PGL proteins self associate and bind RNPs to mediate germ granule assembly in C. elegans

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Germ granules are germ lineage–specific ribonucleoprotein (RNP) complexes, but how they are assembled and specifically segregated to germ lineage cells remains unclear. Here, we show that the PGL proteins PGL-1 and PGL-3 serve as the scaffold for germ granule formation in Caenorhabditis elegans. Using cultured mammalian cells, we found that PGL proteins have the ability to self-associate and recruit RNPs. Depletion of PGL proteins from early C. elegans embryos caused dispersal of other germ granule components in the cytoplasm, suggesting that PGL proteins are essential for the architecture of germ granules. Using a structure–function analysis in vivo, we found that two functional domains of PGL proteins contribute to germ granule assembly: an RGG box for recruiting RNA and RNA-binding proteins and a self-association domain for formation of globular granules. We propose that self-association of scaffold proteins that can bind to RNPs is a general mechanism by which large RNP granules are formed.

Introduction

In many organisms, germ cells have electron-dense cytoplasmic organelles, generally referred to as “germ granules,” which are believed to play roles in germ cell specification and differentiation (Eddy, 1975; Saffman and Lasko, 1999). They are large RNA-enriched nonmembranous organelles, and historically called by diverse names such as P granules in Caenorhabditis elegans, polar granules in Drosophila, and germinal granules in Xenopus (Eddy, 1975). In animals in which the germline is preformed (including the above three organisms), germ granules are maternally inherited by the fertilized egg and then specifically segregated to the germ lineage during early embryogenesis. Germ granules contain specific mRNAs and proteins, some of which are conserved among species (e.g., Drosophila Vasa [Hay et al., 1988a,b; Lasko and Ashburner, 1988] and its homologues [Raz, 2000]), but many appear to be species specific. Despite the evolutionary divergence of their molecular composition, the majority of the protein components of germ granules are implicated in various aspects of RNA metabolism, which has led to the prediction that the common biochemical function of germ granules is to regulate the translation efficiency and/or stability of mRNAs in the germline (Seydoux and Braun, 2006; Strome and Lehmann, 2007).

In Caenorhabditis elegans, germ granules are called P granules, and some of the specific mRNAs and ~40 protein components (mostly RNA-binding proteins) of P granules have been identified (Strome, 2005). Among them, GLH-1–4 (Drosophila Vasa homologues; with DEAD-box helicase motifs; Gruidl et al., 1996; Kuznicki et al., 2000), PGL-1 and PGL-3 (C. elegans–specific germ granule components; each with an RGG box [multiple Arg-Gly-Gly repeats]; Kawasaki et al., 1998, 2004), and DEPS-1 (Spike et al., 2008) are the only known components that exclusively localize to P granules at all developmental stages; other components localize to P granules transiently, and many of them are present in somatic lineages as well (Strome, 2005). Although many P-granule components have been identified, how the granules are assembled (and disassembled in somatic cells) is not well understood.

Here we established an assay to analyze the granule-forming ability of P-granule components using cultured mammalian...
Table I. Percentage of CHO cells with granules containing the indicated P-granule components

| P-granule component | Localization to P granules | Motifs | ① Formation of amorphous aggregates | ② Formation of globular granules | ③ Colocalization with PGL-3 granules |
|---------------------|---------------------------|--------|-----------------------------------|---------------------------------|----------------------------------|
| PGL-3               | Constitutive               | RGG box| 4                                 | 96                              | N.A.                             |
| PGL-1               | Constitutive               | RGG box| 10                                | 82                              | 100                              |
| GLH-1               | Constitutive               | DEAD box, 4 CCHC fingers | 2                      | 0                              | 4                               |
| GLH-3               | Constitutive               | DEAD box, 2 CCHC fingers | 4                      | 0                              | 20                              |
| MEX-1               | Transient                 | 2 CCHC fingers | 20                                | 0                              | 100                              |
| MEX-3               | Transient                 | 2 KH domains | 4                                 | 0                              | 92                              |
| POS-1               | Transient                 | 2 CCHC fingers | 50                                | 0                              | 82                              |
| OMA-1               | Transient                 | 2 CCHC fingers | 8                                 | 0                              | 90                              |
| OMA-2               | Transient                 | 2 CCHC fingers | 10                                | 0                              | 92                              |
| SPN-4               | Transient                 | RNP motif | 8                                 | 0                              | 98                              |
| GLD-1               | Transient                 | KH domain | 10                                | 0                              | 92                              |
| CGH-1               | Transient                 | DEAD box | 16                                | 0                              | 80                              |
| GLD-3               | Transient                 | KH-related domains | 4                                | 0                              | 16                              |
| IFE-1               | Transient                 | Translation initiation factor 4E | 0                                | 0                              | 6                               |

①Percentage of cells in which the expressed GST-tagged P-granule components formed amorphous aggregates.
②Percentage of cells in which the expressed GST-tagged P-granule components formed globular granules.
③Percentage of cells in which the expressed GST-tagged P-granule components colocalized with the granules formed by 6xHis-tagged PGL-3.

