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ARTICLES

Regulation of PLC-mediated signalling *in vivo* by CDP-diacylglycerol synthase

Louisa Wu, Barbara Niemeyer, Nansi Colley, Michael Socolich & Charles S. Zuker*

Howard Hughes Medical Institute and Departments of Biology and Neurosciences, University of California at San Diego, La Jolla, California 92093-0649, USA

CDP-diacylglycerol synthase (CDS) is an enzyme required for the regeneration of the signalling molecule phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$) from phosphatidic acid. A photoreceptor cell-specific isoform of CDS from *Drosophila* is a key regulator of phototransduction, a G-protein-coupled signalling cascade mediated by phospholipase C. *cds* mutants cannot sustain a light-activated current as a result of depletion of $PtdInsP_2$. Overexpression of CDS increases the amplitude of the light response, demonstrating that availability of $PtdInsP_2$ is a determinant in the gain of this pathway. *cds* mutants undergo light-dependent retinal degeneration which can be suppressed by a mutation in phospholipase C. Thus, enzymes involved in $PtdInsP_2$ metabolism regulate phosphoinositide-mediated signalling cascades *in vivo*.

PHOSPHOINOSITIDE-MEDIATED signalling pathways are a ubiquitous mode of intracellular signal transduction in eukaryotic cells. Phosphoinositides and their cleavage products are a class of second messengers that can be found downstream of many tyrosine kinase receptors and G-protein-coupled seven-transmembrane-helix receptors[1,2]. These second messengers are involved in cell growth and oncogenesis[3,4], differentiation and development[5,6], the action of neurotransmitters and hormones[7], and sensory perception (olfaction, taste and vision)[8–10]. The signals from many of these different cascades converge on the activation of a phospholipase C (PLC). PLC catalyses the hydrolysis of the minor membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$) into the second messengers inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG)[1,2]. $InsP_3$ mobilizes internal stores of calcium, which affects and modulates many cellular processes[11]; DAG activates members of the protein kinase C (PKC) family of proteins[12].

Given the central role of $PtdInsP_2$ in signalling, it may be expected that its levels would be tightly modulated in the cell. Although $PtdInsP_2$-mediated signalling pathways have been studied in great detail in a number of systems, little is known about the metabolic machinery that couples $PtdInsP_2$ synthesis with its availability for signalling. Recent studies have demonstrated that in order to reconstitute PLC activity in permeabilized cells, phosphatidylinositol transfer protein (PtdIns-TP), a protein required for the transfer of phosphoinositides between intracellular membranes, must be added back to the preparation[13]. These results argue that $PtdInsP_2$ is synthesized on demand (as opposed to being stored) and suggest that its metabolism plays an important and direct role in the regulation of phosphoinositide-mediated cascades.

Failure to regulate components of phosphoinositide signalling cascades can lead to severe cellular dysfunction[14]. For instance, uncontrolled signalling from a PLC-mediated pathway can lead to calcium cytotoxicity[15]. Hyperactivation of PKC may lead to uncontrolled cell growth and tumorigenesis[12]. Also, it is hypothesized that the therapeutic effects of lithium in manic-depression therapy involve the depletion of internal inositol[16]. This may limit the amount of $PtdInsP_2$ that can be made in these 'overactive' brain cells and restore signalling to normal levels. Thus, enzymes involved in $PtdInsP_2$ metabolism may serve as important targets for pharmacological intervention of normal and abnormal signalling pathways.

Phototransduction in *Drosophila* is an ideal model system for the study of G-protein-coupled phospholipase-C-mediated sig-

* To whom correspondence should be addressed.

