Development 124, 2591-2602 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5119

The *Drosophila* G-protein-coupled receptor kinase homologue *Gprk2* is required for egg morphogenesis

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SUMMARY

G protein signaling is a widely utilized form of extracellular communication that is mediated by a family of serpentine receptors containing seven transmembrane domains. In sensory neurons, cardiac muscle and other tissues, G protein-coupled receptors are desensitized through phosphorylation by a family of kinases, the G protein-coupled receptor kinases (GRKs). Desensitization allows a cell to decrease its response to a given signal, in the continued presence of that signal. We have identified a *Drosophila* mutant, *gprk2^{6936}* that disrupts expression of a putative member of the GRK family, the *G protein-coupled receptor kinase 2* gene (*Gprk2*). This mutation affects *Gprk2* gene expression in the ovaries and renders mutant females sterile. The mutant eggs contain defects in several anterior eggshell structures that are produced by specific subsets of migratory follicle cells. In addition, rare eggs that become fertilized display gross defects in embryogenesis. These observations suggest that developmental signals transduced by G protein-coupled receptors are regulated by receptor phosphorylation. Based on the known functions of G protein-coupled receptor kinases, we speculate that receptor desensitization assists cells that are migrating or undergoing shape changes to respond rapidly to changing external signals.

Key words: *Drosophila, Gprk2,* G protein signaling, ovarian development, cell signaling

INTRODUCTION

Patterning a *Drosophila* oocyte requires the highly integrated activities of 15 interconnected nurse cells and an overlying layer of epithelial follicle cells (reviewed by Spradling, 1993; Ray and Schüpbach, 1996). Signaling events between follicle and germline cells precede the initial formation of new egg chambers (stage 1) and continue throughout development of the mature egg (stage 14). Using maternal information, the anteroposterior axis is specified very early, prior to egg chamber formation. *hedgehog* (*hh*) production by small groups of somatic cells at the tip of the ovary plays a role in establishing this axis, and in controlling egg chamber assembly (Forbes et al., 1996a). A likely Hedgehog receptor, encoded by the gene *smoothened* (*smo*), is a protein homologous to mammalian G protein-coupled serpentine receptors (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). The dorsal-ventral axis is laid down much later, following the relocation of the oocyte nucleus to the future dorsal side. The TGF-α family member, *gurken,* is expressed locally around the oocyte nucleus and binds to the *Drosophila* EGF-receptor homologue, Torpedo, on the surface of the overlying dorsal follicle cells. This initiates a signaling pathway that defines embryonic dorsal-ventral polarity (Chasan and Anderson, 1993; Neuman-Silberberg and Schüpbach, 1993).

The Gurken signal not only establishes the dorsal-ventral axis, but it also participates in patterning the anterior end of the egg. Eggs contain specialized structures in this region, including a micropyle (to allow sperm entry), two dorsal appendages (to allow embryonic respiration), and an operculum (to allow the mature embryo to escape from the egg shell). These structures are shaped by special subsets of migrating follicle cells. The 'centripetally migrating' cells produce the operculum and collaborate with the 'border' cells to make the micropyle. A distinct subset of 'dorsal appendage' follicle cells migrate anteriorly where they lay down the tubular dorsal appendages. At least one other major signaling pathway, involving the TGF-β family member *decapentaplegic* (*dpp*), also influences anterior eggshell production by these cells (Twombly et al., 1996). *dpp* is expressed by a set of 40-50 follicle cells that overlie the nurse cells and by the centripetally migrating follicle cells. Mutations reducing the activity of *dpp* or of the Dpp receptors encoded by *saxophone* (*sax*) and *thick veins* (*tkv*) cause eggs to be produced that have shortened dorsal appendages and other anterior defects. Overexpression of *dpp* causes an expansion of these anterior structures (Twombly et al., 1996).

We have identified a new mutant, *gprk2^{6936},* that produces anterior eggshell defects that resemble those seen in flies with reduced levels of Dpp signaling. The gene disrupted in this mutant encodes a putative member of the family of kinases that phosphorylate G protein-coupled receptors. Activation of G protein-coupled receptors leads to the dissociation of G proteins into α and βγ subunits, which in turn activate effector molecules including adenylyl cyclase and protein kinase A, phospholipases, ion channels, and the Ras/MAP kinase pathway (Gilman, 1987;

Logothetis et al., 1987; Birnbaumer, 1992; Luttrell et al., 1995; van Biesen et al., 1996). G protein-coupled receptor kinases (GRKs) rapidly downregulate these pathways by phosphorylating activated receptors at multiple sites which in turn allows the binding of proteins of the arrestin family. Arrestin binding uncouples the receptor from its associated G protein (Wilden et al., 1986) and contributes to resensitization of receptors through sequestration and recycling (Ménard et al., 1996; Yu et al., 1993)

The GRK family contains six members from mammals (reviewed in Premont et al., 1995), two from *Drosophila* (Cassill et al., 1991), and one whose existence is suggested from sequencing of the *C. elegans* genome (accession no. U22833). The biochemistry and cell biology of GRKs have been determined primarily through analysis of the original members of the family: rhodopsin kinase and β-adrenergic receptor kinase (βARK; Benovic et al., 1986; Palczewski et al., 1988). All of the mammalian family members phosphorylate agonist-bound G protein-coupled receptors, but which receptors they activate and the role of desensitization in vivo is not known with certainty. The *Drosophila* GRKs (GPRK1 and GPRK2) were identified by homology to βARK1 (Cassill et al., 1991) but no mutations had been previously identified. In addition to *smoothened*, several other *Drosophila* genes have been identified that encode putative G protein-coupled receptors (Shapiro et al., 1989; Saudou et al., 1992; Arakawa et al., 1990; Li et al., 1991, 1992; Monnier et al., 1992; Gotzes et al., 1994; Colas et al., 1995; Feng et al., 1996; Han et al., 1996), including a putative Wingless receptor Frizzled-2 (Bhanot et al., 1996). Our studies of the *gprk2*^{6936} mutant suggest that a signaling pathway involving a G protein-coupled receptor functions during egg chamber maturation.

MATERIALS AND METHODS

Drosophila stocks and crosses

Flies were maintained on yeasted cornmeal/molasses media under standard culture conditions. *ry*^{506} stocks were used as controls in all experiments (Lindsley and Zimm, 1992). The *gprk2*^{6936} mutant, *fs(3)06936*, and a lethal *dpp* allele, *l(3)10638*, were recovered from a P element mutagenesis screen (Karpen and Spradling, 1992). The *dpp* allele is described by Twombly et al. (1996). Balancer chromosomes are as described by Lindsley and Zimm (1992). The *BS3.0*, *dpp-lacZ* enhancer line, which was kindly provided by Dr. W. Gelbart, gives the complete pattern of *dpp* staining in the blade of the imaginal wing disc (Blackman et al., 1991).

To test for an interaction between *gprk2*^{6936} and *dpp* two sets of crosses were carried out. To examine wing imaginal discs, the homozygous *BS3.0 dpp-lacZ* enhancer line was crossed to *gprk2*^{6936}/TM3, *Sb*. *Sb*$^{+}$ male progeny were crossed to *gprk2*^{6936}/TM6, *Tb* and *Tb*$^{+}$, third-instar larvae were dissected open and stained for *lacZ* activity. Tissues were fixed and stained as described by Margolis and Spradling (1995). Homozygous *gprk2*^{6936} larvae were identified by the strength of β-gal staining in the central nervous system.

To examine the effects of the *gprk2*^{6936} mutation on *dpp* expression in the ovaries, a *gprk2*^{6936} chromosome that harbors the recessive markers *thread* (*th*), *scarlet* (*st*) and *curved* (*cu*) was crossed to the *dpp* allele, *l(3)10638*. *l(3)10638*/+; *gprk2*^{6936}, *th st cu*/+ males were crossed to *gprk2*^{6936}/, *th st cu*/TM3, *Sb* females and *gprk2*^{6936} homozygotes were identified by the presence of *th*, *st* and *cu*. Ovaries were dissected, fixed and stained for β-gal activity.

Molecular methods

Recovery of genomic DNA flanking the P element insertion was carried out as described by Cooley et al. (1988). A 2.0 kb fragment flanking the *fs(3)06936* insertion was used to screen a Charon 4A library of Canton-S genomic DNA (Maniatis et al., 1978) to obtain overlapping clones for the 5′ region of the gene. The same library was probed with the B6936 cDNA (see below) to obtain clones for the 3′ region of the gene. These hybridizations were carried out in 6× SSC + 0.5% dry milk, as described by Sambrook et al. (1989). All probes were generated by random hexamer priming.

A 3.1 kb genomic fragment from the 5′ region of the gene was used to screen a *Drosophila* ovary cDNA library (Stroumbakis et al., 1994). Hybridizations were carried out as above. Three identical clones of approximately 2.5 kb were isolated (named T6936). A 1.0 kb 3′ T6936 fragment was used to probe a 0-4 hour, cDNA embryo library (Brown and Kafatos, 1988) and two clones of approximately 3.5 kb were isolated. These clones were judged to be identical based on restriction mapping and sequencing of 5′ and 3′ ends, and only one of these clones was further characterized (named B6936). P1 clones from the 5′ and 3′ ends of the gene were obtained from the Berkeley *Drosophila* Genome Project (BDGP). P1 DNA was isolated according to the BDGP protocol.