50 cells were randomly selected in each expression experiment, and the number of the cells forming granules was counted. In the coexpression experiments, we counted cells with granules that contained both PGL-3 and the corresponding component. N.A., not applicable.

cells and somatic *C. elegans* cells. We show that PGL proteins autonomously form cytoplasmic granules in heterologous cells, and that RNA and some protein components are recruited to granules in a manner dependent on the RGG box. In contrast, GLH proteins do not exhibit autonomous granule formation ability. Our findings demonstrated that PGL proteins play a crucial role as scaffolds in the assembly of P granules in *C. elegans*.

Results and discussion

To identify the components that play a major role in P-granule assembly, each component of P granules was examined for its ability to form granules in cultured mammalian cells in which no other *C. elegans* proteins were present. 14 P-granule components were used for this study: four were constitutive P-granule components—PGL-1 (Kawasaki et al., 1998), PGL-3 (Kawasaki et al., 2004), GLH-1 (Gruidl et al., 1996), and GLH-3 (Kuznicki et al., 2000)—and ten were transient components: MEX-1 (Guedes and Priess, 1997), MEX-3 (Draper et al., 1996), POS-1 (Tabara et al., 1999), OMA-1 (Dettwiler et al., 2001; Shimada et al., 2002), OMA-2 (Dettwiler et al., 2001; Shimada et al., 2002), SPN-4 (Ogura et al., 2003), GLD-1 (Jones et al., 1996), GLD-3 (Eckmann et al., 2002), CGH-1 (Navarro et al., 2001), and IFE-1 (Amiri et al., 2001; Table I).

Although the majority (12/14) of the P-granule components were scattered in the cytoplasm or formed amorphous aggregates of diverse shapes with unclear boundaries, GST-tagged PGL-1 and PGL-3 formed globular granules—either spherical or ellipsoidal shape with clear boundaries, reminiscent of P granules—in the cytoplasm of CHO cells (Fig. 1, A–C; Table I; and Fig. S1). Granules were detected in 92% (46/50) and 100% (50/50) of the GST::PGL-1– and GST::PGL-3–positive cells, respectively. Notably, two other constitutive P-granule components, GLH-1 and GLH-3, were dispersed in the cytoplasm. PGL-1 and PGL-3 are paralogues with 77% amino acid similarity and they interact directly with each other (Kawasaki et al., 2004). When coexpressed in CHO cells, they colocalized in the same granules (Fig. 1 D), suggesting that both of these PGL proteins participate in globular granule assembly. Granule formation by PGL-3 was confirmed in four other mammalian cell lines (NIH3T3, HeLa, MDCK, and HEK293; unpublished data). We refer to granules formed by PGL-1 and/or PGL-3 as “PGL granules.”

To examine whether PGL proteins can form granules in *C. elegans* cells in the absence of germ cell–specific factors, PGL-1 and PGL-3 were individually expressed in *C. elegans* somatic cells. In embryonic somatic cells (PGLs expressed via the pes-10 promoter; Seydoux and Fire, 1994) as well as in adult pharyngeal cells (PGLs expressed via the myo-2 promoter; Okkema et al., 1993), GFP::PGL-1 and GFP::PGL-3 formed cytoplasmic granules (Fig. 1, E and F; and unpublished data). In contrast, GFP and GFP::GLH-1 were dispersed in the cytoplasm, and no granules were detected (Fig. 1, G and H; and unpublished data). Thus, consistent with the results obtained with CHO cells, PGL proteins, but not GLH-1, have the ability to form granules autonomously in the absence of other germline-specific factors in *C. elegans*.