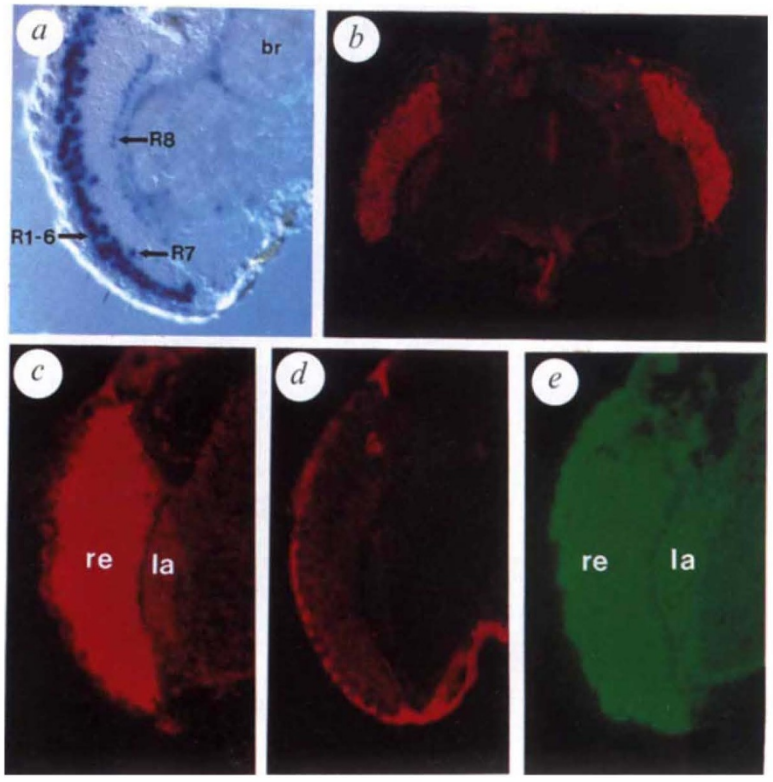


FIG. 1 CDS is a photoreceptor-specific gene. *a*, The enhancer trap line, *cds*^{1}, displays photoreceptor-specific expression of a lacZ reporter containing a nuclear localization signal. Note specific localization of β-galactosidase to the nuclei of the R1-6, R7, and R8 photoreceptor cells. No staining is seen in other tissues; br, brain. *b*, *c*, Indirect immunofluorescence staining of CDS protein in frozen tissue sections of wild-type flies. Note specific expression in the retina. *d*, Immunolocalization of CDS protein in *cds*^{1} mutants, and *e*, *cds*^{1} mutants transformed with a wild-type copy of the eye-CDS cDNA; re, retina; la, laminae.
METHODS. Enhancer trap lines were generated in the laboratories of G. M. Rubin and C. Goodman (UC Berkeley) and shipped to our laboratory for testing. These lines were first prescreened for expression of the lacZ reporter in the head. The lacZ staining was done as described[29] with the following modifications. The sections were dried at room temperature and fixed in 1% glutaraldehyde in PBS for 20 min. For immunocytochemistry, the sections were fixed in 1% paraformaldehyde, in PBS[41] and washed once with 0.1% Triton X-100. Sections were indirectly immunolabelled with an antibody directed to the C-terminal peptide of CDP-DAG synthase (see legend to Fig. 5) followed by a rhodamine or fluorescein-conjugated goat anti-rat antibody (Jackson ImmunoResearch).

nalling processes[10]. Light activation of rhodopsin activates a heterotrimeric G protein of the Gq family[17], which activates a phospholipase C encoded by the *norpA* gene[18,19]. Because the eye is not required for viability, the mechanism of visual transduction in *Drosophila* photoreceptor neurons has been amenable to classical mutational analysis. We and others have done comprehensive genetic and physiological screens to identify and isolate molecules involved in the function and regulation of this cascade[20–24]. Many genes encoding components of this pathway have been characterized; of particular value are those whose role could not have been predicted on biochemical grounds but in which a genetic approach provided fundamental insight as to their functional requirement[10,25]. We now report the isolation and characterization of mutations in an eye-specific CDP-diacylglycerol synthase (CDS), an enzyme required for the generation of $PtdInsP_2$ from phosphatidic acid (PA)[26–28]. The mutant photoreceptor cells show severe defects in their signalling properties which can be genetically rescued by reintroduction of the cloned CDS complementary DNA, and pharmacologically rescued by adding back $PtdInsP_2$. We show that CDS is required to maintain a steady supply of $PtdInsP_2$ during signalling, demonstrating its essential role during PLC-mediated transduction. CDS mutants also show dramatic light-dependent retinal degeneration, demonstrating the pathological effects of the failure to regulate signalling molecules.

Isolation of *cds*

To identify novel regulators of the phototransduction cascade, we screened a collection of P-element enhancer trap lines for retinal lacZ expression and tested positive lines for their electrophysiological response to light stimuli. Of 4,000 lines[29], 270 showed expression of lacZ in the head, and 23 of those demonstrated eye-specific expression. Twelve of these 23 displayed both retinal expression and abnormal electroretinogram responses

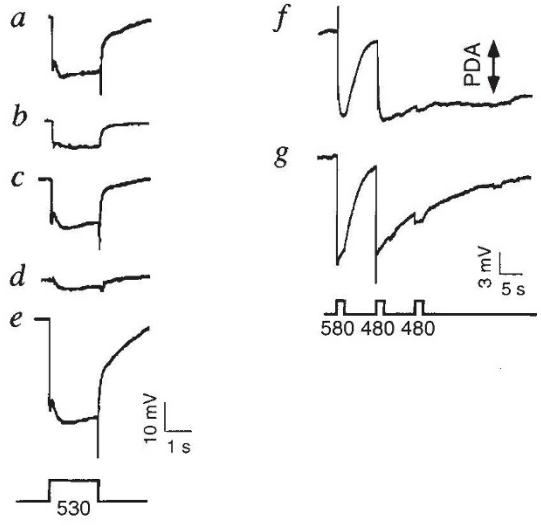


FIG. 2 *cds* mutants show defective light responses. *a*–*e*, Shown are electroretinograms (ERGs) in response to 2 s of 530 nm light. *a*, Control wild-type flies; *b*, *cds*^{1} mutants showing reduced sensitivity; *c*–*e*, responses from three transposase-induced excision classes showing wild-type revertants (*c*), mutants with a reduced response (*d*), and mutants with a much greater response to light (*e*). In all cases, similar results were obtained with all individuals of a given stock. *f* and *g*, PDA recordings from *arr2*^{3} mutants and *arr2*^{3}, *cds*^{1} double mutants. Flies used for assaying the PDA were raised in the dark to prevent retinal degeneration, and aged for 3 days before testing. *arr2*^{3} mutant photoreceptors readily enter a PDA upon blue light stimulation (480 nm)[22], while *arr2*^{3}, *cds*^{1} double mutants are unable to enter or maintain a PDA, even after successive or prolonged blue light stimulation.

