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Drosophila retinal degeneration A gene encodes an eye-specific diacylglycerol kinase with cysteine-rich zinc-finger motifs and ankyrin repeats

(signal transduction/phosphatidylinositol metabolism)

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ABSTRACT **The *Drosophila* visual mutant, carrying the retinal degeneration A gene (*rdgA*), has photoreceptor cells that degenerate within a week after eclosion. Morphological studies suggested that this mutant harbors abnormalities in membrane turnover of the photoreceptor cells. Biochemically, the rdgA mutant lacks an eye-specific and membrane-associated diacylglycerol kinase (DGK; EC 2.7.1.107) activity in a gene-dosage-dependent manner, suggesting that *rdgA* gene encodes a DGK. We report the molecular cloning and characterization of a DGK gene, which maps to the *rdgA* locus. This gene, designated as DGK2, has a single open reading frame that encodes 1454 amino acids. Like porcine DGK, DGK2 has two cysteine-rich zinc-finger motifs as well as a DGK catalytic domain. The DGK2 protein contains four ankyrin-like repeats at the C-terminal region, suggesting that DGK2 is likely anchored to the membrane or cytoskeleton. Northern blot analysis and tissue *in situ* hybridization to adult sections revealed that DGK2 is expressed exclusively in the adult retina and that the amount of its mRNA is reduced in some of the rdgA mutant alleles. Furthermore, in two *rdgA* alleles, *rdgA*^{1} and *rdgA*^{2}, nonsense and missense mutations occur within their DGK2 gene, respectively. Thus, we conclude that *rdgA* encodes an eye-specific DGK, the absence of which leads to rhabdomere degeneration due to defective phospholipid turnover.**

Hereditary retinal degeneration has been described in human, mouse, and the fruit fly *Drosophila melanogaster* (1). Molecular analyses have revealed that some forms of vertebrate retinal degeneration are due to structural defects in visual transduction molecules, such as rhodopsin in human retinitis pigmentosa (2) and the β subunit of cGMP phosphodiesterase in retinal degeneration (*rd*) mouse (3). The retinal degeneration phenotype in the retinal degeneration slow (*rds*) mouse is caused by a defect in peripherin, which functions in maintaining the structure of the rod disc membranes (4). Retinal degeneration mutants of *Drosophila* provide further understanding of the molecular mechanisms of hereditary retinal degenerations.

The *Drosophila* retinal degeneration A (rdgA) mutant has photoreceptor cells that differentiate normally but degenerate rapidly after eclosion (5). Morphological observations of rdgA mutant eyes demonstrated that the subrhabdomeric cisternae (SRC), which are vital for the transport of photoreceptive membrane components to rhabdomere microvilli, are absent at early stages of degeneration (6). This defect may cause an insufficient supply of photoreceptive membrane, leading to retinal degeneration. On the other hand, biochemical analysis showed that an eye-specific diacylglycerol kinase (DGK; EC 2.7.1.107) activity is defective in rdgA mutants (7). The DGK activity depends upon *rdgA*$^+$ gene dosage, suggesting that the *rdgA* gene encodes a DGK enzyme (8).

Upon cell stimulation, DGK initiates resynthesis of phosphatidylinositols by phosphorylation of diacylglycerol (DG) to make phosphatidic acid (PA) (9). Furthermore, this enzyme reduces the level of DG to regulate protein kinase C (PKC) activity. DGK activity is known to exist in a wide variety of tissues and organisms, from *Escherichia coli* to mammals. In mammalian cells, the 80-kDa species was initially purified from pig brain and later from thymus cytosol (10). Cloning of the porcine DGK cDNA revealed that the protein has EF hand and cysteine-rich zinc-finger motifs (11). Previously, we cloned a *Drosophila* DGK gene expressed predominantly in the nervous system and muscle (designated as DGK1) (12). DGK1 maps to cytogenetic position 43F1, which is distinct from that of *rdgA*.