These results raised the possibility that PGL proteins serve as a scaffold for P-granule formation in *C. elegans*. To test this possibility, we next examined whether PGL granules recruit other known P-granule components in CHO cells. PGL granules were stained positively by the DNA/RNA-specific dye, SYTOX, and this staining disappeared when the fixed cells were pretreated with ribonuclease A (RNase A; Fig. 2, A and B), suggesting that PGL granules contain RNA. In addition, endogenous poly(A)-binding protein (PABP), which normally localizes throughout the cytoplasm (Fig. 2 C), was also enriched in PGL...
PGL proteins autonomously form RNP granules that contain endogenous RNA, PABP, and certain coexpressed 
C. elegans P-granule components. To test whether the PGL proteins serve as the scaffold for 
P-granule assembly in C. elegans, we depleted the PGL proteins from early C. elegans embryos via a combination of genetic 
mutations and RNAi, and then examined the localization of other P-granule components. In wild-type early embryos, POS-1 
and MEX-3 (both transient P-granule components) are dispersed in the cytoplasm of somatic cells, whereas in the germ 
lineage they form granules that predominantly colocalize with PGL proteins, with some diffuse signal still detectable in the 
cytoplasm (Fig. 3, B and D; Draper et al., 1996; Ogura et al., 2003). In pgl-1(RNAi);pgl-3(RNAi) embryos in which both 
PGL-1 and PGL-3 were undetectable, granules formed by POS-1 were vastly decreased compared with wild-type em-
bryos (Fig. 3 C; number of granules of >1.5-µm diameter in 

Figure 1. PGL proteins autonomously form globular granules. (A–D) Immunofluorescence images of CHO cells expressing C. elegans P-granule components. (A) GST::PGL-3, (B) GST::MEX-3, (C) GST::GLH-1, (D) coexpression of PGL-3::6×His and GST::PGL-1. (E–H) Transgenic C. elegans expressing GFP-tagged proteins. (E and F) GFP::PGL-3 ectopically expressed via the pes-10 [E] or myo-2 [F] promoter. (G and H) GFP::GLH-1 ectopically expressed via the 

granules (Fig. 2 D). Furthermore, when coexpressed with 
PGL-3, 8 of 12 P granule components showed substantial co-
localization with the granules formed by PGL-3 (Table I and 

Figure S2). For example, when MEX-3 was expressed by itself or co-
expressed with LacZ, it was generally dispersed in the cytoplasm 
(Fig. 1 B). When MEX-3 was coexpressed with PGL-3, how-
ever, 92% (46/50) of the cells had PGL granules that contained 

MEX-3 (Table I and Fig. 2 E). GLD-3, and the constitutive 
P-granule components GLH-1 and GLH-3, were recruited to PGL 
granules in <25% of cells (Table I, Fig. 2 F, and Fig. S2). More 
efficient recruitment of GLH-1 and GLH-3 to PGL granules is 
observed in C. elegans intestinal cells, perhaps revealing an 
effect of cell origin or type on this association (Updike et al., 
2011). IFE-1 was recruited to PGL granules only when they 
contained PGL-1 (Fig. S2), consistent with previous reports 
that PGL-1, but not PGL-3, directly binds to IFE-1 (Amiri 
et al., 2001; Kawasaki et al., 2004). Thus, in mammalian cells, 
PGL proteins autonomously form RNP granules that contain 
endogenous RNA, PABP, and certain coexpressed C. elegans 
P-granule components.

To test whether the PGL proteins serve as the scaffold for 
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Localization of GLH proteins was also affected by depletion of PGL proteins. In wild-type embryos, GLH-1 almost completely colocalizes with PGL granules (Fig. 3 F; Gruidl et al., 1996). When both PGL-1 and PGL-3 were depleted, however, GLH-1 was dispersed in the cytoplasm of both germline and somatic embryonic cells (Fig. 3 G), suggesting that PGL proteins are essential for GLH-1 to be incorporated into granular structures. GLH-1 and GLH-4 have partially redundant functions that are necessary for PGL proteins to form granular

P2 cell [mean ± SD]: wild type, 40.0 ± 10.0 [n = 8]; pgl-1(RNAi);pgl-3(RNAi), 12.4 ± 3.5 [n = 7]). Granules formed by GEP::MEX-3 were also significantly decreased (Fig. 3 E; wild type, 48.3 ± 9.0 [n = 4]; pgl-1(RNAi);pgl-3(RNAi), 13.8 ± 3.6 [n = 6]). We speculate that the POS-1– or MEX-3–containing granules in the absence of PGL proteins are different types of RNP granules, such as P bodies (Gallo et al., 2008). These results support the model that PGL granules recruit other components to assemble P granules.
structures in the *C. elegans* adult germline (Kawasaki et al., 1998; Kuznicki et al., 2000). We confirmed this in early embryos; in *glh-1(RNAi) glh-4(gk225)* embryos in which both GLH-1 and GLH-4 were undetectable, PGL-3 was dispersed in both somatic and germline cytoplasm, and cytoplasmic granules smaller than wild-type P granules were detected throughout embryos (Fig. 3 H). These results demonstrate that PGL and GLH proteins are mutually required to form granular structures and indicate that, although PGL proteins have the ability to self-aggregate, GLH proteins are essential in the early germline to maintain the multicomponent granular structures formed by PGL proteins.

