Squid, Cup, and PABP55B function together to regulate gurken translation in Drosophila

K. Nicole Clouse, Scott B. Ferguson, Trudi Schüpbach *

Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

Received for publication 24 April 2007; revised 7 November 2007; accepted 7 November 2007
Available online 19 November 2007

Abstract

During Drosophila melanogaster oogenesis, the proper localization of gurken (grk) mRNA and protein is required for the establishment of the dorsal–ventral axis of the egg and future embryo. Squid (Sqd) is an RNA-binding protein that is required for the correct localization and translational regulation of the grk message. We show that Cup and polyA-binding protein (PABP) interact physically with Sqd and with each other in ovaries. We show that cup mutants lay dorsalized eggs, enhance dorsalization of weak sqd alleles, and display defects in grk mRNA localization and Grk protein accumulation. In contrast, pAbp mutants lay ventralized eggs and enhance grk haploinsufficiency. PABP also interacts genetically and biochemically with Encore. These data predict a model in which Cup and Sqd mediate translational repression of unlocalized grk mRNA, and PABP and Enc facilitate translational activation of the message once it is fully localized to the dorsal–anterior region of the oocyte. These data also provide the first evidence of a link between the complex of commonly used trans-acting factors and Enc, a factor that is required for grk translation. 

© 2007 Elsevier Inc. All rights reserved.

Keywords: Drosophila; Oogenesis; gurken mRNA; Translational control; Axis specification

Introduction

Many cells display inherent asymmetries, and polarity is often accompanied by restricting the expression of certain mRNAs to specific regions of the cell by localizing the RNAs and regulating their translation. In Drosophila melanogaster oogenesis, RNA localization followed by localized translation plays an important role in the establishment of the major body axes of the egg and future embryo. Localization of gurken (grk) mRNA during mid-oogenesis establishes the dorsoventral axis, and localization of oskar (osk) and bicoid (bcd) mRNAs results in the formation of the embryonic anterior–posterior axis (reviewed in Huynh and St Johnston, 2004). Similar to localized RNAs in other systems, these RNAs are packaged into large protein complexes that facilitate both their microtubule-dependent transport and their translational control (Chekulaeva et al., 2006; reviewed in Johnstone and Lasko, 2001; Wilhelm and Smibert, 2005).

Dorsoventral asymmetries in the Drosophila egg are easily observed in the eggshell. The dorsal surface of the egg is marked by two respiratory appendages, and the ventral surface is much more rounded than the dorsal side. The asymmetries seen in the mature egg are initiated during oogenesis as the egg chamber develops (reviewed in Ray and Schupbach, 1996). Dorsal fate is established when the epidermal growth factor receptor (Egfr) is activated by the TGF-α-like ligand, Gurken (Grk) (Neuman-Silberberg and Schupbach, 1993; Price et al., 1989; Schupbach, 1987). Egfr is expressed uniformly in the follicle cells overlying the oocyte and nurse cells (Sapir et al., 1998). In contrast, grk mRNA is tightly localized to the future dorsal–anterior region of the oocyte to produce a local supply of ligand. As a result, Egfr is activated in only a subset of follicle cells, and these specific cells adopt dorsal fates (Neuman-Silberberg and Schupbach, 1993, 1996; Nilson and Schupbach, 1999).

The distribution of Grk is controlled at the level of both RNA localization as well as translational control, and mutants have been identified that disrupt both processes. In squid (sqd) and fs (1)K10 (K10) mutants, grk mRNA is mislocalized along the entire anterior cortex of the oocyte, and the mislocalized RNA is
translated, resulting in ectopic activation of Egfr and dorsalized eggshells (Kelley, 1993; Wieschaus et al., 1978, 1979). Sqd is a heterogeneous nuclear ribonucleoprotein, or hnRNP, a family of proteins that has been implicated in many processes including RNA processing and transport (and Dreyfuss et al., 2002; reviewed in Dreyfuss et al., 1993) and whose members are often able to shuttle between the nucleus and cytoplasm (Michael et al., 1997; Mili et al., 2001; Pinol-Roma and Dreyfuss, 1992; reviewed in Shyu and Wilkinson, 2000).

Previous studies have shown that Sqd is required for the regulated nuclear export, cytoplasmic localization, and translational control of grk mRNA and have led to a model for Sqd in grk expression (Goodrich et al., 2004; Norvell et al., 1999). In this model, grk mRNA is transcribed and processed in the nucleus and assembled into a protein complex that contains Sqd and other RNA processing factors, such as Hrb27C/Hrp48. The grk message is exported from the nucleus, where Sqd is required to mediate translational repression of grk until the mRNA is properly localized. This repression is reversible, however, and upon localization to the dorsal–anterior region of the oocyte, grk mRNA is translated and the protein is available to activate Egfr in the overlying follicle cells.

