Retinal Targets for Calmodulin Include Proteins Implicated in Synaptic Transmission*

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Ca\textsuperscript{2+} influxes regulate multiple events in photoreceptor cells including phototransduction and synaptic transmission. An important Ca\textsuperscript{2+} sensor in Drosophila vision appears to be calmodulin since a reduction in levels of retinal calmodulin causes defects in adaptation and termination of the photoresponse. These functions of calmodulin appear to be mediated, at least in part, by four previously identified calmodulin-binding proteins: the TRP and TRPL ion channels, NINAC and INAD. To identify additional calmodulin-binding proteins that may function in phototransduction and/or synaptic transmission, we conducted a screen for retinal calmodulin-binding proteins. We found eight additional calmodulin-binding proteins that were expressed in the Drosophila retina. These included six targets that were related to proteins implicated in synaptic transmission. Among these were the diacylglycerol-binding protein, UNC13, and a protein, CRAG, related to Rab3 GTPase exchange proteins. Two other calmodulin-binding proteins included Pollux, a protein with similarity to a portion of a yeast Rab GTPase activating protein, and Calossin, an enormous protein of unknown function conserved throughout animal phylogeny. Thus, it appears that calmodulin functions as a Ca\textsuperscript{2+} sensor for a broad diversity of retinal proteins, some of which are implicated in synaptic transmission.

Influx of Ca\textsuperscript{2+} in Drosophila photoreceptor cells, occurs in two spatially and temporally distinct phases. The initial Ca\textsuperscript{2+} influx is activated in response to light stimulation of rhodopsin and is mediated by the TRP and TRPL cation channels situated in the microvillar portion of the photoreceptor cells, the rhabdomeres (reviewed in Ref. 1). TRP- and TRPL-dependent Ca\textsuperscript{2+} entry contributes both to the depolarization of the photoreceptor cells as well as to Ca\textsuperscript{2+}-mediated negative-feedback regulation. A second phase of Ca\textsuperscript{2+} entry in fly photoreceptor cells presumably occurs in the active zones in the presynaptic terminals and is the result of opening of voltage-gated Ca\textsuperscript{2+} channels. Ca\textsuperscript{2+} influx via the voltage-gated channels leads to fusion of synaptic vesicles with the plasma membrane and release of neurotransmitter (reviewed in Ref. 2). Thus, fluxes in Ca\textsuperscript{2+} levels regulate multiple processes that are critical for vision including negative feedback regulation, termination of the light-induced current, and synaptic transmission. Nevertheless, identification of the Ca\textsuperscript{2+} sensors which regulate these processes is quite incomplete.

Some of the proteins that mediate the various Ca\textsuperscript{2+}-regulated processes in fly photoreceptor cells are likely to do so through direct interaction with Ca\textsuperscript{2+}. These include protein kinase C, which functions in adaptation and termination of the photoresponse (3, 4), and synaptotagmin, a synaptic vesicle protein that may be one of the Ca\textsuperscript{2+} sensors that functions in release of neurotransmitter (reviewed in Ref. 5). Other Ca\textsuperscript{2+}-binding proteins implicated in synaptic transmission are the protein phosphatase calcineurin (6), which binds both Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/calmodulin, and rabphilin (7, 8), a peripheral membrane protein that binds Rab3, a small GTPase that is associated with synaptic vesicles (reviewed in Ref. 2). Many additional retinal proteins may be controlled indirectly by Ca\textsuperscript{2+} through association with the Ca\textsuperscript{2+}-binding regulatory protein calmodulin. A number of studies have indicated that there exists a plethora of retinal calmodulin-binding proteins in vertebrates (9–11) several of which, such as the cGMP-gated ion channel, have been identified (12). Calmodulin is expressed at very high levels in Drosophila photoreceptor cells (13) and mutations and experimental conditions that lower the intracellular calmodulin levels result in defects in negative feedback regulation and termination of the photoresponse (14–17).

NINAC p174 and p132, proteins that consist of protein kinase and myosin head domains (18), are the major calmodulin-binding proteins expressed in Drosophila photoreceptor cells (13). Disruption of the NINAC/calmodulin interaction causes a decrease in the intracellular concentration of calmodulin, a dramatic alteration in the normal spatial distribution of calmodulin and consequently, defects in adaptation and termination of the photoresponse (13, 14). Calmodulin also binds to the light-sensitive ion channels, TRP and TRPL (19, 20), and disruption of the calmodulin association with TRPL causes an impairment in inactivation (17). Two other retinal proteins shown to bind calmodulin are RIC, a protein with homology to RAS (21), and INAD (22), a PDZ containing scaffold protein which binds many of the proteins required in phototransduction (reviewed in Ref. 23).