DNA sequencing was carried out manually with Sequenase (United States Biochemical) or with Taq DyeDeoxy terminator Cycle AutoSequencing (Applied Biosystems Model 373A). Genomic and P1 fragments and cDNA clones B6936 and T6936 were subcloned into Bluescript-(KS−). Sequencing was carried out using T3, T7 and *Gprk2* primers on subcloned DNA, as well as on templates generated by nested deletions. cDNA B6936 was sequenced entirely on both strands whereas the T6936 3′ fragment and genomic fragments were sequenced on one strand and compared to the B6936 sequence.

Northern blot analysis

To obtain RNA from *Drosophila* ovaries, females were dissected in *Drosophila* Ringers and their ovaries were frozen immediately on dry ice. Tissues were thawed and homogenized in extraction buffer containing 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5 % SDS, and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 ratio). Tissues were repeatedly extracted until the protein interface was minimal and the RNA was precipitated by the addition of sodium acetate and ethanol.

To obtain RNA from staged embryos and third instar larvae, tissues were frozen on dry ice and homogenized in 8 M guanidine isothiocyanate buffer using a polytron homogenizer (as described by Sambrook et al., 1989). Poly(A)$^{+}$ RNA was purified on oligo(dT)-cellulose columns (Collaborative Research), size-fractionated on formaldehyde agarose gels, and transferred to GeneScreen nylon membranes in 10× SSC. Hybridization was carried out using the buffers described by Church and Gilbert (1984). Relative amounts of RNA on northern blots were determined by hybridization with β-tubulin probes.

The ovary northern blot (Fig. 6A) contained 4 μg of poly(A)$^{+}$ RNA per lane. The blot was probed with fragments of B6936 cDNA or the entire T6936 cDNA. The developmental northern blot (Fig. 6B) contained 1 μg of poly(A)$^{+}$ RNA per lane. The blot was probed first with a T6936-specific probe and exposed for 10 days. Next the blot was probed (without stripping) with the entire B6936 cDNA and exposed to film overnight. Finally, the blot was probed (again without stripping) with β-tubulin and exposed to film overnight. The T6936-specific probe was generated by digesting the T6936 cDNA insert with *Hin*fI, and isolating a 453 bp internal fragment by electrophoresis. This fragment has no sequences in common with B6936.

Western blot analysis and tissue immunohistochemistry

Purified antibodies to GPRK2 were generously provided by Charles Zuker and are described by Cassill et al. (1991). The antigen used to generate the antibody was an expressed protein containing amino acids 429-714 (according to the numbering in this article).

For western blot analysis, tissues were dissected in *Drosophila* Ringers and immediately frozen on dry ice. Tissues were thawed and homogenized in sample buffer (10 mM sodium phosphate buffer, 6 M urea, 2% SDS, 1% β-mercaptoethanol, 10 mM dithiothreitol, and 20

μg/ml PMSF). Samples were boiled for 10 minutes, electrophoresed on 7% SDS-PAGE gels, and transferred to nitrocellulose (Schleicer and Schuell) using the Bio-Rad MiniProtean system. Complete transfer was assessed by prestained molecular weight standards (Bio-Rad) and by staining of transferred proteins with amido black.

Nitrocellulose filters were preincubated in blocking solution (3% dry milk and 3% BSA in PBS) for 2 hours at room temperature or overnight at 4°C. Primary antibody (1:100 of anti-GPRK2) was added to the blocking solution and allowed to incubate overnight at room temperature. Washes were carried out in PBS + 0.1% Tween 20 at room temperature. Incubations in horseradish peroxidase-coupled, secondary antibody (Amersham; diluted 1:5000 in blocking solution) were carried out for 1-2 hours at room temperature. Antibody binding was visualized using the Amersham ECL kit.

For immunohistochemistry, tissues were dissected in *Drosophila* ringers and fixed in 4% paraformaldehyde (e.m. grade, Ted Pella) diluted in PBS. Optimal times of fixation were derived for each tissue: 10 minutes for ovaries, 20 minutes for imaginal discs, and 40 minutes for central nervous systems. Fixed tissues were washed in PBS + 0.3% Triton X-100 and blocked for 4 hours at room temperature or overnight at 4°C in PBS, 0.3% Triton X-100, 3% normal goat serum (NGS). Primary antibody was added to the blocking solution at a dilution of 1:50 or 1:100 and allowed to incubate 1-2 nights at 4°C. Tissues were washed 3 times for 5 minutes and then 6 times for 20 minutes in PBS + 0.3% Triton X-100. Secondary antibody coupled to horseradish peroxidase (Jackson Labs) was diluted 1:300 in PBS, 0.3% Triton X-100, 3% NGS and incubated with the tissue overnight at 4°C. Tissues were washed as described above and antibody binding was visualized by reaction with diaminobenzidene (DAB; Sigma Chemical). In some cases the anti-GPRK2 antibody was pre-absorbed against *gprk2*^{6936} ovaries; this treatment reduced cytoplasmic staining in the nurse cells but did not affect membrane-associated staining in any tissue. Due to the weakness of GPRK2 staining, tissues were viewed with brightfield rather than interference-contrast microscopy.

Double staining in wing discs was carried out using the GPRK2 antibody and an Engrailed (En) monoclonal antibody that was kindly provided by N. Patel (4F11, Patel et al., 1989). Expression of *dpp* was assessed using the enhancer trap staining of *l(3)10638* (Twombly et al., 1996) or the *dpp-lacZ* enhancer line, *BS3.0* (Blackman et al., 1991) visualized by a β-galactosidase antibody. The En antibody was used at a concentration of 1:2 and the β-gal antibody was used at a concentration of 1:5000. Staining with these antibodies was carried out as described above for anti-GPRK2 and according to Patel (1994) for anti-En and anti-β-gal. To achieve two-color staining, the first antibody (anti-GPRK2) was visualized using DAB intensified with nickel chloride and the second antibody (anti-En or anti-β-gal) using DAB with no intensification.

In situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Yue and Spradling, 1992).

Embryo cuticle preparations

To collect staged embryos, females were allowed to lay eggs on agar plates for 4 hours. After 24 hours, unhatched eggs were dechorionated, mounted in Hoyer's medium, and heated to 50°C for several hours. Cuticles were visualized and images were captured under brightfield optics. Pseudo-darkfield images were generated by inverting the image colors using Adobe Photoshop.

RESULTS

fs(3)06936, a new mutation that disrupts egg shape

Adult viable mutations of *decapentaplegic (dpp)*, or its putative receptor *saxophone (sax)*, cause homozygous females to produce short rounded eggs with abnormal anterior structures (Twombly et al., 1996). We identified a new female sterile mutation, *fs(3)06936*, during a single-P element mutagenesis screen (Karpen and Spradling, 1992) that caused similar effects on egg shape. Mature oocytes produced by homozygous *fs(3)06936* females are slightly shorter and more rounded than wild type (compare Fig. 1A with Fig. 1B-D). Although pos-

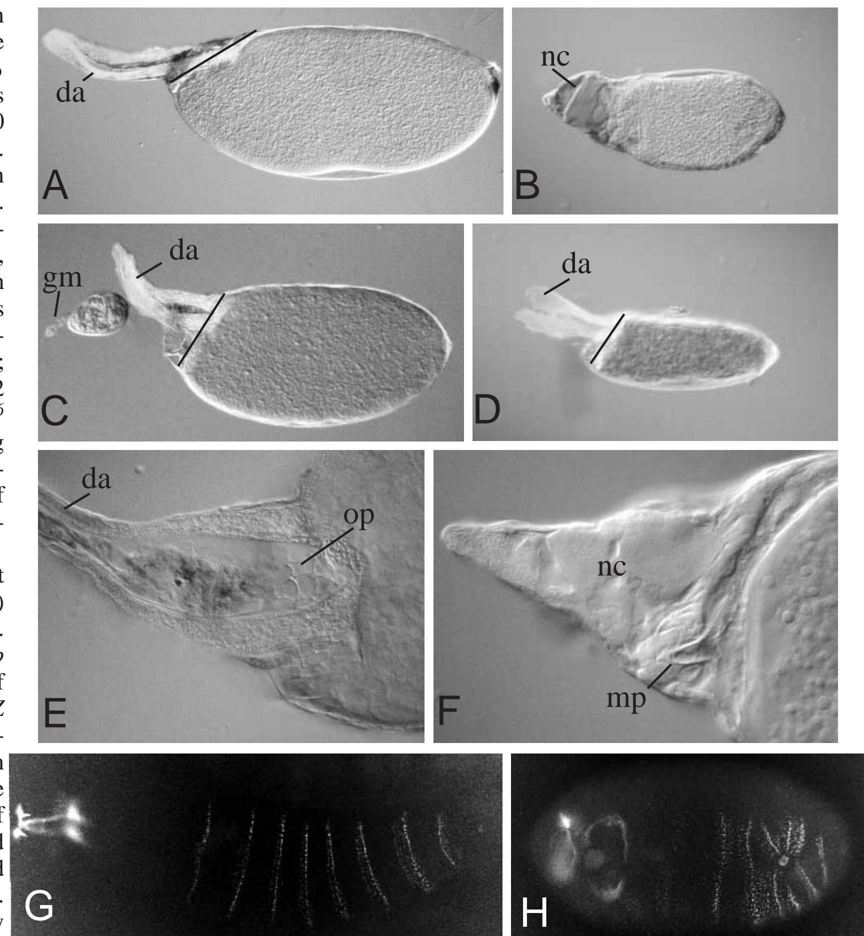

Fig. 1. Developmental phenotypes of *gprk2*^{6936}. Anterior is to the left in A-H. (A) A wild-type stage 14 egg chamber. (B-D) Examples of late stage egg chambers from *gprk2*^{6936} homozygous mothers. *gprk2*^{6936} oocytes are smaller and more rounded than wild-type oocytes, with dorsal appendages that are short and broad (*da* in C,D). As a result of the altered egg shape, the operculum is at a more vertical angle (compare angle of bars in A,C, and D). In some examples, nurse cells are slightly dumpless (*nc* in B and F). Note the severely reduced number of egg chambers and the small germarium (*gm*) in some *gprk2*^{6936} ovarioles (C). (E,F) Operculum chorion patterns (*op* in E) and micropyles (*mp* in F) are still formed in many affected mutant oocytes. (G) Embryo from a homozygous *gprk2*^{6936} mother and a wild-type *ry*^{506} father showing a wild-type cuticle pattern. (H) Embryo from a homozygous *gprk2*^{6936} mother and a homozygous *gprk2*^{6936} father showing fusion of adjacent segments and holes in the dorsal and ventral cuticle.

