, are associated with P granules throughout development and thus are likely to be “core” granule components. Within the PGL family, PGL-1 was discovered through a genetic screen for mutants with altered P-granule staining by a monoclonal antibody (Kawasaki *et al.* 1998), and PGL-2 and PGL-3 were discovered by sequence analysis of the *C. elegans* genome and by a yeast two-hybrid screen for proteins that interact with PGL-1. PGL proteins are novel and do not resemble any of the known components of germ granules in other organisms.
to bind RNA. PGL-2 lacks an RGG box and also differs from PGL-1 and PGL-3 in being undetectable in embryos. PGL-1 appears to be the most important of the three for germline development, as loss of PGL-1 alone results in sterility at elevated temperature, and, among the three PGL proteins, only PGL-1 can bind to IFE-1, a germline-enriched isoform of eukaryotic initiation factor 4E (eIF4E) that is required for spermatogenesis (Amiri et al. 2001). Loss of PGL-2 alone or PGL-3 alone does not cause sterility. However, loss of both PGL-1 and PGL-3 results in more severe germline defects at elevated temperature and in sterility at lower temperatures as well, indicating that PGL-3 functions redundantly with PGL-1. Even though loss of PGL-2 does not cause any obvious germline problems in worms grown in the laboratory, it may contribute to fertility in the wild.

The GLH proteins were identified as *C. elegans* homologs of Drosophila Vasa, a DEAD-box RNA helicase that is a component of Drosophila germline granules (Hay et al. 1988; Lasko and Ashburner 1988; Roussell and Bennett 1993; Gruidl et al. 1996; Kuznicki et al. 2000). Of the four GLH proteins, GLH-1 appears to serve the most important role in the germline; reduction of glh-1 function results in sterility at elevated temperature, while reduction of glh-2, glh-3, or glh-4 function does not result in significant sterility at any temperature (Gruidl et al. 1996; Kawasaki et al. 1998; Kuznicki et al. 2000; Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). Similar to the PGL-1-PGL-3 relationship, GLH-4 appears to serve a redundant role with GLH-1, as loss of both glh-1 and glh-4 functions results in sterility at low temperature (Kuznicki et al. 2000). GLH-2 and GLH-3, like PGL-2, may contribute in subtle ways to fertility.

**P-granule assembly:** Results of molecular epistasis analyses indicate that each GLH protein can assemble into P granules independently of the other GLHs (Kuznicki et al. 2000) and that at least GLH-1 can assemble independently of the PGLs. Each PGL protein can assemble into P granules independently of the other PGLs, but all three PGLs depend upon GLH-1 for their efficient recruitment to or retention by P granules (this article; Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). IFE-1, a germline-enriched isoform of elf4e that binds mRNA caps, requires PGL-1 to assemble into P granules (Amiri et al. 2001). Consistent with this, IFE-1 interacts with PGL-1 but not with PGL-2 or PGL-3 in GST pull-down assays (Amiri et al. 2001; this article). These findings generally support a P-granule assembly pathway in which GLH-1 is early in the pathway, the three PGLs are downstream of GLH-1, and IFE-1 is downstream of PGL-1. Interestingly, no mutant has been discovered that prevents P-granule assembly altogether. However, RNAi depletion of core splicing factors, such as the Sm proteins, U2AF, and U170K, disrupts the localization of all three PGLs to granules and may disrupt granule integrity altogether (Barbee et al. 2002; I. Kawasaki, unpublished result), suggesting that mRNA processing or export from the nucleus is required to build, stabilize, or localize P granules. The finding that at least some of the Sm proteins associate with P granules raises another possibility: that the physical presence of the proteins in P granules, and not necessarily their splicing functions, is critical to granule integrity.

