Localization of *Drosophila retinal degeneration B*, a Membrane-associated Phosphatidylinositol Transfer Protein

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Abstract. The *Drosophila retinal degeneration B* (*rdgB*) mutation causes abnormal photoreceptor response and light-enhanced retinal degeneration. Immunoblots using polyclonal anti-rdgB serum showed that rdgB is a 160-kD membrane protein. The antiserum localized the rdgB protein in photoreceptors, antennae, and regions of the *Drosophila* brain, indicating that the rdgB protein functions in many sensory and neuronal cells. In photoreceptors, the protein localized adjacent to the rhabdomeres, in the vicinity of the subrhabdomeric cisternae. The rdgB protein's amino-terminal 281 residues are >40% identical to the rat brain phosphatidylinositol transfer protein (PI-TP). A truncated rdgB protein, which contains only this amino-terminal domain, possesses a phosphatidylinositol transfer activity in vitro. The remaining 773 carboxyl terminal amino acids have additional functional domains. Nitrocellulose overlay experiments reveal that an acidic amino acid domain, adjacent to the PI transfer domain, binds $^{45}Ca^{+2}$. Six hydrophobic segments are found in the middle of the putative translation product and likely function as membrane spanning domains. These results suggest that the rdgB protein, unlike the small soluble PI-TPs, is a membrane-associated PI-TP, which may be directly regulated by light-induced changes in intracellular calcium.

To date, molecular and genetic analyses have identified only one protein that is used in multiple invertebrate sensory systems. The *Drosophila retinal degeneration-B* (*rdgB*)[1] mutant affects both the visual and olfactory sensory systems. The *rdgB* mutant was initially identified by defects in the compound eye, in that *rdgB* mutant flies undergo light-enhanced photoreceptor cell degeneration (Hotta and Benzer, 1970). The initial evoked light response of *rdgB* photoreceptors is defective and deteriorates further with light exposure (Harris and Stark, 1977). Genetic evidence suggests that *rdgB* gene product acts within the light-triggered phosphoinositide cascade responsible for phototransduction. Mutations at the *norpA* locus, which encodes a phospholipase C (Bloomquist et al., 1988), suppress *rdgB* degeneration (Harris and Stark, 1977; Stark et al., 1983). The allele-specific suppression of $rdgB^{KS222}$ degeneration by $norpA^{sull}$ further suggests that the rdgB protein may interact directly with the *norpA*-encoded phospholipase C (Harris and Stark, 1977). Mutations at an eye-specific protein kinase C gene also suppress degeneration in *rdgB* flies (Smith et al., 1991). Biochemical experiments are also consistent with *rdgB* playing a role in the phosphoinositide cascade: a water soluble phorbol ester (phorbol 12,13-dibutyrate), thought to activate protein kinase C, causes photoreceptor degeneration of dark-protected *rdgB* flies (Minke et al., 1990). Assignment of a role for the *rdgB* gene product in the *Drosophila* olfactory system comes from genetic studies. An *rdgB* allele was recovered in a screen for *Drosophila* olfaction mutants, and some previously isolated *rdgB* alleles were subsequently demonstrated to be defective in olfaction (Woodward et al., 1992). Study of the *rdgB* gene and protein product should help identify the biochemical defect responsible for retinal degeneration and specify its roles in the visual phosphoinositide cascade and the olfactory system.

We found previously that the *rdgB* gene encodes at least five different mRNAs expressed in the retina and other head tissues (Vihtelic et al., 1991). *rdgB* expression in head tissues outside the retina differentiates it from other characterized visual transduction components, and is consistent with a role in olfaction. The putative translation product has several hydrophobic segments and a highly acidic region, suggesting that the rdgB protein is a calcium-binding, integral membrane protein.

In this report, we show that the rdgB protein is an integral membrane protein expressed in photoreceptor cells, chemosensory neurons, and sensory processing centers of the central brain. In photoreceptor cells, the protein is localized to the subrhabdomeric cisternal membranes adjacent to the rhabdomeres. The rdgB protein shares sequence identity with the rat brain phosphatidylinositol transfer protein

1. *Abbreviations used in this paper:* MBP, maltose binding protein; NFDM, non-fat dry milk; PI-TP, phosphatidylinositol transfer protein; *rdgB*, retinal degeneration-B; SB, standard buffer; SRC, subrhabdomeric cisternae.

(PI-TP; Dickeson et al., 1989). Yeast mutants lacking the functional PI-TP gene *sec14* are defective in vesicular transport (Bankaitis et al., 1989). We demonstrate that this region of rdgB possesses phosphatidylinositol transfer activity in vitro. The region adjacent to the PI-TP domain binds $^{45}Ca^{2+}$ on overlays. The subcellular location of the rdgB protein within photoreceptor cells, and its homology with PI-TP, suggests that rdgB is involved in membrane transport to the rhabdomeres.

Materials and Methods

Polyclonal Antiserum to the rdgB Protein

To obtain antiserum for immunolocalization of the rdgB protein, a BamHI fragment (residues 1046–2989 in Fig. 4; Vihtelic et al., 1991) that includes the six putative transmembrane domains of the rdgB protein was subcloned into the pGEMEX-1 plasmid (Stratagene Inc., La Jolla, CA), and expressed as a T7 gene 10 fusion protein. The orientation and ORF of the construct was confirmed by DNA sequence analysis. SDS-PAGE analysis of bacterial extracts confirmed production of the fusion protein at the appropriate molecular weight. The fusion protein band was excised from the SDS-PAGE, purified by electroelution, and used as an immunogen in mice (Catty and Raykundalia, 1988). The antiserum obtained from the immunized mouse recognized the purified protein in Western blot analyses and elicited a positive signal in *Drosophila* head sections. The preimmune serum did not stain *Drosophila* head sections.

To generate polyclonal antiserum to the amino terminal portion of the rdgB protein, a cDNA encoding the amino terminal 449 amino acids of rdgB was subcloned into the pMal-cRI vector (New England Biolabs, Inc.) using a two-step strategy involving site-directed mutagenesis and PCR amplification. First, an EcoRI restriction site was altered at nucleotide No. 1218 by site-directed mutagenesis (Kunkel, 1987) using primer rdgB3 (5′ CGGATGAGGAGTTCTTTGATTGCC). Second, the mutagenized cDNA template was PCR amplified between nucleotides −14 and 1358, which is capable of encoding amino acids Nos. 1–449 (Vihtelic et al., 1991). This portion of the rdgB protein contains both the PI-TP domain and the acidic domain. The forward primer (5′ CACGCGGTGAATTCATGCTGATCAAGG) contains nucleotides −14 to +13, which encodes the rdgB protein's putative translation initiation codon and is modified to introduce an EcoRI restriction site. The reverse primer (5′ CTTGCCGCGAATTCCCTCACCCACCCAC) contains nucleotides 1339 to 1358, which was modified to introduce an EcoRI restriction site and a translational termination codon. PCR reactions were carried out using 100 ng of template DNA and primers at 1 mM each according to the TA Cloning System instructions (Invitrogen Corp.). The PCR product was digested with EcoRI, agarose gel purified and ligated into the EcoRI digested pMal-cRI plasmid. Plasmid constructs were transformed into DH5α cells and the orientation of the subcloned insert was determined by restriction digest and DNA sequencing.

A similar construct, but which contains only the rdgB PI-TP domain (amino acids No. 1–296) was generated by an analogous PCR reaction as described above. The same forward primer was used, but the reverse primer (5′ GCCGGAGCGGAATTCTTTCACTCGCTGC) corresponding to nucleotides 881 to 908 of the rdgB protein (Vihtelic et al., 1991) and modified to introduce an EcoRI restriction site and a translational termination codon was used. The PCR reaction and subcloning were as described above.

