**Diacylglycerol Kinase Defect in a Drosophila Retinal Degeneration Mutant rdgA**

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The *Drosophila* visual mutant *rdgA* is known to show age-dependent retinal degeneration with defective diacylglycerol (DG) kinase activity. In this study we examined DG kinase activity of several visual mutants and found that only *rdgA* mutant eyes showed the lack of DG kinase activity in a gene dosage-dependent manner. The enzyme activity is already absent at the time of eclosion from pupal case when the degeneration is not yet apparent. To examine whether *rdgA* gene dosage effect holds for other enzymes related to the phosphatidylinositol turnover, phospholipase C and phosphoinositide turnover, phospholipase C was analyzed which did not show any gene dosage effect. Therefore, it is strongly suggested that *rdgA* gene correlates closely with DG kinase activity, and the defect of DG kinase activity is a primary cause of retinal degeneration in *rdgA* mutant.

The structure of the retina is affected by a variety of visual mutations in *Drosophila*. In many of the cases, photoreceptor cells are severely affected, as in a *Drosophila* mutant which causes age-dependent retinal degenerations (*rdgA*), or one whose retinal degeneration is triggered by light irradiation (*rdgB*) (1).

In *rdgA* retina, photoreceptors degenerate during the first week of adult life (2–4). On the other hand, photoreceptors in the *rdgB* retina show light-induced degeneration (2, 5). It is also noteworthy that photoreceptors of visual mutant *norpa* (no receptor potential) which lack electrical responses, appear to degenerate relatively late in adult life (1). A biochemical mechanism for the degeneration has not yet been analyzed.

Recently retina of the *rdgA* and *norpa* mutants have been found to be deficient in phosphatidic acid (PA) because of abnormally low activity of diacylglycerol (DG) kinase (6, 7). This observation implied that the abnormal low activity of DG kinase may be related to the neuronal degeneration or to the lack of receptor potential.

The aim of this paper is to provide evidence that *rdgA* is a structural gene coding for DG kinase and that the lack of DG kinase is the primary cause of rhabdomere degeneration in *rdgA*. We demonstrate that (a) $V_{max}$ of DG kinase is affected by *rdgA* mutations, (b) DG kinase activity depends on gene dosage of wild type *rdgA* gene, and (c) DG kinase deficiency precedes the morphological change.

**EXPERIMENTAL PROCEDURES**

**Materials**—Canton-S (CS) strain of *Drosophila melanogaster* was used as a standard, normal strain, from which all the mutations were induced with an alkylating agent, ethyl methanesulfonate. The independently isolated alleles of *rdgA* (retinal degeneration A) genes used were *rdgA*<sub>014</sub>, *rdgA*<sub>3886</sub>, *rdgA*<sub>8512</sub>. Hereafter, they are designated as K014, KS60, and BS12, respectively. The mutants called *rdgB* (causing light-induced retinal degeneration), *norpa* (no receptor potential), *ora* (outer rhabdomere absent), and *so* (*sine oculis*, without eyes) were also used. A deletion strain Df(1)B14::Lacks X-chromosomal bands between 7P1–2 and 8C6, including the *rdgA* locus. Mutants and their origin used in this experiment are listed in Table I.

**Chemicals**—[γ-<sup>32</sup>P]ATP (specific activity; 3000 Ci/mmol) and [γ-<sup>32</sup>P]orthophosphate (P) (carrier-free) were purchased from Du Pont-New England Nuclear, and [γ-<sup>32</sup>P]inositol phosphatidylinositol (PI) (specific activity; 270 Ci/mm) was from Amer sham Corp. 1,2-diolein and PI were obtained from Serdary Research Inc.

**Assay of DG Kinase Activity**—Flies were collected within 1 day after eclosion and kept on a fresh cornmeal-agar-yeast medium until they become 4–7-days old. Then they were etherized lightly, frozen, and kept in a deep-freezer (−80 °C) until the enzyme assay. Heads were detached manually on dry ice. DG kinase activity was assayed as described previously (6). The standard reaction mixture contained 100 mM KCl, 10 mM NaCl, 10 mM KF, 1 mM EGTA, 20 mM phosphate buffer, pH 6.8, 2 mM 1,2-diolein, 1 mM [γ-<sup>32</sup>P]ATP and head homogenate (20 heads) in a final volume of 50 μl. Immediately before the reaction was started, DG was sonicated for 5 min using a microtip. Dioxylate (1 mM) was added to the DG suspension in some experiments. The incubation was carried out for 5 min at room temperature and the reaction was terminated by adding 0.5 ml of 12% ice-cold perchloric acid. The reaction mixture was centrifuged and the supernatant was counted by scintillation counting.