Studies in a variety of organisms indicate that calmodulin functions in synaptic transmission. Calmodulin binds to the synaptic vesicle protein synapsin I, from vertebrates and Drosophila (24–26), as well as to mammalian Rab3A (27). Furthermore, association of Rab3A with synaptic membranes is disrupted in vitro by Ca\textsuperscript{2+}/calmodulin (27). Several calmodulin-dependent protein kinases appear to have roles in synaptic transmission. Ca\textsuperscript{2+}/calmodulin-dependent protein kinases I

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and II (CaM kinase I and II) appear to function in synaptic transmission in a variety of neurons and are known to phosphorylate several components associated with synaptic vesicles (reviewed in Ref. 28), such as the synapsin I-like protein expressed in Drosophila photoreceptor cells (29). Additionally, inhibition of myosin light chain kinase (MLCK) disrupts synaptic transmission in rat sympathetic neurons (30). Further evidence that calmodulin may also function in synaptic transmission in the Drosophila visual system is that a reduction in calmodulin levels in photoreceptor cells affects a feature of the electroretinogram that emanates from activity post-synaptic to calmodulin levels in photoreceptor cells (29). Additionally, 100–500 nm (in H2O) for 5 min, cooling quickly on ice, and adding 2 μl of 10 × DIG Labeling Mixture (Boehringer Mannheim catalog number 1277–065), 2 μl of 10 × hexanucleotide mixture (Boehringer Mannheim catalog number 1277–081), and Klenow fragment in a total volume of 20 μl. After incubating at room temperature for 3–16 h, 20 μg of carrier DNA was added and the DNA was ethanol precipitated and resuspended in 75 μl of hybridization buffer (0.1 M NaCl, 40 μM sodium dodecyl phosphate buffer (pH 6.8), 0.1% Denhardt’s, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 5 mM MgCl2). After hybridizing to polytene chromosomes, the probes were detected using a 1:500 dilution of anti-digoxigenin-P(O) (Boehringer Mannheim catalog number 1207–733).

RNA Blots—Tissue from various developmental stages (0.5 g each, w/m-strain) were collected and total RNAs were isolated with RNAzol (Tel-Test, Inc.). According to the RNAzol protocol, total RNA (1 μg) was purified from total RNA using an mRNA isolation kit (Boehringer Mannheim). 50 μg of total RNA or 5 μg of poly(A)+ RNA from each tissue was fractionated on formaldehyde–agarose gels (1.2% agarose), transferred to a nylon membrane, probed with 32P-labeled cDNA fragments and the signals were detected using a PhosphorImager (Fujifilm). The DNA probe for hybridizing the blots encoded the following amino acids indicated in parentheses: UNC13 (80–405), PLX (164–1379), CRAG (588–1209), CALO (1415–1118). The blots were stripped by incubating at 95°C in 0.1% SDS and re-probed with the full-length RP49 DNA probe.

Generation of GST Fusion Proteins—All the fusion proteins were expressed as glutathione S-transferase (GST) fusions using pGEX vectors (Pharmacia Biotech Inc.). Unless specified otherwise, the fragment used for subcloning was generated by polymerase chain reaction (PCR). The dUnc13 fragments were inserted between the BamHI/EcoRI sites of pGEX-5X-3 and the Crag sequences were subcloned into pGEX-5X-2 as XhoI/Xhol fragments. The fusions constructed using Lyncein, the bovine homolog of plx, were generated using a Lyncein cDNA (B61) isolated from a bovine retinal ZAP library (gift of Donald Zack, JHU-SOM). pPollux.9 and pLyncein-L1 through pLyncein-L6 were created by inserting BamHI/EcoRI fragments into pGEX-5X-3. pLyncein-L7 was generated by digesting with restriction enzymes and then re-cloning non-directionally into pGEX-5X-3. pLyncein-L8 was obtained by digesting B61 with XhoI and EcoRI and cloning into pGEX-5X-3. pPollux.3 was generated by cloning the original plx isoform, dl265, into the XhoI/EcoRI sites of pGEX-5X-3 and then digesting with BamHI and religating. pPollux.2 was obtained by subcloning between the BamHI/EcoRI sites of pGEX-5X-3. The amino acid residues encoded by the probe were indicated in the following enzymes and restriction enzymes: pLyncein-L1 (388–664), pCrMaC.2 (655–944), pCrMaC.3 (935–1209), pCrMaC.2a (655–822), pCrMaC.2b (812–944), pUnc13-370/131 (64–229), pUnc13.2 (89–229), pUnc13.3 (63–103), pUnc13.4 (64–88), pPollux.2 (595–701), pPollux.3 (180–733), pPollux.9 (657–680), and pPollux.dm265 (180–1379). The residues encoded in the following Lyncein clones indicate the corresponding residues in PLX: pLyncein-L1 (657–675), pLyncein-L2 (657–680), pLyncein-L5 (657–701), pLyncein-L6 (650–701), pLyncein-L7 (887–1013), and pLyncein-L8 (615–886). Calmodulin Binding Assays—The fusion proteins were expressed in freshly transformed Escherichia coli BL21(DE3) cells as described (Pharmacia Biotech Inc.). To perform the calmodulin overlay assay, 20 μl of bacterial cell supernatant were added to wells containing 20 μl of 0.1% SDS sample buffer for 5 min. The samples were then fractionated by SDS-PAGE (10% gel) and transferred to a nylon membrane, probed with 32P-labeled cDNA fragments and the signals were detected using a PhosphorImager (Fujifilm). The fusion proteins were expressed in Drosophila embryos (reviewed in Ref. 28). The cDNAs were used for subcloning were generated by polymerase chain reaction (PCR). The dUnc13 fragments were inserted between the BamHI/EcoRI sites of pGEX-5X-3 and the Crag sequences were subcloned into pGEX-5X-2 as XhoI/Xhol fragments. The fusions constructed using Lyncein, the bovine homolog of plx, were generated using a Lyncein cDNA (B61) isolated from a bovine retinal ZAP library (gift of Donald Zack, JHU-SOM). pPollux.9 and pLyncein-L1 through pLyncein-L6 were created by inserting BamHI/EcoRI fragments into pGEX-5X-3. pLyncein-L7 was generated by digesting with restriction enzymes and then re-cloning non-directionally into pGEX-5X-3. pLyncein-L8 was obtained by digesting B61 with XhoI and EcoRI and cloning into pGEX-5X-3. pPollux.3 was generated by cloning the original plx isoform, dl265, into the XhoI/EcoRI sites of pGEX-5X-3 and then digesting with BamHI and religating. pPollux.2 was obtained by subcloning between the BamHI/EcoRI sites of pGEX-5X-3. The amino acid residues encoded by the probe were indicated in the following enzymes and restriction enzymes: pLyncein-L1 (388–664), pCrMaC.2 (655–944), pCrMaC.3 (935–1209), pCrMaC.2a (655–822), pCrMaC.2b (812–944), pUnc13-370/131 (64–229), pUnc13.2 (89–229), pUnc13.3 (63–103), pUnc13.4 (64–88), pPollux.2 (595–701), pPollux.3 (180–733), pPollux.9 (657–680), and pPollux.dm265 (180–1379). The residues encoded in the following Lyncein clones indicate the corresponding residues in PLX: pLyncein-L1 (657–675), pLyncein-L2 (657–680), pLyncein-L5 (657–701), pLyncein-L6 (650–701), pLyncein-L7 (887–1013), and pLyncein-L8 (615–886). Calmodulin Binding Assays—The fusion proteins were expressed in freshly transformed Escherichia coli BL21(DE3) cells as described (Pharmacia Biotech Inc.). To perform the calmodulin overlay assay, 200 μl of bacterial cell supernatant were added to wells containing 20 μl of 0.1% SDS sample buffer for 5 min. The samples were then fractionated by SDS-PAGE (10% gel) and transferred to a nylon membrane, probed with 32P-labeled cDNA fragments and the signals were detected using a PhosphorImager (Fujifilm). The fusion proteins were expressed in Drosophila embryos (reviewed in Ref. 28).
of pre-equilibrated beads. Protein blots were performed with goat anti-GST antibodies (Pharmacia).