In contrast to sqd and K10 mutants, in which both grk RNA localization and translational repression are disrupted, mutations in encore (enc) result in a partial mislocalization of grk mRNA, yet the RNA is maintained in a state of translational repression (Hawkins et al., 1997). Enc protein co-localizes with grk mRNA (Van Buskirk et al., 2000), and taken together, these data suggest that Enc assists in mediating the translational activation of grk mRNA once it is fully localized to the dorsal–anterior region of the oocyte. However, a specific mechanism for Enc’s function in grk translational activation has not been described.

Precise spatial expression of many proteins is accomplished by physical localization of the RNA as well as tight translational control of the message (Dreyfuss et al., 2002). Unique expression patterns can be generated by the integration of input from RNA-specific and more general translation control factors. For instance, Smg and Glo bind specifically to the RNA, while Smibert, 2005). In contrast, PABP is thought to mediate translational activation by interacting with the translational initiation machinery (reviewed in Mendez and Richter, 2001; and Richter and Sonenberg, 2005), but a specific role for PABP in Drosophila oogenesis has not been previously described.

Here we report the isolation of Cup and PABP through biochemical interactions with Sqd. We show that these interactions are sensitive to RNase A digestion and are therefore likely bridged by an RNA molecule. We confirm the functional significance of these biochemical data by demonstrating that cup mutants lay dorsalized eggs, and pAbp55B mutants lay ventralized eggs. These data are consistent with a severe perturbation of grk signaling. In addition, we observed genetic interactions between pAbp55B and grk as well as between cup and sqd. cup mutants also display improper Grk protein accumulation and compromised grk mRNA localization in the cytoplasm. Finally, we have demonstrated a genetic interaction between pAbp55B and enc. Taken together, these data support a model in which the regulation of grk expression occurs in the context of multilobed, dynamic RNA–protein complexes. In this model, Cup functions with Sqd and Hrb27C/Hrp48 in a protein complex that mediates the translational repression of grk mRNA before it is properly localized. We hypothesize that once the RNA has reached the future dorsal–anterior region of the oocyte, PABP55B and Enc facilitate the translational activation of grk mRNA. Significantly, the PABP55B–Enc interaction is the first direct biochemical link between a factor that is specifically required for the regulation of grk RNA and the complex containing factors (Cup, PABP55B, and Sqd) that function more generally in the regulation of multiple RNAs during oogenesis.

Materials and methods

Fly stocks

Akira Nakamura provided the cupΔ777 allele, which was generated by mobilizing the P element in cup11080 (Keyes and Spradling, 1997) and resulted in a deletion of the N-terminal third of the Cup protein product, including most of the eIF4E-binding domain (Nakamura et al., 2004). cup1 and cup2 are EMS-generated alleles (Schupbach and Wieschaus, 1991). pAbp55B101106 is a lethal P-element insertion residing in the 5’ UTR of pAbp55B, 102 base pairs upstream of the start codon, and precise excision of the P[hsFlp] element restores viability and reverts the morphological phenotypes of pAbp5101106 heterozygotes to wild type (Sigrist et al., 2000). pAbp55B101106 is a viable P-element insertion in the 5’ UTR of the RA, RF, and RH transcripts of pAbp55B, 762 base pairs upstream of the start codon.

Germline clones of pAbp5101106 were generated using the FLP-DHS (yeast flipase recombination target-site-specific recombinase–dominant female sterile) system previously described (Chou and Perrimon, 1992, 1996). Progeny from yw hsFLP;ovoD FRTG13/Cyo×FRTG13-pAbp5101106/Cyo were heat shocked at 37 °C for 2 h a day for 4 days during the second and third larval instar. sqdΔ is a P-element insertion that specifically disrupts germine expression during oogenesis (Kelley, 1993; Matunis et al., 1994; Norvell et al., 1999; Yano et al., 2000). pAbp5101106 is an EMS-generated null allele. enc124 and enc123 are strong, EMS-generated hypomorphic alleles and enc124 was generated by P-element excision (Hawkins et al., 1997).

Eggs were collected and eggshell morphology was determined as previously described (Schupbach and Wieschaus, 1991). Eggshell characterization represents multiple independent collections scored by two investigators.
Antibodies