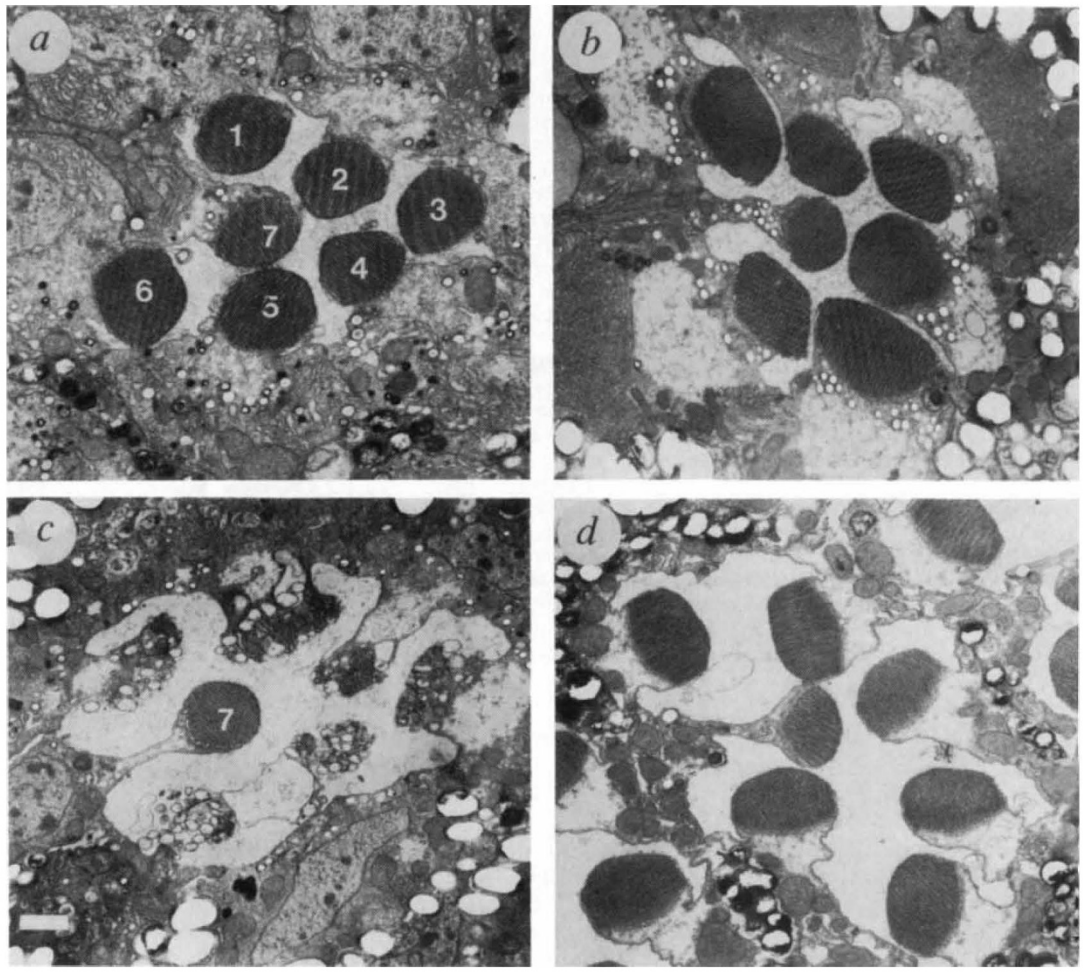


FIG. 3 *cds* mutant photoreceptors undergo severe light-dependent degeneration. Flies were either raised on a 12:12 light–dark cycle or in constant darkness. *a*, Cross-section of a cds^1 retina from a newly enclosed fly. These photoreceptor cells are indistinguishable from wild-type. Numbers identify the rhabdomeres of the R1 to R7 photoreceptor cells. *b*, Retina of cds^1 mutants grown for 10 days in constant darkness. Note the lack of retinal degeneration. *c*, Retina of cds^1 mutants grown for 10 days in the light. Note the massive photoreceptor cell degeneration. The UV-sensitive R7 cells are more resistant to degeneration probably because of their shift in spectral sensitivity[42]. *d*, cds^1; $norpA^{P41}$ double mutants confer protection from the light-dependent degeneration (flies were grown in the light for 10 days). Scale bar, 0.5 μm. METHODS. Adult heads were fixed and processed according to ref. 43. The fixed tissue was dehydrated in serial changes of ethanol followed by propylene oxide and embedded in Spurr's medium (Polysciences). Ultrathin sections were obtained on a Reichert Ultracut E ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate and viewed at 80kV on a JEOL 1200EX electron microscope. For all genotypes described, at least 5 individual heads were sectioned and 100 ommatidia were scored from each eye. Sections taken from the top and the bottom of the eye were observed to ensure that the phenotype was consistent from the apical to the basal regions of the eye.

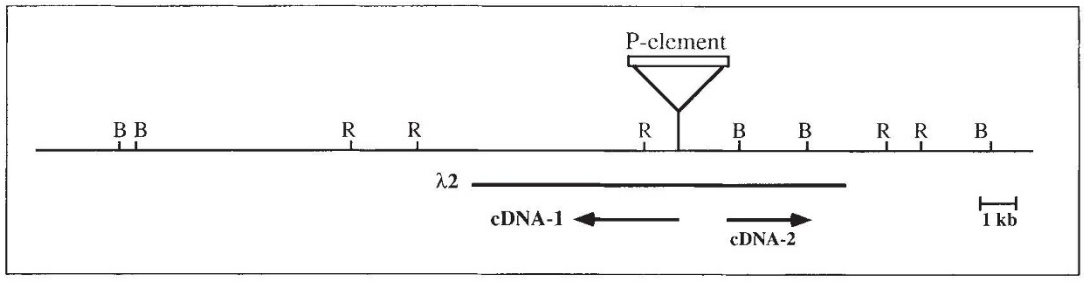
(ERG); ERGs are extracellular recordings of light-induced electrical activity in the eye[20]. None of the 23 inserts mapped to the chromosomal location of any previously known visual mutants. From this set, we focused on the 'cds' line (chromosomal map position 66B[10,11]). cds^1 showed lacZ staining specific to the photoreceptor neurons, including R1-6, R7 and R8 (Fig. 1*a*), and exhibited a significant reduction in light sensitivity (Fig. 2*a*, *b*). Mobilization of the P element by hybrid dysgenesis produced four classes of revertants. The first and most abundant class (24/39), represents precise excisions of the P element; these restore wild-type ERGs (Fig. 2*c*). This class confirmed that the P[w+, lacZ] insertion is indeed the cause of the ERG phenotype. The second class (6/39), still showed a reduced ERG response (Fig. 2*d*). These lines represent imprecise excisions and internal deletions of the P element (data not shown). The third class (6/39) are an unexpected group which, instead of a reduced ERG response, displayed a much greater response to light (super-depolarizing; Fig. 2*e*). This phenotype may represent mis-regulation of the gene tagged by the P[w+, lacZ] insertion because of aberrant excision events, which may not be unexpected because P elements often insert into regulatory regions[30]. This result suggests that the *cds* gene product may be important in regulating the gain of this signalling cascade (see below). The fourth and final class is represented by three lethal lines, demonstrating a new lethal gene in this region of the genome (see discussion).