The fusion protein was expressed as recommended (New England Biolabs, Inc., Beverly, MA) and analyzed by SDS-PAGE. The bacterial lysate was loaded onto an amylose resin affinity column and fusion protein eluted and concentrated according to the recommended protocol (New England Biolabs, Inc.). The affinity-purified fusion protein was injected into mice and antiserum collected and analyzed as described above. The antiserum recognized the purified protein in Western blot analyses and elicited a positive signal in *Drosophila* head sections equivalent to the polyclonal antisera generated against the larger rdgB fusion protein described above. No signal was evident when preimmune serum was used.

Immunohistochemistry

Immunofluorescent detection of antibody-stained *Drosophila* heads was performed essentially as described (Fujita et al., 1982). Wild-type and *rdgB* adult heads were frozen in OCT (Miles Laboratories) and 8-μm sections were cut on a Zeiss Microm cryostat (Carl Zeiss, Oberkochen, Germany). Sections were retrieved onto Superfrost Plus slides (Fisher Scientific Co., Pittsburgh, PA) and allowed to dry for at least 30 min at room temperature before staining or storage at −20°C over silica. Sections were fixed for 30 min in phosphate buffered 2% formalin and then washed for 5 min in 0.05% Tween-20/TBS. Primary antibody was diluted 1:1,000 in TBS and allowed to incubate on the slides, in a humidified chamber, for 20 min at room temperature. The slides were washed for 5 min in 0.05% Tween-20/TBS and incubated with a 1:50 dilution of FITC-conjugated goat anti-mouse secondary antibody for 15 min at room temperature and then washed for 5 min in 0.05% Tween-20/TBS. Coverslips were mounted with 90% glycerol and 0.1% phenylenediamine/TBS.

Electron Microscopy

Localization of the rdgB protein by EM was carried out according to protocols described by Van Vactor et al. (1991). Fly heads were removed and prefixed in PLP (0.075 M lysine, 2% paraformaldehyde, 0.01 M $NaIO_4$, 0.037 phosphate buffer, pH 7.4) after which the retinal tissue was dissected from the corneal layer, washed in PBS, and then incubated 2 × 10 min in PBS containing 0.01% saponin. The tissue was then incubated overnight in primary antibody (1:500 antibody in PBS–saponin and 10% horse serum). After two washes in PBS–saponin, the tissue was incubated for 4 h in secondary antibody (1:50 goat anti-mouse-HRP and 10% horse serum in PBS–saponin), followed by a PBS–saponin wash, and then two washes in PBS only. The tissue was fixed in 2% glutaraldehyde for 20 min, washed twice in PBS, and developed with DAB (Vectastain Substrate Kit, Vector Laboratories, Burlingame, CA). The DAB reaction was stopped with 0.01% thimerosol in PBS. Silver intensification was carried out as described by Van Vactor et al. (1991) with the following modifications. The tissue was poststained in 2% OsO_4 in PBS for 60 min, washed 2 × 10 min in PBS and dehydrated by an ethanol series (10-min steps of 30, 50, 70, 80, 95, and 3 × 100% EtOH). the tissue was incubated for 15 min in 1:1 xylene/ethanol, xylene only, 3:1 xylene/Polybed, 1:1 xylene/Polybed, and Polybed resin where it was left overnight at 18°C. The sections were transferred to embedding molds, left at 30°C overnight, 45°C for 8 h, and then 60°C overnight. EM sections were poststained for 4 min in Reynold's lead citrate. Because staining could be detected in only two or three ommatidia adjacent to an exposed edge of tissue (see Fig. 3 *a*), it is clear that infiltration of the tissue is limited in this protocol.

Western Blot Analysis

Protein extract was obtained by homogenizing 10 fly heads in 20 μl of extraction buffer (2% SDS, 0.2 M KCl, 3% urea, 10 mM Tris pH 8.0, 2 mM EDTA, 2 mM EGTA, and 5 mM DTT) and incubated for 12 min at 55°C. Tissue debris was pelleted in a microfuge and 15 μl of supernatant removed and mixed with 5 μl of sample buffer. The sample was boiled for 2 min and 12 μl was separated on a 7.5% SDS-PAGE and electroblotted overnight onto nitrocellulose (Nitro ME, Micro Separations, Inc., Westboro, MA). The membrane was blocked in 5% non-fat dry milk (NFDM) for 1 h at room temperature, and then rinsed in TBS + 0.05% Tween-20 (TTBS). Primary antibody was diluted 1:1,000 in 2% NFDM/TTBS and incubated with the membrane for 2 h at room temperature. After three 10-min washes in TTBS, areas of primary antibody binding were detected with ^{125}I-labeled Protein A (specific activity of 1×10^8 dpm/ml) in 2% NFDM/TTBS for 2 h. After washes in TTBS as above, the membrane was dried and exposed to x-ray film for at least 18 h.

The membrane fraction of head tissue was prepared by homogenizing 60 heads in 1 ml 0.25 M sucrose at 4°C. The homogenate was centrifuged at 1,000 *g* for 10 min, the supernatant was saved and the pellet washed in 1 ml of the sucrose buffer and recentrifuged. The two 1-ml supernatants were combined, mixed, and then split into two 1-ml vol, and centrifuged at 150,000 *g* for 60 min. One of the pellets thus obtained was resuspended in 75-μl extraction buffer (see above) and incubated at 55°C for 15 min. 12 head equivalents (30 μl) were combined with 10 μl of 4× sample buffer for electrophoresis on 7.5% SDS-PAGE. 12 head equivalents of cytosol extract was loaded on the gel after concentration (Centricon-10 microconcentrator, Amicon, Inc., Beverly, MA) of the ultracentrifuged supernatant. For alkaline extraction of the membrane fraction, the second membrane pellet obtained above, was resuspended in 1.5 ml of 50 mM $NaHCO_3$ (pH 11), and incubated on ice for 20 min. The membrane was repelleted by centrifugation at 150,000 *g* for 60 min. The pellet and supernatant were treated the same as the initial membrane fraction, and electrophoresed as above.

$^{45}Ca^{2+}$ Binding

The ability of the acidic domain of the rdgB protein to bind calcium was shown by incubation of Western transferred fusion protein with $^{45}CaCl^{2+}$ (Maruyama et al., 1984). Approximately 10 μg of affinity purified fusion protein was separated on a 10% SDS-PAGE. The proteins were electrotransferred at 70 V for 3 h in transfer buffer consisting of 39 mM glycine, 48 mM Tris base (pH 8.3), 0.037% SDS, and 20% methanol. The membrane was then incubated in three changes of overlay buffer (60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl [pH 6.8]) for 1 h followed by incubation in the same buffer containing 1.2 μCi/ml $^{45}Ca^{2+}$ for 10 min. The membrane was washed in 50% ethanol for 5 min, dried thoroughly, and exposed to x-ray film for about 48 h. The amount of $^{45}Ca^{2+}$ bound per mole of fusion protein was estimated by scanning laser densitometry.

Transcriptional Fusion Construct

Expression of recombinant protein for determination of phosphatidylinositol transfer activity was accomplished by subcloning the PI-TP domain of rdgB into a T7 RNA polymerase/promoter vector; pT7-5 (Tabor, Department of Biological Chemistry, Harvard Medical School). This 2.404-kb vector places the T7 RNA polymerase promoter 14-bp upstream of the EcoRI site within the polylinker region. The β-lactamase gene is oriented opposite to transcription by T7 RNA polymerase such that expression by the induced polymerase is limited exclusively to cloned genes.