We further isolated a second *Drosophila* DGK gene (DGK2), which maps to the cytogenetic position 8C1—that is, the *rdgA* locus.§ DGK2 has an amino acid sequence highly homologous to the DGK catalytic domain. Furthermore, DGK2 has two cysteine-rich zinc-finger motifs like porcine DGK. As an interesting feature, DGK2 has four ankyrin-like repeats in the C terminus, through which DGK2 may interact with the membrane cytoskeleton. RNA blot analysis revealed that the 9-kb DGK2 mRNA is detected in the adult head and that it is reduced in some of the rdgA mutants. By sequencing two rdgA mutant genomes, we found nonsense and missense mutations within the DGK2 gene. Tissue *in situ* hybridization indicated that DGK2 is expressed exclusively in the compound eye. Thus, we conclude that the DGK2 is an eye-specific DGK enzyme that is coded by the *rdgA* locus.

MATERIALS AND METHODS

Fly Stocks. Flies were reared on yeast/cornmeal/agar medium at 25°C. Canton-S was used as a wild-type strain. Four *rdgA* alleles (*rdgA*^{1}, *rdgA*^{2}, *rdgA*^{KS60}, and *rdgA*^{3}) were induced by ethyl methanesulfonate (5).

Screening of Library and Nucleotide Sequencing. A genomic library was constructed with lambda-Dash (Stratagene). A λgt11 cDNA library constructed from adult head mRNA was provided by Konrad Zinsmaier (California Institute of Technology). Low-stringency conditions used for cDNA library screening were hybridization in buffer as described (13) at 42°C and washing the filters in 0.5× standard saline/citrate (SSC) (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 0.1% SDS at 42°C. High-stringency conditions for genomic library screening were identical, except that washing was done at 65°C. Genomic and cDNA clones isolated were subcloned

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Abbreviations: rdgA, retinal degeneration A; DGK, diacylglycerol kinase; DG, diacylglycerol; PA, phosphatidic acid; PKC, protein kinase C; SRC, subrhabdomeric cisternae.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D17315).

into pBluescript II SK⁻ (Stratagene) and sequenced with Sequenase (United States Biochemical). A series of deletion plasmids was prepared by using the exonuclease III/mung bean nuclease (Takara Shuzo, Kyoto) and they were sequenced.

Chromosome *in Situ* Hybridization. Chromosome squashes for hybridization were prepared as described by Engels *et al.* (14). The procedure for hybridization and posthybridization was according to de Frutos *et al.* (15).

RNA Blot Analysis. Poly(A)⁺ mRNA was electrophoresed in a formaldehyde/1% agarose gel, and transferred to a nylon membrane (16). The filter was hybridized at 42°C for 12 hr with ^{32}P-labeled DGK2 cDNA probe in buffer as described (13). Extensive washing was done in 0.5× SSC/0.1% SDS at 65°C.

Tissue *in Situ* Hybridization. DGK2 cDNA clones were used as probes for tissue *in situ* hybridization. Probe was labeled using digoxigenin-11-rUTP with either T7 RNA polymerase or T3 RNA polymerase according to the protocol of the Boehringer Mannheim nucleic acid labeling kit. Frozen tissue sections (8 μm) of white adult flies were cut on a cryostat, transferred to gelatin-subbed slides, fixed in 4% (wt/vol) paraformaldehyde, and dehydrated in ethanol. Tissue sections were pretreated according to the method of Hafen *et al.* (17). After pretreatment and air-drying, mild alkali hydrolysis of the RNA probe, overnight RNA–RNA hybridization at 50°C, posthybridization washes, and immunological detection of the hybridized probe were done essentially as described by Coen *et al.* (18).

Identification of the Mutational Site in rdgA Mutants. The genomic DNA of the rdgA mutants was amplified by the PCR and sequenced after subcloning into pBluescript II SK⁻ (Stratagene). The genomic fragments cover the entire coding region of the DGK2 gene. Two independently isolated subclones from different PCR reactions were sequenced to eliminate possible errors during PCR amplification.

RESULTS

Isolation of the DGK2 Gene. Previously, we reported a *Drosophila* DGK gene that is expressed in the nervous system and muscle (designated DGK1) (12). To clone the *rdgA* gene, we screened a *Drosophila* adult-head cDNA library with the conserved region of the DGK1 gene under low-stringency conditions. We isolated several cDNA clones of another DGK gene (designated DGK2). Two of the largest cDNA clones, 9 and B1 (Fig. 1), which together cover the full-length of the DGK2 transcript, were sequenced. The DGK2 cDNA sequence contains a single open reading frame that encodes 1454 amino acids (Fig. 2). Furthermore, we screened a genomic library with DGK2 cDNA and isolated the genomic region corresponding to the DGK2 transcription units. The DGK2 transcript is derived from 15 exons spreading over >30 kb of genomic DNA (Fig. 1).