RESULTS

Summary of Screen for Retinal Calmodulin-binding Proteins—To identify calmodulin-binding proteins expressed in the Drosophila retina, we probed a retinal expression library with 125I-labeled calmodulin. The positive clones isolated in the screen prior to the current work (see below). Thus, it appears that at least two of the four novel calmodulin-binding proteins are expressed in the retina but were previously cloned in Drosophila. Approximate 50 isolates cross-hybridized with trpl and five were subjected to DNA sequence analysis. All five showed 100% identity to trpl.

A. Drosophila retinal proteins known to bind CaM previous to the current report

B. Fly homologs of vertebrate CaM-binding proteins not previously known to be expressed in the retina (but were previously cloned in Drosophila)

C. Not previously identified in Drosophila but related to vertebrate CaM-binding protein

D. Novel calmodulin proteins

Drosophila retinal proteins known to bind CaM previous to the current report

1. NINAC 1 28A1–3 Pak 65/myosin 1 >10:1a Porter et al. (13)
2. TRPL — 46B1–2 TRP >10:1a Phillips et al. (19)
3. TRP 0 99C5–6 TRPL >10:1a Chevesich et al. (20)
4. INAD 0 59B1–4 hINAD >10:1a Xu et al. (22)
5. RIC 3 52E3 ND Wes et al. (21)

A protein related to a Rab3 GDP/GTP exchange protein; Table I) due to its similarity to a domain in the recently identified rat Rab3 GDP/GTP exchange protein (rRab3 GEP) (31) and the C. elegans homolog, AEX-3 (32), which has been implicated in synaptic vesicle release (Figs. 1B and 2A). The sequences of AEX-3 and the rRab3 GEP were published contemporaneously and were therefore not directly compared. AEX-3 and the rRab3 GEP (1409 and 1602 amino acids, respectively) contained three regions of homology, the first of which (~500 residues) was conserved in CRAG, AEX-3, rRab3 GEP (CAR domain; Fig. 1A) and the human homolog, MADD (death domain MAP kinase activator; Ref. 46). The latter two regions, were conserved in AEX-3 and rRab3 GEP (AR1 and AR2), but not CRAG, and were shorter (~100 and 300 residues, respectively) than the CAR homology. The CAR domain in CRAG was ~36 identical over 321 amino acids (residues 95–415) to either rRab3 GEP or AEX-3. In addition, there was weak homology (16%) in the flanking sequences that extended the CAR domain in CRAG to residues 73–490. The C-terminal ~800 residues of CRAG did not share significant primary amino acid sequence homology with the rRab3 GEP, AEX-3, or any other protein in the data banks.