The following antibodies were used: monoclonal α-Grk serum (ID12; 1:10 dilution for immunohistochemistry) (Queenan et al., 1999), monoclonal α-Sqd serum (8F3; 1:7 dilution for IP; 1:200 for western blot) (Goodrich et al., 2004), monoclonal α-Dorsal serum (7F12; 1:7 dilution for IP) (provided by Ruth Steward), monoclonal α-pAbp55B ascites (6E2; 1:140 dilution for IP; 1:1000 for western blot) (Matunis et al., 1992), polyclonal α-Cup (#219; rat against amino acids 1–225; 1:70 dilution for IP; 1:10,000 for western blot) (Nelson et al., 2004), polyclonal α-Cup (rabbit against amino acids 597–975; 1:28 dilution of affinity purified antibody for IP; 1:1000 dilution of non-affinity purified serum for western blot) (Nakamura et al., 2004), polyclonal α-Cup (#27; rabbit; 1:70 dilution for IP; 1:500 for western blot) (Verrotti and Wharton, 2000), polyclonal α-Enc (rabbit and rat against amino acids 133–340; 1:28 dilution of rabbit for IP; 1:1000 dilution of rat for western blot) (Van Buskirk et al., 2000), polyclonal α-Hrb27C/Hrp48 (rabbit; 1:20 dilution for IP) (Siebel et al., 1994), monoclonal α-CycB (F2; 1:20 dilution for IP; 1:30 for western blot) (Knoblich and Lehner, 1993), polyclonal α-silbo (rat; 1:500 for immunohistochemistry), (Jekely et al., 2005), HRP-conjugated goat α-mouse (1:10,000; Jackson ImmunoResearch), HRP-conjugated goat α-rabbit (1:7500, Pierce), and HRP-conjugated goat α-rat (1:10,000, Jackson ImmunoResearch).

Immunoprecipitations

Immunoprecipitations were performed as described (Van Buskirk et al., 2000) with the following modifications: instead of adding protease inhibitors individually to the lysis buffer, a complete mini protease inhibitor cocktail tablet was used (Roche); ovarian lysates were not pre-cleared with pre-immune serum but were sometimes pre-cleared with Protein A/G agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C; and ovarian lysates were first incubated with antibodies for 1–2 h at 4 °C and then beads were added to the mixture for an additional 1–2 h at 4 °C. Beads were rinsed 4× in cold lysis buffer before electrophoresis. When indicated, RNase A was included in the lysis and wash buffers at a final concentration 1 mg/mL.

For immunoprecipitations performed for mass spectrometry analysis, the gels were fixed in 7% acetic acid+10% methanol for 30 min, incubated in Sypro Ruby Protein Gel Stain (Molecular Probes) overnight, and destained in fix solution for 1 h prior to UV visualization. Gel slices containing Sqd-interacting proteins were analyzed by the Princeton SynSeq facility.

For immunoprecipitations performed for western blotting, the samples were transferred to nitrocellulose (Amersham), blocked in TBST (Tris-buffered saline+0.1% Tween-20)+5% milk+1% BSA overnight, and incubated in primary antibody for 1–2 h at room temperature. Blots were washed in TBST for 2–3 h and incubated in secondary antibody for 1 h at room temperature, washed in TBST for 2–3 h, and detected by ECL (Amersham or Pierce).

Immunohistochemistry

Ovaries were dissected in ice cold 1× PBS and fixed for 20 min at room temperature in 4% paraformaldehyde in PBST (PBS+0.3% Triton X-100) and heptane. Ovaries were rinsed three times with PBST and blocked in PBS+1% Triton X-100+2.5% BSA for 1 h at room temperature. Ovaries were incubated for 1 h at room temperature in 1:500 α-silbo and/or 1:10 α-Grk diluted in block buffer and washed overnight at 4 °C in PBST. Ovaries were incubated for 1 h at room temperature in block buffer containing AlexaFluor 488-conjugated goat α-rat and/or AlexaFluor 568-conjugated goat α-mouse secondary (Molecular Probes) diluted 1:1000+Hoechst diluted 1:10,000+Oregon Green or AlexaFluor 633-conjugated phalloidin (Molecular Probes) diluted 1:1000. Ovaries were washed for 2–3 h at room temperature in PBST and mounted in 1:1 PBS-glycerol or Aqua Poly/Mount (Polysciences, Inc.).

In situ hybridization

Ovaries were dissected in ice cold 1× PBS and fixed for 20 min at room temperature in 4% paraformaldehyde in PBS+heptane+DMSO. Subsequent steps were performed as previously described (Tautz and Pfeifle, 1989) using a grk RNA probe.

Results

Sqd co-purifies with Cup and PABP55B in ovarian extracts

In order to elucidate the role of Sqd in the regulation of grk expression, we prepared ovarian extracts and performed immunoprecipitations using either an α-Sqd antibody or a negative control antibody. Eleven bands were pulled down using the α-Sqd antibody but not by the negative control antibody (Fig. 1A, arrows). The identities of these bands were determined by mass spectrometry. One band of 150 kDa was identified as the translational repressor Cup, and a 65-kDa protein was identified as the translational activator polya-A-binding protein (PABP55B, encoded by CG5519). The negative control antibody, α-Dorsal (α-Dl) was used because it is a monoclonal antibody that was generated in the same facility as α-Sqd, but DI is not expressed during oogenesis and therefore should not specifically pull down any ovarian factors.