itioned normally, dorsal appendages in *fs(3)06936* eggs are generally shorter and broader than wild type (Fig 1C,D), and the two appendages on a single egg chamber frequently differ in length. The operculum is oriented more vertically than in wild type, giving the eggs a 'square-ended' appearance (compare Fig. 1A with Fig. 1C,D), but the chorion within the operculum retains its distinctive appearance (Fig. 1E) and the micropyle forms (Fig. 1F). Nurse cells often fail to completely transfer their contents into the oocyte, leaving residual material that may interfere with anterior end formation (Fig. 1B,F). Thus *fs(3)06936* appears to affect specific aspects of egg formation without grossly altering the major pattern axes of the egg.

Two additional ovarian defects suggested that *fs(3)06936* also functions at earlier stages of oogenesis. First, homozygous *fs(3)06936* egg chambers degenerate during vitellogenic stages (stages 8-10A) much more frequently than expected. 26.8% of ovarioles (n=594) from 4-day-old *fs(3)06936* females contained a degenerating vitellogenic chamber compared to only 0.7% of wild-type ovarioles (n=276). Second, egg chamber formation slows or ceases entirely within a significant number of *fs(3)06936* ovarioles. 5.2% of the mutant ovarioles (n=594) contained only 0-2 egg chambers instead of the 6-7 that are present in wild type (Fig. 1C). Germaria in such ovarioles were often smaller and thinner than in wild type, like the germaria of agametic ovarioles (see Margolis and Spradling, 1995). Although cyst production normally declines in old females, 4-day-old wild-type females contained no similar ovarioles (n=276).

The *fs(3)06936* mutant exhibited additional defects that indicate roles for this gene outside of the ovaries. Homozygous *fs(3)06936* females lay a small number of eggs, but those that are laid display a maternal effect that is partially rescued by zygotic *fs(3)06936*$^+$ expression. 23.7% of embryos (n=300 eggs laid) produced by homozygous females hatch when crossed to wild-type males compared to only 10.3% (n=390) following crosses to homozygous males. The unhatched eggs displayed a wide variety of defects including twisted gastrulation, fused adjacent segments, and perforated dorsal and ventral cuticle (Fig. 1G,H). These defects were more severe when the embryos lacked both maternal and zygotic *fs(3)06936*$^+$ function (as seen in Fig. 1H).

fs(3)06936 encodes a putative member of the family of G protein-coupled receptor kinases (GRKs)

To further analyze the *fs(3)06936* mutation, we cloned the gene disrupted in this strain. The single P element insertion in the *fs(3)06936* mutant was localized to cytological position 100C by in situ hybridization (data not shown). Seventy-two of eighty-six lines in which the P element was excised reverted to fertility, confirming that the insertion was responsible for the oogenic defect. We first cloned genomic DNA flanking the insertion by plasmid rescue (Cooley et al., 1988), and used it to isolate overlapping clones from a lambda genomic library (Fig. 2). One of these genomic clones, λMgB, contains the P element insertion site and hybridized in situ at position 100C, confirming that the genomic DNA derived from the appropriate chromosomal region (not shown).

We used the genomic map to identify candidate transcripts encoded by the *fs(3)06936* gene. A 3.1 kb fragment containing the insertion site was used to select a clone, T6936, from an ovary cDNA library (Stroumbakis et al., 1994) that hybridizes to transcripts of 4.0, 5.0 and 5.5 kb in length in ovarian poly(A)$^+$ RNA. The sizes of the T6936-complementary RNAs were drastically altered in *fs(3)06936* mutant ovaries (see below), indicating that they were good candidates for *fs(3)06936* transcripts. A 3′ segment of T6936 was used to probe a cDNA library prepared from 0-4 hour embryos (Brown and Kafatos, 1988) and a longer 3.5 kb cDNA, B6936, was recovered. The 3547 nucleotide sequence of B6936 (Fig. 3) predicts a protein of 714 amino acids preceded by a long 5′ untranslated region (1118 bp). Nucleotides 1719 through 3532 are identical to a previously described cDNA sequence, the *G protein-coupled receptor kinase 2* gene (*Gprk2*, Cassill et al., 1991). However, the B6936 cDNA extends further in the 5′ direction than the previously described clone, and predicts a larger protein due to an extended amino-terminal region. The P element insertion was located between nucleotides 142 and 143 of B6936, within the 5′ untranslated region of the gene. These observations imply that the gene disrupted in *fs(3)06936* corresponds to *Gprk2*; and we will subsequently refer to *fs(3)06936* as *gprk2*^{6936}.

The *Gprk2* gene was originally isolated based on its sequence similarity to the human *βARK1* gene (Cassill et al., 1991). The predicted protein from *Gprk2* is 47% identical to βARK1 protein in the kinase domain. Outside of this region there is no significant similarity. In contrast, the GPRK2 protein predicted by the B6936 sequence shares a high level of sequence identity with two other mammalian members of the GRK family, GRK5 and GRK6 (Fig. 4). This similarity extends over the entire protein, except for a 121 amino acid region that is unique to GPRK2. The physiological functions of GRK5 and GRK6 have not yet been established, although both proteins are able to phosphorylate and desensitize activated G protein-coupled receptors.

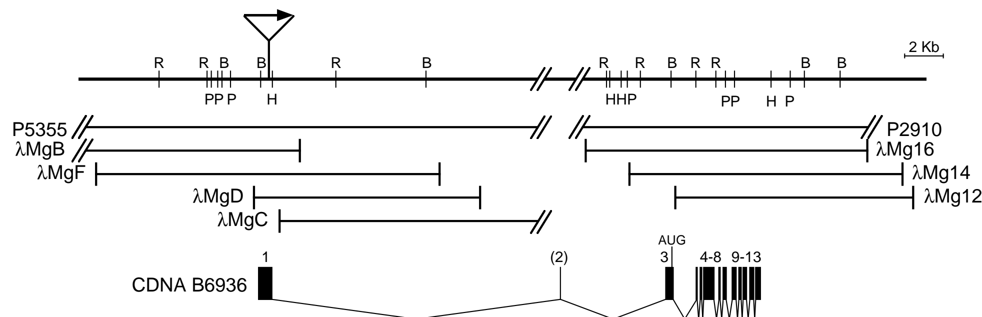

Fig. 2. Structure of the *Gprk2* gene. Top line: a restriction map of the *Gprk2* gene. The location and orientation of the P element is shown by the inverted triangle. Double lines denote gaps of unknown size. Middle line: genomic and P1 clones from the *Gprk2* locus. Bottom line; the deduced structure of the B6936 cDNA. Exons are shown by black boxes and thin lines represent intronic regions. The presumed initiator methionine is encoded by exon 3. The putative exon II, which is predicted by the B6936 cDNA, is shown in parentheses. Restriction sites: (B) *Bam*HI, (R) *Eco*RI, (H) *Hin*dIII, and (P) *Pst*I.

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   1 GAGAAGACTCCAATATTTTTCCGAGCAGGCAATTCGCGGATCCATCGTTAATTTTGGGCACTTGCAGAAAGCCCAGTTGCGAACGTC
  88 GTCGTGTGTCGTCTTAGAAAAGCCACTCCTCCAACCGGAAAACCGCAAGAGCGTCGAGCCCTTGTTTTG\/CGAGCCTTTTCGAAAA
 173 AAGCTGGGAATTTCACGCCGTATTTTTCGTATCTTTTCCGTCAACGAAACGCCGCGTGTGTCGTAGTGTGCGTGTGTGTGTTCTGTG
 260 TGCCCGCCCATCTGTGCATGTGTGAGAGCGCGCCTGTGTGTGTGAATTTTGGTGAGAGCCGCAACAACAACCGAAAAGAAAACCAAA
 347 ATCGACAAATGCGGTCAAATTGAAAAGAAAAACAAAAAATCAAAGAGTATTCTTGCGCAAAGCCAAAAGGCAAAGTCAAAAAAGAGC
 434 GCAAAGCCAAAAAAAAAGCAGATTTCCCAACGAAAAGGCCGGAAAATTGCCCACTAGGCGTGTCGCTGCTTCTTCTTGTTCTTTCAAA
 521 TTGTTTATGCTGCAATTGCAGTGGTGTGCGAAAGGAGTGGTAGAGAGAGAGAGAGAGAAGGAAAAGCGTCTTATCAACGCCCGCTTT
 608 CTTATCAGAACCCACACATTTGTTGCGAAAATTCCAACCAGCCGAAACTCACAGTGAAGCTTTAAATAGCACATCAAACAAACAGgt
 693 aagt..intron 1..TTGCCCAGCTATCAGCGAATCGGTCAATTTCATTTGGAGAAGCCAAAG..intron 2..cagATTGAGGA
 749 AATCCATTGACCGACGGTTTTACAGCATTCAATCAAGCGCAAATTGGCTCAACTTGCAAAAAGGGCACATTTTCAGCCTTGCAAATT
 836 CCAATCTTGAAACTAAATGACGCCTGTGTCTGAAACCGAGACCCTGCTGCCAAATACCGCAACGCTGGACGCCAGACGACGGCCACA
 923 AATTAGCCAGCCAGCCGTAGATTTGCAAGTCTAAGGAGTCGCCCTGCCCACCAGAACGCAGAAGGGGATCCCTGTTCAAAGGAGCCA
1010 GCGAAGGCCACACACAGGAGAAAGATCAAAAGGAGCACCAGCCAGTGTGACTGCGACTATTTTTCGGGAGCGCCAAAGGATTCATTA
1097 AAAAACCGCCAACCTGGAACTA