Degeneration of *cds* mutants

An indication of the importance of *cds* in photoreceptor cell signalling came from the observation that *cds* mutants undergo dramatic light-dependent retinal degeneration. To characterize the degeneration, we examined the ultrastructure of photoreceptor cells by transmission electron microscopy (Fig. 3): at day 1 post-eclosion, cds^1 photoreceptors display normal morphology (Fig. 3*a*), but by 10 days in the light, these cells have undergone dramatic cellular degeneration (Fig. 3*c*). In contrast, *cds* mutants grown in the dark are indistinguishable from wild-type controls (compare Fig. 3*a* and *b*). To demonstrate that the light-dependent retinal degeneration of cds^1 mutants results from the activation of the visual cascade, we generated flies mutant for both *cds* and the structural gene for the effector molecule of this signalling cascade, a phospholipase C encoded by the *norpA* (*no receptor potential A*) locus[18,19]. The *norpA* mutation protects cds^1 flies from retinal degeneration (Fig. 3*d*), demonstrating that the events responsible for degeneration occur downstream of phospholipase C activation.

cds encodes a CDP-DAG synthase

To gain insight into the nature of the defect in *cds*, we isolated the gene tagged by the P[w+, lacZ] transposon. A plasmid rescue of the P element provided an entry for a chromosomal walk of the region (Fig. 4). A 10-kilobase (kb) genomic fragment from the P insertion site (clone λ2 in Fig. 4) was reintroduced into cds^1



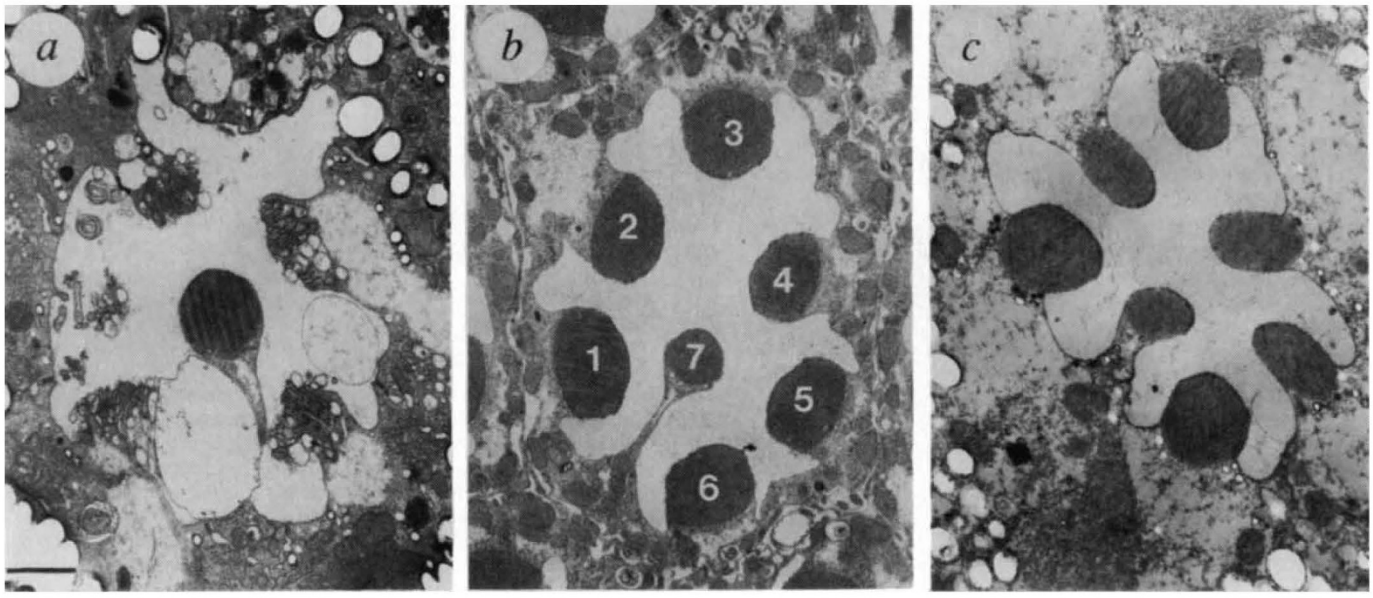


FIG. 4 Genomic walk of the 66B[10,11] region and identification of the CDS cDNA. The diagram shows a 30 kb interval from this genomic region and indicates the genomic DNA used in the rescue experiments (λ2). cDNA-1 fully rescues the morphological and physiological defects of *cds*^{1} mutants. *R*, *Eco*RI and B, *Bam*HI. *a*, Electron micrograph of a cross-section from a *cds* retina after 10 days in the light. *b*, Photoreceptor cells from a wild-type revertant grown for 10 days in the light, demonstrating that the P-element insertion is the cause of the phenotype. *c*, Retina from mutant animals transformed with cDNA-1. The transgene fully rescues the retinal degeneration. Scale bar, 1 μm.

mutants and shown to rescue fully the light-dependent retinal degeneration of *cds*^{1} mutants (data not shown). Genomic DNA from this fragment was used to screen RNA blots and a retinal cDNA library. Two transcriptional units running in opposite directions from each other, and encoding two main transcripts of 2.5 and 2.4 kb, respectively, were identified (arrowheads in Fig. 4). The original P-element insertion is located within the 2.5-kb transcriptional unit (cDNA-1 in Fig. 4). A cDNA corresponding to the 2.5-kb transcript was placed under the control of a photoreceptor-cell specific promoter[22] and reintroduced into the *cds* mutants by P-element-mediated germline transformation. This cDNA fully rescued the ERG and retinal degeneration phenotype of *cds* mutants (Fig. 4*c*). Identical results were obtained with the P-element jumpout lines that led to precise excision of the P element (Fig. 4*b*).