To verify the identity of the bands sequenced by mass spectrometry, we performed co-immunoprecipitations using antibodies that recognize Sqd (Goodrich et al., 2004), Cup (Keyes and Spradling, 1997; Nakamura et al., 2004; Nelson et al., 2004; Verrotti and Wharton, 2000), or PABP55B (Matunis et al., 1992). In these assays, α-Sqd, but not α-Dl, immunoprecipitates Cup and PABP55B in an mRNA-dependent manner (Figs. 1B and C), confirming the results of the mass spectrometry analysis. We further characterized the approximate stoichiometry of the Sqd/Cup interaction and found that sqd is complexed with approximately 5–7% of the Cup in ovarian lysates (Supplemental Fig. 1). Given that Cup is known to regulate other RNAs, this level of interaction is significant. We also wanted to determine whether the interaction between Sqd and Cup or PABP55B is specific for one of the Sqd isoforms (Kelley, 1993), so we used an antibody that recognizes the HA epitope to perform immunoprecipitations out of ovarian extracts expressing either HA-SqdA or HA-SqdS. In these experiments, Cup and PABP55B were able to interact with both Sqd isoforms, indicating that these interactions are not isoform-specific (data not shown). Given that the hnrNP Hrb27C/Hrp48 interacts with Squid (Goodrich et al., 2004), we tested whether it also interacts with Cup and PABP55B. Indeed we were able to immunoprecipitate both Cup and PABP55B with antibodies against Hrb27C/Hrp48 (Fig. 1E).

Cup and PABP55B females lay eggs with dorsoventral patterning defects

Cup has been shown to interact with other proteins in oogenesis, and cup mutations have multiple phenotypes, indicating that Cup regulates a number of targets in oogenesis (Keyes and Spradling, 1997; Nakamura et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004). To determine whether the physical interactions between Cup, Squid and PABP were relevant to grk function, we examined the eggs laid by pAbp55B or cup mutant females and observed that these eggs display dorsoventral patterning defects. We obtained two P-element insertion alleles in the 5’ UTR of pAbp55B (pAbp55B715 and pAbp55B724). The
$pAbp^{1010}$ insertion is homozygous and hemizygous lethal whereas the $pAbp^{EY11561}$ insertion is homozygous viable. We examined the eggs that were laid by $pAbp^{1010}/pAbp^{EY11561}$ trans-heterozygous and $pAbp^{EY11561}$ homozygous females. Significantly, these females lay 3.9±0.7% ($n=355$) and 8.4±1.2% ($n=286$) ventralized eggs, respectively. These eggs are charac-
Grk protein is inefficiently localized in cup ovaries

To test whether the eggshell phenotypes seen in cup mutants were due to defective grk expression, we monitored Grk protein by indirect immunofluorescence. Consistent with the dorsalized eggshells, Grk protein localizes inefficiently in cup mutants (Fig. 3). cup mutants exhibit a range of oogenesis defects, such as abnormally small oocytes and nurse cell chromosomes that fail to properly disperse (Keyes and Spradling, 1997), therefore defining the stage of these egg chambers was extremely difficult. For this reason, the egg chambers were not staged according to traditional definitions in these experiments, but instead Grk localization was analyzed and scored in every egg chamber in which the oocyte nucleus had achieved an asymmetric localization within the oocyte, indicating that the egg chamber had at least reached stage 8 of oogenesis. In the wild-type controls, only stage 8 and 9 egg chambers were counted, whereas stage 10 and older egg chambers were disregarded because they were over-represented in the wild-type samples relative to stage 10 egg chambers in cup alleles. Omitting stage 10 egg chambers from wild-type scoring increases the percentage of egg chambers displaying unlocalized Grk in the wild-type control, thus increasing the stringency of this control.

In scoring, Grk localization was categorized as either localized properly to the future dorsal–anterior of the oocyte (Fig. 3A), dispersed throughout the oocyte, but somewhat accumulated at the future dorsal–anterior (Fig. 3B), or evenly dispersed throughout the oocyte (Fig. 3C). Grk protein was localized to the future dorsal–anterior of about 86% (n = 548) of wild-type egg chambers, in contrast to 31% (n = 196) of cupΔ Δ and 61% (n = 163) of cupΔ A212 egg chambers. The degree of severity of this defect was greater for cupΔ Δ mutants than for cupΔ A212 mutants, which is also consistent with the degree of severity of the eggshell phenotypes (Fig. 3).

We also further confirmed this phenotype by staining egg chambers homozygous for cupΔ with the antibody to Grk as well as an antibody to the Slbo protein (Jekely et al., 2005). By assessing the location of the border cells, which migrate through the nurse cell cluster during stage 9 of oogenesis, we were able to stage the cupΔ Δ egg chambers by this independent criterion. The squamous morphology of the follicle cells associated with the nurse cells was also used to indicate whether the egg chambers were in early or late stage 9. However, very few egg chambers ever reached late stage 9 in the cupΔ Δ homozygous mutant. Nevertheless, a mislocalization of Gurken protein could be seen in these egg chambers, whereas in the wild-type control, Grk protein was always dorsally localized (data not shown).
grk mRNA is localized less efficiently in cup mutants

In order to determine whether the ectopic Grk protein expression is a result of unrestrained translation alone, we analyzed the localization of grk mRNA in cup mutants (Fig. 4). In contrast to wild-type egg chambers, in which about 71% (n=349) of stages 8–9 egg chambers demonstrate grk mRNA localized to the future dorsal–anterior of the oocyte, only 27% (n=271) of cup5 and 54% (n=259) of cupΔ212 egg chambers display this localization. Consistent with the eggshell phenotypes and Grk protein localization data, grk mRNA localization was less severely disrupted in cupΔ212 than in cup5 (Fig. 4). This might suggest that Cup is required for grk mRNA localization; however, this effect of cup mutants may also be indirect. Perhaps removing Cup from the localization/repression complex built upon grk mRNA compromises the stable architecture of the complex, resulting in less efficient localization.