1119 ATGGAATTAGAGAATATTGTGGCCAATACGGTCTACTTGAAAGCGAGAGAAGgtaagt..intron 3..cagGTGGTTCCGACAGC
      M  E  L  E  N  I  V  A  N  T  V  Y  L  K  A  R  E                        G  G  S  D  S   22
1185 AACAAGGGAAAGAGCAAAAAATGGCGCAAAATATTGCAATTCCCACACATATCGCAATGCATCAACCTGAAAGACAAATTAGgtgag
      N  K  G  K  S  K  K  W  R  K  I  L  Q  F  P  H  I  S  Q  C  I  N  L  K  D  K  L         49
1267 c..intron 4..cagATATAAGCTATGGCTACGTGATAGATCAGCAACCCATTGGTCGTGAGCTCTTCCGTCTGTTTTGCGAGAAC
                      D  I  S  Y  G  Y  V  I  D  Q  Q  P  I  G  R  E  L  F  R  L  F  C  E  N   73
1338 AAACGGCCCGTCTACTTTCGCTACATCACCTTCCTGGATGAGGTGGTCAAgtatgt..intron 5..cagATACGAGATCGAGTAC
      K  R  P  V  Y  F  R  Y  I  T  F  L  D  E  V  V  K                        Y  E  I  E  Y   95
1404 ATCTCGAACCGCATATTCATTGGACATGACATCGGCCGTCGCTTCTTGGATGTCGAGGCACAGCTGGAGCTGCGCAACGGGAGCGGG
      I  S  N  R  I  F  I  G  H  D  I  G  R  R  F  L  D  V  E  A  Q  L  E  L  R  N  G  S  G  124
1491 GGCGACGCCTTGGACGCCGAGACGCAGGAGGAGCTGCTGCTCAACAGCAGCAATGCCAATCCAACTGAAACAGCTGAGACTGAACAC
      G  D  A  L  D  A  E  T  Q  E  E  L  L  L  N  S  S  N  A  N  P  T  E  T  A  E  T  E  H  153
1578 TGCAACAATACCACCGCCAACAACTGCAACAACATCAACAACAGCAACAACTCGCAGCACAGCAGCGACATCAATCACAAGAAGCTG
      C  N  N  T  T  A  N  N  C  N  N  I  N  N  S  N  N  S  Q  H  S  S  D  I  N  H  K  K  L  182
1665 GACACGCGCAATCACAACGGCGACGACGCCACTGGGAACGGGAGCAGCCACCAGGACGACGGGGACGAGAGCGTCAAGTGCCAGGAG
      D  T  R  N  H  N  G  D  D  A  T  G  N  G  S  S  H  Q  D  D  G  D  E  S  V  K  C  Q  E  211
1752 GGCCATGACGATGCGGAAAAGGGTGGTGGCGGAGAGGGAGGCGGCGGCGGAAAGTGTGTCCACGTGGGCGGGTATCCTGACGAACTC
      G  H  D  D  A  E  K  G  G  G  G  E  G  G  G  G  G  K  C  V  H  V  G  G  Y  P  D  E  L  240
1839 GTGTTGGACGTGCTTAACGATGATCTCATTGCGCAAGTGCGTAACAAACTCAACAGCGGCGGCAAGGACATCTTTGCCCAGTGTGTG
      V  L  D  V  L  N  D  D  L  I  A  Q  V  R  N  K  L  N  S  G  G  K  D  I  F  A  Q  C  V  269
1926 AACGCGGTTAAGGCGTTCCTGGCCGGCGAACCGTTCCGGGAGTTTGAGAGCTCTATGTATTTTCACCGgtacgt..intron 6..c
      N  A  V  K  A  F  L  A  G  E  P  F  R  E  F  E  S  S  M  Y  F  H  R                     292
1994 agATACTTGCAATGGAAGTGGCTTGAGGCGCAGCCCATCACCTACAAAACGTTTCGCATGTACCGGGTGCTCGGAAAGGGCGGCTTC
         Y  L  Q  W  K  W  L  E  A  Q  P  I  T  Y  K  T  F  R  M  Y  R  V  L  G  K  G  G  F  320
2079 GGCGAGGTGTGCGCCTGTCAGgtaagc..intron 7..cagGTGCGGGCCACTGGGAAAATGTACGCGTCCAAAAAGCTGGAGAAA
      G  E  V  C  A  C  Q                          V  R  A  T  G  K  M  Y  A  S  K  K  L  E  K  342
2145 AAGCGCATCAAGAAGCGCAAGGGCGAGTCGATGGTGTTAATCGAGAAGCAGATACTGCAGAAAATCAACTCGCCGTTCGTGGTCAAT
      K  R  I  K  K  R  K  G  E  S  M  V  L  I  E  K  Q  I  L  Q  K  I  N  S  P  F  V  V  N  371
2232 CTGGCCTACGCGTACGAGACAAAGGACGCCCTCTGCCTGGTGCTGACCATAATGAATGgtgggt..intron 8..cagGCGGCGAT
      L  A  Y  A  Y  E  T  K  D  A  L  C  L  V  L  T  I  M  N                          G  G  D  393
2298 TTGAAATTCCACATTTACAACATGGGCGGCGAACCAGGCTTTGAACTGGAGCGTGCCCGCTTCTATGCCGCCGAGGTGGCCTGTGGA
      L  K  F  H  I  Y  N  M  G  G  E  P  G  F  E  L  E  R  A  R  F  Y  A  A  E  V  A  C  G  422
2385 CTGCAGCACCTGCACAAGCAGGGCATCGTCTACCGGGATTGCAAGCCGGAAAACATCCTCCTTGACGATCACGGCCATGTGCGCATT
      L  Q  H  L  H  K  Q  G  I  V  Y  R  D  C  K  P  E  N  I  L  L  D  D  H  G  H  V  R  I  451
2472 TCCGACCTGGGACTGGCCGTCGAGATACCGGAGGGCGAGATGGTGCGCGGCCGGGTGGGCACAGTGGgtaagt..intron 9..ta
      S  D  L  G  L  A  V  E  I  P  E  G  E  M  V  R  G  R  V  G  T  V                        473
2539 gGCTACATGGCCCCCGAGGTCATCGACAACGAGAAGTACGCCTTCTCCCCGGACTGGTTCAGCTTCGGCTGCCTGCTGTACGAGATG
      G  Y  M  A  P  E  V  I  D  N  E  K  Y  A  F  S  P  D  W  F  S  F  G  C  L  L  Y  E  M  502
2625 ATCGAGGGCCAAGCGCCGTTCCGGATGCGCAAGGAGAAGGTCAAGCGCGAGGAGGTGGACAGGCGCGTTAAGgtcggt..intron
      I  E  G  Q  A  P  F  R  M  R  K  E  K  V  K  R  E  E  V  D  R  R  V  K                  526
2697 10..cagGAGGACCCCGAAAAGTATTCAAGCAAGTTTAATGATGAAGCCAAATCAATGTGCCAACAGCTGTTAGCTAAATCGATAAA
                 E  D  P  E  K  Y  S  S  K  F  N  D  E  A  K  S  M  C  Q  Q  L  L  A  K  S  I  K  553
2777 GCAGCGCCTGGGCTTCCGCAACGGTCGCATGGGCGGGCAGGATGTGATGGCCCACCCGTTTTTCCACTCCACCCAGCTCAATTGGCG
       Q  R  L  G  F  R  N  G  R  M  G  G  Q  D  V  M  A  H  P  F  F  H  S  T  Q  L  N  W  R 582
2864 TCGCCTGGAGGCTGGTATGCTGGAGCCACCCTTTGTGCCAGACgtgagt..intron 11..cagCCGCACGCCGTTTACGCCAAAG
       R  L  E  A  G  M  L  E  P  P  F  V  P  D                             P  H  A  V  Y  A  K  603
2929 ATGTGCTCGATATTGAACAGTTCTCTACGGTGAAGGGCGTCAATATCGACGAATCCGACACGAATTTCTATACGAAATTCAACACAG
     D  V  L  D  I  E  Q  F  S  T  V  K  G  V  N  I  D  E  S  D  T  N  F  Y  T  K  F  N  T    632
3016 GCTCCGTGTCCATTTCCTGGCAGAACGAGATGATGGAGACCGAGTGCTTTCGGGAACTGAATGTTTTTGGTCCCGAGGAGTGTCCCA
     G  S  V  S  I  S  W  Q  N  E  M  M  E  T  E  C  F  R  E  L  N  V  F  G  P  E  E  C  P    661
3103 CGCCAGATTTGCAAATTAATGCAGCGCCCGAACCAGACAAGGCGGGCTGTTTCCCCTTTCGCCGGAAGgtttgt..intron 12..
     T  P  D  L  Q  I  N  A  A  P  E  P  D  K  A  G  C  F  P  F  R  R  K                      684
3171 gagAAGAAGCAGCCGGCTCGCACTCAGCCCATACCCATTCCGGAGCATCTGTTAACCACTAGTCACAGCGTGTCCTCCACGACGGTC
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3255 GAAAGCTGATAGCAGAGATCAGTCGAGGATGCCTTTAAGCGGACGCCTGACGTAGACGTCGACACATAGCACAAATTGCGCACAGTT
      E  S  *                                                                                  714
3342 ACCCCGGATCGGAAGAGTTCAGCAGGAGCATAATAACTTTAGAGCACAACCTAATTATAAAACCGAAACTACATCGGTTTATCCTTC
3429 AGTTGACGCGCAAGAAGCATTTTTGAAATTGTACTTTTACATCAAATTTTCTAATGAAAGTTGTTCAAGCTGATTTCGACACACAAT
3516 ATATATTTATAAATGTTAAAAAAAAAAAAAAA
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Fig. 3. DNA sequence and predicted protein of the B6936 cDNA. The numbers along the left refer to nucleotides in the B6936 cDNA whereas numbers along the right refer to amino acids in the predicted protein. Coding regions of the gene are in upper case and intronic regions are in lower case. The position of the P element insertion is marked by the symbol \/. The kinase domain of the predicted protein is underlined, potential autophosphorylation sites are denoted by bold face, and amino acids that are conserved in serine/threonine kinases have double underlines.