A PCR reaction to amplify the rdgB PI-TP domain (amino acids No. 1-296) was performed using the primer and reaction conditions described above. The PCR product was digested with EcoRI, agarose gel purified, and ligated into the EcoRI-digested T7-5 plasmid. Plasmid constructs were transformed into DH5α cells and orientation of the subcloned insert was determined by restriction digest. The plasmid construct was transformed into BL21(DE3) cells and successful induction of a 5-ml culture was shown by Coomassie-stained SDS-PAGE and confirmed by Western blot using the antibody against a fusion protein which includes the PI-TP domain (see Polyclonal antiserum to the rdgB protein). The induced protein migrated near the expected molecular weight of 33 kD.

As a negative control, cells were transferred with the T7-5 plasmid which lacked any subcloned sequences. All procedures including induction, SDS-PAGE analysis, Western blot analysis and FPLC chromatography were performed in parallel using both negative and recombinant protein containing extracts.

Expression and Purification of a Truncated rdgB Protein from E. coli

A recombinant, truncated form of the rdgB protein was induced in *E. coli* in order to purify quantities useful in the transfer assay. 4 liters of LB, containing ampicillin (100 μg/ml), were inoculated with 1/200 dilution of overnight culture and grown to $O.D._{600}$=1.5. IPTG (0.1 M) was added to 0.4 mM and the culture grown for 3 h more. Cells were harvested by centrifugation at 1,935 *g* for 20 min at 4°C, resuspended in 2× the cell pellet weight in lysis buffer (10 mM phosphate [pH 7.0], 30 mM NaCl, 0.25% Tween-20, 10 mM β-ME, 10 mM EDTA, 10 mM EGTA) and frozen at −20°C. After a slow thaw, the cell suspension was sonicated in 30-s bursts until the A_{600} was <10% of the initial A_{600}. The sonicated suspension was diluted with lysis buffer to 10× initial cell pellet weight and 10% polyethyleneimine (pH 8.0), added dropwise, while stirring on ice, to 0.1% final concentration and the stirring continued for 30 min. The extract was then centrifuged at 9,750 *g* for 45 min at 4°C and the supernatant saved. Protein in the post-PEI extract (140 ml total volume) was selectively precipitated by addition of ammonium sulfate to 40%, stirred on ice for 60 min, divided into four aliquots, and centrifuged at 12,000 *g* for 1 h. The four pellets were each resuspended in 5 ml standard buffer (SB0) (10 mM Tris-HCl [pH 8.0], 2 mM $CaCl_2$, 0.1 mM EDTA, 1.4 mM β-ME) and dialyzed overnight at 4°C against the same buffer plus 50 mM KCl (SB50). Dialyzed samples were centrifuged at 4,640 *g* for 10 min to remove any precipitate. The resulting solutions contained 10–15 mg/ml protein (Bradford, 1976) and were subjected to Mono Q FPLC chromatography.

The Mono Q column was equilibrated with SB50 buffer and the proteins eluted in 0.5-ml fractions over a linear gradient of KCl from 0.2 to 0.6 M (SB50 with 1 M KCl). The recombinant protein, as determined by Western analysis, eluted at approximately 0.4 M KCl. Four fractions were identified which contained the recombinant protein between 70 and 110 μg total protein per fraction. Four equivalent fractions from FPLC chromatography of the negative extract were selected based on elution at 0.4 M KCl. They contained 56 to 142 μg total protein per fraction.

Phosphatidylinositol Transfer Assay

Liposomes were prepared by drying 142 nmoles total phospholipid containing 0.25 μCi L-3-phosphatidyl[2-^{3}H]inositol (19.1 Ci/mmol; Amersham Corp., Arlington Heights, IL), 6.29 μg phosphatidylinositol, 103 μg phosphatidylcholine, and 0.023 μCi cholesteryl [1-^{14}C] oleate (53.9 mCi/mmol; Amersham Corp.) under a nitrogen atmosphere followed by lyophilization overnight. The dried lipids were resuspended by vortexing in 1.0 ml of 10 mM Hepes (pH 7.4), 140 mM KCl, 1 mM DTT, 1 mM Na_2EDTA, and 1% BSA (assay buffer with BSA). Small unilamellar vesicles were formed by sonication in a water bath sonicator at room temperature until the solution cleared (Zilversmit and Johnson, 1973). Large liposomes were removed by centrifugation at 2,500 *g* for 10 min at room temperature.

Mitochondria were isolated from fresh beef heart muscle that was finely minced and suspended in an equal volume of homogenization buffer (0.3 M sucrose, 5 mM MOPS, 1 mM $CaCl_2$, 5 mM KH_2PO_4, 0.1% BSA) containing 7 mg of collagenase per 100 ml of tissue solution and incubated on ice for 1 h. EGTA was added to the solution (2 mM final concentration) to terminate the collagenase reaction. The tissue was drained and 300 ml of fresh homogenization buffer was added per 100 g of tissue. The tissue was homogenized with a Potter-Elvejhem homogenizer using 5 to 8 strokes at 500 to 1,000 rpm. The homogenate was filtered through cheesecloth and centrifuged at 1,500 *g* for 10 min at 4°C. The mitochondrial pellet was washed three times with assay buffer, before finally resuspending in assay buffer (Rickwood et al., 1987). Before use, the mitochondria were heated at 80°C for 20 min, and washed once with assay buffer (Zilversmit and Hughes, 1976).

The phosphatidylinositol transfer assay consisted of 14.2 nmoles of liposomes, 400 μg of mitochondrial protein, and either the truncated rdgB protein, the negative protein mixture, or no additional proteins in a total volume of 500 μl of assay buffer with BSA. Incubations were carried out at 37°C in a shaking water bath. The reactions were terminated by placing the reaction on ice for 2 min, followed by pelleting the mitochondria by centrifugation at 2,500 *g* for 10 min. 450 μl of the supernatant was combined with 5 ml of Liquiscint (National Diagnostics, Manville, NJ) scintillation cocktail. The mitochondrial pellet was washed two times with assay buffer with BSA before being resuspended in 250 μl of assay buffer with BSA and 200 μl 3.0% SDS. The mitochondrial solution was vortexed and added to 5 ml of Liquiscint (Zilversmit and Hughes, 1976). The transfer of ^{3}H-

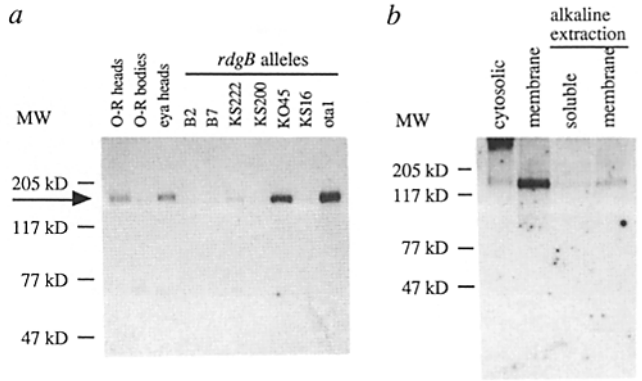

Figure 1. Western blot analysis of the rdgB protein. (a) Analysis of protein isolated from wild type strain Oregon R (*O-R*) heads, O-R bodies, *eya* heads, and heads of seven *rdgB* alleles. A protein band is detected in wild-type, *eya*, $rdgB^{KO45}$ and $rdgB^{ota1}$ heads at 160 kD (*arrow*). This protein is reduced in wild-type bodies and $rdgB^{KS222}$ heads, and not detected in several *rdgB* alleles that also exhibit negative fluorescent antibody staining ($rdgB^{KS200}$, $rdgB^{2}$, $rdgB^{7}$, and $rdgB^{KS16}$). (b) Analysis of the protein in membrane and soluble protein fractions. Oregon-R heads were homogenized in a sucrose buffer and centrifuged to generate soluble and membrane head fractions (see Materials and Methods). rdgB protein is found predominantly in the membrane fraction (lane *2*). The membrane fraction was incubated in an alkali solution to remove peripheral membrane proteins. The rdgB protein remains in the membrane fraction after the alkali extraction (lane *4*); it is not detected in the supernatant (lane *3*).