POLLUX (PLX) is a protein previously reported to be 732 amino acids in length and required for viability (47). The protein is predicted to have a transmembrane domain and a leucine zipper (Fig. 2B; Ref. 47). We found that PLX was 1379 amino acids in length and the formerly assigned initiator methionine corresponded to residue 648 (Fig. 1C). A protein related to PLX was TBC1 (48), a mouse protein which had homology to the majority of PLX. The region in PLX that contained the greatest similarity to TBC1 was a 337-amino acid segment (51% identity, residues 676–1012) that included the putative transmembrane domain (Figs. 1C and 2B). Of particular interest, the region most highly conserved between PLX and TBC1 included a 153-amino acid domain (residues 811–963) that displayed moderate homology to the yeast Rab family GTPase-activating proteins, GYP6 or GYP7 (37). GYP7 was ~29% identical to this domain in either PLX or TBC1; however, if two gaps of 18 and 36 amino acids were introduced in PLX and TBC1, the 29% homology extended to over 222...
FIG. 1. Deduced amino acid sequences of calmodulin-binding proteins. The sequence alignment was generated using a program, Pileup, from the Sequence Analysis Software Package by Genetics Computer, Inc. (the GCG Package; version 7). Identical amino acids are shaded and a tally of amino acids and acronyms for each protein is indicated to the right and left of each sequence. Dots indicate gaps in one sequence relative to the other(s). Only those regions in other proteins that are related to the Drosophila calmodulin-binding proteins are shown. The regions containing sequences that are not shown are indicated by dashes. Numbers flanked by dashes indicate the number of residues not included in this figure. Accession numbers are indicated below.

A, Drosophila CaM kinase I (dCKI) compared with rat CaM kinase I (rCKI).

B, CRAG lined-up with the C. elegans AEX-3 and the rat Rab3A GEP.

C, PLX is displayed with mouse TBC1 and yeast GYP7. The overline indicates the CBS. The sequence of the Lyncein (LYN) CBS is shown. The asterisk indicates the methionine (residue 648, indicated in bold) that was assigned as the first residue in PLX in a previous report. A histidine (residue 1286) was identified as a glutamine in the prior sequence.

D, dUNC13 compared with rat mUNC13-1.

E, CALO shown with a related protein in C. elegans and a variety of mouse ESTs that were artificially fused together to create one sequence.
amino acids (742–963). This ~200 amino acid sequence corresponded to the domain previously referred to as a TBC domain due to its similarity to segments in the TRE-2 oncogene and the yeast regulators of mitosis, BUB2 and CDC16. Neither this nor any other domain in PLX shared primary amino acid sequence homology with the recently described Rab3 GTPase activating protein (49).

A Calmodulin-binding Protein Homologous to a Diacylglycerol-binding Protein Implicated in Synaptic Transmission, UNC13—A third protein, not previously known to bind calmodulin, was a Drosophila homolog of UNC13 (dUNC13; Table I), a diacylglycerol-binding protein which may be required for release of neurotransmitter from the presynaptic terminal (36, 50). dUNC13 was expressed as at least two alternatively spliced forms encoding proteins of 1304 (dUNC13A) and 1724 (dUNC13B) amino acids (Fig. 1D). dUNC13A and dUNC13B shared a common C-terminal region of 1216 amino acids and differed due to unique N-terminal sequences (158 and 508 residues, respectively). dUNC13 contained extensive homology (68%) with the C. elegans UNC13 and rat homologs (mUNC13) beginning in the unique region of dUNC13A and extending over the entire region common between both isoforms.

Fig. 1—continued

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forms (residues 72–1304; Figs. 1D and 2C). UNC13 and mUNC13-1 shared a similar level of homology over the same region and are only weakly related over the N-terminal; 500 amino acids. The 508 amino acids specific to dUNC13B were not homologous to the UNC13 proteins or any proteins in the data banks.

Features common between dUNC13 and other members of the UNC13 family include strong homology to two conserved sequence motifs, C1 and C2, originally recognized in various protein kinase C isoforms (51). A large variety of other signaling proteins, such as RAF, diacylglycerol kinase, RAS GTPase-activating protein, synaptotagmin, and phospholipase C, were subsequently found to contain these domains (see Refs. 52 and 53). C1 domains typically bind diacylglycerol and many C2 domains are Ca\(^{2+}\)-binding regulatory domains. Some C2 domains also bind phospholipids and do so in a Ca\(^{2+}\)-dependent manner. Other C2 domains confer Ca\(^{2+}\) dependence to functions, such as protein kinase activity, mediated by domains distinct from C2. Biochemical analyses of UNC13 demonstrates that it is a bona fide Ca\(^{2+}\)-dependent phorbol ester-binding protein (52). The putative C1 domain in dUNC13 (residues 182–232) included six invariant cysteines (Fig. 1D) as well as a seventh cysteine conserved among all UNC13 proteins. Overall, the C1 domain was 92% identical to the corresponding region in mUNC13 (34). The two C2 domains present in each of the three other UNC13 proteins (C2-1 and C2-2) were also found in dUNC13. C2-1 (residues 299–393) and C2-2 (residues 1170–1264) were 76 and 67% identical with the same motifs in mUNC13-1.