**Assay of Other Enzyme Activities**—Phospholipase C activity for PI was assayed as described previously (8). The reaction mixture contained 50 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM [γ-<sup>32</sup>P]PI and 100 mM HEPES buffer, pH 7.4, and the homogenate of 20 heads in a final volume of 100 μl. The incubation was carried out for 5 min at room temperature. The reaction was stopped by the addition of 0.2 ml of ice-cold methanol and 0.4 ml of chloroform. After shaking the mixture, 0.15 ml of water was added. The mixture was shaken well and centrifuged. An aliquot of the upper layer was taken, and the radioactivity was counted. Phospholipase D activity was assayed as described in Bockino et al. (9) using microsomal fraction with endogenous substrates. The fraction was isolated from [γ-<sup>32</sup>P]-labeled head of normal and KO14 as described previously (8). After incubation for a desired period, [γ-<sup>32</sup>P]-
DG Kinase Defect in a Drosophila Mutant

**Results**

DG Kinase Activity of Various Visual Mutants—We examined DG kinase activity in rdgA, rdgB, ora, norpA, and so using head homogenates. The results are shown in Table II. All three haploid alleles of rdgA, whose retinular cells degenerate during the first week of adult life, were shown to have very low level of the enzyme activity, although expressivity of the retinal degeneration varies among these alleles. A mutant, so, which has no eyes, showed an extremely low activity of DG kinase as reported previously (6). In the case of rdgB mutant, however, the retinular cells also degenerate as in rdgA under normal environment, but as high as 36% of the normal DG kinase activity was detected. Furthermore, ora was found to possess almost normal enzyme activity, even though outer rhabdomeres are absent in the mutant. Therefore, it is evident that DG kinase is not localized only in rhabdomeres but is distributed in the cell body of the retinular cells and the enzyme deficiency in rdgA is not simply due to the absence of degeneration of rhabdomere.

Ontogenic Changes of DG Kinase Activity after Eclosion—According to morphological examination, small rhabdomeres were observed in rdgA mutant eyes immediately after eclosion from pupal case (4, 14). They then disappear within a few days. If the lack of DG kinase is the cause of the degeneration, the enzyme deficit should precede the morphological change. To prove this, we collected K014 flies of various stages, from immediately after eclosion (day 0) to 3 days later (day 3). The enzyme activities of the mutants were found to be consistently low (5-7% of the normal) regardless of the age after eclosion. On the other hand, DG kinase activity of the normal flies showed a time-dependent change; the value on day 3 was 60% and that on day 3 was 90% of the enzyme activity of the normal fly 4 days after eclosion. From these results we conclude that DG kinase in rdgA mutant was already deficient at the time of eclosion when the degeneration is not yet apparent.

Gene Dosage Dependence of DG Kinase—When DG kinase activity in heterozygotes of various mutants was measured, the enzyme activity of rdgA/+ female, having one normal and one rdgA carrying chromosome, was about half of the normal level. The same is not true for other mutants, such as rdgB, norpA, ora, and so in which DG kinase activity recovers to almost normal levels in heterozygotes (Table III). In other words, the enzyme defect is semidominant in rdgA, but is recessive in other mutants. Fig. 1 shows the effect of DG concentration on DG kinase activity both in normal and K014/+ heads. The apparent $K_a$ values for normal and K014/+

| Symbol | Phenotype | Genotype DG kinase activity |
|--------|-----------|----------------------------|
| rdgA   | Retinal degeneration | Normal: 6.18 ± 1.71 (14) |
| rdgB   | Retinal degeneration | rdgAB: 0.03 ± 0.06 (8) |
| norpA  | No receptor potential | norpA: 0.24 ± 0.06 (4) |
| ora    | Outer rhabdomere absent | ora: 0.18 ± 0.06 (4) |
| so     | Sine oculis | so: 2.24 ± 0.30 (3) |