Sequence analysis of the 2.5-kb cDNA showed that it encodes a predicted polypeptide of 447 amino acids (M_r 49K). A search of the protein and nucleotide databases revealed that CDS shares 31% amino-acid identity with the bacterial enzyme CDP-diacylglycerol synthase (CDS) (Fig. 5*a*)[31]. We have overexpressed CDS in bacteria and demonstrated that it functions as a CDP-DAG synthase (Fig. 5*d*). CDS catalyses the synthesis of CDP-diacylglycerol (CDP-DAG) from phosphatidic acid and CTP (Fig. 5*b*)[26,27]. CDP-DAG is an essential precursor in the biosynthesis of phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylinositols[26,27]. This *Drosophila* CDS is the first eukaryotic CDP-DAG synthase cloned so far.

Given the importance of anionic phospholipids, and notably inositides, in cellular metabolism, it may be expected that CDP-DAG synthase mutants be non-viable. However, *cds*^{1} mutants are fully viable and only display a visual defect phenotype, suggesting that CDS may represent a tissue-specific isoform of the enzyme. To determine the tissue distribution of CDS, we generated polyclonal antibodies to selected peptides based on the predicted amino-acid sequence of CDS, and used these antibodies to examine CDS distribution in wild-type and mutant animals. The antibodies recognize a 49K protein that is expressed in the fly head but not in the body (Fig. 5*c*). Within the head, the protein is expressed primarily in the retina. Importantly, this protein is missing in the *cds*^{1} mutants but is restored in the transgenic lines (Fig. 5*c*). To determine more precisely the cellular distribution of CDS, we did immunocytochemical analysis of frozen tissue sections of adult heads. The antibodies show that CDS is localized to the photoreceptor neurons, both in the compound eyes and ocelli (Fig. 1*b–e*, and data not shown). We have correspondingly renamed CDS as eye-CDS.

Eye-CDS is essential for phototransduction

The finding that eye-CDS is a photoreceptor-specific protein is particularly interesting because fly phototransduction is a PLC-mediated signalling cascade[10,25]. This suggests that eye-CDS may be required to provide a continuous supply of $PtdInsP_2$ in this pathway. To determine whether eye-CDS mutants have a defect in their signalling properties, we assayed wild-type and mutant