Structure of DGK2 Protein. Schematic comparison of the *Drosophila* DGK1, DGK2, and porcine DGK putative amino acid sequences is shown in Fig. 3*A*. The *Drosophila* DGK2 protein contains a domain that is homologous to and at a comparable position with the porcine DGK catalytic domain. In this region, DGK2 has ≈42% amino acid identity with both the porcine DGK and DGK1 (Fig. 3*B*). In the N-terminal region of the catalytic domain, DGK2 has two cysteine-rich zinc-finger motifs, of which cysteine and histidine residues are at similar positions as those in PKC (Fig. 2). These motifs in PKC are known as the binding sites of phorbol ester and probably of DG (19). The low similarity in these motifs between porcine DGK and *Drosophila* DGK2 may be related to the functional difference between the two DGK species. The EF hand motif was not found in DGK2, suggesting that, unlike porcine DGK, the DGK2 activity is not regulated by Ca^{2+} ions. No homology could be detected in the N-terminal 590-amino acid sequences by search of the Swiss-Prot data base (European Molecular Biology Laboratory) using the SDC-GENETYX program (Software Development, Tokyo) (20). Partial purification of a DGK protein from *Drosophila* eyes showed that a 115-kDa protein correlated with the enzyme activity (21), suggesting a possibility that the real gene product may be smaller. If Met-402 is the translation-initiation site, molecular size would be estimated as 116 kDa.

Additionally, four ankyrin-like repeats were found at the C terminus of DGK2 (Fig. 2). These repeats are known to exist in a variety of proteins, such as yeast-cell-cycle control proteins [cdc10/SWI6 (22) and SWI4 (23)], tissue-differentiation proteins of *Drosophila* and *Caenorhabditis elegans* [Notch (24), *glp-1* (25), and *lin-12* (26)], and ankyrin (27). Fig. 3*C* shows a comparison of the aligned ankyrin-related repeats among the four repeats in DGK2 with several homologous proteins, indicating the conserved consensus sequences in DGK2.

Mapping of the DGK2 Gene. By chromosome *in situ* hybridization using the DGK2 genomic DNA as a probe, the DGK2 gene was mapped to cytogenetic position 8C1 on the X chromosome (data not shown). The DGK2 locus coincides well with the *rdgA* locus known to be within 8A4–8C6 by deficiency mapping, suggesting that DGK2 is the *rdgA* gene.

Expression of the DGK2 Gene. Northern blot analysis with the DGK2 cDNA detected a 9-kb mRNA in adult heads (Fig. 4). The amount of this transcript is reduced in $rdgA^2$, and it is not detected in $rdgA^{KS60}$. The mutant flies used for isolation of poly(A)⁺ RNA were collected within 1 day after eclosion, when the retinal degeneration is not yet apparent. Thus, the low level of mRNA in the *rdgA* alleles could not be secondary to the morphological degeneration.

Tissue *in situ* hybridization to adult head sections revealed that the DGK2 gene is expressed exclusively in the adult compound eye but is not expressed in the brain or in the muscle (Fig. 5*A*). Furthermore, the DGK2 expression could not be observed in $rdgA^3$ retina by RNA blot analysis or tissue *in situ* hybridization (Fig. 5*B*). These data show that DGK2 expression is eye-specific and that its expression is defective in rdgA mutants, strongly supporting our previous notion (8) that *rdgA* encodes retina-specific DGK.

Sequencing of the Mutant Genome. We isolated the DGK2 gene from two severe rdgA mutants, $rdgA^1$ and $rdgA^2$ (28),