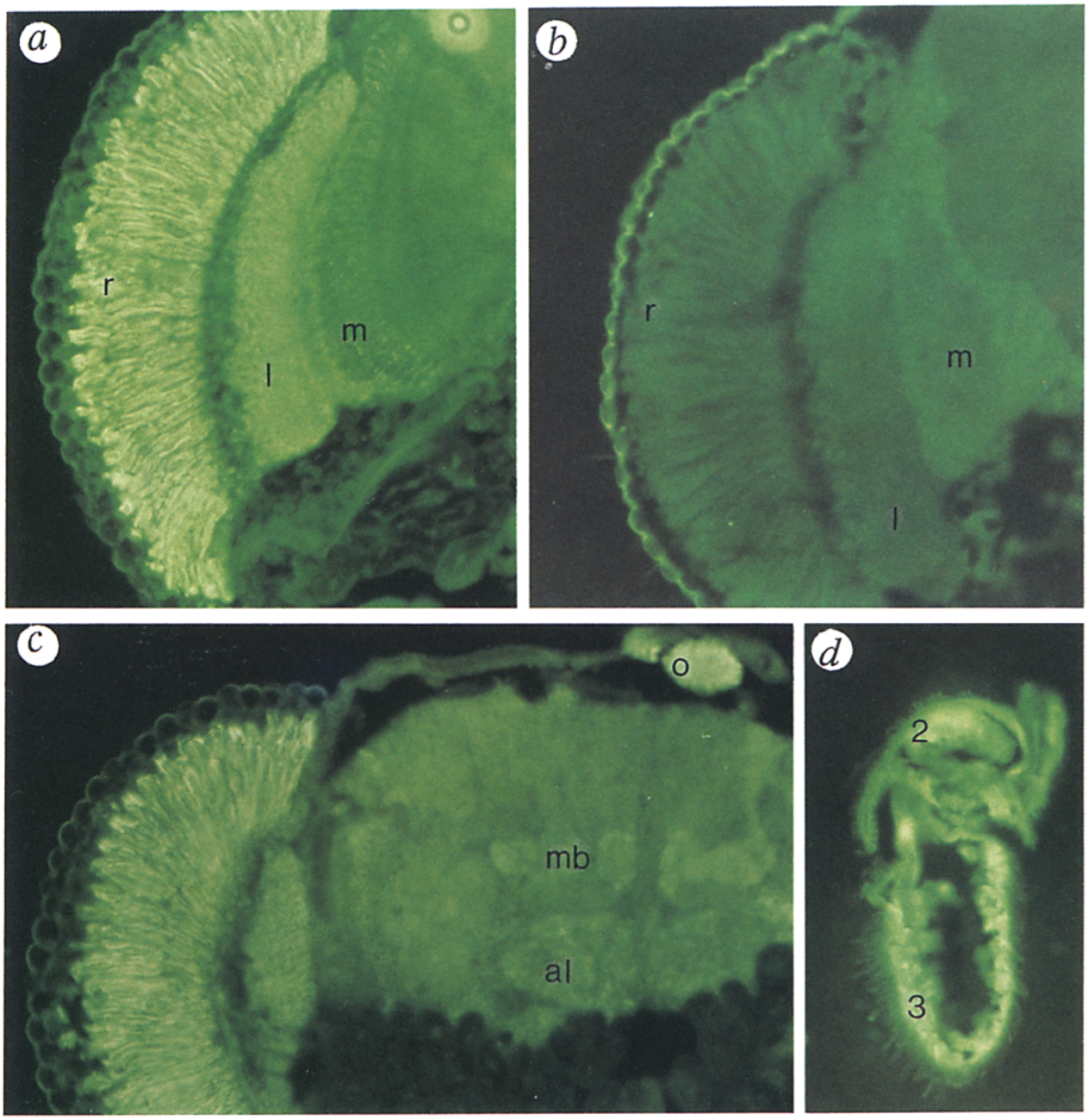

Figure 2. Distribution of the rdgB protein in wild-type and mutant *Drosophila* heads. (a) Frontal section showing retina, lamina, and medulla. The antiserum stains the entire depth of the retina (*r*). Staining is also seen in the lamina (*l*) and medulla (*m*). (b) Frontal section of rdgB^2 shows retina (*r*), lamina (*l*), and medulla (*m*). Note complete lack of staining. (c) Frontal section showing the retina and central brain. The antiserum stains the ocelli (*o*) and regions of the central brain, primarily the antennal lobes (*al*) and the mushroom bodies (*mb*). (d) Longitudinal section showing staining in the second (*2*) and third (*3*) antennal segments.

labeled phosphatidylinositol from the liposomes to mitochondria was determined relative to the [^{14}C]cholesteryl oleate, a nontransferable marker.

Results

Immunoblot Analysis of rdgB Alleles

A rdgB fusion protein, containing 55% of the proposed rdgB protein (Vihtelic et al., 1991) was expressed in the inducible T7 expression vector, pGEMEX and was used to generate a polyclonal antiserum (see Materials and Methods). To determine the size and expression of the rdgB protein in wild-type and mutant flies, we performed an immunoblot analysis. The antiserum detected a 160-kD protein in both wild-type and *eyes absent* (*eya*; Sved, 1986) heads (Fig. 1 *a*, lanes *1* and *3*). The protein is expressed at much lower, but detectable, levels in bodies (Fig. 1 *a*, lane *2*). This is consistent with previous RNA Northern blots in which the *rdgB* mRNAs could be detected in wild-type and *eya* heads, but not in bodies (Vihtelic et al., 1991). The specificity of the antiserum is confirmed by its failure to detect the 160-kD protein in *rdgB*^{2} and *rdgB*^{7} mutant head extracts (Fig. 1 *a*,