The protein-protein interactions that have been documented are PGL-1-PGL-1, PGL-1-PGL-3, PGL-2-PGL-2, PGL-2-PGL-3, and PGL-1-IFE-1. PGL-3 appears to associate specifically with the higher-molecular-weight band of the two PGL-1 bands present in embryo extracts. Interestingly, the higher-molecular-weight PGL-1 band is specifically enriched in the nuclear membrane fraction during the course of subcellular fractionation of both embryo extracts and adult worm extracts, whereas the lower PGL-1 band is detected mainly in the cytoplasmic fraction (I. Kawasaki, unpublished result). These results raise the possibility that post-translational modification, such as phosphorylation, affects recruitment of components to perinuclearly localized P granules.

Drosophila polar granules differ from *C. elegans* P granules in several respects. The Drosophila genome lacks an obvious pg1 homolog, and the *C. elegans* genome lacks an obvious oskar homolog. Thus, those germ-granule components are not shared between flies and worms. In flies, germ-granule components are not encoded by multi-gene families, and mutations in numerous polar-granule genes (e.g., oskar and vasa) prevent the assembly of polar granules during oogenesis (reviewed in Williamson and Lehmann 1996). The “polar-granule null” phenotype appears to be failure to form primordial germ cells and failure to correctly pattern the embryo (Lehmann and Nusslein-Volhard 1986). The latter phenotype is due to a role for Nanos in translational regulation in the somatic embryo as well as in primordial germ cells (Kobayashi et al. 1996; Forbes and Lehmann 1998).

**The PGL-null phenotype:** Since no mutant isolated to date abolishes P granules altogether, the “P-granule null” phenotype is not known at present. This article presents analysis of the phenotype seen in the absence of the entire PGL family. It is useful to consider the defects observed as worms growing at elevated temperature experience progressively declining levels of PGL protein(s) (Kawasaki et al. 1998; this article). pg1/pg1 F1 progeny from pg1/+ mothers contain a maternal load of PGL-1 protein, which remains detectable in P granules until at least the L3 larval stage. By adulthood, we expect that P granules in these F1 worms will be virtually devoid of PGL-1, and probably as a result, ~40% of the F1’s are sterile: their germlines are well proliferated and contain gametes, but they produce no viable progeny. The “fertile” F1’s actually have greatly reduced fertility: they produce reduced numbers of F2 embryos, ~35% of which die during embryogenesis or
larval development. The few F₂ worms that survive to adulthood are sterile: their germlines are significantly underproliferated and generally lack gametes. The simultaneous loss of PGL-3 enhances all aspects of the phenotype described above for pgl-1. Somewhat surprisingly, the PGL-null (i.e., pgl-2; pgl-1; pgl-3 triple mutant) phenotype remains a mix of zygotic sterility, maternal-effect lethality, and maternal-effect sterility. We think that this reflects the progressive loss of PGL proteins from P granules in F₁ worms and the progressive compromise of P-granule function(s) in F₁ and F₂ worms.

The temperature sensitivity of Pgl phenotypes: We previously speculated that loss of PGL-1 results in sterility predominantly at elevated temperature because redundant factors are sufficient to confer fertility at low temperatures. Indeed, PGL-3 appears to serve just such a redundant role. However, the phenotype of pgl-1; pgl-3 double (and of pgl-2; pgl-1; pgl-3 triple) mutants is still more pronounced at high temperature. The temperature sensitivity of Pgl phenotypes may reflect a role for PGL proteins in maintaining the stability and function of multi-protein complexes, most likely P granules. P-granule complexes may be unstable or prone to inactivation, especially at elevated temperature. The presence of PGL-1 in the complex may contribute to maintaining the structure/function of granules at all temperatures. In the absence of PGL-1, PGL-3 may serve this role adequately at low temperatures but not at 26°C. In the absence of both PGL-1 and PGL-3, P-granule complexes may tend to dissociate or lose activity, and elevated temperature may further promote granule dissociation/inactivation. An alternative scenario is that the process(es) in which the PGL proteins participate (e.g., export of mRNAs from germ nuclei to the cytoplasm) is inherently sensitive to temperature: in the absence of PGL function, the process(es) can occur with reasonable fidelity or efficiency at lower temperatures but not at elevated temperatures. Whatever the molecular explanation, it is noteworthy that the phenotypes caused by loss of other P-granule components (e.g., glh-1 and ife-1) are also more pronounced at elevated temperature (Kawasaki et al. 1998; Amiri et al. 2001; N. Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). Interestingly, germline expression of transgenes from extra-chromosomal arrays is also sensitive to temperature (e.g., Strome et al. 2001).