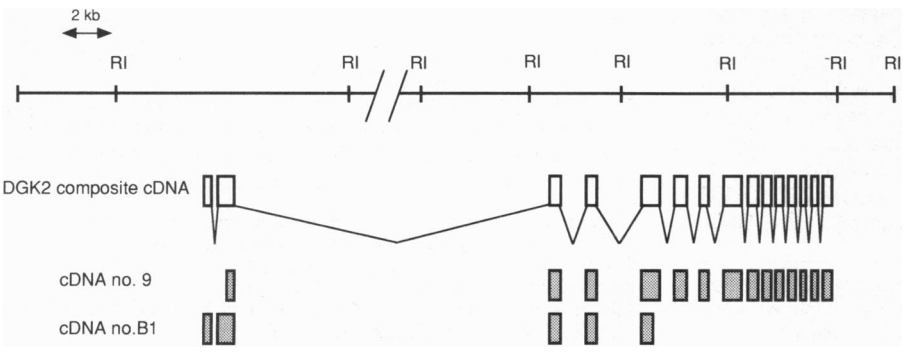

FIG. 1. Restriction map of the DGK2 genomic region and location of the composite cDNA. Restriction endonuclease *Eco*RI sites are shown as RI. The genomic region between the second and third exons contains long repetitive sequences, so that length of the gap shown here is not precisely determined. The two groups of stippled boxes below the composite cDNA correspond to cDNAs 9 and B1.