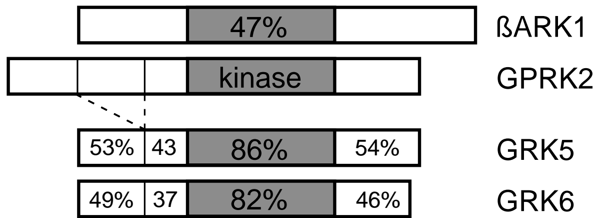

Fig. 4. *Gprk2* is a member of the family of G protein-coupled kinases. Each bar represents the predicted protein of a GRK family member. The kinase domain is shown as a gray box whereas the remainder of the open reading frame is shown in white. At the top is βARK1, the kinase that was originally used to clone GPRK2 (Cassill et al., 1991). The numbers within βARK1, GRK5, and GRK6 represent the level of identity between these proteins and the corresponding region of GPRK2. The similarity between GPRK2 and human βARK1 is limited to the kinase domain. In contrast, the similarity between GRK5 and GRK6, and GPRK2 is much higher and extends to all portions of the protein, with the exception of a 121 amino acid region that is not present in GRK5 or GRK6.

Organization and expression of *Gprk2* transcripts

Comparing genomic DNA clones with cDNA B6936 revealed that only the first exon (where the P element had inserted) derives from the genomic region that we had analyzed. To obtain genomic clones containing the rest of the gene, we used a 3′ fragment of B6936 as a probe to re-screen the genomic phage library. In addition, P1 clones of this region were obtained from the Berkeley Drosophila Genome Project. Mapping of the genomic and P1 clones to B6936 showed that *Gprk2* is encoded by at least 13 exons (Fig. 2). Phage clones that mapped 3′ to exon 1 and 5′ to exon 3 did not overlap suggesting that these two exons are at least 15 kb apart. According to the cDNA sequence, there are 48 nucleotides between exon 1 and 3 that are encoded in this uncloned region. This is the only portion of the cDNA B6936 for which the genomic counterpart was not sequenced. We have putatively designated this 48 nucleotide sequence as exon 2, although it could be composed of more than one mini-exon. Sequencing of the 3′ end of cDNA T6936 demonstrated that it ends in a polyadenylation site that is 750 nucleotides 3′ to the B6936 polyadenylation site. The 5′ end of T6936 was not fully characterized, but hybridization analysis demonstrated that it contains sequences from the 5′ end of B6936. This suggests that the middle portion of the gene is not present in T6936, either due to alternative exon processing or to deletions that were generated during construction of the cDNA library.

To determine how the P element disrupts expression of the *Gprk2* gene, we analyzed RNA expression using probes from both ends of cDNA B6936 and from the 3′ end of cDNA T6936 (Fig. 5A). All three probes hybridized to transcripts of 4.0, 5.0, and 5.5 kb in length in poly(A)$^+$ RNA from wild-type ovaries. In mutant ovaries, the full-length transcripts were no longer detectable. However, with the 5′-most probe, new transcripts of 1.3 and 0.8 kb were recognized. This suggests that transcription begins normally but is terminated by the P element (Horowitz and Berg, 1995). A developmental analysis of expression (Fig. 5B) showed that the 4.0 and 5.0 kb transcripts are abundantly expressed in 0-3 hour embryos, as a result of maternal expression. The 5.0 kb transcript is no longer detectable after 0-3 hours while the 4.0 kb transcript continues to be expressed at a low level. The 5.5 kb transcript is expressed

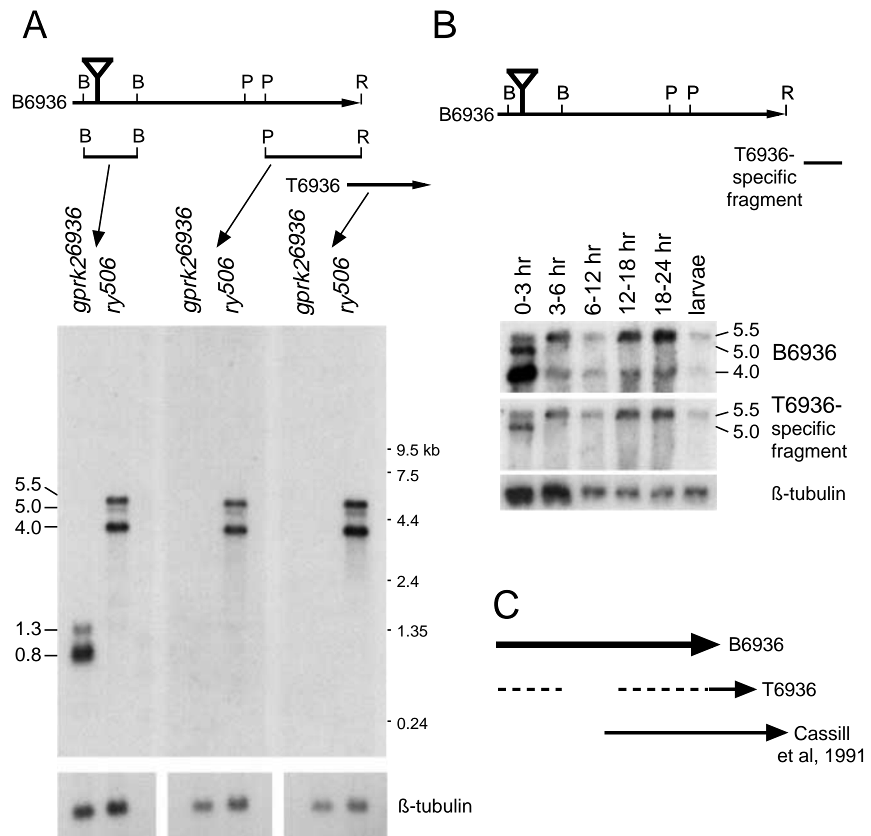

Fig. 5. Northern blot analysis of *Gprk2*. (A) Identical northern blots of wild-type and *gprk2*^{6936} poly(A)$^+$ RNA probed with 5′ and 3′ fragments of cDNA B6936, and a 3′ fragment of cDNA T6936. All three fragments hybridize to 4.0, 5.0 and 5.5 kb transcripts in wild-type ovary RNA. In contrast, in *gprk2*^{6936} ovary RNA these bands are no longer detectable. Only the 5′ B6936 fragment containing the site of the P element insertion (shown by the inverted triangle) hybridizes to smaller transcripts of 0.8 and 1.3 kb. (B) Developmental analysis of *Gprk2* transcription. Wild type poly(A)+ RNA from embryos and third instar larvae were probed with the entire B6936 cDNA or with a 3′ fragment of T6936 that is not represented in B6936. The B6936 cDNA hybridizes to three transcripts in 0-3 hour embryos (as in the ovaries). The 5.0 kb transcript is no longer detectable after 0-3 hours, and the level of the 4.0 kb transcript is greatly reduced. The T6936-specific cDNA hybridizes to the 5.5 and 5.0 kb transcripts only, suggesting that the B6936 cDNA represents the 4.0 kb transcript. In both A and B, the amount of RNA was indicated by re-hybridization to a β-tubulin probe. (C) A comparison of B6936, T6936, and the previously published cDNA (Cassill et al., 1991). The dashed line in T6936 refers to regions that were inferred from hybridization analysis rather than determined by sequencing.