Calossin, a Novel Calmodulin Protein Conserved throughout Animal Phylogeny—The fourth novel calmodulin-binding protein is referred to as Calossin (CALO) due to its interaction with calmodulin and colossal molecular mass (predicted 450 kDa). Several overlapping cDNAs were obtained resulting in the identification of a single open reading frame encoding 4118 amino acids (Fig. 1E). Several hydrophobic regions were predicted according to a computer algorithm (54); however, it is unclear if any is sufficiently long to span a lipid bilayer. CALO was related to a predicted C. elegans protein (cCalossin) of similar size (3864 residues) which was identified as part of the C. elegans Genome Sequencing Consortium. The homology between CALO and cCALO was not uniform but concentrated in several domains. The longest continuous region of identity began at amino acid 2460 and extended; 1650 residues to near the C terminus. In addition, there were two shorter stretches of similarity between residues 604 and 1150. The highest levels of identity (each >70%) were in three 50–100 amino acid regions: 1) residues 604–649; 2) residues 2587–2638; 3) residues 3087–3130.
Fig. 2. Domain organization of CRAG, PLX, dUNC13, and related proteins. Scale bars above each group of proteins indicate lengths in amino acids. The black boxes show the positions of the domains containing the calmodulin-binding sites (CBS). A, CRAG and related proteins. Most of the CAR domain was conserved in all three proteins; however, the AR1 and AR2 domains are conserved only in AEX-3 and the Rab3 GEP. The location of the death domain (DD), identified in MADD, is shown. There are several insertions in the Rab3 GEP relative to AEX-3; the approximate position of the largest (~100 residues) is indicated by the arrowhead. B, PLX, TBC1, and LYNCEIN (LYNC). The domain similar to the yeast Rab GTPases (GAP), the putative TMD (TMD), and the leucine zipper (LZ) are indicated. C, UNC13 family. The domain organization of the dUNC13-A isoform is depicted. The N-terminal C2 motif unique to mUNC13-1 is only distantly related to other C2 domains. The region in mUNC13-1 which binds Doc2a is represented by the gray box. The syntaxin (Stx)-binding site, indicated by the thin black line, maps to a region within the Doc2a-binding domain.

3276–3380. The first two of these conserved regions were cysteine-rich domains, CRD1 and CRD2, respectively (Fig. 1E), that resembled different classes of zinc finger domains (55). CRD1 was most similar to the zinc finger family defined by Requiem, a protein required for apoptosis (56), while CRD2 shared features equally well with several families of zinc family proteins and could not be included within a single group.

Several mouse and human expressed sequence tags (ESTs) were also related to CALO. In general, CALO was more highly related to the mammalian ESTs (mCalossin) than to cCALO. In some stretches, the homology to mCALO was twice as high as to cCALO (e.g., residues 2460–2578; 67 and 32% identity, respectively). Thus, although CALO was not related to any protein of known function, it appeared to be conserved from C. elegans to humans.

Expression of mRNAs Enriched in Adult Eyes—The mRNAs encoding each of the calmodulin-binding proteins described above appeared to be expressed in the retina since the cDNAs were isolated by screening a retinal expression library with calmodulin. To ascertain whether the mRNAs were enriched in the adult visual system, we probed Northern blots containing portions of the adult head. Although CaM kinase I was only slightly enriched in wild-type heads (2-fold), an adult-specific ~3.5 kb mRNA was not detected in sine oculis heads. Consequently, this CaM kinase I mRNA appeared to be eye-specific.

Mapping Calmodulin-binding Sites—To confirm that dUNC13, CRAG, and PLX associate with calmodulin and to map the binding sites, we performed gel overlay and pull-down assays. The overlap between the two original dUNC13 calmodulin-binding clones included amino acids 64–229 (Fig. 4A). To further map the calmodulin-binding site(s), we generated several GST-dUNC13 fusion proteins and performed gel overlay experiments by fractionating the fusions by SDS-PAGE, transferring them to membranes, and probing with biotinylated calmodulin in the presence of Ca^{2+} or the Ca^{2+} chelator, EGTA. Calmodulin-binding was detected in the presence of Ca^{2+} but not EGTA (Fig. 5A). Furthermore, the calmodulin-binding site mapped to residues 64–88, a portion of dUNC13 specific to the unique N-terminal domain of dUNC13A (Fig. 1D). The absence of a calmodulin binding signal with the fusion missing residues 64–88 was not due to absence of the protein since the appropriate size GST-dUNC13 fusion was detected upon reprobing the filter with anti-GST antibodies (data not shown). The region of dUNC13 that bound calmodulin included
some homology to mUNC13-1, but no sequence similarity to mUNC13-2, mUNC13-3, or UNC13.

The gel overlay assay described above tested for interaction between calmodulin and dUNC13 immobilized on a membrane. To address whether dUNC13 was capable of binding to calmodulin in solution, we performed a pull-down assay by incubating a GST-dUNC13 fusion protein with agarose beads linked to calmodulin. The GST-dUNC13 fusion protein, bound calmodulin and did so in a Ca\textsuperscript{2+}-independent manner (Fig. 6A). GST alone did not bind calmodulin in the presence of Ca\textsuperscript{2+} or EGTA and neither GST-dUNC13 or any other fusion protein described below bound to agarose beads under any Ca\textsuperscript{2+} conditions (data not shown; Ref. 21).