Likely function(s) of P granules and PGL proteins: Schisa et al. (2001) observed that perinuclear P granules are rich in RNA and contain even higher levels of RNA under conditions that result in reduced levels of PGL-1, GLH-1, and GLH-2. On the basis of these and other findings, they hypothesized that a primary role of perinuclear P granules in the germ line is in export of mRNAs from nuclei to the cytoplasm and that the PGL and GLH proteins promote export. An attractive scenario is that the progressive and variable defects observed in pgl-2; pgl-1; pgl-3 triple mutants are due to progressive and variable loss of the ability of P granules to export mRNAs to export mRNAs. In F₁, pgl/pgl worms from pgl/+ mothers, export may be only mildly impaired, especially early in larval development when some maternal PGL product persists. Some P granules may be more impaired than others, and the export of some mRNAs may be affected more than the export of other mRNAs, leading to variability in defects. By adulthood, those F₂, pgl/pgl worms may have lost the ability to export and express at least some mRNAs needed for oogenesis and early embryogenesis, leading to production of defective oocytes and embryos (i.e., sterility and maternal-effect lethality). Certainly any F₂ embryos that survived would be expected to have severely compromised P granules, which might be fully impaired in mRNA export. This could lead to the problems observed in proliferation of the germ line in F₂ worms and to their sterility (i.e., maternal-effect sterility).

Another potential role for P granules is delivery of maternal proteins and mRNAs to particular blastomeres during early embryogenesis. Among the proteins that are known to transiently associate with P granules in early embryos are the CCCH-type zinc-finger proteins, PIE-1 (Mello et al. 1992, 1996), MEX-1 (Guedes and Priess 1997), and POS-1 (Tabara et al. 1999), and the KH-domain proteins, MEX-3 (Draper et al. 1996) and GLD-1 (Jones et al. 1996). PIE-1, MEX-1, and POS-1 become enriched in the germline blastomeres and participate in preventing the germline blastomeres from adopting somatic fates. Studies of PIE-1 localization illustrate the diversity of mechanisms that can be used to concentrate a protein in the germline blastomere; PIE-1 associates with germ-line-destined P granules, PIE-1 is selectively retained on the centrosome destined for the germline blastomere, and PIE-1 in the cytoplasm of somatic blastomeres is degraded (Mello et al. 1996; Reese et al. 2000; DeRenzo et al. 2003). It is currently not known whether P granules serve a major or minor role in the delivery of specific proteins to the germline blastomeres and whether the proteins that transiently associate with P granules in the early embryo are important for P-granule functions.

Finally, P granules may recruit translation factors and in that way activate or repress translation. For example, the association of IFE-1, a germline-enriched isoform of eIF4E, with P granules may activate translation of some granule-associated mRNAs. Alternatively, the association of IFE-1 with P granules may inhibit translation: IFE-1 may be sequestered away from the translation machinery, or the binding of IFE-1 to PGL-1 may prevent the binding of IFE-1 to eIF4G, which is essential for initiation of translation (Sonenberg 1996). Indeed, PGL-1 and PGL-3 possess the amino acid motif YXXXLφ (where φ is a hydrophobic amino acid and X is any amino acid), which is used by eIF4G, eIF4E-binding proteins, and Maskin to bind eIF4E (Mader et al. 1995; Amiri et al. 2001; I. Kawasaki, unpublished result). However, our
GST pull-down experiments revealed that IFE-1 interacts with PGL-1 but not with PGL-3. Furthermore, a PGL-1 fragment C-terminal of the YXXXXL motif appears to mediate binding of PGL-1 to IFE-1 (N. Meyer and S. Strome, unpublished result).

Future identification of the RNAs to which the PGL proteins bind will help elucidate how the PGLs and P granules function during germline development.

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