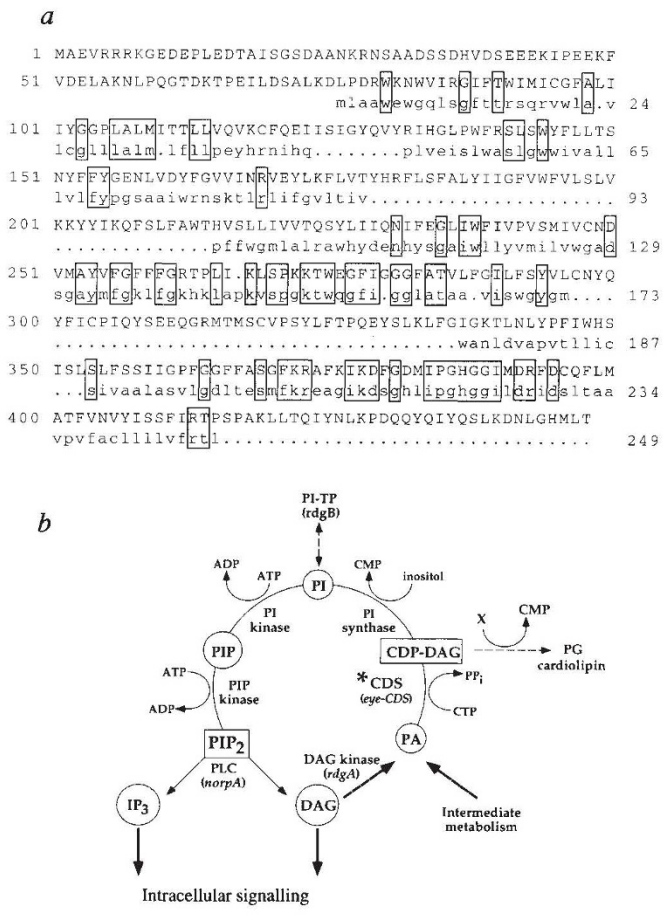


FIG. 5 The *cds* gene encodes a photoreceptor-specific CDP-diacylglycerol synthase. *a*, An alignment of the amino-acid sequence of CDS (top line, upper case) to bacterial CDP-diacylglycerol synthase (bottom line, lower case)[31]. The proteins display 31% sequence identity, and 58% amino-acid similarity. This enzyme converts phosphatidic acid (PA) into CDP-DAG. These are intermediates in the regeneration of $PtdInsP_2$ from DAG. *b*, $PtdInsP_2$ (PIP_2) regeneration cycle[26–28]. Upon activation, phospholipase C (PLC) hydrolyses $PtdInsP_2$ to yield $InsP_3$ (IP_3) and DAG. To regenerate the $PtdInsP_2$, DAG is phosphorylated by diacylglycerol kinase to produce phosphatidic acid (PA). CDP-diacylglycerol synthase adds on CMP to phosphatidic acid. This product, CDP-diacylglycerol, is the activated donor of the phosphatidyl group to inositol. The phosphatidylinositol (PI) is phosphorylated by PI kinase and PtdInsP (PIP) kinase to yield $PtdInsP_2$. Listed in parentheses are the sites of action of known photoreceptor cell-specific genes in *Drosophila*[18,19,44,45]. *c*, CDS is a photoreceptor-specific protein. Antibodies raised against an N-terminal peptide of the CDS protein recognize a 49K protein that is present in the head of flies, not in the body and greatly diminished in the heads of flies with no eyes (*eya* mutants). As expected, the protein is missing in *cds* mutant flies (flies were raised in the dark to prevent retinal degeneration). cds^1 represents the original enhancer trap allele, cds^7 is one of the three lethal alleles. Note the lack of protein in the cds^1/cds^7 heterozygous animal. P[cds^+] refers to transgenic flies carrying a copy of the cloned *cds* cDNA under control of the Arr2 promoter.* Dimers of CDS. *d*, CDS functions as a CDP-diacylglycerol synthase. The CDS protein was overexpressed in bacteria using the T7 expression system[46], and assayed as described previously[47]. Shown is an activity histogram of enzyme assays before (U) and after (I) induction ($n = 4$). METHODS. Nested deletions of the eye-CDS cDNA were generated by exonuclease III digestion[48]. Dideoxy DNA sequencing[49] was done using the Sequenase kit (US biochemicals). The CDS N-terminal peptide, EVRRRKGEDEPLEDT and C-terminal peptide, KPDQQYQIYQSLKDN were used to generate monospecific polyclonal antibodies. Similar results are obtained with both antibodies. The antibodies were generated and affinity purified as described[50]. With the exception of the body lane (1 body per lane), all lanes contain protein extract from 10 heads. The CDS assays were done as described[47], with the following modifications. The crude bacterial extracts were sonicated in 20 mM Tris pH 7.8, 0.1 mM PMSF, 0.001 mg ml^{-1} leupeptin, 0.001 mg ml^{-1} pepstatin on ice. The CDS assays were done in 0.1 M Tris pH 7.5, 0.2 M KCl, 0.01 M $MgCl_2$, 1 mg ml^{-1} BSA, 5 mM Triton X-100, 0.25 mM dithiothreitol, 1 mM phosphatidic acid (Sigma, PA mixture), 1 mM dCTP and 5 mM [α-^{32}P]dCTP.

animals for their ability to maintain a continuously activated state of the photoreceptor cells. Such a state would require the continuous availability of the second messenger $PtdInsP_2$. We used the following strategy to test this hypothesis. We have previously shown that in the absence of arrestin, a protein required for the inactivation of G-protein-couped receptors, light-activated photoreceptors readily enter a continuously activated state known as a prologned depolarized afterpotential or PDA, due to the presence of activated metarhodopsin and no inactivation mechanisms[22]. We generated cds^1; $arr2^3$ double mutants and compared their response to those of control *arr2* flies. Figure 2*f* shows that, although *arr2* mutants readily enter and maintain a PDA, cds^1;*arr2* double mutants cannot maintain a PDA (Fig. 2*g*). Indeed, *cds* mutants cannot enter a PDA even in a non-arrestin mutant background, nor can they sustain a light response following strong light stimulation (data not shown). These results suggest that light activation depletes the pool of a transmitter (or transmitter precursor) necessary for excitation that cannot be replenished in *cds* mutants.

Using whole-cell patch-clamp recordings[32,33], we analysed in detail the electrophysiological responses of wild-type and *cds* photoreceptors. We recorded under conditions where the lifetime of the patch is not critical and where negative feedback and calcium-dependent inactivation mechanisms can be excluded. Control and mutant cells were dissected and transferred to a bath solution with nominally zero calcium. The excitation mechanisms were then depleted before patching by subjecting the cells to 40 min of a light-pulse protocol, consisting of 3 s of intense light pulses followed by 3 min in the dark[34]. If wild-type cells are patched after the depletion protocol, the recovery of the light response depends on the inclusion of calcium in the patch pipette. Pipette solutions with 10 nM free Ca^{2+} do not rescue the light response, but when the internal calcium is raised to 700 nM, the light response reliably recovers in wild-type cells