The portion of the PLX protein that was isolated in the screen extended from residues 180–1379. Using a series of overlapping GST fusion proteins and the gel overlay assay, the calmodulin-binding site(s) contained in the original fusion protein was further mapped to residues 657–680 (Figs. 4B and 5B). The sequence of the calmodulin-binding site was not conserved in the mouse homolog, TBC1 (Fig. 1C), but was in several human ESTs (data not shown). A bovine homolog of PLX (Lyncein), which we isolated from a bovine retinal library, was highly conserved in the calmodulin-binding domain (Fig. 1C) despite having no higher overall sequence conservation to PLX than TBC1 (data not shown). Moreover, a fusion protein containing the conserved sequence in Lyncein bound calmodulin (data not shown; Fig. 4B). PLX also bound to calmodulin in a pull-down assay; although this interaction was Ca\textsuperscript{2+}-independent (Fig. 6B).

Both gel overlay and pull-down assays were also used to confirm that CRAG bound calmodulin and to further localize the domain which contained the binding site(s). The initial fusion protein identified in the screen extended from residues 388 to 1209 (Fig. 4C). Using the gel overlay technique, the domain containing the calmodulin-binding site was narrowed to residues 655–822 (Fig. 4C and 5C), a region that was not conserved in AEX-3 or the rRab3 GEP. As was the case with PLX and dUNC13, the association with calmodulin was Ca\textsuperscript{2+} dependent in the gel overlay assay, but Ca\textsuperscript{2+}-independent in the pull-down experiments (Fig. 6C).

Many, but not all, calmodulin-binding sites resemble basic amphipathic helices and/or conform to the IQ consensus motif, a sequence of ~25 amino acids containing the core consensus IQXXXRGXXXR (57–59). However, no such sequences were...
detected in dUNC13, CRAG, or dUNC13 and there was no obvious similarity among these proteins within the calmodulin-binding domains. Furthermore, although we have mapped calmodulin-binding sites within each of these three proteins, it remains possible that there exist additional sites that were not detected in these analyses.

**DISCUSSION**

Prior to the current screen, five calmodulin-binding proteins were known to be expressed in the *Drosophila* retina, four of which, NINAC, TRP, TRPL and INAD, function in phototransduction. However, we found that at least six out of the eight additional calmodulin-binding proteins expressed in the *Drosophila* retina contained domains related to proteins implicated in synaptic transmission. Two of the proteins were related to components implicated in synaptic transmission which were not previously known to bind calmodulin and four were known calmodulin-binding proteins: calcineurin, MLCK, CaM kinase I, and CaM kinase II. A seventh protein had weak homology to two yeast RAB GTPase-activating proteins and may function in exocytosis. The eighth protein, Calossin, is the largest known calmodulin-binding protein. Although the function of CALO could not be inferred from the sequence, it appeared to be conserved throughout animal phylogeny.

**CRAG May Provide a Mechanism for Ca$^{2+}$-regulated GDP/GTP Exchange of Rab3**—One of the novel calmodulin-binding proteins identified in the screen, CRAG, contained significant homology to the largest of the three conserved domains in the recently identified Rab3 GEPs (31, 32). Moreover, CRAG was similar in size to Rab3 GEPs. While it remains to be determined if CRAG is also a Rab3 GEP, such a finding would have interesting implications regarding the mechanism by which GTP exchange on Rab3 is regulated. Rab3 binds to synaptic

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**FIG. 5. Calmodulin overlay assay.** Various GST fusions indicated in Fig. 4 or GST alone were fractionated by SDS-PAGE and probed with biotinylated calmodulin in the presence of 0.5 mM CaCl$_2$ (upper panel) or in the presence of 5 mM EGTA (lower panel). The proteins loaded are indicated above each lane. The positions of protein size markers (kDa) are shown to the left. A, calmodulin overlay with dUNC13. Lanes were loaded with the following fusion proteins: UNC13.1, dUNC13.2, dUNC13.3, and dUNC13.4. B, calmodulin overlay with PLX. Representative data with POLLUX.9 and the vector (v) expressing GST alone are shown. C, calmodulin overlay with CRAG. CRAG.1, CRAG2, CRAG3, CRAG2a, and CRAG2b.

**FIG. 6. Calmodulin-agarose binding assay.** Soluble fractions of bacterial lysates were incubated in batch with calmodulin-agarose in the presence of 5 mM EGTA (−Ca$^{2+}$) or 5 mM EGTA and 5.5 mM CaCl$_2$ (+Ca$^{2+}$). These latter conditions are equivalent to ~100 μM free Ca$^{2+}$. The unbound (U) and bound (B) fractions were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-GST antibodies. The fusion proteins used were as follows: A, dUNC13.4; B, PLX.9; B, CRAG.2. GST alone did not bind calmodulin-agarose using either −Ca$^{2+}$ or +Ca$^{2+}$ conditions (data not shown).
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vesicles; however, this association only occurs in resting nerve terminals and requires Rab3 in the GTP bound state. Mutation of the C. elegans Rab3 GEP, AEX-3, causes accumulation of Rab3 in neuronal cell bodies and an impairment in release of neurotransmitter (32). Thus, the Rab3 GEP appears to play a critical role in association of Rab3 with synaptic vesicles and in synaptic transmission. The observation that CRAG binds calmodulin implies that the putative GEP activity of this protein could be regulated by changes in Ca\textsuperscript{2+} levels which are spatially restricted to microdomains near the active zones in presynaptic terminals.