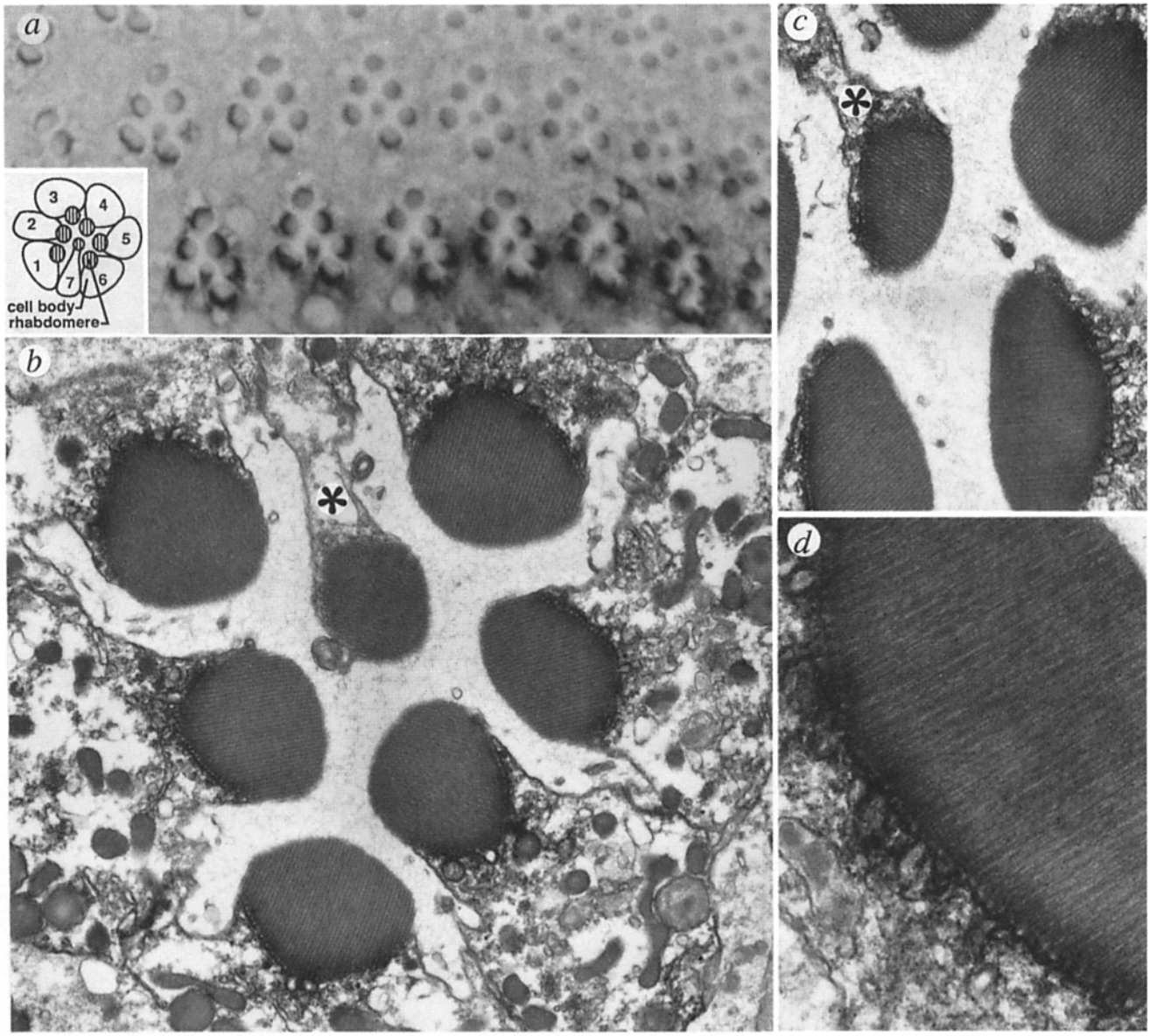

Figure 3. Localization of the rdgB protein in photoreceptor cells. (a) Light microscopic view of a cross-sectioned retinal cell layer following silver intensification of the HRP-detected rdgB antibody. The inset diagram identifies photoreceptors 1–7 within an ommatidial cluster. (b) Electron microscopic section obtained from the same tissue shows that the rdgB protein is located adjacent to the rhabdomeres in R1-6 photoreceptors. The R7 photoreceptor cell is identified by an asterisk (*) in b and c. (c) EM view with the R7 photoreceptor cell. These cells show similar localization of the rdgB protein, but at a lower level than the R1-6 cells. (d) Magnified view of the rhabdomere–cytoplasmic interface in an R1-6 photoreceptor. The heaviest labeling occurs on membranes that are near, but not part of, the rhabdomeric membranes.

lanes *4* and *5*, respectively), even upon longer exposures. These two alleles lack the *rdgB* gene region that is expressed in the fusion protein (Vihtelic et al., 1991).

Two other mutant alleles, $rdgB^{KS200}$ and $rdgB^{KS16}$, lacked detectable rdgB protein in the Western analysis (Fig. 1 *a*, lanes *7* and *9*). These independently isolated EMS-induced alleles have severe mutant phenotypes (Harris and Stark, 1977). The identification of two independently isolated EMS-induced *rdgB* alleles lacking detectable protein further supports the specificity of this antiserum for the rdgB protein. Three additional alleles possess a protein species that migrates at the same molecular weight as the *rdgB* protein. $rdgB^{KO45}$ and $rdgB^{ota1}$ have near normal levels of the protein and $rdgB^{KS222}$ has reduced protein expression (Fig. 1 *a*, lanes *6*, *8*, and *10*).

There are six potential transmembrane domains within the deduced amino acid sequence of the rdgB protein (Vihtelic et al., 1991). To determine whether the rdgB protein is membrane bound, we separated soluble and membrane fractions from wild-type heads and assayed for rdgB protein on Western blots. The rdgB protein was predominantly found in the membrane fraction and only weakly detected in the soluble fraction (Fig. 1 *b*, lanes *1* and *2*, respectively). To determine whether the rdgB protein is a peripheral or integral membrane component, membrane fractions were alkaline extracted to remove peripheral membrane proteins (Hub-

bard and Ma, 1983). Because the alkaline extraction failed to remove rdgB protein from the membrane (Fig. 1 *b*, lanes *3* and *4*), rdgB is likely an integral membrane protein.

Distribution of the rdgB Protein in the Adult Head

We used the antiserum to localize the rdgB protein in frozen *Drosophila* head sections. The antiserum stained the retina and optic lobes (Fig. 2 *a*), the ocelli and the central brain (Fig. 2 *c*), and the antennal segments (Fig. 2 *d*). The antiserum also gave a low level of general staining throughout the brain. The staining in all these tissues, including the low level brain signal, was due to expression of the rdgB protein, as no tissue staining was seen in $rdgB^2$ and other alleles (Fig. 2 *b*) and pre-immune serum failed to stain any of these tissues (data not shown).

The third antennal segment is covered by chemoreceptive sensilla, whose morphology and distribution have been described (Venkatesh and Singh, 1984). On the posterior surface of the segment is a sensory pit, the sacculus, that is lined with one of the three types of sensilla. Fig. 2 *d* demonstrates that the rdgB protein is found in the periphery of the antennal tissue in the region occupied by the tightly packed chemoreceptors of the third segment (Stocker and Gendre, 1988). An inward protrusion of the stained tissue is located dorsal-laterally in the region of the sacculus. This figure also shows the rdgB protein is expressed in the second antennal segment, which functions in mechanoreception (Strausfeld, 1989).

The antennal nerve is formed by axons originating in the three antennal segments (Venkatesh and Singh, 1984) and a cross section of the nerve trunk is easily seen within the brain, ventral–lateral to the antennal lobes (Strausfeld, 1976). The antennal nerve lacked detectable staining by the antiserum, suggesting that the rdgB protein is not found in the antennal receptor axons (not shown). The antennal chemosensory receptor axons terminate in the antennal lobes, the first synaptic neuropil of the antennal sensory system. The antennal lobes are a collection of glomerular-like structures, and consist of chemosensory receptor terminals, dendrites of first order interneurons, and terminals from second order neuropil (Strausfeld, 1976). The fluorescent signal detecting rdgB protein highlighted the characteristic round, clustered appearance of the lobes (Fig. 2 *c*).

The rdgB protein was also found in higher order neuropil structures of the chemosensory compartment. The paired mushroom bodies have been implicated in olfactory memory (Erber et al., 1980; Heisenberg et al., 1985; Nighorn et al., 1991). Fig. 2 *c* shows concentrated rdgB staining in the β and γ lobes of the mushroom bodies; signal was also detected in the α lobes.

EM Localization of the rdgB Protein in Photoreceptors

At the light microscopic level, we found the rdgB protein in a dense linear pattern extending the depth of the retina (Fig. 2 *a*). To confirm and extend this observation, we labeled wild-type heads in situ with anti-rdgB serum and detected the antibody immunohistochemically (see Materials and Methods). Cross sections of photoreceptors showed the protein predominantly localized to the crescent-shaped interface between the rhabdomeric membranes and the cytoplasm of the cell (Fig. 3 *a*). The signal was present in all photoreceptors, though the R7 cell did not stain as intensely as the R1-6 cells. The lack of rhabdomeric staining in these preparations could have been due to the poor permeability of the antibody into these regions of the tissue. However, the same localization results were obtained from frozen head sections (data not shown), establishing that rdgB is not found in the rhabdomeres.