<table>
<thead>
<tr>
<th></th>
<th>dorsal-anterior only</th>
<th>dispersed; dorsal-anterior bias</th>
<th>completely dispersed</th>
</tr>
</thead>
<tbody>
<tr>
<td>OreR n=548</td>
<td>85.8 ± 8.4%</td>
<td>13.0 ± 4.8%</td>
<td>1.3 ± 3.6%</td>
</tr>
<tr>
<td>cup5 n=196</td>
<td>30.6 ± 9.2%**</td>
<td>46.9 ± 4.1%**</td>
<td>22.4 ± 13.3%**</td>
</tr>
<tr>
<td>cupΔ212 n=163</td>
<td>60.7 ± 18.7%**</td>
<td>35.6 ± 21.3%**</td>
<td>3.7 ± 2.6%*</td>
</tr>
</tbody>
</table>

Fig. 4. grk mRNA is localized less efficiently in cup mutants. In situ hybridization using a grk RNA probe was performed on OregonR, cup5, and cupΔ212 egg chambers. grk mRNA distribution was categorized as either dorsal–anterior only (A), an anterior ring with a dorsal–anterior bias (B), or an anterior ring (C). ± indicates standard deviation of multiple microscopy sessions. Binomial probabilities for the frequency of each classification in cup mutants were calculated relative to OregonR. *p<0.05; **p<0.01.
Importantly, the grk mRNA localization data show that Cup is required for the translational repression of unlocalized grk mRNA. This effect is also seen in sqd mutants (Norvell et al., 1999) and is in contrast to many ventralizing mutants in which grk mRNA is mislocalized but remains in a translationally repressed state, such as encore or the spindle class genes (Ghribial et al., 1998; Gonzalez-Reyes et al., 1997; Hawkins et al., 1997).

Genetic and biochemical interactions with cup and PABP55B

Females trans-heterozygous for a weak allelic combination of sqd (sqd<sup>1</sup>/sqd<sup>k12</sup>) lay only 13% wild-type eggs (n=355), and the remaining eggs display mild to strong dorsalized phenotypes. Females homozygous for the weak allele sqdk<sup>k12</sup> lay only 5% weakly dorsalized eggs (n=160), but the frequency and severity of dorsalization in both sqd allelic combinations was dramatically enhanced by heterozygosity for cup<sup>20</sup> (Fig. 5), a strong cup allele (Keyes and Spradling, 1997). This effect has been observed over multiple independent experiments, and representative data are shown (Fig. 5). This synergistic genetic interaction presents further evidence that the observed Cup–Sqd biochemical interaction is functionally relevant for grk translational repression.

Females heterozygous for a null allele of grk, grk<sup>HF48</sup>, lay 3% weakly ventralized eggs (n=1146), and reducing pAbp<sup>55B</sup> by one copy was able to greatly enhance this ventralization (n=1212, Fig. 5). This synergistic genetic interaction is consistent with PABP55B functioning positively in grk translation.

Immunoprecipitation experiments show that α-PABP55B is able to specifically pull down Cup protein (Figs. 1D and E) and α-Cup pulls down PABP55B (Fig. 1E). Considering that Cup and PABP55B interact biochemically with each other and with Sqd, cup and pAbp<sup>55B</sup> females lay eggs with opposite phenotypes, and cup–sqd and pAbp<sup>55B</sup>–grk interact genetically, we propose that Cup and PABP55B work antagonistically to regulate grk mRNA expression.