A variety of evidence suggests that the absolute level of Rab3-GTP bound to synaptic vesicles regulates the rate of exocytosis by limiting the number of vesicles that can be fused with the plasma membrane. Thus, formation of Rab3-GTP appears to be a crucial step in synaptic transmission. The mechanisms controlling the GDP-GTP exchange are not known but one possibility is that CRAG is a Rab3 GEP and the associated calmodulin provides a sensor to differentiate between the lower Ca\textsuperscript{2+} levels in resting nerve terminals and higher levels resulting from Ca\textsuperscript{2+} influx. While it remains to be determined if CRAG is a Rab3 GEP and whether the exchange activity is regulated through the associated calmodulin, an exchange factor for another small GTPase, RAS, binds to and is regulated by Ca\textsuperscript{2+}/calmodulin (60).

CRAG bound to calmodulin in solution in either the presence of absence of Ca\textsuperscript{2+}. However, using the gel overlay assay, calmodulin-binding was strictly Ca\textsuperscript{2+} dependent. The gel overlay technique involves fractionating the proteins by SDS-PAGE resulting in extensive protein denaturation. Although the basis for the differences in calcium dependence in the gel overlay and column binding assays is unclear, many other proteins bind calmodulin in a Ca\textsuperscript{2+}-independent manner under native conditions but show Ca\textsuperscript{2+}-dependent binding using denaturing conditions. Examples include the vertebrate brain myosin V (formerly referred to as p190; 61), phosphorylase kinase (62), and the small GTP-binding protein RIC (21). Thus, CRAG probably binds calmodulin in a Ca\textsuperscript{2+}-independent manner since the solution binding assays are more likely to reflect physiological binding conditions. While Ca\textsuperscript{2+} is typically required for association of calmodulin with its targets, several others also bind in a Ca\textsuperscript{2+}-independent manner. These include some unconventional myosins, neuromodulin and neurogranin (reviewed in Ref. 63).

UNC13 Proteins May Have Dual Ca\textsuperscript{2+} Sensors—In addition to aex-3, several other mutations have been identified in C. elegans that appear to disrupt exocytosis of synaptic vesicles and release of neurotransmitter. One such mutation is in the gene encoding the diacylglycerol-binding protein, UNC13. In the current work, we found that one of the calmodulin-binding proteins is a highly conserved Drosophila homolog of the C. elegans and mammalian UNC13 proteins. Although the specific function of UNC13 remains unclear, it may operate in docking and/or fusion of synaptic vesicles since the rat brain-specific mUNC13-1 protein binds directly to two proteins, syntaxin and Doc2a, which function in Ca\textsuperscript{2+}-dependent exocytosis (64, 65).

The C2 domains present in UNC13 homologs could potentially serve as a Ca\textsuperscript{2+} sensor which responds to the Ca\textsuperscript{2+} influx required for exocytosis. Therefore, the question arises as to the function of a potential second type of Ca\textsuperscript{2+} sensor provided by the binding of calmodulin to dUNC13. One possibility is that each UNC13 protein really has only one Ca\textsuperscript{2+} sensor and that it is supplied in some isoforms by the C2 domain and in others through Ca\textsuperscript{2+}/calmodulin. Consistent with this proposal, the calmodulin-binding domain is not conserved in UNC13 suggesting that the C2 domain provides the only Ca\textsuperscript{2+} detector in this protein. The reverse may be the case in mUNC13-1 since this protein does not appear to contain Ca\textsuperscript{2+}-binding C2 domains (34) but does show sequence similarity to the dUNC13 calmodulin-binding site.

An alternative proposal, which we favor, is that some UNC13 proteins may be regulated by Ca\textsuperscript{2+} via both C2 domains and calmodulin. Such dual regulation may provide a mechanism for extremely rapid as well as sustained responses to highly transient increases in Ca\textsuperscript{2+}. The rise in Ca\textsuperscript{2+}, resulting from opening of the voltage-gated channels in synaptic terminals, occurs in microdomains and collapses within microseconds after closing of the ion channels (reviewed in Ref. 2). C2 domains comprise an unusual Ca\textsuperscript{2+} binding motif in that Ca\textsuperscript{2+} appears to regulate this domain through a shift in electrostatic potential rather than a conformational change (reviewed in Ref. 2). As such, C2 domains have the potential to respond very quickly, but transiently, to the rapid Ca\textsuperscript{2+} flux in the active zones of presynaptic terminal. Although fusion and release of neurotransmitter is extremely rapid (submilliseconds to milliseconds), there is some latency between the opening and closing of the ion channels and these latter events. Ca\textsuperscript{2+} binding to calmodulin, which induces a conformational change, may induce a more delayed but sustained response to Ca\textsuperscript{2+} than provided by the C2 domain. Thus, dual binding of Ca\textsuperscript{2+} to calmodulin and C2 domains may enable UNC13 proteins to sense the Ca\textsuperscript{2+} rise within a few microseconds and sustain the response for several hundred microseconds to several milliseconds.

Potential Roles of Pollux in Photoreceptor Cells—One of the calmodulin-binding proteins identified in the screen was PLX, a protein suggested to be a novel cell adhesion molecule of 732 residues (47). Based on the deduced amino acid sequence, PLX is predicted to contain at least one transmembrane domain and a leucine zipper. Spatial localization of the protein showed that it is found on the cell surface as well as the lumen of the trachael system and on the plasma membrane of axons in the central nervous system (47). A related mouse homolog, TBC1, described in a contemporaneous report, was 1141 residues and was found to be a nuclear protein (48). Thus, TBC1 and PLX have very disparate spatial distributions.