The transmission electron microscope was used to refine the subcellular localization of the rdgB protein. The rdgB protein was detected immediately adjacent to the R1-6 photoreceptor rhabdomeres (Fig. 3 *b*). Weaker staining is detected in the R7 cell at an analogous position (Fig. 3 *c*). Fig. 3 *d* shows a magnified view of the stained region in an R1-6 photoreceptor. The heaviest labeling occurred on membranes closely associated with, but not part of, the base of the rhabdomeric microvilli. This localization suggests that the rdgB protein is associated with the subrhabdomeric cisternae (SRC), an extensive network of membranes derived from the RER or Golgi apparatus and thought to play a critical role in maintenance of the rhabdomeric membranes (Matsumoto-Suzuki et al., 1989). A lower level of staining may be associated with the base of the rhabdomeric microvilli.

The rdgB Protein Possesses a Phospholipid Transfer Domain

The *rdgB* gene encodes a putative protein of 1,054 amino acids that contains six potential transmembrane domains. The amino-terminal 281 amino acids of the rdgB protein are 41% identical and 11% conserved substitutions (Fig. 4) when compared to the entire rat brain PI-TP (Dickeson et al., 1989). There is no significant homology between any portion of the rdgB protein and a bovine liver phosphatidylcholine exchange protein (Moonen et al., 1980) nor a bovine liver nonspecific lipid transfer protein (Westerman and Wirtz, 1985). The first 65 amino acids show the highest degree of homology to PI-TP with 75% identity or conserved substitutions. In addition, 82% (9/11) of the prolines and 57% (8/14) of the glycines in the PI-TP are conserved in rdgB. Because proline and glycine residues are important determinants of β structure, the putative PI-TP domain of rdgB is likely to have the same secondary structure as rat brain PI-TP. The absence of a cleavable signal peptide in the rdgB sequence (Vihtelic et al., 1991) before the six putative transmembrane domains suggests that the amino-terminus, including the PI-TP homologous domain, is within the cytoplasm (Engelman and Steitz, 1981). Fig. 7 shows the proposed topology of the rdgB protein based on the biochemical extractions (Fig. 1 *b*) and conceptual translation of the cDNAs.

To determine if rdgB's PI-TP domain has PI transfer activity, we assayed the ability of a truncated form of the rdgB protein to transfer phosphatidylinositol in vitro. Amino acids 1-296 were expressed in *E. coli* under the transcriptional control of the T7 RNA polymerase promoter. The rdgB protein was partially purified from an *E. coli* strain containing the expressed protein and a control strain lacking a rdgB insert in the vector (see Materials and Methods). The rdgB protein was followed through the purification steps by immunoblotting. A protein fraction containing the expressed rdgB PI-TP domain transfers PI over sixfold faster than the control

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Drosophila rdgB   MLIKEYRIPLPLTVEEYRIAQLYMIAKKSREESHGEGSGVEIIINEPY-KDGPGGNGQYTKKIYHVGNHLPGWIKS   75
rat brain PI-TP   MVLLKEYRVILPVSVDEYQVGQLYSVAEASKNET-GGGEGVEVLVNEPYEKDD-GEKGQYTHKIYHLQSKVPTFVRM

Drosophila rdgB   LLPKSALTVEEEAMECYPYTRTRYTCPFV-EKFSLDIETYYYPDNGYQDNVFQLSGSDLRNRIVDVIDI-VKDQLW  149
rat brain PI-TP   LAPEGALNIHEKAWNAYPYCRTVITNEYMKEDFLIKIETWHKPDLGTQENVHKLEPEAWKHVEAIYIDIADRSQVL

Drosophila rdgB   GGDYVKEEDPKHFVSDKTGRGPLAEDWLEEYWREVKGKKQPTPRNMSLMTAYKICRVEFRYWGMQTKLEKFIHDVA  224
rat brain PI-TP   SKDYKAEEDPAKFKSIKTGRGPLGPNWKQEL---VNQKDCPY------MCAYKLVTVKFKWWGLQNKVENFIHKQE

Drosophila rdgB   LRKMMLRAHRQAWAWQDEWFGLTIEDIRELERQTQLALAKKMGGGEECSDDSVSEP----773 a.a.-------* 1054
rat brain PI-TP   KRLFTNF-HRQLFCWLDKWVDLTMDDIRRMEEETKRQLDE-MRQKDPVKGMTADD*
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Figure 4. rdgB protein homology. Amino acid comparison between rdgB and rat brain phosphatidylinositol transfer protein (*PI-TP*). The sequences are aligned to maximize identity. Identical amino acids are marked (●) and conserved amino acid change marked (○). There is 41% identity with PI-TP over the amino-terminal 281 amino acids of the rdgB protein, and conservative substitutions represent an additional 11% of identified matches. The first of six putative rdgB transmembrane domains begins at residue 499 (Vihtelic et al., 1991).

fraction (Fig. 5 *a*). When the protein concentration was varied, the rdgB containing fraction transferred 80-fold more PI than the control fraction at the highest protein concentration (98 μg, Fig. 5 *b*).

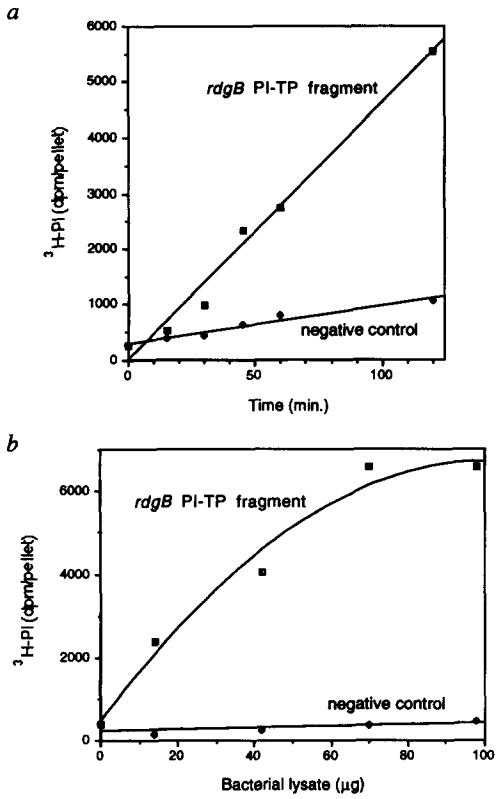

Figure 5. Assay of phosphatidylinositol transfer activity of a particularly purified truncated form of the rdgB protein. (a) Bacterial lysates from strains either expressing or not expressing a recombinant protein corresponding to the amino terminal 296 residues were partially purified (see Materials and Methods). 20 μg of protein was assayed for PI-TP activity as described in Materials and Methods. A fraction containing the recombinant rdgB's PI-TP domain (rdgB protein) transferred phosphatidylinositol over sixfold faster than a control fraction lacking the rdgB sequences (*negative control*). The data shown is representative of three independent analyses performed on different days using different mitochondrial membrane preparations as the acceptor vesicle. (b) The same fractions used in a were assayed by varying the amount of protein over a fixed 60-min reaction. At the highest protein concentration examined (98 μg), the rdgB containing lysate transferred approximately 80-fold more phosphatidylinositol than the negative control.