Table A

<table>
<thead>
<tr>
<th></th>
<th>wild-type like</th>
<th>weakly dorsalized</th>
<th>moderately dorsalized</th>
<th>strongly dorsalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>cup&lt;sup&gt;20&lt;/sup&gt; / +</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>sqd&lt;sup&gt;k12&lt;/sup&gt; / sqd&lt;sup&gt;1&lt;/sup&gt; (n=355)</td>
<td>13.2%</td>
<td>4.2%</td>
<td>54.1%</td>
<td>28.5%</td>
</tr>
<tr>
<td>cup&lt;sup&gt;20&lt;/sup&gt; / + ; sqd&lt;sup&gt;k12&lt;/sup&gt; / sqd&lt;sup&gt;1&lt;/sup&gt; (n=443)</td>
<td>0%</td>
<td>6.8%</td>
<td>8.6%</td>
<td>84.7%</td>
</tr>
<tr>
<td>sqd&lt;sup&gt;k12&lt;/sup&gt; / sqd&lt;sup&gt;k12&lt;/sup&gt; (n=160)</td>
<td>94.4%</td>
<td>1.3%</td>
<td>4.4%</td>
<td>0%</td>
</tr>
<tr>
<td>cup&lt;sup&gt;20&lt;/sup&gt; / + ; sqd&lt;sup&gt;k12&lt;/sup&gt; / sqd&lt;sup&gt;k12&lt;/sup&gt; (n=524)</td>
<td>25.2%</td>
<td>37.8%</td>
<td>28.8%</td>
<td>8.2%</td>
</tr>
</tbody>
</table>

Table B

<table>
<thead>
<tr>
<th></th>
<th>wild-type like</th>
<th>weakly ventralized</th>
<th>moderately ventralized</th>
<th>strongly ventralized</th>
</tr>
</thead>
<tbody>
<tr>
<td>grk&lt;sup&gt;HF48&lt;/sup&gt; / + (n=1146)</td>
<td>97.3 ± 1.2%</td>
<td>2.7 ± 1.2%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>grk&lt;sup&gt;HF48&lt;/sup&gt; / pAbp&lt;sup&gt;10109&lt;/sup&gt; (n=1212)</td>
<td>45.1 ± 5.0%</td>
<td>43.4 ± 6.3%</td>
<td>10.0 ± 2.0%</td>
<td>0%</td>
</tr>
<tr>
<td>pAbp&lt;sup&gt;10109&lt;/sup&gt; / + (n=767)</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Fig. 5. sqd interacts genetically with cup, and grk interacts genetically with pAbp. Heterozygosity for cup<sup>20</sup> enhances the moderately dorsalized phenotype of sqd<sup>k12</sup>/sqd<sup>1</sup> trans-heterozygotes and of sqd<sup>k12</sup>/sqd<sup>12</sup> at 29 °C (A). Eggs were characterized as either wild-type-like, weakly dorsalized (single, broad fused appendage), moderately dorsalized (widely spaced appendages), or strongly dorsalized (crown of appendage material). In addition, grk<sup>HF48</sup>/pAbp<sup>10109</sup> trans-heterozygotes lay an increased percentage of and more severely ventralized eggs at 25 °C than does either heterozygote alone (B). Eggs were characterized as either wild-type-like, weakly ventralized (appendages fused at the base), moderately ventralized (single, slender fused appendage), or strongly ventralized (no appendage material). ± indicates standard deviation of multiple egg collections scored by independent investigators. Binomial probabilities for the frequency of each eggshell classification were calculated for cup<sup>20</sup> / + ; sqd<sup>k12</sup>/sqd<sup>12</sup> relative to sqd<sup>k12</sup>/sqd<sup>12</sup>, cup<sup>20</sup> / + ; sqd<sup>k12</sup>/sqd<sup>k12</sup> relative to sqd<sup>k12</sup>/sqd<sup>k12</sup>, and grk<sup>HF48</sup>/pAbp<sup>10109</sup> relative to grk<sup>HF48</sup>/+. All p-values were less than 0.01.*
repressed state and ventralized eggs are laid (Hawkins et al., 1997). These data are consistent with a role for Enc in the translational activation of grk mRNA.

Enc has previously been shown to be a large cytoplasmic protein that interacts with subunits of the proteasome in early oogenesis (Ohlmeyer and Schupbach, 2003; Van Buskirk et al., 2000). Considering its function early in oogenesis, and given its cortical localization in the oocyte during mid-oogenesis, Enc may act as a scaffolding protein mediating the transition from translational repression to activation of grk mRNA. To test this hypothesis, we performed immunoprecipitations and showed that α-Enc specifically immunoprecipitates the translational activator PABP55B. Furthermore, the data suggest that the interaction may be direct and not bridged by an RNA molecule, as α-Enc is able to immunoprecipitate PABP55B even when RNase A is added to the extract (Fig. 6A).

In addition to the biochemical interaction, pAbp55B and enc also interact genetically. enc mutants are cold-sensitive for ventralization (Hawkins et al., 1997) and display much weaker ventralization at 25 °C than at 18 °C. However, heterozygosity for pAbp510109 enhances the both the frequency and severity of ventralized eggs laid by enc homozygotes at 25 °C (Fig. 6B). Although this enhancement is a subtle phenotype, the effect has been observed reproducibly over several independent experiments using two different enc alleles (encQ4 and encUU3). This genetic interaction suggests that the PABP55B–Enc biochemical interaction is functionally relevant in grk expression.

In addition to the enhanced ventralization, heterozygosity for pAbp510109 dramatically reduces the number of eggs that are laid by enc homozygotes and increases the percentage of collapsed eggs for which the eggshell phenotype cannot be determined. In fact, heterozygosity for pAbp510109 causes encR17 and encR1 homoyzogous females to lay no eggs. Taken together, the genetic interaction and RNA-independent physical association between Enc and PABP55B suggest that these proteins may function together to activate translation of grk mRNA.