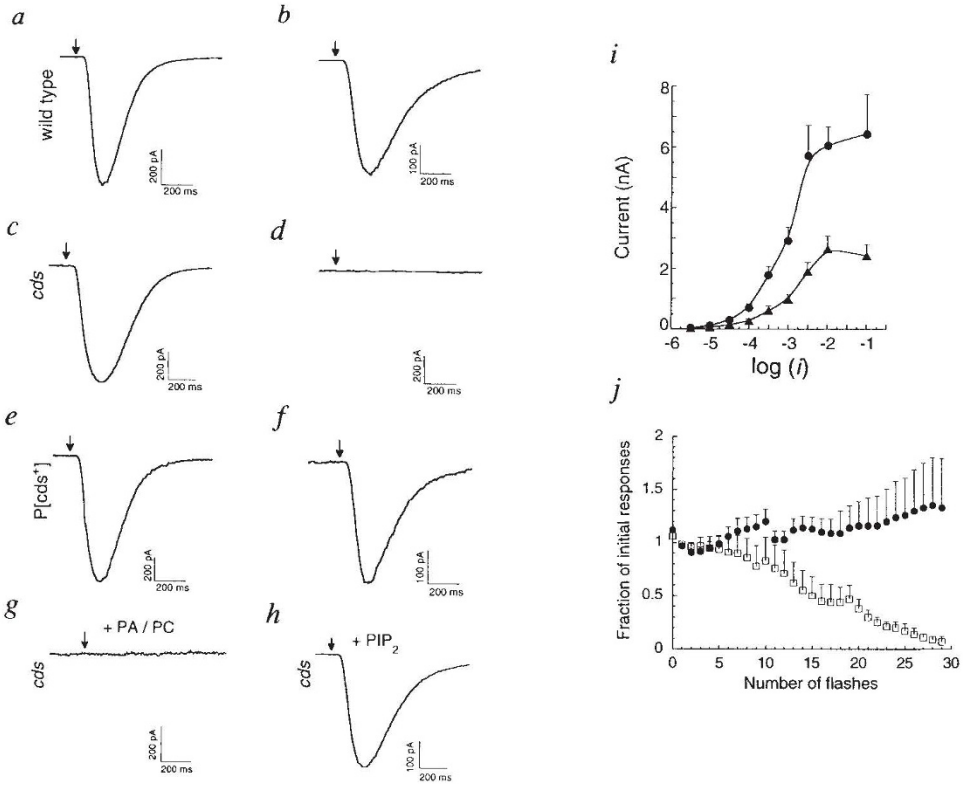


FIG. 6 Defects shown by *cds* mutants in photoreceptor cell function. *eye-cds* mutants fail to recover light responsiveness after light-induced depletion (see text for details), but can be rescued by adding back either a PtdIns/PtdInsP/PtdInsP$_2$ mixture or PtdInsP$_2$. *a*, *c*, *e*, Responses of wild-type, *cds*^{1} and P[cds$^+$] photoreceptors before depletion, respectively. After depletion, 700 nM [Ca^{2+}]$_{in}$ in the patch pipette restores the light response in wild-type cells (*b*) and rescue cells (*f*), but not in the *cds*^{1} mutants (*d*). A phospholipid mixture of PA and PtdC does not restore responsiveness (*g*). However, depleted *cds*^{1} mutants can be rescued by supplying PtdInsP$_2$ through the patch pipette (*h*). Arrows indicate the position of the stimulating light flash. *i*, *j*, Demonstrate that PtdInsP$_2$ availability regulates the gain of the phototransduction cascade. *i*, Intensity–response functions for wild-type (triangles, $n=10$) and P[Arr-cds] transgenic flies (circles, $n=10$) to 10-ms flashes of increasing light intensities. Plotted are averaged amplitudes ±s.e.m. *j*, Time course of response exhaustion in control (rescue or wild type, circles, $n=9$) and *cds*^{1} mutant photoreceptors (squares, $n=7$). Shown are peak amplitudes ±s.e.m. Note that mutant photoreceptors lose 50% of their responsiveness by 15 consecutive flashes.

METHODS. *a–h*, Dissociation of dark-raised photoreceptor cells was according to ref. 33. Cells were transferred into bath solution with nominally zero Ca^{2+}. Internal Ca^{2+} was depleted in unclamped cells using 3-s light pulses (log $I=-0.5$) every 3 min for at least 40 min[34]. Pure PtdInsP$_2$ (Sigma) was weighted into internal solution for a total pipette concentration of 150 μM and sonicated with 0.2% DMSO and 0.04% Pluronic (Molecular Probes) for 1 min in a bath sonicator (Branson) immediately before loading into the pipette. Equivalent results were obtained when using a phosphoinositide mixture of phospholipids (Sigma, P6023); in this case we used 0.56 mg ml^{-1} phospholipid in internal solution plus 0.2% DMSO, 0.04% Pluronic (5/8 cells responded, data not shown). Controls using PA and PtdC used dicapryl phosphatidic acid and phosphatidylcholine (Avanti Polar Lipids). Stock solutions (10 mg ml^{-1} and 25 mg ml^{-1}, respectively) were evaporated and resuspended in internal solution plus 0.1% DMSO, 0.004% Pluronic to a final phospholipid concentration of 100 μM (each at 50 μM). Internal solution: 140 mM KCl, 2.1 mM $MgSO_4$, 10 mM HEPES, 4.8 mM EGTA, 3.7 mM $CaCl_2$, pH 7.15 (free [Ca^{2+}]$_{in}$ = 700 nM). Bath solution: 120 mM NaCl, 5 mM KCl, 8 mM $MgSO_4$, 10 mM HEPES, 29.5 mM sucrose, 5 mM proline, pH 7.15. Within 90 min after depletion, photoreceptors were stimulated with 10-ms flashes of 580 nm light from a 75 W xenon arc lamp at a holding potential of −60 mV. Relative log order of light intensity (log I/I_0) = −0.4. I_0 is the maximum intensity at 580 nm produced by the light source (0.09 mW cm^{-2}). Photoreceptors were counted as responders if the response amplitude reached at least 100 pA. For the intensity–response studies (*i*), dissociated photoreceptors were transferred to physiological bath solution (1.5 mM $CaCl_2$), and cells were stimulated with 580 ± 10 nm light at a holding potential of −60 mV. Maximal amplitudes were averaged and unpaired, two-tailed *t*-tests were done for each intensity; $P(I=\log -3.5)<0.02$, $P(I=\log -3.0)<0.003$, $P(I=\log -2.5)<0.007$, $P(I=\log -2.0)<0.005$, $P(I=\log -1.0)<0.03$. $I_0=0.09$ mW cm^{-2}. Equally significant results were obtained when normalizing amplitudes of each cell to the whole-cell capacitance (an indirect measure of photoreceptor maturity). *j*, Dissociated photoreceptors were transferred to physiological bath solution with nominally zero $CaCl_2$. After establishing whole-cell configuration with an internal solution containing 700 nM free Ca^{2+} (see above), photoreceptors were stimulated with 10-ms flashes of 580 ± 10 nm light every 10 s for the duration of a stable seal. We used stimulating light that evoked non-saturating response amplitudes. The first three responses were averaged and each response was normalized to the averaged initial value (±s.e.m.). Holding potential −40 mV.

(Fig. 6*a*, *b*; 6/6 cells responded). If the same depletion protocol is applied to *cds* mutant cells, the light response does not recover even if 700 nM Ca^{2+} is supplied through the pipette (Fig. 6*c*, *d*; 16/17 cells did not respond). This phenotype is due exclusively to a defect in *eye-cds*, because introduction of the wild-type eye-CDS cDNA into mutant hosts fully restores wild-type physiology (Fig. 6*e*, *f*; 9/10 cells). These results indicate that *cds* mutants are depleted in another factor (messenger or messenger precursor) besides calcium. To determine whether this defect is indeed due to a depletion of PtdInsP$_2$ or its precursors, we added a mixture of PtdIns$_s$, PtdInsP and PtdInsP$_2$ to the patch pipette. We also tested a mixture of phosphatidic acid (the substrate of CDP-DAG synthase; see Fig. 5*b*) and phosphatidylcholine (PtdC, a phospholipid abundant in cell membranes), or purified PtdInsP$_2$ alone. Figure 6*g*, *h* demonstrates that although phosphatidic acid (and PtdC) fails to rescue the *cds* phenotype (0/4 cells), PtdInsP$_2$ is sufficient to restore signalling in the depleted *cds* mutants (6/8 cells). Together, these results prove that the inability of *cds* mutants to maintain a light response is due to the depletion of the intracellular messenger PtdInsP$_2$, and demonstrate that CDP-DAG synthase is essential for 'on-line' PLC-mediated signalling *in vivo*.