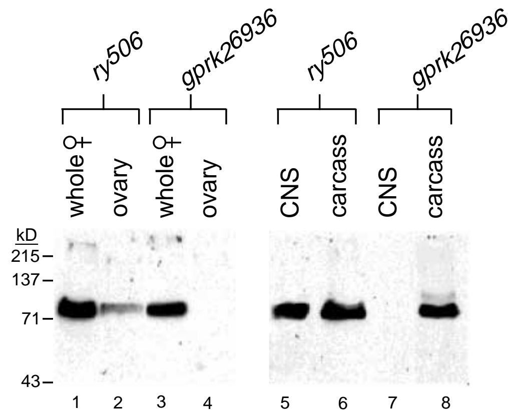

Fig. 6. Immunoblot analysis of GPRK2 expression. Tissue homogenates from 1 whole female (lanes 1 and 3), 2 ovary pairs (lanes 2 and 4), 8 larval CNS (lanes 5 and 7), and two larval carcasses (whole animal minus the CNS; lanes 6 and 8). The GPRK2 antibody detects a band of approximately 80 kDa in all wild-type tissues tested (lanes 1, 2, 5 and 6). This staining was also present in whole females and carcasses from *gprk2*^{6936}. In contrast, ovary and CNS expression was not detectable in *gprk2*^{6936} homozygotes (lanes 7). Equal amounts of protein were present in lanes 2 and 4 and 5 and 7 (see Methods). The higher molecular weight band in lane 8 was not seen consistently. Molecular weight markers are shown along the left side.

at a low level in early embryogenesis, with an increase in expression from 12 to 24 hours. Thus the three transcripts are differentially expressed during development.

A probe derived from the 3′ region that is specific to T6936 hybridized to only the two largest transcripts (data not shown). This suggests that B6936 corresponds to the smallest (4.0 kb) transcript, which is consistent with the fact that this cDNA was isolated from a 0-4 hour library. The cDNA sequence reported by Cassill et al. (1991) did not terminate in a poly(A) tail, however it extended further 3′ than either B6936 or T6936 suggesting that the *Gprk2* gene utilizes at least three polyadenylation sites (Fig. 5C). Whether use of these three sites gives rise to the three transcript sizes will require further confirmation.

Expression of GPRK2 protein

The expression of GPRK2 protein was analyzed using a polyclonal antibody that was kindly provided by Charles Zuker. This antibody was generated against a bacterially expressed protein which contained the final 145 amino acids of the kinase domain and the entire 139 amino acid carboxyl region (Cassill et al., 1991). DNA probes from the corresponding genomic region hybridize to all three transcripts (not shown). In immunoblot analysis of adult tissues, we detected a single band of 80 kDa in ovaries and whole females, in close agreement to the 80.7 kDa protein predicted by the B6936 cDNA. This band was absent in ovaries from *gprk2*^{6936} homozygotes whereas expression in whole females remained (Fig. 6). Thus, as expected, GPRK2 is expressed in the ovary and its expression is disrupted in the *gprk2*^{6936} mutant. In larval tissues, we again observed an 80 kDa band in both the central nervous system (CNS) and in carcasses (the entire animal minus the CNS). In homozygous *gprk2*^{6936} larvae, expression was no longer

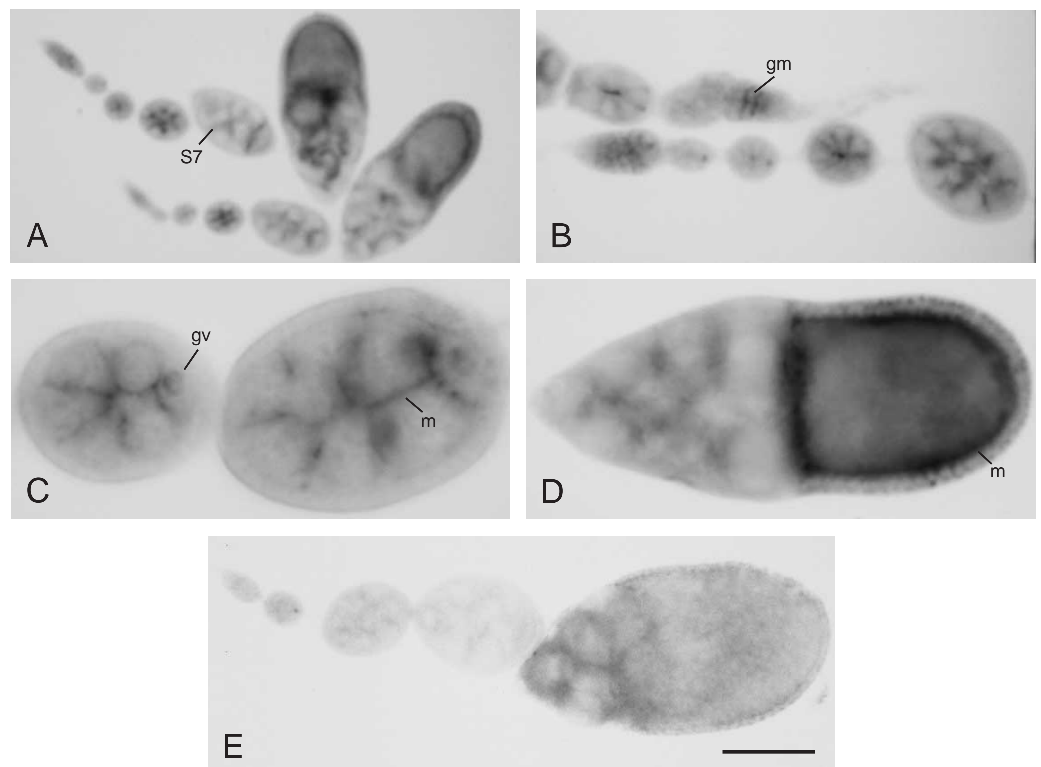

Fig. 7. Membrane-associated expression of GPRK2 in the ovaries. Staining in wild-type (A-D) and *gprk2*^{6936} (E) ovaries. Anterior is to the left in all cases except the top ovariole in B. (A) Expression is first detectable in the germarium and increases in nurse cells through stage 6. Staining diminishes in stage 7 (*S7*), and increases again in the oocyte in stages 8-11. (B) Germarial expression is first detectable in region 2A of the germarium (*gm*). (C) GPRK2 staining is associated with the membrane between nurse cells (*m*) and between nurse cells and the oocyte, but not between the nurse cells and the follicle cells. Diffuse staining is sometimes seen around the germinal vesicle (*gv*) and in the nurse cell cytoplasm. (D) In stage 10 egg chambers, staining is no longer detectable along nurse cell membranes although cytoplasmic staining remains. Strong staining is apparent around the circumference of the oocyte (*m*). (E) Membrane-associated staining is not detectable in *gprk2*^{6936} egg chambers although staining remains in the cytoplasm of nurse cells and around the germinal vesicle. The scale bar corresponds to 100 μm in A and E, 10 μm in B and C, and 50 μm in D.

detectable in CNS tissue but appeared to be unaffected in carcasses. These results demonstrate that *gprk2*^{6936} selectively disrupts *Gprk2* expression in some but not all tissues.

The tissue distribution of the protein was studied by staining whole-mount tissues with the same antibody. The anti-*Gprk2* antibody labeled developing wild-type egg chambers (Fig. 7), and most of the staining was eliminated in *gprk2*^{6936} mutant ovaries. Expression was first seen in region 2B of the germarium, the stage where germline cysts are being enveloped by follicle cells (Fig. 7B, *gm*). In early egg chambers, membranes between adjacent nurse cells and between nurse cells and the oocyte were labeled (Fig. 7A-C, *m*). In addition, staining around the germinal vesicle was sometimes observed (Fig. 7C, *gv*). Membrane-associated staining persisted through stage 6 but decreased in intensity at stage 7, except between the nurse cells and the oocyte (Fig. 7A, *S7*). In these early stages we observed no staining in follicle cells or in the region of the nurse cells that lies adjacent to the follicle cells. During stages 8-11 membrane-associated staining decreased in the nurse cells and appeared around the entire circumference of the oocyte (Fig. 7A and D, *m*). We also observed weak cytoplasmic staining in nurse cells and follicle cells of these stages; however, the cytoplasmic (and germinal vesicle) staining sometimes persisted in *gprk2*^{6936} ovaries (Fig. 7E). Thus GPRK2 protein appears to be preferentially associated with nurse cell and oocyte plasma membranes during much of oogenesis. Although in situ hybridization studies also detected *gprk2* RNA only in the nurse cells (not shown), a lower level of expression in somatic cells can not be ruled out by these experiments.

Specific staining using anti-GPRK2 was also detected in non-ovarian tissues, consistent with a role for this gene outside of the ovaries. The central nervous system (CNS) of both larvae and adults stained intensely. In larvae, staining was present in axon fascicles, especially large ones, including nerves projecting to the optic lobes, the longitudinal connectives, and portions of the mushroom bodies (*ol*, *lc*, and *mb* in Fig. 8A). We detected little staining in cell bodies within the CNS. However, we consistently observed strong staining in the cell bodies and nerves of the corpus allatum of the ring gland (*rg* in Fig. 8A). In the adult CNS, staining was restricted to two major structures within the brain. First, the nerves within and projecting to the ellipsoid body of the central complex were consistently stained (*eb* in Fig. 8C). The ellipsoid body contributes to higher brain function in flies, such as locomotion (Strauss and Heisenberg, 1993). Second, we also detected strong staining in the mushroom bodies that included the Kenyon cells and nerve processes within the peduncles and the alpha lobes (not shown). The mushroom bodies have been implicated in memory and learning. Interestingly, the learning mutants *dunce*, *rutabaga*, and *DCO*, which all disrupt genes involved in G protein signaling, are predominately expressed in the mushroom bodies (reviewed in Davis et al., 1995). In the mutant, GPRK2 staining was abolished from both the larval and adult CNS, and from the larval ring gland (Fig. 8B,D).

Most of the other tissues in the larva and adult were only stained weakly and it was not possible to determine if staining was affected in the mutant. One exception to this was in the wing imaginal disc where we observed staining in the notum and in a stripe that paralleled the anterior/posterior boundary of the wing blade (Fig. 8E, *A/P*, and 9A). Within this stripe, staining was always weakest in the region corresponding to the tip of the wing. This staining was eliminated in *gprk2*^{6936} wing discs (Fig. 8F).