Table II: DG kinase activity of various visual mutants

DG kinase was measured in crude head homogenate as described under "Experimental Procedures." The activity is expressed as nmol/5 min/mg protein. Values are the means ± S.D. Number of experiments is given in parentheses.
Relative DG kinase activity in heterozygote of various visual mutants

DG kinase was measured using crude head homogenate as described under "Experimental Procedures." The activity is expressed as relative to normal value, 6.18 ± 1.71 nmol/5 min/mg protein. Values are mean ± S.D. Number of experiments is given in parentheses.

| Genotype                | Relative DG kinase activity |
|-------------------------|----------------------------|
| rDG5/ko14             | 0.58 ± 0.14 (8)             |
| rDG5XSM/ko14          | 0.50 ± 0.17 (4)             |
| rDG5XBB/ko14          | 0.42 ± 0.10 (4)             |
| rdgAKo14/ko14         | 1.13 ± 0.18 (6)             |
| norpAko14/ko14        | 0.91 ± 0.11 (3)             |
| ota/ko14              | 1.04 ± 0.06 (4)             |
| so/ko14               | 0.85 ± 0.08 (6)             |

We analyzed DG kinase activities in flies with different doses of the normal and mutant rdgA gene. In this experiment, flies having equivalents of 0, 0.5, and 1.0 times the normal dose of rdgA+ gene were examined. Females with one normal chromosome and one deficiency chromosome (Df(1)KA14/+) exhibit 50% of normal activity levels because each X chromosome female retained is known to be half as active as the one X in male Drosophila. As shown in Table IV, DG kinase activity is expressed relative to normal value of the normal rdgA gene at three different doses. These results indicate that rdgA gene codes for DG kinase or the protein that regulates the synthesis or activity of the enzyme.

Gene Dosage Test for Phospholipase C—In order to test whether the rdgA gene dosage effect holds for other enzymes related to phospholipid metabolism, phospholipase C, hydrolyzing PI to DG and inositol phosphate, was assayed with rdgA and rdgA/+ flies. As is shown in Table V, phospholipase C activity was reduced to 30–40% of normal value in three alleles of rdgA but recovered to normal level in the heterozygotes. In other words, phospholipase C deficits in rdgA mutant are not dependent on the rdgA+ gene dosage. The reduction of phospholipase C, however, cannot explain the decreased level of PA, because alleles of norpA mutant, norpA<sup>Me1</sup>, and norpA<sup>5BT</sup> were found to have more than half of the normal PA content (18), in spite of the absence of phospholipase C activity (6).

These results support the idea that reduction of DG kinase activity in rdgA is a direct effect of the mutant gene, while the phospholipase C defect is caused more indirectly.

**DG and Phospholipids in rdgA Mutants**—It is anticipated generally proportional to the number of structural genes for the enzyme in a Drosophila genome (17).

**Fig. 1.** a, effect of DG concentration on DG kinase in normal (○) and ko14/+(□). The activity of ko14 at 2 mM DG was also indicated (●). DG kinase activity was measured without deoxycholate. Details are described under "Experimental Procedures." b, Lineweaver-Burk plot of the data shown in a.

+ were almost the same (0.32 mM), but V<sub>max</sub> for ko14/+ was 1.8 nmol/min/mg protein, which was 60% of the normal level (3.0 nmol/min/mg protein). These values of V<sub>max</sub> and K<sub>m</sub> for ko14 cannot be determined because the activity was as low as 5% to the normal. The effect of deoxycholate on DG kinase activity was examined because some investigators suggested the use of DOC promotes the activity of DG kinase (15, 16). It was found that DG kinase activities in the presence of 2 mM DG were enhanced about twice by the addition of deoxycholate in normal, ko14/+, and ko14. Under these conditions, V<sub>max</sub> for ko14/+ was found to be 49% of that for the normal.

This suggests a possibility that rdgA gene directly codes for DG kinase, since it is known that an enzyme activity is generally proportional to the number of structural genes for the enzyme in a Drosophila genome (17).
that the DG content of rdgA mutant eye may be high because DG cannot be used as a substrate of DG kinase. Indeed a DG kinase mutant in Escherichia coli was reported to accumulate 15–30-fold of DG intracellularly (19). Therefore, we examined DG content of Drosophila heads using HPLC. The amount of DG in normal fly heads was found to be 60 ± 9 µg/500 heads (mean ± S.D.) and that in rdgA was almost the same in three alleles of the mutant, 62 ± 9 µg/500 heads (mean ± S.D.). In order to estimate DG content in the eye of whole head, the amount of DG in so mutant (without eyes) heads was measured. The DG content of so heads was about half of the normal. These results suggest that DG is not accumulated significantly in the mutant eyes, even though the mutant lacks DG kinase in the eye.