The rdgB Protein Possesses a Ca^{2+} Binding Domain

We previously noted that two potential Ca^{2+} binding sites were located adjacent to what is now identified as the PI-TP domain (Vihtelic et al., 1991). Both of these sites are located in a highly acidic region of the protein (amino acids 316-335). To determine if this rdgB region binds Ca^{2+}, regions of the rdgB protein were translationally fused to the maltose binding protein (MBP) and purified (see Materials and Methods). Purified MBP, MBP-rdgB PI-TP domain fusion protein, and MBP-rdgB PI-TP and acidic domain fusion protein, were separated by SDS-PAGE (Fig. 6, lanes *1–3*) and electrotransferred to nitrocellulose. The nitrocellulose was incubated with $^{45}Ca^{2+}$ and autoradiographed to determine the levels of $^{45}Ca^{2+}$ bound. Because the maltose binding protein failed to bind any $^{45}Ca^{2+}$ (Fig. 6, lane *4*), any signal observed with the remaining proteins is attributable to the rdgB portion of the fusion protein. An additional negative control, the *Drosophila* $G_{q\alpha}$ sequence (Lee et al., 1990) fused to the T7 gene 10 protein (pGEMEX vector; Promega Biotech, Madison, WI), also failed to bind $^{45}Ca^{2+}$ (data not shown). In this assay, the PI-TP domain appeared to bind $^{45}Ca^{2+}$ at a low level (Fig. 6, lane *5*), but the combination of the PI-TP

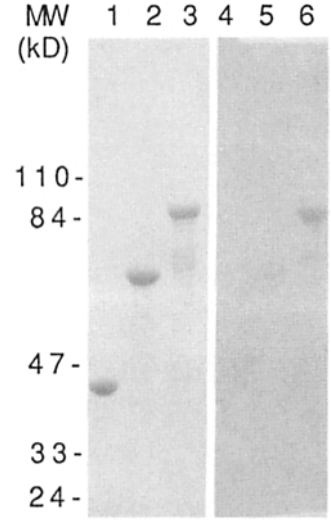

Figure 6. Identification of a Ca^{2+} binding activity in the rdgB protein. Translational protein fusions of portions of the rdgB protein and the maltose binding protein were constructed (see Materials and Methods). The purified maltose binding protein (MBP, lane *1*), purified MBP fused to the PI-TP domain of the rdgB (lane *2*), and the purified MBP fused to the PI-TP and acidic domains of the rdgB (lane *3*) were electrophoresed and transferred to nitrocellulose to be examined for the ability to bind $^{45}Ca^{2+}$. While the MBP fused to the PI-TP and acidic domains strongly bound the $^{45}Ca^{2+}$ (lane *6*), the MBP fused to the PI-TP only weakly bound the $^{45}Ca^{2+}$ (lane *5*), and the MBP failed to bind any detectable $^{45}Ca^{2+}$ (lane *4*). The sizes of protein molecular weight markers are shown along the left side in kilodaltons (*kD*).

and acidic domains bound significantly more $^{45}Ca^{2+}$ (Fig. 6, lane *6*). We estimate that the PI-TP and acidic domains are capable of binding approximately seven times more Ca^{2+} per mole of protein than the PI-TP domain alone. These results support the suggestion that the region from amino acids 296–449 contains a calcium-binding domain.

Discussion

To begin an analysis of the rdgB protein's role in the cell, we investigated the protein's spatial expression and biochemical properties. We used immunoblots to determine the size and location of the rdgB protein. The antiserum's specificity was demonstrated by its failure on immunoblots to detect the rdgB protein in four independently isolated *rdgB* alleles and its detection of altered expression in one additional allele. Unlike other proteins previously identified by their role in visual cell physiology (O'Tousa, 1990), the rdgB protein's expression is not restricted to the visual system, but rather is expressed in multiple primary sensory structures and integration centers of the head. The protein is detected in photoreceptors of both the compound eye and the ocelli, as expected from cell autonomy studies establishing that the gene product is required in photoreceptors to prevent degeneration (Hotta and Benzer, 1971). The lamina and medulla, the first and second optic neuropil, also show rdgB protein expression. It seems likely that expression in these structures is due to the presence of rdgB in the axons of the photoreceptor cells because the medulla shows a regular repeating array of individual units, consistent with the positions and morphologies of the R7 and R8 axons (Zipursky et al., 1984). The protein's axonal localization is consistent with the observation that *rdgB* induces degeneration in the laminal receptor terminals (Stark and Sapp, 1987) and suggests that the rdgB protein is required in the photoreceptor axons. The protein is also expressed in the second and third antennal segments, which contain chemoreceptors and mechanoreceptors. The expression of rdgB within the antenna is consistent with the olfaction defects associated with some *rdgB* alleles (Woodard et al., 1992). Prominent staining is also seen in the antennal lobes and many central brain structures including the mushroom bodies, which appears to process olfactory information (Homberg, 1984).

In photoreceptor cells, the rdgB protein is concentrated in the vicinity of the SRC. The SRC is composed of elaborate extensions of the ER running the length of the photoreceptor (Matsumoto-Suzuki et al., 1989; Baumann and Walz, 1989). The SRC likely plays a role in rhabdomere maintenance by transporting membrane proteins to the rhabdomeric microvilli (Matsumoto-Suzuki et al., 1989) and acting as a source of the Ca^{2+} released during phototransduction (Payne et al., 1988; Baumann et al., 1991). The rdgB protein is not essential for establishing the SRC's structure, because the $rdgB^{EE170}$ allele, which lacks detectable rdgB protein, has a normal SRC ultrastructure before the onset of degeneration (Matsumoto-Suzuki et al., 1989).

The 160-kD rdgB protein band identified by immunoblot analysis is larger than the 116-kD protein predicted from the primary sequence (Vihtelic et al., 1990). The increased apparent molecular weight of the protein may be due to the hydrophobic nature of the potential transmembrane domains, the acidic amino acid regions and/or glycosylation events. However, we are unable to detect any evidence of N-linked glycosylation in the rdgB protein using N-glycosidase F treatment of *Drosophila* head extracts followed by Western blot analysis (Milligan, S., and D. R. Hyde, unpublished results).

The 281 amino terminal residues of the rdgB protein are >40% identical over the entire length of the rat brain PI-TP. Three major classes of phospholipid transfer proteins are distinguishable by the specificity of the phospholipid ligand that is exchanged between lipid bilayers in vitro (Wirtz, 1991). The PI-TP class transports both phosphatidylinositol and phosphatidylcholine, though in vitro the protein shows a marked preference for phosphatidylinositol. We determined that a partially purified *E. coli* protein fraction expressing the PI-TP domain of the rdgB protein catalyzes the exchange of phosphatidylinositol between two membrane compartments in vitro. A control *E. coli* protein fraction lacking the truncated rdgB protein fails to transfer PI. Because of its amino acid similarity to PI-TP, its lack of homology to PC-TP and the nonspecific lipid transfer protein, and its ability to transfer PI, we propose the rdgB protein is a membrane-associated member of the PI-TP class.

All previously characterized phospholipid transfer proteins are cytosolic. Three observations suggest that the rdgB protein is an integral membrane protein. First, the rdgB protein was recovered in the membrane fraction of fly heads. Alkaline extraction of the membrane fraction to remove peripheral membrane proteins (Hubbard and Ma, 1983) failed to liberate the rdgB protein. Second, the rdgB protein is localized to specific regions of the photoreceptor cells, and not within the cytoplasm. Third, the primary sequence of the rdgB protein contains six potential transmembrane domains. The rdgB protein is much larger (160 kD) than other phospholipid transfer proteins (11–36 kD). Although the amino-terminal 25% of the rdgB molecule contains the entire PI-TP homology, molecular analyses of *rdgB* mutations suggest that the COOH-terminal region of the rdgB molecule is critical for proper protein function (Vihtelic et al., 1991). This is also substantiated by the confirmation that amino acids 296–449 are capable of binding $^{45}Ca^{2+}$ on nitrocellulose overlays. We previously suggested that this region of the rdgB protein may possess a Ca^{2+} binding activity (Vihtelic et al., 1991). Although we have not proven that the protein binds Ca^{2+} in vivo, it is interesting to note that activation of the invertebrate visual transduction cascade causes an increase in intracellular Ca^{2+} (Payne, 1986; Payne et al., 1986). This change of free intracellular Ca^{2+} may modulate the PI-TP activity or a related activity associated with the rdgB protein. However, the PI-TP and Ca^{2+} binding domains were biochemically examined independent of other rdgB protein domains. It is possible that other regions of the rdgB protein could mask or modify the biochemical activities that are described here.