To understand the molecular mechanism by which PGL proteins mediate RNP granule formation, we performed a structure–function analysis of PGL-3 in CHO cells (Fig. 4 A). The only recognizable motif in PGL-1 and PGL-3 is a C-terminal RGG box that acts as an RNA-binding domain (Godin and Varani, 2007). PGL-3 lacking the RGG box (PGL-3ΔRGG; lacking amino acid residues 633–693) formed globular granules approximately the same size as those formed by the full-length PGL-3 (Fig. 4, B and C). Endogenous RNA, however, was undetectable in the granules formed by PGL-3ΔRGG (unpublished data), and endogenous PABP and other coexpressed P-granule components (MEX-1, MEX-3, POS-1, OMA-1, OMA-2, SPN-4, GLD-1, and CGH-1) were not present in the granules (Fig. 4 C and Fig. S3). These results indicate that the RGG box of PGL-3 is dispensable for the formation of globular granules but necessary to capture and incorporate RNA and RNA-binding proteins into the granules.

Although PGL-3(1–318) did not form globular granules on its own (Fig. 4 D), when coexpressed with the full-length PGL-3, PGL-3(1–318) colocalized with granules formed by the
Next, to investigate the importance of the RGG box for recruitment of other P-granule components in *C. elegans* em−bryos, GFP::PGL-3 or GFP::PGL-3 RGG was expressed in the absence of endogenous PGL-1 and PGL-3 (pgl-1(RNAi);pgl-3(bn104)) in the early embryos, and we examined colocalization of GFP::PGL-3 or GFP::PGL-3 RGG with a transient P-granule component, POS-1. GFP::PGL-3 formed granules in the germ line cells and these granules contained POS-1 (Fig. 4 H). GFP::PGL-3 RGG also formed granules in germ line cells, but these granules did not contain POS-1 (Fig. 4 I). As in pgl-1;pgl-3 embryos (Fig. 3, C and E), POS-1–containing granules were significantly decreased, and they did not contain GFP::PGL-3 RGG (Fig. 4 I). These results further confirmed the finding in CHO cells that the RGG box of PGL-3 is dispensable for granule formation but essential for recruitment of other P-granule components (presumably RNPs) in *C. elegans*.

Our results reveal that PGL-3 proteins have two distinct regions for RNP granule assembly: one for globular granule assembly via self-interaction, and the RGG box for recruiting RNA and RNA-binding proteins (Fig. 4 A).

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Our results reveal that PGL-3 proteins have two distinct regions for RNP granule assembly: one for globular granule assembly via self-interaction, and the RGG box for recruiting RNA and RNA-binding proteins (Fig. 4 A). Because RGG boxes of various proteins have been shown to bind RNA with low sequence preference (Godin and Varani, 2007), PGL proteins...
are likely to bind via the RGG box to diverse mRNAs and/or mRNA protein complexes (mRNPs) that contain transient protein components of P granules. As most of the transient P-granule components contain various types of RNA-binding motifs and all of the tested components in this study were excluded from granules formed by PGL-3ΔRGG in CHO cells (Fig. S3), we speculate that this RNA-dependent recruitment is the major mechanism of incorporation of transient components into P granules. Because some RNA-binding proteins (e.g., GLH-1) were not recruited to PGL-3 granules, there seems to be some specificity for the recruitment of RNPs, but how it is achieved is currently unclear (some P-granule components, such as IFE-1, appear to be recruited to PGL granules by direct protein–protein interactions [Amiri et al., 2001]). These properties of PGL proteins raise a two-step model for P-granule formation: in the first step, PGL proteins bind to various mRNPs through interaction with RGG boxes; in the second step, globular granules are formed by the self-interaction domain of PGL proteins (Fig. 5); the order of the two steps can be opposite.

A recent report showed that P granules in early *C. elegans* embryos are in dynamic equilibrium with their soluble components, and a conceptual model was proposed in which assembly and disassembly of P granules are regulated by the concentration of soluble P-granule components and their saturation point, which would be lower in the germ lineage than in the soma (Brangwynne et al., 2009). Our data indicate that PGL proteins are the key components directing P-granule assembly/disassembly, and that GLH proteins may be involved in shifting the equilibrium toward granule assembly, possibly by lowering the saturation point of free PGL proteins in the germ lineage.