Discussion

Sqd binds to RNA localization and translation factors in ovarian extracts

We have taken a direct approach to identify proteins that interact with Sqd protein in ovaries. Using an Sqd antibody, we performed immunoprecipitations out of ovarian extracts, isolated proteins that specifically interacted with Sqd, and identified those proteins by mass spectrometry. Four of the Sqd-interacting proteins were positively identified in the mass spectrometry analysis: Cup, PABP55B, Imp, and Hrb27C/Hrp48. The remaining bands were not identified with certainty. Imp and Hrb27C/Hrp48 are two factors that have previously been shown to be involved in RNA localization (Geng and Macdonald, 2006; Goodrich et al., 2004; Munro et al., 2006), and both Hrb27C/Hrp48 and Imp bind to grk mRNA (Geng and...
shown that 2003; Zappavigna et al., 2004), and independent studies have shown that osk localization and translation, such as Exu, Yps, elf4E, Me31B, and Bruno (Nakamura et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004), and independent studies have shown that osk mRNA is prematurely translated in cup mutants (Chekulaeva et al., 2006; Nakamura et al., 2004; Wilhelm et al., 2003). Cup co-localizes with the cap-binding protein, elf4E, and elf4E is not properly localized to the oocyte posterior pole in cup mutants (Wilhelm et al., 2003; Zappavigna et al., 2004). Cup competes away elf4G, another translation initiation factor, for binding to elf4E (Nelson et al., 2004; Zappavigna et al., 2004), thereby repressing translation. Together, these data are consistent with the following model for Cup-mediated translational repression; Cup represses the translation of RNAs containing BREs through interactions with Bruno. In this complex, Cup binds directly to elf4E and interferes with elf4G binding to elf4E. Because elf4G binding to elf4E is a prerequisite for translation initiation, Cup represses translation by blocking this interaction (reviewed in Richter and Sonenberg, 2005). Direct biochemical data supporting this model have recently been obtained by Chekulaeva et al. (2006). We propose that Cup represses grk translation by a similar mechanism prior to its localization to the dorsal–anterior of the oocyte. Cup activity is used by several transcript-specific factors to mediate translational repression of that RNA in a developmentally appropriate context. For instance, Cup is required to mediate the translational repression of the nanos (nos) transcript. Cup has been shown to interact with Nos protein and co-localizes with Nos in the germarium. cup and nos also interact genetically, as heterozygosity for cup suppresses nos-induced phenotypes in early oogenesis (Verrotti and Wharton, 2000). Later in development, Cup binds to Smaug, a factor that specifically binds to nos RNA and is required for its translational repression in embryos (Crucs et al., 2000; Dahanukar et al., 1999; Smibert et al., 1996). In this example, Cup is required for Smaug to interact with elf4E and mediate nos repression. Consistent with this biochemical model, Smaug-mediated translational repression is less efficient in cup mutants (Nelson et al., 2004).

Here we have shown that Cup is also required for grk translational repression. This contrasts with previous reports that grk expression is normal in cup mutants (Nakamura et al., 2004; Wilhelm et al., 2003), but these earlier reports used relatively weak cup alleles and monitored Grk levels by immunofluorescence. In contrast, we were able to use alleles that allowed us to assess the eggshell phenotype in cup mutants, which provides the most sensitive assay for defects in Grk levels. Our own analyses showed that the different cup alleles vary greatly in phenotypic strength and range of phenotypes (data not shown).
that is used for oligomerization of PABP55B on polyA tails (Kuhn and Pieler, 1996). Once PABP55B is bound to RNA, it binds to eIF4G, and this interaction helps to increase the affinity of eIF4G for eIF4E. With this increased affinity, eIF4G is able to effectively compete with Cup for binding to eIF4E, and translation is able to begin (reviewed in Richter and Sonenberg, 2005; Tarun and Sachs, 1996; Tarun et al., 2005).

There are at least three polyA-binding proteins in the Drosophila genome (CG5119 at 55B, CG4612 at 60D, and CG2163 at 44B), which are predicted to function as general translation factors, so it is conceivable that PABP55B could regulate a subset of RNAs. CG2163 has also been designated as PABP2 and has been shown to have essential roles in germ line development and in early embryogenesis (Benoit et al., 2005). Here we have shown that PABP55B mediates the translational activation of fully localized grk mRNA. Specifically, heterozygous pAbp55B mutants lay ventralized eggs in certain genetic combinations, and heterozygosity for pAbp55B also enhances the weakly ventralized phenotype of grk heterozygotes, consistent with a role in translational activation of grk.