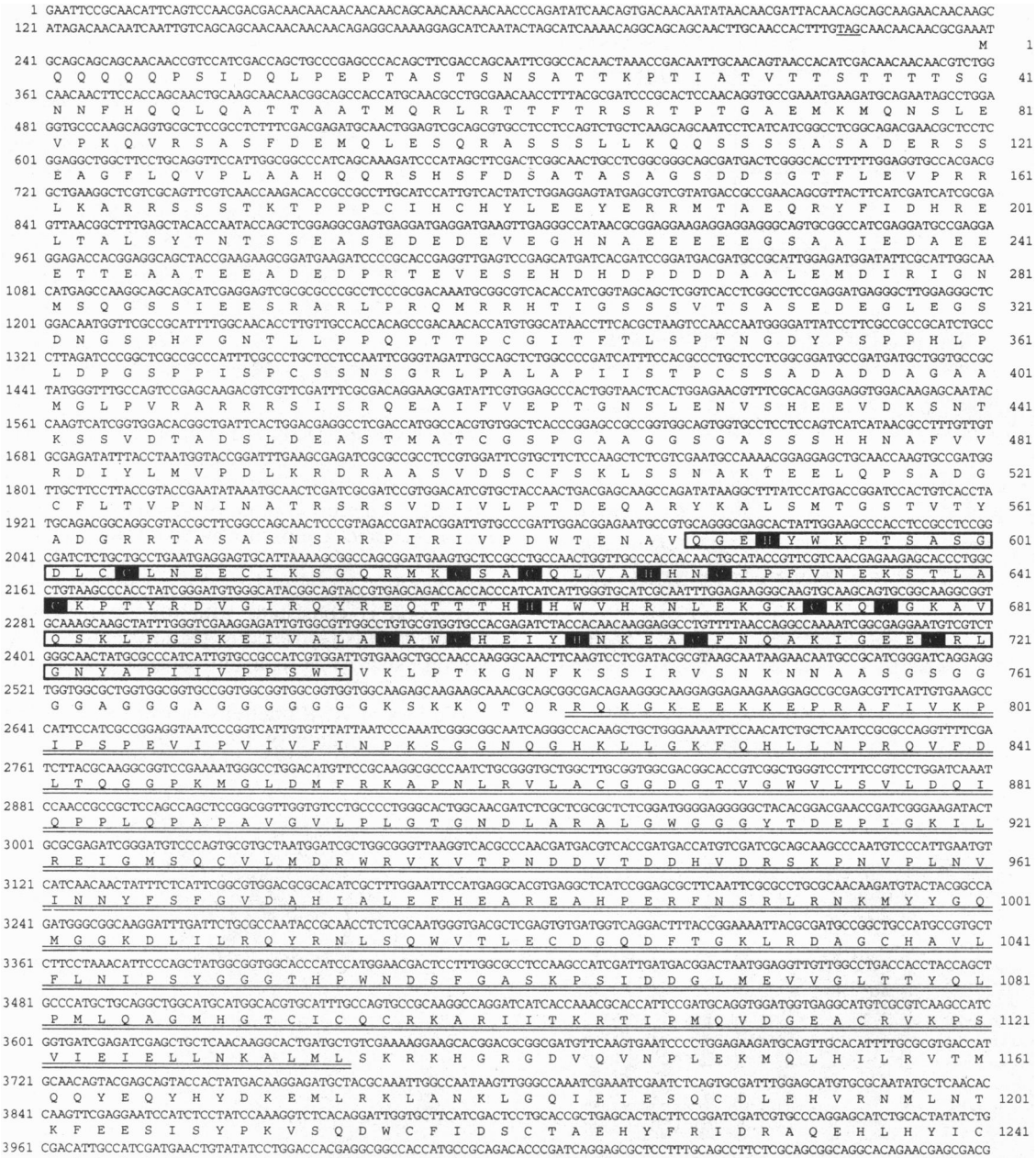

FIG. 2. Nucleotide and deduced amino acid sequences of the composite cDNA derived from cDNAs 9 and B1. A single open reading frame of 4362 nt encodes 1454 amino acids. The in-frame termination codon that precedes the putative translation initiation site is underlined at position 221. The DGK catalytic domain is doubly underlined. The cysteine-rich zinc-finger motifs are boxed, and conserved cysteine and histidine residues are printed in reverse type. Four ankyrin-like repeats in the C-terminal region are indicated by boldly outlined boxes. The last nucleotide positions of preceding exons at exon–intron boundaries are as follows: 493, 1929, 2071, 2275, 2402, 2787, 2968, 3378, 3672, 3899, 4121, 4173, 4237, 4395, and 4713.

and determined their nucleotide sequences. The rdgA[1] mutant has a nonsense mutation (CAG → TAG) at nt 3695. Because this nonsense mutation is located at residue 1153 between the catalytic domain and ankyrin repeats, this gene product must have a truncated C terminus, resulting in the loss of its ankyrin repeats in spite of the intact catalytic domain. In the rdgA[2] mutant, a nucleotide substitution (GGC → GAC) occurs at position 2844, resulting in a Gly-869 → Asp substitution. This substitution occurs at the highly conserved amino acid in the catalytic domain, suggesting that this