We found that PLX was 1379 residues rather than 732 amino acids as previously reported. The additional sequence was not due to a chimeric cDNA since multiple plx cDNAs were obtained and TBC1 shared similarity to PLX both N- and C-terminal to the formerly assigned initiating methionine at residue 648. Of particular relevance here, we found that PLX bound calmodulin and did so in a Ca\textsuperscript{2+}-independent manner. Although the sequence of the calmodulin-binding site was not conserved in TBC1, the region was very similar in Lyncein, a homolog isolated from a bovine retinal library. Furthermore, the Lyncein sequence also bound calmodulin. Thus, it appears that a PLX homolog is expressed in the vertebrate retina.

A possible clue as to the function of PLX in the retina is that it shares some similarity to two yeast Rab GAP proteins (37); although, there was no homology to the Rab3 GAP expressed in the rat brain (49). Nevertheless, the observation that PLX contains a domain related to Rab GAPs combined with the finding that it appears to be localized to the plasma membrane and lumen of the trachael system raises the possibility that PLX may be involved in exocytosis. In the Drosophila visual system, exocytosis is important not only in synaptic transmission but in turn-over of the microvillar membrane of the photoreceptor cells. Shedding of membrane does not occur uni-
formally during the diurnal cycle, but occurs maximally soon after dawn (66). Thus, an increase in the exocytotic process is correlated with the light dependent rise in Ca^{2+} and therefore might be regulated in part by a Ca^{2+} sensing component in a Rab cycle. Alternative potential functions for PLX in photoreceptor cells include also processes that involve vesicular trafficking such as insertion of new membrane in the microvilli and the budding, targeting, and fusion of rhodopsin carrier vesicles with the plasma membrane. These latter events involve a variety of Rab proteins (67) and also appear to be regulated during the daily light cycle (66).

Concluding Remarks—With the identification of the calmodulin-binding proteins in the current screen, there exists a minimum of 13 targets for calmodulin in the Drosophila retina. Furthermore, each of the calmodulin-binding proteins was enriched in the retina. Drosophila Rab3 is also enriched in the adult heads (68) and may bind calmodulin since vertebrate Rab3 associates with calmodulin (27). Interestingly, most of the same proteins that were identified in the Drosophila retina are also expressed in the vertebrate retina. The great diversity and abundance of retinal calmodulin-binding proteins indicates the particular importance of calmodulin as a Ca^{2+} sensor in the retina. Currently, there is strong evidence that retinal calmodulin functions in phototransduction. However, Ca^{2+}/calmodulin may also be utilized to regulate a broad range of vesicular trafficking events in the retina. Such processes may include membrane turnover, protein transport and especially synaptic transmission.

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REFERENCES

1. Montell, C. (1997) Mol. Pharmacol. 52, 755–763
2. Geppert, M., and Südhof, T. C. (1998) Mol. Pharmacol. 53, 75–95
3. Smith, D. P., Ranganathan, R., Hardy, R. W., Marx, J., Tsuchida, T., and Zuker, C. S. (1991) Science 254, 1478–1484
4. Hardie, R. C., Peretz, A., Suss-Toby, E., Rom-Glass, A., Bishop, S. A., Selinger, Z., and Minke, B. (1993) Nature 363, 654–657
5. Littleton, J. T., and Belles, H. J. (1995) Trends Neurosci. 18, 177–183
6. Liu, J.-P., Sim, A. T. R., and Robinson, P. J. (1994) Science 265, 970–973
7. Li, C., Takei, K., Geppert, M., Daniell, L., Stenius, K., Chapman, E. R., Jahn, R., De Camilli, P., and Südhof, T. C. (1994) Neuron 13, 885–898
8. Shiraiata, H., Kainai, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1994) Mol. Cell. Biol. 14, 2061–2068
9. Nagle, B. W., and Burnside, B. (1984) Eur. J. Cell Biol. 33, 248–257
10. Nageo, S., Yamanashi, A., and Bitensky, M. W. (1987) Biochemistry 26, 1659–1665
11. Morelli, A., Damonte, G., and Pepe, I. (1989) Biochem. Biophys. Res. Commun. 163, 363–368
12. Liu, M., Chen, T.-Y., Ahamed, B., Li, J., and Yau, K.-W. (1994) Science 262, 1348–1354
13. Porter, J. A., Yu, M., Doberstein, S. K., Pullard, T. S., and Montell, C. (1993) Science 260, 1034–1042
14. Porter, J. A., Minke, B., and Montell, C. (1995) EMBO J. 14, 4450–4459
15. Arnon, A., Cook, M., Montell, C., Selinger, Z., and Minke, B. (1997) Science 275, 1119–1121
16. Arnon, A., Cook, B., Gillo, B., Montell, C., Selinger, Z., and Minke, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5894–5899
17. Scott, K., Sun, Y., Beckham, K., and Zakeri, C. S. (1997) Cell 91, 375–383
18. Montell, C., and Rubin, G. (1988) Cell 52, 757–772
19. Phillips, A. M., Bull, A., and Kelly, L. E. (1992) Neuron 8, 631–642

3 M. Yu and C. Montell, unpublished observations.