It is also expected that PA content in the rdgA mutant eye must be low, since DG is not phosphorylated into PA in the mutant. We compared phospholipid composition of rdgA mutant eyes with those of normal flies by feeding 32P-labeled P, for 24 h. The two-dimensional TLC autoradiographs of 32P-labeled phospholipids extracted from the eye and brain of normal and rdgA are given in Fig. 2. PA in rdgA mutant eye was hardly labeled, whereas phospholipids other than PA were essentially normal.

Recently another pathway for PA production was proposed in animal cells. This is mediated by phospholipase D for two other phospholipids, phosphatidylethanolamine and phosphatidylcholine (9). To test if the phospholipase D is involved in the Drosophila phospholipid metabolism, we assayed the enzyme activity with endogenous substrates. 32P-labeled microsomal fraction including plasma membrane was prepared from normal and KO14 heads, respectively, and the radioactivity of PA was measured with incubation time. We could not find any increase in 32P-labeled PA after 30 min of incubation in both normal and in rdgA mutant. The results were not affected by the addition of GTPγS (100 µM) in the incubation medium. This result indicates that phospholipase D is not involved in the PA synthesis in Drosophila eye.

**FIG. 2. Autoradiographs of two-dimensional TLC pattern of in vivo labeled phospholipids.** Radioactive phospholipids were extracted from (a) normal eyes, (b) normal brains, (c) rdgA1104 eyes, and (d) rdgA114 brains. All phospholipids were labeled in vivo for 24 h by feeding [32P]Pi. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylinositol; PA, phosphatidic acid.

**DISCUSSION**

In this report, we show that absence of DG kinase activity and/or PA in the eyes of rdgA mutant precedes the retinal degeneration. This fact suggests that the phospholipid metabolism defect may be a primary cause of the retinal degeneration. We can exclude a possibility that deficiency in DG kinase activity was a result of photoreceptor degeneration in rdgA mutant. This is because rdgB mutants have a fair amount of DG kinase activity, although their rhabdomere had already degenerated when examined biochemically (2).

In order to obtain further evidence, we carried out two independent examinations: a dependence of DG kinase on the gene dosage, and an analysis of ontogenic changes in the enzyme activity. As demonstrated in Tables III and IV, DG kinase activity in rdgA mutant was found to be dependent on the rdgA+ gene dosage. Also DG kinase activity in rdgA mutant was deficient when the degeneration is not yet apparent. Recently, it was reported that acyl chain composition of DG affects DG kinase activity directly (20). According to our previous work, it was found that the predominant fatty acids of DG extracted from normal fly head were palmitic, palmitoleic, oleic, and myristic acid, but arachidonic acid was not detected (21). The fatty acid composition of K014 was indistinguishable from that of normal flies. Therefore, it is concluded that DG kinase abnormality in the mutant is in no way related to its endogenous substrates’ anomaly.

From the data described above, it is suggested that a deficiency of DG kinase activity is a cause of the retinal cell degeneration in Drosophila eyes. If this is the case, what is the role of DG kinase in the cell degeneration? DG kinase can catalyze formation of PA from DG and is also known to distinguishable from that of normal flies. Therefore, it is concluded that DG kinase abnormality in the mutant is in no way related to its endogenous substrates’ anomaly.

As shown in Fig. 2, only PA was found to be affected in rdgA mutant eyes. On the other hand, incorporations of [32P]Pi into PI, phosphatidylethanolamine, phosphatidylserine, and phosphatidylethanolamine were normal. These results indicate that most phospholipids, even PI, might be produced from PA made via de novo synthesis, i.e. acylation of monoacylglycerolphosphate with acyltransferase in de novo pathway. Therefore, it is anticipated that the deficiency of DG kinase activity may result in alteration of glycerophospholipid content because PA is known as an important intermediate in the formation of the various glycerophospholipids.