Phospholipid transfer proteins may be responsible for intracellular phospholipid trafficking (Kent et al., 1991). The *Saccharomyces cerevisiae sec14* gene encodes a PI-TP that has similar in vitro catalytic properties as mammalian PI-TP. *sec14* mutants are defective in transport of secretory glycoproteins from a late Golgi compartment (Bankaitis et al., 1989; Franzukoff and Schekman, 1989). These findings suggest that one role of PI-TPs is to stimulate membrane transport from the Golgi, which is hypothesized to require an

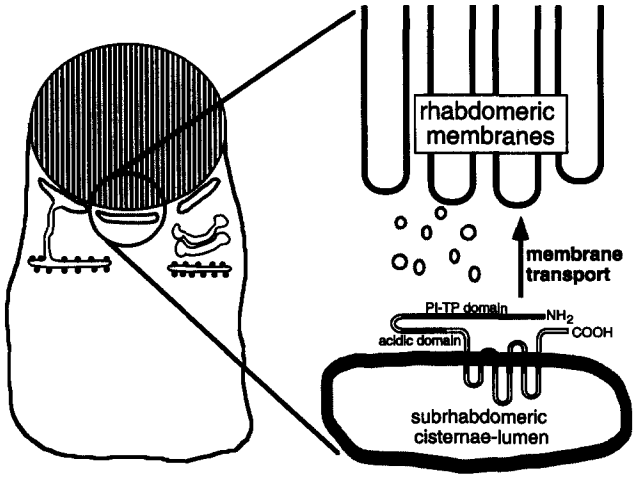

Figure 7. Working model of the function of the rdgB protein in the photoreceptor cell. Model shows a photoreceptor cell and various subcellular structures, such as the ER, Golgi, and the subrhabdomeric cisternae. The SRC is often connected to the rough endoplasmic reticulum by tubules (Matsumoto-Suzuki et al. 1989). Integral membrane proteins, such as rhodopsin, appear to pass through the SRC enroute to the rhabdomeric microvilli (Suzuki and Hirosawa, 1991). The expanded view to the right shows a SRC at the rhabdomere's base. The rdgB protein is situated within the SRC membrane, with the PI-TP and acidic domains located on the cytoplasmic face. These domains are in position to respond to visual transduction messengers, such as intracellular Ca^{2+} levels. This subcellular localization and the PI-TP homology suggests that the rdgB protein regulates membrane transport from the SRC to replenish rhabdomeric proteins and phospholipids.

elevated phosphatidylinositol/phosphatidylcholine ratio for secretory competence (Cleves et al., 1991). One model of rdgB action is that it may promote membrane transport from a specific intracellular compartment (Fig. 7). In photoreceptor cells, rdgB protein is found in the region where the SRC juxtaposes to the rhabdomeric membranes, suggesting that it is involved in membrane movement from the SRC to the rhabdomere. Many essential phototransduction components are expected to move to the rhabdomere through such a transport process (Matsumoto-Suzuki et al., 1989). For example, rhodopsin has been localized in the SRC, presumably on its way to the rhabdomere (Suzuki and Hirosawa, 1991). Additionally, the vesicles that transport rhodopsin to the rhabdomeres will likely be enriched in PI, which can be phosphorylated to produce PIP_2, the substrate for PLC. The rdgB protein may regulate membrane transport to the rhabdomere in response to the intracellular Ca^{2+} level, which is increased by the light-activated visual cascade. A second model supposes that phosphatidylinositol and/or its phosphorylated derivatives may bind as a ligand to the rdgB protein to activate an unidentified function of the rdgB protein. The location of the rdgB protein on the SRC adjacent to the base of the rhabdomere microvilli is consistent with it playing a role in the visual transduction cascade.

The allele-specific suppression of $rdgB^{KS222}$ by $norpA^{null}$ previously suggested that the rdgB protein may be involved in a direct protein-protein interaction with the *norpA*-encoded phospholipase C (Harris and Stark, 1977). Recently, the *norpA* gene product was localized within the rhabdomere (Schneuwly et al., 1991). However, the rdgB protein is localized primarily in the SRC, adjacent to the rhabdomere, and possibly at the proximal plasmalemmal invaginations of the rhabdomeric microvilli. Therefore, only the subset of phospholipase C proteins that are found at the rhabdomere's cytosolic face are able to directly interact with the rdgB protein. This suggests that the allele-specific suppression must be mediated through a mechanism other than a direct protein-protein interaction. An alternative model for the allele-specific suppression is based on the evidence that both $rdgB^{KS222}$ and $norpA^{null}$ are hypomorphs. The $rdgB^{KS222}$ allele is considered the weakest *rdgB* allele of the allelic series and the $norpA_{null}$ allele, unlike other *norpA* alleles, possesses a normal ERG response (Harris and Stark, 1977). A general model for *rdgB* degeneration is that light activation of the cascade, via stimulation of norpA protein activity, results in the alteration of a metabolite's level such that it causes retinal degeneration. The role of the wild-type rdgB product is to reestablish a non-toxic level of this metabolite. Degeneration is blocked in the $norpA^{null};rdgB^{KS222}$ double mutant, because the opposing hypomorphic norpA and rdgB enzyme activities never allow the metabolite to reach a toxic level. For example, the rdgB product could be required to move phosphatidylinositol into the rhabdomeric microvilli, where it is phosphorylated to PIP_2. In *rdgB* mutants, light activation of PLC hydrolyzes PIP_2. Because PI cannot be replenished in the rhabdomeres of a *rdgB* mutant, the membrane experiences a decrease in PI or PIP_2 levels, thereby leading to degeneration. In the $rdgB^{KS222};norpA^{null}$ double mutant, reduced PLC hydrolysis of PIP_2 combined with the reduced replenishment of PI by rdgB results in little net change of PI or PIP_2 levels and therefore no degeneration occurs.

While our work established potential activities associated with the rdgB protein, the mechanism for *rdgB*-induced retinal degeneration remains elusive. Our working model is that *rdgB* is required to transport PI or proteins involved in phototransduction from the SRC to the photoresponsive rhabdomeric membranes. Because *rdgB* mutants show an aberrant light response before there is substantial degeneration (Harris and Stark, 1977), the SRC and rhabdomeres must not have established proper conditions for phototransduction. Matsumoto-Suzuki et al. (1989) have shown that the SRC degenerates before the rhabdomeres, and suggest that the rhabdomeres are gradually lost as a consequence of inadequate maintenance by the SRC. Localization of the rdgB protein to the SRC membrane is consistent with the importance of this organelle in *rdgB* degeneration. Thus, we imagine that regulated interactions between the SRC and rhabdomeres normally establish conditions for phototransduction, and the regulation of these are aberrant in *rdgB* mutants. As a consequence, a cascade of events, perhaps involving a rise in intracellular Ca^{2+} via voltage-gated Ca^{2+} channels (Sahly et al., 1992), leads to degeneration.

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