An essential role for scaffolding proteins in RNP granule assembly has been reported. For example, in the case of formation of processing bodies (P-bodies, a class of cytoplasmic RNP granules) in yeast, a multi-step assembly mechanism was proposed: initially, an individual mRNA associates with various proteins to form an mRNP, and then individual mRNPs form P-bodies via self-aggregation of a scaffolding protein(s)–Edc3p and/or Lsm4p (Decker et al., 2007). In *Drosophila*, Tudor protein is thought to function as a scaffold for germ granule (polar granule) assembly (Arkov et al., 2006). Because some germ plasm–specific proteins and RNAs can form small RNPs (“pre-particles”) even in the absence of Tudor, it was proposed that the role of Tudor in germ plasm formation may be to bind these small RNPs and assemble them into a larger granule (Arkov et al., 2006). Thus, we propose that self-association of scaffold proteins that can bind to RNPs might be one of the general mechanisms by which large RNP granules are formed.

**Materials and methods**

Cloning and constructs of tagged P-granule components

The full-length coding region of each P-granule component was PCR amplified from the corresponding cDNA clones from Y. Kohara, National Institute
In brief, embryos were collected by cutting gravid hermaphrodite adults, perished by freeze-cracking, and fixed with methanol–aceton (−20°C methanol for 5 min, −20°C aceton for 5 min). The samples were rehydrated before antibody staining by passing the slide through an aceton series (90, 70, 50, and 30%) at room temperature before transfer into PBS + 0.5% (wt/vol) Tween 20 (PBST). For immunostaining, the slides were incubated for 1–2 h at room temperature in PBST containing 0.5% BSA + 0.5% skim milk. Endogenous PGL-3 was visualized with mAb KT3 (1:4 dilution; Takeda et al., 2008) or polyclonal rabbit or anti-PGL-1 (1:20,000; MBL; generated using His-tagged PGL-3 as antigen). Endogenous PGL-1 was visualized with mAb KT4 (1:4 dilution; Takeda et al., 2008); mAb KT6 (1:500 dilution; Wood et al., 1984), or rabbit polyclonal anti-PGL-1 (1:10,000; MBL; generated using GST-tagged PGL-1 as antigen). Specificity of the PGL-3 antibodies and PGL-1 antibody generated in this study was confirmed by Western blotting and immunostaining of samples from wild type and pgl-3 or pgl-1 mutants. Anti-GFP (1:200 dilution; MBL) was used to visualize GFP::MEK3. Anti-GH-L1 (Kawasaki et al., 2004) was used at 1:2,000 dilution. For secondary antibodies, goat anti–mouse IgG(H+L) Alexa Fluor 488 and 594, goat anti–rabbit IgG(H+L) Alexa Fluor 488 and 594, donkey anti–rat IgG(H+L) Alexa Fluor 594, and goat anti–mouse IgM Alexa Fluor 594 (Invitrogen) were used. Secondary antibodies [1:100–1:200 dilution] were preadsorbed with C. elegans acetone powder and incubated for 1 h at room temperature. After final washes, each sample was mounted with VECTASHIELD (Vector Laboratories).

Microscopy
Immunofluorescence images of cultured cells and embryos were acquired with a DSU disk-scanning confocal microscope system (BX61; Olympus) with a 100× objective lens (UPlanApo Oil l/3, 1.35 NA). For each cultured cell and embryo, Z-series images (0.5–1.0 μm steps) were acquired and projected using a maximum intensity algorithm to produce a single integrated image using MetaMorph software (Molecular Devices). Images were processed with Photoshop (Adobe) software.

RNAi
RNAi was performed by the soaking method described previously (Moeda et al., 2001). In brief, L4 worms were soaked in dsRNA solution (−2 μg/ml for each RNA species) for 24 h and then cultured on plates for 24 h at 25°C. Adult worms were then cut open, and embryos were immunostained. dsRNA was transcribed in vitro from cDNA clones yk847b03 for pgl-1, yk1437e06 for pgl-3, and yk514b11 for gh-1.

Online supplemental material
Fig. S1 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and LacZ::6xHis. Fig. S2 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and PGL-3::6xHis or PGL-1::6xHis and PGL-3::6xHis. Fig. S3 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and 6xHis::PGL-3::RGG. Table S1 lists the PCR templates and expression vectors used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201010106/DC1.

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