Cellular and Subcellular Localization, N-terminal Acylation, and Calcium Binding of Caenorhabditis elegans Protein Phosphatase with EF-hands*

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The RdgC/PPEF family of serine/threonine protein phosphatases is distinguished by the presence of C-terminal EF-hands and neuron-specific expression, including frequent expression in primary sensory neurons. Here we report that the sole Caenorhabditis elegans PPEF (CePPEF) homolog is also highly expressed in primary sensory neurons and is not found outside the nervous system. Neurons expressing CePPEF include the ciliary chemosensory neurons AWB and AWC; and within these neurons, CePPEF is highly enriched in the sensory cilia. In transgenic C. elegans and in transfected 293 cells, CePPEF is membrane-associated, and the N terminus of CePPEF is necessary and sufficient for this membrane association. [3H]Myristate and [3H]palmitate labeling studies in 293 cells demonstrated that this association was mediated by myristoylation at Gly and palmitoylation at Cys. Introducing the G2A or C3S mutation into CePPEF greatly reduced membrane association in 293 cells and in transgenic nematodes. A recombinant C-terminal fragment of CePPEF containing two putative EF-hands bound between one and two Ca\(^{2+}\) ions/protein, and mutation of residues presumed to ligand calcium in the two putative EF-hands led to diminished calcium binding. These results establish the first direct evidence for fatty acylation and calcium binding of a PPEF family member and demonstrate a remarkable conservation of sensory neuron expression among the members of this distinctive family of protein phosphatases.

Phosphorylation and dephosphorylation of serine and threonine residues are known to be important in several types of sensory neurons. In vertebrate photoreceptors, where light-dependent phosphorylation and dephosphorylation have been extensively characterized, light-induced rhodopsin activation of transducin is terminated by phosphorylation of multiple serine and threonine residues at the rhodopsin C terminus and the subsequent binding of arrestin to phosphorhodopsin (1–3). Recycling of rhodopsin back to the dark state involves replacement of the all-trans-retinal chromophore with 11-cis-retinal, release of arrestin, and dephosphorylation (4, 5). A similar phosphorylation-dephosphorylation cycle occurs in Drosophila photoreceptors (6, 7). In Drosophila, rhodopsin dephosphorylation appears to be catalyzed by the protein product of the retinal degeneration C gene rdgC, and hyperphosphorylation of rhodopsin due to rdgC mutation produces a rapid light-dependent photoreceptor degeneration of the R1–R6 photoreceptors (8–11).

High throughput sequencing and database homology searches from a variety of species have revealed several predicted protein sequences with extensive homology to RdgC (12, 13). Collectively, these proteins are referred to as PPEFs (for protein phosphatases with EF-hands) in recognition of the most distinctive features of their primary sequence: a serine/threonine protein phosphatase domain and two or more C-terminal EF-hand calcium-binding motifs. Members of this family identified to date include the single Drosophila PPEF (RdgC), two mammalian PPEFs (PPEF-1 and PPEF-2), a pufferfish PPEF, and a single Caenorhabditis elegans PPEF (CePPEF). As rdgC mutants show elevated levels of phosphorylated rhodopsin (10, 14), and the dephosphorylation of Drosophila rhodopsin in photoreceptor extracts is stimulated by calcium (14), the C-terminal EF-hands of RdgC have been postulated to regulate calcium-dependent rhodopsin dephosphorylation. However, direct evidence that calcium binds to the EF-hand motifs has not been reported for any PPEF family member.

Mammalian and Drosophila PPEFs are expressed principally or exclusively within the nervous system and are highly enriched in a subset of primary sensory neurons. Drosophila RdgC has been immunolocalized to photoreceptors and the mushroom bodies within the brain (9); PPEF-1 transcripts have been localized by in situ hybridization to the inner ear, the dorsal root ganglia, and several brainstem nuclei in the developing mouse (13); and PPEF-2 has been localized by in situ hybridization and immunostaining to photoreceptors and pinealocytes in the adult rodent (12). These observations suggest that the PPEFs may play a conserved role in diverse sensory systems.