The disc staining was particularly interesting because *dpp* is also expressed along the anterior/posterior boundary of the wing disc. To determine how GPRK2 staining corresponds to the anterior/posterior boundary, we compared expression of GPRK2 with Engrailed (En) and *dpp*. En protein, which is expressed in the posterior compartment of the wing disc, was visualized with a monoclonal antibody (4F11, Patel et al., 1989). *dpp* expression was visualized by β-gal immunoreactivity in flies bearing a *dpp-lacZ* fusion gene (*BS3.0*; Blackman et al., 1991). GPRK2 and *dpp* are co-expressed along the anterior/posterior boundary with two exceptions (Fig. 9C). First, the stripe of GPRK2 staining is thinner, about one cell in thickness as opposed to a 2-3 cell thickness of *dpp* expression. The stripe of GPRK2 expression coincided with the *dpp*-expressing cells closest to the boundary. Second, near the wing tip, *dpp* is not expressed in the cells immediately adjacent to the boundary. In this region GPRK2 staining was present in cells that do not express *dpp*.

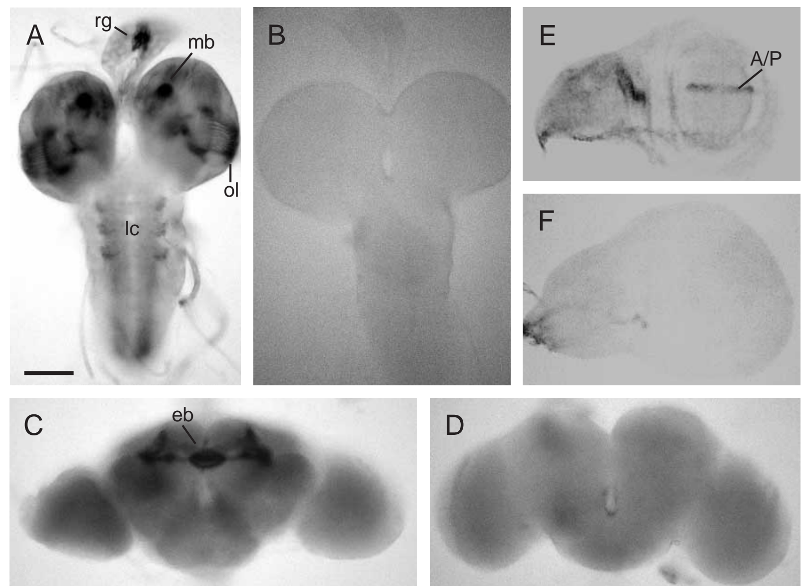

Fig. 8. GPRK2 expression in non-ovarian tissues. Anterior is towards the top in all panels. (A) Larval CNS staining is localized to axons projecting to the optic lobes (*ol*) and the mushroom bodies (*mb*), in the longitudinal connectives (*lc*), and in cell bodies and nerves of the corpus allatum of the ring gland (*rg*). (C) Adult CNS staining is detectable only in cell bodies and processes associated with the ellipsoid body of the central complex (*eb*) and portions of the mushroom bodies (not shown). There is no staining in the ventral nerve cord of adults. (B,D) CNS staining is not detectable in *gprk2*^{6936} larvae and adults. (E) In the wing disc, GPRK2 expression is confined to a stripe that parallels the anterior/posterior boundary of the wing blade (*A/P*) and the hinge region, and weak expression in the prospective notum. (F) Wing disc staining is not detectable in *gprk2*^{6936} wing discs. The scale bar corresponds to 75 μm in A-D and 50 μm in E-F.

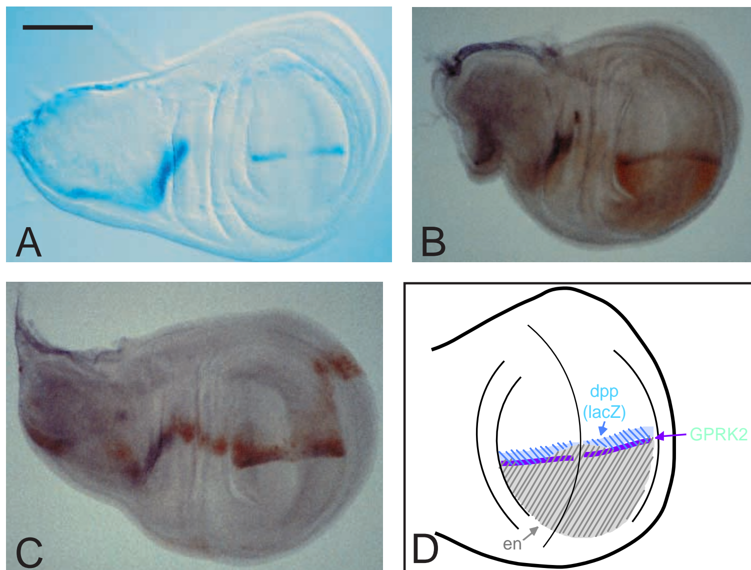

Fig. 9. Comparison of GPRK2 expression with Engrailed and *dpp*. Proximal is to the left and anterior is towards the top. (A) Enhancer trap staining of *Gprk2* in the wing disc. Enhancer trap staining is stronger than but identical to GPRK2 immunoreactivity in this tissue, and better reveals the pattern of expression. (B) Double staining with anti-GPRK2 and anti-Engrailed antibodies in ry^{506} wing discs. GPRK2 is shown in purple and engrailed in brown. Engrailed expression overlaps GPRK2 in the wing margin and at the lateral extremes of the wing blade. In other areas the two expression domains abut one another. (C) Double staining with anti-GPRK2 and anti-β-gal antibodies in the *l(2)10638* enhancer trap line. GPRK2 is shown in purple and *dpp* (β-gal) in brown. The two patterns overlap in the wing blade except in the region of the wing margin. (D) Schematic of GPRK2, En, and *dpp* in the wing disc.

At the tip of the wing En staining crosses the anterior/posterior boundary, as previously described (Blair, 1992; Raftery et al., 1991). Therefore, in this region GPRK2 and En were co-expressed whereas outside of this region the patterns of the two proteins abutted (Fig. 9B). These results (summarized in Fig. 9D) confirm that GPRK2 is expressed along the anterior side of the anterior/posterior border in a pattern that overlaps *dpp*.

The *gprk2*^{6936} mutant does not affect the expression of *dpp*-enhancer lines

One result of signaling through G protein-coupled receptors is the induction of cAMP which activates cAMP-dependent protein kinase (PKA). PKA has been shown to repress *dpp* expression in imaginal discs (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). If PKA plays a similar role in egg chambers, then *Gprk2* may control the level of *dpp* expression in discs and ovaries by influencing PKA activity. To test this hypothesis we followed the expression of *dpp-lacZ* enhancer lines in *gprk2*^{6936} homozygotes. *l(3)10638* and the *BS3.0, dpp-lacZ* enhancer line were used to assess *dpp* expression in the ovaries and wing imaginal discs, respectively. We observed no differences in *dpp* expression in either ovaries or wing imaginal discs in these studies (not shown).

DISCUSSION

A putative G protein-coupled receptor kinase encoded by *Gprk2* functions during development

We characterized a *Gprk2* mutation, *gprk2*^{6936}, and showed that *Gprk2* activity is required during oogenesis and embryogenesis. *Gprk2* encodes a putative member of the G protein-coupled receptor kinase (GRK) family. The hallmark of GRKs is that they phosphorylate G protein-coupled receptors when they are in the active (agonist-bound) state (Premont et al., 1995). Although we have not demonstrated biochemical activity of GPRK2, several characteristics suggest that it is a true member of the GRK family. First, the sequence similarity in the kinase domain between GPRK2 and other family members with known activity is high, ranging from 47% with βARK1 to 86% with GRK5. Second, the GPRK2 kinase domain contains amino acids that are conserved in all other members of the GRK family as well as residues that are required for the activity of all serine/threonine kinases (Fig. 3; Hanks and Quinn, 1991; Taylor et al., 1993). Finally, GPRK2 and its putative human homologue, GRK5, share extensive sequence similarity outside of the kinase domain including a serine/threonine pair that is autophosphorylated in GRK5 (Kunapuli et al., 1994). One difference between GPRK2 and the other GRKs is the length of the amino-terminal region. Most GRKs have an amino terminus of 183-188 amino acids. GPRK2 has a 121 amino acid insertion with respect to the GRK5 sequence, generating an amino terminus of 309 amino acids. This region of the protein is thought to play a role in substrate specificity.

Despite the presence of multiple transcripts, our studies identified only a single GPRK2 protein of 80 kDa. Previously, Cassill et al. (1991) predicted a GPRK2 protein of 50 kDa by assuming that translation initiates at a methionine just prior to the kinase domain. In our sequence this methionine lies at amino acid 288, and the predicted size of the protein is 80.7 kDa. Because only the 80 kDa protein was detected using the antibodies reported in Cassill et al. (1991), and because this band was specifically eliminated in *gprk2*^{6936} mutant ovaries and CNS, we believe that it corresponds to the *Gprk2* gene product. Comparing the GPRK2 sequence predicted from the B6936 cDNA with GRK5 provided further support that we had analyzed the full-length protein. The first 114 amino acids share 53% identity with GRK5 including the first 19 amino acids, 18 of which are identical between GPRK2 and GRK5.

Does GPRK2 act in a G protein-coupled receptor pathway that utilizes cAMP as a second messenger?