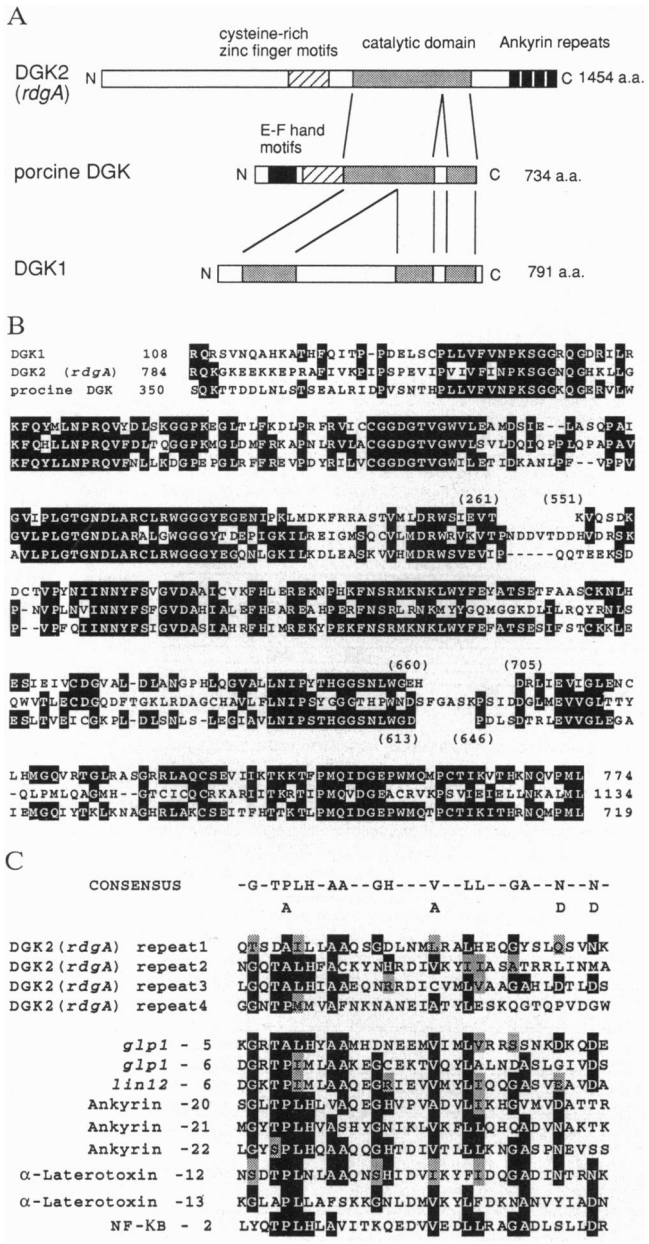

FIG. 3. (*A*) Schematic comparison among the *Drosophila* DGK1, DGK2, and porcine DGK proteins. The light-shaded boxes indicate homologous regions. The cysteine-rich zinc-finger motifs and ankyrin-like repeats are marked and shown with hatched boxes and small filled boxes, respectively. A filled box in porcine DGK indicates EF hand motifs. (*B*) Amino acid alignment of the putative catalytic domain between the *Drosophila* DGK2 and other two DGKs. Identical residues are indicated in reverse type. (*C*) Comparison of the aligned ankyrin-like repeats with the complete erythrocyte ankyrin consensus sequence. Four ankyrin-like repeats from DGK2 are compared with those of other repeat-containing proteins including glp-1, lin-12, ankyrin, α-laterotoxin, and NF-κB. The consensus sequence is at top. Residues identical to the consensus sequence are in reverse type; conserved residues are stippled. Conservative substitutions are defined as pairs belonging to one of the following groups; S, T, and G; P and A; N and Q; D and E; H, R, and K; M, I, L, and V; and F and W.

conservative glycine residue must be important for the DGK catalytic activity.

DISCUSSION

Our previous biochemical studies on rdgA mutants showed that the DGK activity in adult eyes is proportional to the $rdgA^+$ gene dosage, suggesting that *rdgA* encodes a photoreceptor-specific DGK. To support this hypothesis, we isolated the *Drosophila* DGK2 gene and mapped it to the *rdgA* locus. Northern blot analysis revealed that the DGK2 expression is altered in three *rdgA* alleles. By sequencing the mutant genome, a nonsense mutation was found in the DGK2 gene of the most severe allele, $rdgA^1$. These findings indicate that the *rdgA* gene encodes DGK2. The DGK2 retina-specific expression is consistent with the observation that rdgA degeneration is restricted to photoreceptors, whereas other neurons, such as those in optic ganglia, maintain their normal morphology (30).

The loss of DGK2 activity in rdgA eyes should result in either accumulation of DG or lack of PA, or both. One possibility for rdgA degeneration is that accumulation of DG

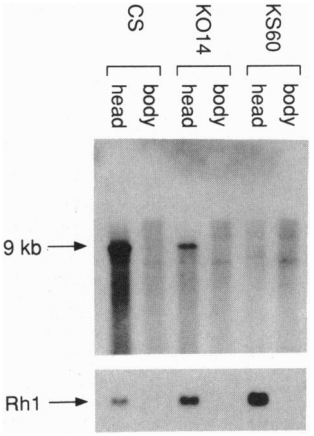

FIG. 4. Expression of the *Drosophila* DGK2 gene in wild-type and rdgA mutants. Poly(A)$^+$ selected RNA was prepared from adult body and head separately (28). CS, Canton-S wild-type strain; KO14, $rdgA^2$; KS60, $rdgA^{KS60}$. Ten micrograms of poly(A)$^+$ RNA was applied to each lane. Rhodopsin (Rh1) (29) was used as an eye-specific control probe to confirm the existence of photoreceptor cells, as well as the integrity of the mRNA.