Aside from Drosophila rhodopsin, the identities of PPEF substrates have remained elusive. By analogy, it would be plausible to suppose that mammalian PPEF-2 specifically dephosphorylates phosphorhodopsin, but current evidence argues against this idea. In particular, protein phosphatase 2A activity has been detected in rod outer segment extracts and can

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¶ The abbreviations used are: CePPEF, C. elegans protein phosphatase with EF-hands; FL-GFP, full-length green fluorescent protein; kb, kilobase pair(s); aa, amino acid(s); NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; MBP, maltose-binding protein; PBS, phosphate-buffered saline.
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efficiently dephosphorylate phosphorhodopsin (5, 15–17). Moreover, immunolocalization of one isoform of PPEF-2 that contains the EF-hand motifs localizes this protein to photoreceptor inner segments rather than outer segments (12). (The subcellular localization of a second PPEF-2 isoform that lacks the C-terminal domain is unknown.) These observations suggest that, in mammals, protein phosphatase 2A is the rhodopsin phosphatase and therefore that some aspects of PPEF function may not be conserved between vertebrate and invertebrate photoreceptors.

C. elegans contains a wide variety of sensory neurons (18–20), and the C. elegans genome is known to contain several hundred putative G protein-coupled receptors (21). Expression analysis has shown that these are widely distributed inside and outside the nervous system, with many receptors localizing to primary sensory neurons, including the three neurons responsible for responding to volatile odorants: AWA, AWB, and AWC (22–25). In this report, we show that expression of CePPEF, the sole member of the PPEF family in C. elegans, is restricted to several neurons in the head and tail, including the primary sensory neurons AWB and AWC. Within these sensory neurons, CePPEF is highly enriched in sensory cilia, and this localization depends on sequences at the extreme N terminus, which site-directed mutagenesis shows are the sites of both myristoylation and palmitoylation. Finally, we show that the two consensus EF-hand motifs within the C terminus of CePPEF bind calcium. These observations reveal a mechanism for membrane association of the PPEFs; strengthen the idea that PPEF activity is directly regulated by calcium binding; and suggest that some substrates of CePPEF are found in the sensory cilia, possibly including G protein-coupled receptors in and suggest that some substrates of CePPEF are found in the sensory cilia, possibly including G protein-coupled receptors in

C. elegans Immunostaining—Animals were synchronized by collecting N2 hermaphrodites from a single 10-cm plate, mixing gently in 10% bleach + 1 mM NaOH for 1 min, washing several times in M9 medium, and then gently rocking at room temperature for 4 h to allow all embryos to hatch. Approximately 100 L1 larvae were placed on a Probe-On Plus glass slide (Fisher), coverslipped, and then freeze-cracked under slight pressure. Animals were fixed in −20 °C methanol for 30 min, followed by 15 min in −20 °C acetone. Freeze-cracked animals were blocked for several hours in Tris-buffered saline with 0.5% normal goat serum, 0.3% Triton X-100, 0.1% sodium azide, and 10% normal goat serum (or 1% normal rabbit serum) to allow all antibodies to penetrate the sample.

Antibody Preparation and Purification—Fusion proteins between the bacteriophage gene 10 protein and the C-terminal 142 amino acids of CePPEF were expressed in E. coli, purified to apparent homogeneity on an amionose resin (New England Biolabs Inc.), covalently cross-linked to Affi-Gel 15 (Bio-Rad), and used to affinity purify the rabbit polyclonal antibodies.

Immunostaining of C. elegans Lysates—Three-cm plates of wild-type N2 animals or transgenic animals with the roller phenotype were resuspended in PBS and washed extensively. Nematodes were lysed in PBS with 1% Triton X-100; sonicated; and centrifuged at 731 × g at 4 °C to remove cell debris, genomic DNA, and cuticle fragments. The protein concentration in the supernatants was determined using the Bradford assay (Bio-Rad), and 20 μg of each lysate were subjected to SDS-PAGE analysis and electrophoretic transfer. Immunoblots were visualized with the Supersignal West Pico system (Pierce) after sequential incubation with the CePPEF C terminus-specific antibody and a horseradish peroxidase-conjugated goat anti-rabbit antibody.

293 Subcellular Fractionation—Twenty-four hours after transient transfection using the calcium phosphate method, cells were washed in PBS, dissociated in PBS with 5 mM EDTA, collected by centrifugation at 418 × g, resuspended in 6 ml of PBS with protease inhibitors (1 μg/ml each of chymostatin, leupeptin, aprotinin, and pepstatin A and 100 μg/ml phenylmethylsulfonyl fluoride), and disrupted with a Polytron 10/35 homogenizer (Kinematica). Nuclei were removed by centrifugation at 731 × g for 5 min at 4 °C, and a portion of the post-nuclear supernatant was saved as the lysate fraction. The remaining sample was centrifuged at 58,450 × g for 30 min at 4 °C. The high-speed supernatant was recovered, and the pellet was resuspended in 4 ml of 1× SDS sample buffer, sonicated for 1 min, and diluted to 6 ml. 6× SDS sample buffer was added to the lysate and supernatant fractions to a final concentration of 1×, and 20 μl of lysate, pellet, and supernatant fractions were then analyzed by SDS-PAGE and immunoblotting.