If the effects of the *gprk2*^{6936} mutation on oogenesis are caused by the disruption of the second messenger, cAMP, then mutations in genes that lie downstream of G protein-coupled

receptors might also affect female fertility. In fact, three candidate genes have been identified: *dunce* (*dnc*), *rutabaga* (*rut*), and *DCO*. All three genes act in the G protein-coupled receptor pathway that is mediated by the second messenger, cAMP. *dnc* encodes a cAMP-dependent phosphodiesterase, and in *dnc* mutants the level of cAMP is elevated in comparison to wild type. *dnc* homozygous females are sterile although, like *gprk2*^{6936} females, they produce mature oocytes with defects in dorsal appendage formation and nurse cell dumping (Bellen et al., 1987; L. E. S., unpublished observations). The female sterility of *dnc* is suppressed by mutations in the *rut* gene, which encodes Ca^{+}/calmodulin-dependent adenylyl cyclase (Bellen et al., 1987). This suppression occurs by decreasing the levels of cAMP. These phenotypes are consistent with a potential role for *Gprk2* in this classic G protein pathway. If GPRK2 down-regulates a G protein-coupled receptor, then the *gprk2*^{6936} mutant should cause prolonged receptor activity resulting in increased cAMP levels, as seen in *dnc* mutants.

Mutations at the *DCO* locus disrupt the major catalytic component of cAMP-dependent protein kinase (PKA). In ovaries of *DCO* females, late stage nurse cells are often binucleate, although younger egg chambers have no such defects (Lane and Kalderon, 1993). This suggests that the breakdown of nurse cell membranes, which normally begins in stage 11, takes place prematurely in the mutant. In some respects, *gprk2*^{6936} mutant egg chambers have the opposite phenotype since nurse cells do not break down completely. A reduction in GRK activity and a reduction in PKA activity would be expected to exert opposite effects on signaling. Moreover, GPRK2 protein was detected most strongly on nurse cell membranes throughout much of oogenesis and PKA activity is known to be required in the germline.

Our results suggest that GPRK2 may be part of a G protein signaling pathway in the nervous system as well. GPRK2 was strongly expressed in the mushroom bodies, a neural structure that has been implicated in learning and memory. *dnc*, *rut* and *DCO* exhibit defects in olfactory learning (reviewed in Davis et al., 1995), and all three are abundantly expressed in the mushroom bodies (de Belle and Heisenberg, 1994; Erber and Menzel, 1980; Heisenberg et al., 1985). *Gprk2* may act in a common pathway with *dnc*, *rut* and/or *DCO* in both ovaries and mushroom bodies. Since the *gprk2*^{6936} mutation eliminated *gprk2* expression in the CNS, it would be worthwhile testing whether the mutant adults exhibit defects in learning and memory.

What are the likely receptor substrates of GPRK2?

A number of seven-transmembrane receptors that are likely to couple to G proteins have been identified in *Drosophila*. However, most of the known *Drosophila* G protein-coupled receptors, including those for dopamine, serotonin, octopamine, tyramine, tachykinin, and the muscarinic acetylcholine receptor, have been cloned by homology and no mutations are known (Shapiro et al., 1989; Saudou et al., 1992; Arakawa et al., 1990; Li et al., 1991, 1992; Monnier et al., 1992; Gotzes et al., 1994; Colas et al., 1995; Feng et al., 1996; Han et al., 1996). Therefore, it remains uncertain whether these receptors function during development.

Two putative G protein-coupled receptors have been identified recently that play major roles during development. *smoothened* (*smo*), encodes a putative Hedgehog receptor (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) while *frizzled2* (*Dfz2*), specifies a putative Wingless receptor (Bhanot et al., 1996). Both predicted proteins contain seven transmembrane domains although they have little sequence similarity to other G protein-coupled receptors. There are currently three distinct superfamilies of receptors that couple to G proteins: those in the βAR family, the metabotropic glutamate receptors, and members of the GI hormone family (Premont et al., 1995). It is possible that Smo and Dfz2 represent additional families of G protein-coupled receptors.

Are the Smo and Dfz2 receptors likely to be good substrates for GPRK2? GRK5 phosphorylates βAR at 6 sites within the last 40 amino acids of the receptor; no phosphorylation occurs on cytoplasmic loops (Fredericks et al., 1996). The same regions of the Smo and Dfz2 carboxy termini contain 6 or 8 potential phosphorylation sites, respectively. However, Smo may act as the better substrate for GPRK2 because GRK5, like βARK, shows a preference for target residues located within an acidic environment (Fredericks et al., 1996). Dfz2 has no acidic amino acids in the last 40 amino acids of the protein. In contrast, Smo has several acidic residues in the 40 amino acid domain including an acidic pair upstream of the most amino-terminal phosphorylatable residue in the 40 amino acid fragment. This same pattern occurs in receptors that are phosphorylated by GRK5 and βARK. Thus, from a structural standpoint Smo is an excellent candidate to mediate the developmental effects of GPRK2.

Does *Gprk2* act through *hh*, *wg* or *dpp* during oogenesis?

The role played by *hh* and *wg* during oogenesis has been analyzed recently (Forbes et al., 1996a,b). Both genes are expressed in small groups of cells at the anterior tip of each ovariole. The *hh*-expressing cells influence the proliferation of somatic follicle cell precursors and the specification of polar follicle cells. However, effects on oogenesis similar to those caused by *gprk2*^{6936} were not observed following changes in *hh* or *patched* activity. While the ovarian *hh* pathway resembles the pathway in early embryos and imaginal discs (reviewed by Perrimon, 1994; Ingham, 1995) in its requirement for *patched*, ovarian hh signaling is not mediated by *wg* or *dpp*. A separate role for *wg* in oogenesis is suggested by the observation that overexpression of *wg* causes multiple developmental defects in the ovary (Forbes et al., 1996b). However these defects do not mimic the effects of the *gprk2*^{6936} mutation. Consequently, changes in inhibitory phosphorylation of Smo and Dfz2 are not likely to be responsible for the defects we observed in *gprk2*^{6936} females.

The similar effects on oogenesis of disrupting *Gprk2* and *dpp* suggest that these two genes may be involved in a common pathway. Dpp receptors differ significantly from serpentine receptors and are unlikely to serve as GPRK2 substrates. While it is possible that GPRK2 can act directly on targets other than G protein-coupled receptors, mammalian GRK family members are thought to phosphorylate serpentine receptors exclusively. It is more likely that *Gprk2* influences either the expression of *dpp* itself or the effects of Dpp signaling downstream of the receptors. We were unable to detect any change in a *dpp* enhancer line in homozygous *gprk2*^{6936} ovaries, suggesting that *gprk2*^{6936} does not affect *dpp* tran-

scription. It remains a possibility that *gprk2*^{6936} could disrupt Dpp signaling by altering the posttranslational processing of *dpp*. *Gprk2*^{6936} could also affect Dpp signaling by acting downstream of Dpp receptors, a relationship that has been demonstrated between tyrosine kinase receptor and G protein-coupled receptor pathways (Luttrell et al., 1995; Van Biesen et al., 1995; Hafner et al., 1994). This second possibility is favored by our expression studies. *dpp* is expressed most strongly in the anterior follicle cells and its activity is required in these soma. In contrast, GPRK2 is expressed predominantly in the nurse cells although immunoreactivity was also detected in stage 9-10 follicle cells.

The co-expression of GPRK2 and *dpp-lacZ* in the wing imaginal disc suggested that these two molecules interact in the patterning of the wing. However, we have not been able to detect any wing defects or changes in *dpp-lacZ* expression in *gprk2*^{6936} homozygotes. The effects of the *gprk2*^{6936} mutation on *dpp* expression and wing patterning may be too subtle to be detected with the tools that we used. Alternatively, *gprk2*^{6936} may play no role in wing patterning and its expression at the anterior-posterior boundary of the wing disc may play another role altogether.

G protein-coupled signaling may mediate developmental signals that vary in time and space

The finding that a putative G-protein coupled receptor kinase functions during several developmental processes raises the question of the developmental role of receptor desensitization. The primary effect of *gprk2*^{6936} during oogenesis was to disrupt anterior chorion formation, a process mediated by rapidly migrating subpopulations of follicle cells. G protein signaling pathways are known to be important for cell migration and/or shape changes in other developing systems. Examples include the aggregation of *Dictyostelium discoideum* to form a migrating slug and fruiting body (Devreotes, 1994), gastrulation in *Drosophila* embryos which requires a specific Gα subunit encoded by the *concertina* locus (Parks and Wieschaus, 1991), FGF-stimulated endothelial cell migration which is pertussis toxin-sensitive (Sa and Fox, 1994), and Rho-dependent neurite retraction in mammals (Postma et al., 1996).

Because GRKs phosphorylate activated receptors, desensitization provides an ideal mechanism for regulating the responsiveness of receptors based on their previous signaling activity. Based on this idea, we propose three potential developmental roles for desensitization by GRKs. First, desensitization could allow a cell to generate a discrete response to a signal whose presence in the environment is prolonged. This could play an important role for a cell that is using a given signal as a migratory cue as it passes through an area where that signal is constant. Second, expression of a particular GRK could allow cells to respond differentially to particular ligands. In Chinese hamster ovary cells, the neurokinin1 and neurokinin2 receptors display different desensitization responses to substance P and neurokinin A (Garland et al., 1996). Third, desensitization could be the first step in receptor recycling to allow the cell to respond rapidly to changes in the level of an ongoing signal. All of these mechanisms would contribute to the ability of a cell to navigate through a complex and changing environment. Further study of *Gprk2* should help elucidate the detailed role GPRKs play during development.

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(Accepted 30 April 1997)