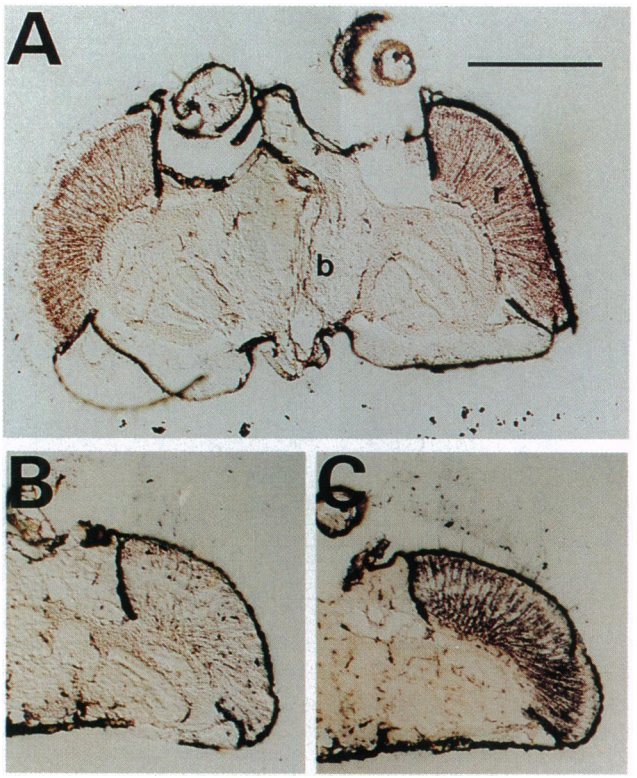

FIG. 5. *In situ* hybridization of the DGK2 cDNA to tissue sections. DGK2 cDNA clones were used as probes. (*A*) Cryostat sections of *Drosophila* wild-type adult heads were hybridized with single-stranded digoxigenin-labeled antisense RNA. Retina (r) and brain (b) are marked. (Scale bar = 200 μm.) No hybridization can be detected with sense-stranded RNA as a control probe. Sections of adult head of $rdgA^3$ *w* flies with an antisense probe of the DGK2 gene (*B*) and the rhodopsin (*Rh1; ninaE*) gene (29) (*C*).

stimulates constitutive activation of a PKC, possibly of an eye-specific PKC (31), leading to retinal degeneration. Recent electrophysiological analysis in *Drosophila* photoreceptors revealed that DG and Ca^{2+} influx are required to regulate PKC to deactivate phototransduction (32). This result suggests that accumulation of DG alone may not be sufficient to activate PKC. Furthermore, the retinal degeneration occurs in rdgA mutants kept in the dark or under the no receptor potential A (*norpA*) mutant background, which prevents accumulation of DG by the action of light-activated phospholipase C (13, 33). These features are in contrast to those of retinal degeneration B (*rdgB*) (34). A more likely possibility is that retinal degeneration arises from the absence of PA. Indeed, PA is relatively enriched in the retinular cell layer in comparison with the brain and it is almost absent in rdgA mutant eyes (8). On the other hand, DG content is not significantly altered in the mutant eyes (8).

In rdgA mutants, the SRC is absent at an early stage of degeneration (6). The SRC network is observed to collapse in the retinular cells of light-adapted rdgB mutants, in which rhabdomere degeneration is light-dependent (35). The loss of the SRC is an initial morphological defect in both mutants, which may trigger abnormal turnover of the components of the rhabdomere microvilli (36). The *rdgB* gene was cloned (37) and shown to be a homologue of phosphatidylinositol-transfer protein, which is localized in the SRC (42). The rdgB protein may control phosphatidylinositol concentration in transport vesicles from the SRC to the rhabdomere, whereas the rdgA mutant defect lies in the supply of membrane components, in particular phospholipids, via the SRC. The relationship between the DGK2 activity and maintenance of SRC is still not clear. PA itself may be an important constituent of the SRC. The lack of PA may block the synthesis of phosphatidylinositols, leading to insufficient supply of phospholipids to the rhabdomere.

Interestingly, DGK2 has four ankyrin repeats in the C-terminal region. Ankyrin repeats are the conspicuous feature of ankyrin, which has an N-terminal domain containing 22 continuous repeats of this motif. Furthermore, a number of proteins including transcription factors [NF-κB (38) and the GA binding protein GABP-β (39)] and a toxin from black widow venom [α-laterotoxin (40)] have been identified as a family containing the ankyrin-like repeats (41). The motifs are considered as interaction sites with other proteins. For example, the motifs in erythrocyte ankyrin are the binding sites with several integral membrane proteins and tubulin, forming a bridge between cytoskeleton and membrane components (41). From the biochemical studies, we also demonstrated that the *rdgA* gene product is a membrane-associated DGK (21). It is highly likely that DGK2 interacts with membrane proteins through the ankyrin repeats and is anchored to the membrane of the rhabdom or SRC. The molecular defect of the severe degeneration allele, $rdgA^1$, suggests that the ankyrin repeats are necessary for the normal function of the DGK2 protein.

In summary, our findings show that the defect in lipid metabolism causes retinal degeneration. Although such retinopathy in forms of human retinitis pigmentosa has not been reported, the phosphatidylinositol-related enzymes, such as phospholipase C and DGK, are also enriched in the vertebrate retina (H. Inoue, T. Yoshioka, and Y.H., unpublished data), indicating the general importance of phospholipid metabolism in function and morphology of photoreceptors. Further studies on *Drosophila* retinal degeneration mutants will provide an understanding of the biological function of retina-specific phospholipid metabolism and the molecular mechanisms of retinal degeneration.

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