Immunostaining of 293 Cells—293 cells on gelatin-coated coverslips were transfected with 0.25 μg each of GFP/pCIS and CePPEF/pCIS using LipofectAMINE (Life Technologies, Inc.). Twenty-four hours after transfection, cells were fixed for 10 min in 4% paraformaldehyde in PBS and then permeabilized for 10 min. Cells were blocked for 1 h in PBS with 5% normal goat serum and incubated overnight at 4 °C in PBS with 5% normal goat serum and a 1:100 dilution of affinity-purified CePPEF C terminus-specific antibody. The following day, CePPEF was visualized by sequential addition of a biotinylated anti-rabbit antibody and Texas Red-streptavidin. Images were analyzed by confocal microscopy using a 1-μm slice thickness.

Calcium Binding Assays—

Generation of Transgenic C. elegans—GFP reporter vectors (60 μg/ml) were mixed with pRF4-Rol6 DNA (100 μg/ml), spun down, and injected into the gonads of L4 or early adult N2 hermaphrodites. Injected animals were recovered and designated as P0. F1 animals with the roller phenotype were isolated and observed for progeny that also showed the roller phenotype. F2 animals with the roller phenotype were generated from at least two different F0 injections for each GFP reporter vector construct analyzed. Adult animals were photographed after anesthetizing in 1 mM levamisole.

Antibody Preparation and Purification—Fusion proteins between the bacteriophage gene 10 protein and the C-terminal 142 amino acids of CePPEF were expressed in Escherichia coli, purified by preparative SDS-PAGE, and used for immunization of rabbits. Fusion proteins between the same CePPEF fragment and maltose-binding protein (MBP) were also produced in E. coli, purified to apparent homogeneity on an amionose resin (New England Biolabs Inc.), covalently cross-linked to Affi-Gel 15 (Bio-Rad), and used to affinity purify the rabbit polyclonal antibodies.

Calcium Binding Assays—Mutations in the C terminus of CePPEF were generated by polymerase chain reaction. MBP fusions with the C-terminal 142 amino acids of wild-type or mutant CePPEF were produced in E. coli. Bacteria were lysed in 10 mM imidazole, pH 7.0, 200 mM KC1, and 0.1 mM EDTA and then purified to near homogeneity by

Experimental Procedures

cDNA and Genomic Sequences—the phage clones encompassing the P23H11.8 (CePPEF) locus were isolated from a C. elegans genomic DNA library and used to prepare transgene constructs. The full-length green fluorescent protein (FL-GFP) transgene was generated by cloning the genomic region from the EcoRI site 3.0 kb 5′ of the FL-GFP/pCIS construct into the StuI site 2 kb 3′ of the stop codon near the end of exon 7 into pPD95.81. pPD96.04 and pPD95.67 were used to generate the I- and II fragment and sequencing. The full-length CePPEF cDNA was obtained by reverse transcription-polymerase chain reaction using random-primed cDNA from N2 hermaphrodites. Following subcloning, the cDNA sequence was confirmed to rule out spurious mutations. The CePPEF cDNA was modified by polymerase chain reaction to insert an optimal Kozak sequence 5′ of the initiator methionine codon and transferred to pCIS, a mammalian expression vector that utilizes the cytomegalovirus promoter. Subsequent mutagenesis of the N-terminal region was achieved by replacing the first 14 codons with synthetic double-stranded DNA segments containing the appropriate mutations.

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amylase affinity chromatography. Fusion proteins were dialyzed extensively against 10 mM imidazole, pH 7.0, and 200 mM KCl, which had been prerun over a Chelex 100 column. Binding assays were performed at 4°C in 10 mM imidazole, pH 7.0, 200 mM KCl, and 0.1 μCi/ml 45CaCl2 (Amersham Pharmacia Biotech) with the indicated concentrations of unlabeled CaCl2. In each binding reaction, the protein concentration was set to 20% of the Ca2+ concentration. The binding mixture was incubated at 4°C for 2 h, and bound calcium was purified by running 50 μl of the binding mixture over a Sephadex G-50 spin column prechilled (in the void volume) from free Ca2+ (retained in the column) occurs within several seconds. Calcium binding at each Ca2+ concentration was assayed in triplicate; a control reaction without protein was analyzed for each Ca2+ concentration and subtracted as background. Binding data were fit to a second-order polynomial equation.