As shown in Fig. 2, only PA was found to be affected in rdgA mutant eyes. On the other hand, incorporations of [32P] Pi into PI, phosphatidylethanolamine, phosphatidylserine, and phosphatidylethanolamine were normal. These results indicate that most phospholipids, even PI, might be produced from PA made via de novo synthesis, i.e. acylation of monoacylglycerolphosphate with acyltransferase, rather than by phosphorylation of DG with DG kinase or hydrolysis of other phospholipids with phospholipase D. This is supported by our previous report that monoacylglycerolphosphate acyltransferase exists in Drosophila heads (6). The other glycerolipid to be affected by the defect in DG kinase activity is DG. DG content in the mutant would be expected to be higher because of the lack of DG kinase. The content of DG in rdgA mutant eyes, however, was found to be within the normal level. One possible explanation about the result is that DG may be supplied not from PI with phospholipase C but from triglyceride with lipase. This is consistent with the data that triglyceride content in Drosophila heads is high (data not shown), and fatty acid component of DG was significantly different from that of PI as we previously reported (21).

These results indicate that the absence of DG kinase activity influences only the PA content of Drosophila eyes. It is pertinent to ask why radioactivity of 32P-labeled PI in reti-

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ular cell of rdgA mutant is maintained at a normal level, even though the content of the precursor lipid [32P]PA is very low. This apparently contradictory fact can be explained by the idea of multiple metabolic pools of inositol lipids, which stem from the studies by Fain and Berridge (24) on blowfly salivary glands in which they obtained quite different results with pulse-chase and equilibrium-labeling experiments. According to Fain and Berridge, there are two kinds of PI pools, responsive and nonresponsive. Responsive PI turns over rapidly upon stimulation, and the responsive PI content in the total PI was estimated to be about 10%. Further demonstrations of the coexistence of two nonmixing PI pools within a single cell came from the studies of a vasopressin-sensitive mammalian cell line (25, 26), and in the studies of thyrotropin-releasing hormone-stimulated GH3 cells (27), and vasopressin stimulated liver cells (28). When we feed [32P]P; to Drosophila, the radioactive PI may be more abundant in nonresponsive PI pool, not in the responsive pool which is synthesized through DG kinase. This probably explains why PI radioactivity was not affected in rdgA mutant eye, as shown in Fig. 2. It also explains why the PI content in Drosophila brain is roughly equal to that in the eye, even though the activity of DG kinase in the brain is extremely low in comparison with that of the eye (10). The detailed mechanisms are still unknown as to how DG kinase defects lead to retinular degeneration in the Drosophila mutants.

If DG kinase can be suppressed by an inhibitor, a similar abnormality will be induced as in the case of rdgA mutant eye. Use of a DG kinase inhibitor, R59022, was attempted by de Charffoy de Corucelles, et al. (29) with intact platelet. They found that the inhibitor caused reduction in the PA formation and increased protein kinase C (Ca2+/phospholipid-dependent enzyme) activity. From the results obtained in their experiments, DG kinase is viewed as a major “switch” that turns off the activity of protein kinase C (22, 30). It is probable that the lack of DG kinase in rdgA mutant perturbs the normal cellular control of protein kinase C. Eventually this may lead to destruction of retinular cells.

If the relation between DG kinase deficiency and retinular cell degeneration discussed above is true, the relation will also hold true in the norpA mutant. As we previously reported (6), DG kinase activity was decreased and PA is not labeled by [32P] in the norpA mutant as in rdgA mutant. However, gene dosage dependence was not shown in the norpA mutant (Table II). This implies that norpA gene does not code DG kinase. DG kinase activity for three alleles of norpA mutant was found to be 58% of normal value in norpA6341, 36% in norpA6387, and 17% in norpA6285. According to Hirosawa,3 the rhabdomere size of norpA6285 decreased to 1/3 of normal. The order of the rhabdomere size among those alleles was consistent with the biochemical findings discussed above. Thus, it is concluded that deficiency in DG kinase of norpA mutant may also be related to the smaller size of rhabdomere.

Another possible link between absence of DG kinase and retinular cell degeneration in rdgA mutants may lie in the altered phospholipid compositions which would alter biophysical properties of specialized, differentiated cell membrane. It is known that phosphatidylserine is a major component of the inner surface of plasma membrane (31, 32), whereas phosphatidylycerine is enriched in endoplasmic reticulum (33). PA may be required for constructing small diameter, high curvature structures, such as microvilli in rhabdomere.

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