**PABP55B functions with Enc to mediate translational activation of grk**

We have also shown that PABP55B binds to Enc in ovarian extracts, and that this interaction may be direct and not bridged by an RNA molecule. Furthermore, heterozygosity for pAbp55B is able to enhance the weakly ventralized phenotype of enc mutants raised at 25 °C. Taken together, the biochemical and genetic interactions suggest that PABP55B and Enc function together to mediate the translational activation of grk mRNA once it is localized to the dorsal–anterior of the oocyte.

Previously, Enc has been shown to be required for activation of grk translation in mid-oogenesis (Hawkins et al., 1997). An effect on osk mRNA localization has also been previously observed in enc mutants, but it is unclear at what level this process is affected, or whether this effect is direct (Van Buskirk et al., 2000). In addition, Enc has been shown to interact with subunits of the proteasome early in oogenesis (Ohlmeyer and Schupbach, 2003). Because of its large size and its ability to interact with several different proteins, Enc may play multiple roles during oogenesis. Considering the function of Enc in grk translational activation (Hawkins et al., 1997) and its localization to the dorsal–anterior region of the oocyte (Van Buskirk et al., 2000), we hypothesize that Enc could function as a scaffolding protein that helps to mediate the transition from translational repression to activation of grk mRNA.

**Sqd, Cup, PABP, and Enc mediate translational control of grk RNA**

We have shown that Cup functions with Sqd in a protein complex that mediates the translational repression of grk mRNA before it is properly localized. It is clear from the analysis of mutants such as spn-F and encore, in which mislocalized grk mRNA is translationally silent, that these two steps can be uncoupled (Abdu et al., 2006; Hawkins et al., 1997). We propose that once the RNA has reached the future dorsal–anterior region of the oocyte, PABP, Sqd, and Enc facilitate the translational activation of grk mRNA (Fig. 7). In Fig. 7, PABP is shown associating with the complex once it is fully localized; however, it is possible that PABP associates with the grk transport complex in an inactive form that is remodeled following its anchorage at the dorsal–anterior of the oocyte.

Previous studies have shown that Bruno (Bru) binds directly to Cup protein (Chekulaeva et al., 2006; Nakamura et al., 2004; Wilhelm et al., 2003) and is required for the translational repression of osk. Bru binds to specific sequence elements in the osk 3′ UTR called Bruno Response Elements (BREs), and mutations in these BREs have been shown to reduce Bru binding and result in ectopic Osk accumulation in the oocyte (Kim-Ha et al., 1995; Webster et al., 1997). Similarly, Bru has also been shown to bind to grk mRNA and to Sqd protein. Overexpression of bru cDNA leads to ventralization of the eggshell, consistent with reduced Grk protein expression in the oocyte. (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995; Norvell et al., 1999). Furthermore, disrupting bru expression in certain genetic contexts has been shown to result in excess Grk protein in the oocyte, consistent with Bru being required to mediate grk translational repression (Yan and Macdonald, 2004). In light of the results presented here, we propose that this phenotype is the result of Bru-mediated repression of grk translation by Cup.

The mechanism of grk translation and the trans-acting factors required for translational control largely parallel the mechanism employed by osk RNA, so an important question to be answered is how these two different RNAs are differentially transported and translationally regulated in distinct parts of the oocyte at the appropriate stage in oogenesis. Since the same group of trans-acting factors is involved in the expression of both RNAs, the specificity could be provided by cis-acting sequences within the RNA molecules themselves that affect the activity of common trans-acting factors. Alternatively, RNA-specificity could be generated by as-yet unidentified trans-acting factors. Given that Enc functions in grk translational activation, but is not required for osk translational activation (Van Buskirk et al., 2000), it is possible that Enc is providing some degree of specificity to the commonly used machinery that mediates translational control of multiple, unrelated transcripts. Currently, Enc is the only factor known to function uniquely in the translational activation of grk mRNA, and our results provide the first evidence of a link between this factor and the general translational control machinery that is used by multiple RNAs in oogenesis.

**Acknowledgments**

The authors would like to thank Akira Nakamura, Craig Smibert, Allan Spradling, Robin Wharton, Pernille Rorth, Don Rio, Ruth Steward, and Gideon Dreyfuss for providing antibodies and fly stocks; Joe Goodhouse for help with confocal microscopy and Saw Kyin for mass spectrometry analysis. We are very grateful to Amanda Norvell, Roshan Jain, and Stas Shvartsman for critical comments on the manuscript and to the
members of the Schüpbach and Wieschaus laboratories for helpful discussions and suggestions. This work was supported by the Howard Hughes Medical Institute and US Public Health Service Grant PO1 CA41086 and RO1 GM077620 as well as a New Jersey Cancer predoctoral fellowship #03-2011-CCR-EO and the DOD fellowship DAMD17-03-1-0393 to KNC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.11.008.

References


Queenan, A.M., Barcelo, G., Van Buskirk, C., Schupbach, T., 1999. The
transmembrane region of Gurken is not required for biological activity, but is necessary for transport to the oocyte membrane in Drosophila. Mech. Dev. 89, 35–42.