Level of eye-CDS limits amplification gain

Phototransduction, like many other G-protein-coupled signalling cascades, relies on large amplification of the original signal for high sensitivity (photoreceptors have single photon sensitivity)[25,35]. Such requirement is often associated with a finely modulated signalling pathway in which the levels of the individual components are tightly regulated. We have shown that the availability of PtdInsP$_2$ for signalling is intimately associated with the availability of eye-CDS. To test whether it is possible to modulate the output of this cascade by manipulating the levels of eye-CDS, we generated transgenic flies that express the eye-CDS cDNA under the control of a strong photoreceptor cell-specific promoter[22], P[arr-CDS], and studied their visual physi-

ology. Figure 6*i* shows that P[arr-CDS] transgenic flies, which express a 4–5-fold increase in eye-CDS, display a large and significant increase in their response amplitudes when compared to wild-type animals. This increase is particularly large at high stimulus intensities (Fig. 6*i*), and is not seen at the lowest light intensities. This can be easily explained by understanding that at low light intensities only a small amount of PLC is activated and so $PtdInsP_2$ levels are not limiting even in wild-type flies. However, at higher stimulus intensities, substrate would become limiting in wild-type animals but not in flies overexpressing CDS. If $PtdInsP_2$ availability indeed regulates the gain of this cascade, *cds* mutants should display a reduction in the amplitude of their response as a function of the number of light flashes (and thus their state of depletion). Figure 6*j* shows that this is the case. These results demonstrate that the gain of this signal transduction pathway (amplification) is dependent on the availability of $PtdInsP_2$ and can be modulated by manipulating the availability of CDS.

Discussion

$PtdInsP_2$ is an important intracellular messenger in a wide range of signalling cascades[1,2]. Although much is known about phosphoinositide signalling pathways, little is known about the regulation of $PtdInsP_2$ availability *in vivo*. Using a molecular genetic screen designed to isolate genes important for the *in vivo* regulation of phototransduction in *Drosophila*, a PLC-mediated signal transduction cascade[10,25], we identified CDP-DAG synthase as a key enzyme in PLC signalling.

Several unexpected findings emerge from this work. First, there is a photoreceptor-specific isoform of CDS: this could be rationalized by understanding the highly specialized role of photoreceptors, and the high signalling demand imposed on these cells[10]. Second, cds^1 mutants have no phenotype other than their defect in photoreceptor function. Thus, different pools of $PtdInsP_2$ are probably involved in phototransduction as opposed to other $PtdInsP_2$ needs of these cells (such as membrane metabolism)[26,27]. Furthermore, other CDS must be present to provide $PtdInsP_2$ in these mutants (see below). Third, the availability of $PtdInsP_2$ and the levels of CDS regulate the gain of the response (Fig. 6*i*,*j*). These findings demonstrate that $PtdInsP_2$ levels are limiting *in vivo*. This is consistent with recent findings in permeabilized mammalian cells which suggested that substrate is limiting in the vicinity of activated PLC[13]. Fourth, mutations in CDS lead to severe signal-dependent cellular degeneration. The finding that a mutation in the phospholipase C effector of this cascade is able to suppress fully this retinal degeneration suggests that the degeneration may be due to the buildup of a signalling intermediate between PLC and eye-CDS, possibly DAG or PA. DAG may activate PKC[12] and PA may interfere with a variety of signalling pathways[36,37]. Interestingly, *Drosophila* mutants defective in a photoreceptor cell-specific DAG-kinase also undergo retinal degeneration[38,39]. To determine whether retinal degeneration in *cds* animals results from inappropriate regulation of PKC, we generated double mutants between cds^1 and a photoreceptor-specific PKC (eye-PKC) encoded by the *inaC* gene[24,40]. cds^1, *ina*C double mutants still undergo light-dependent retinal degeneration (data not shown), demonstrating that the degeneration is not mediated through eye-PKC activation. Genetic screens designed to isolate suppressors of *cds* retinal degeneration may help identify the components required for the degeneration and potential targets for their action.

Given the involvement of PLC signalling cascades in a wide range of cellular responses, animals deficient in a ubiquitous CDS might be expected to be non-viable. Transposase-induced excisions of the P-element insert in the original allele (cds^1) yielded three mutations that are homozygous lethal. These lethal mutations are unable to complement the degeneration and visual physiology of cds^1 mutants, and produced progeny that lacked the eye-CDS polypeptide (Fig. 5*c* and data not shown), indicating that they are alleles of the same gene. This genetic evidence suggests that both a general and the eye-specific CDP-DAG synthase are encoded at the same gene locus. Because the general and the eye-specific CDS should catalyse the same reaction ($PA + CTP \rightarrow CDP\text{-}DAG + PP_i$), having them encoded in the same gene allows them to share exons. Post-transcriptional regulation of the transcript would allow for specialization of different CDS isoforms. Indeed, analysis of the genomic structure of *cds* has confirmed the presence of alternatively spliced CDS isoforms (L.W., Y. Sun and C.S.Z., data not shown). The availability of this and other mutants defective in specific aspects of the phototransduction cascade make it now possible to design rigorous genetic and physiological experiments to dissect the role of the various components required for the functioning and regulation of $PtdInsP_2$ and its metabolites. It is expected that results from these studies will increase our understanding of the nature and pathology of phosphoinositide signalling in a wide range of cell types and transduction pathways. □

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