For analysis of calcium binding under equilibrium conditions, Sephadex G-50 spin columns were pre-equilibrated with a solution that was identical to the binding assay contents described above, except for the last 10 amino acids, fused in-frame to GFP at the indicated StuI site. In the aa-(1)-NLS-GFP-LacZ construct, the first 14 codons of CePPEF are fused in-frame to GFP but with <14 CePPEF N-terminal amino acids were also constructed. For enhanced expression in C. elegans, the coding regions of GFP and lacZ contain 3 and 11 artificial introns, respectively, which are not shown.

RESULTS

Neuronal Localization of CePPEF in Transgenic C. elegans—As a first step in determining the cellular expression pattern of the CePPEF gene in C. elegans, a transgene construct was generated that fused 3.0 kb of DNA sequence upstream of the start codon to a GFP-LacZ reporter carrying an NLS (aa-(1)-NLS-GFP-LacZ) (Fig. 1). The large size of the GFP-LacZ fusion protein facilitates retention in the nucleus. Injection of the aa-(1)-NLS-GFP-LacZ construct into C. elegans revealed reporter gene expression in several anterior neurons, including AWB, AWC, AVA, AVB, AVX, BAG, and URX (Fig. 2F). The ASE neuron showed inconsistent transgene expression. To assess the subcellular localization of the CePPEF protein and to determine whether additional transcriptional regulatory elements exist in intronic sequences, a transgene was constructed in which a 9.5-kb genomic DNA fragment extending from 3 kb 5' of the start codon to a point 32 base pairs upstream of the CePPEF stop codon in exon 7 was fused in-frame to GFP (FL-GFP) (Fig. 1). This FL-GFP transgene directed GFP expression to the same set of neurons as the transgene construct described above (Fig. 2, compare A and B with F), and expression in a single posterior neuron was also more clearly observed (Fig. 2E). Within the expressing cells, the FL-GFP fusion protein localized efficiently to several structures beyond the cell soma, including axons that form the ventral nerve cord (Fig. 2D); dendrites that extend to the anterior tip of the animal (Fig. 2, A and B); and the cilia of neurons AWB, AWC, and BAG (Fig. 2C).

The transgene constructs described above showed minimal expression in non-neuronal cells. However, we observed transgene expression in intestinal, hypodermal, or muscle cells in occasional transgenic lines in which 3.0-kb genomic DNA fragments extending just past the CePPEF start codon directed production of short N-terminal regions of CePPEF fused to GFP (described below). As these expression patterns occurred sporadically, they likely reflect transgene effects rather than an expression pattern relevant to the endogenous CePPEF gene.

Immunolocalization of CePPEF—As a more direct method for determining the pattern of CePPEF protein localization, affinity-purified rabbit antibodies raised against the C-terminal 142 amino acids of CePPEF were used for immunostaining of C. elegans L1 larvae. The anti-CePPEF antibodies recognized a single polypeptide band of ~80 kDa on immunoblots of proteins from 293 cells transfected with CePPEF cDNA, and no bands were seen with proteins from untransfected cells (Fig. 3A). Immunoblots of wild-type C. elegans lysates showed two bands, one at 80 kDa, which corresponds closely to the predicted molecular mass of the full-length protein, and a fainter band of slightly lower molecular mass, which may correspond...
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Role of the CePPEF N Terminus in Membrane Association and Fatty Acylation in 293 Cells—As an independent test of the ability of the CePPEF N terminus to confer membrane association, we examined the properties of CePPEF and its various N-terminal mutants in 293 cells. 293 cells transfected with the wild-type CePPEF cDNA or with a CePPEF cDNA carrying the N-terminal amino acids from CePPEF were fused to an NLS-tagged GFP(aa-(1)-NLS-GFP, aa-(1–5)-NLS-GFP, etc.) (Fig. 1). The NLS-GFP reporter showed little nuclear retention, presumably due to its small size. The aa-(1)-NLS-GFP and aa-(1–5)-NLS-GFP transgenic lines showed localization primarily in the nucleus and cell body and weak or no localization in dendrites and cilia. As an initial step in assessing this possibility, we generated transgenic lines in which 1, 5, 8, 11, or 14 N-terminal amino acids from CePPEF were fused to an NLS-tagged GFP(aa-(1)-NLS-GFP, aa-(1–5)-NLS-GFP, etc.) (Fig. 1).

The CePPEF N Terminus Is Necessary and Sufficient for Localization to Axons, Dendrites, and Cilia—The extreme N terminus of CePPEF contains the myristoylation consensus sequence (MGXXX(S/T) . . . ) as well as the consensus sequence for N-terminal palmitoylation (MGC . . . ) (26, 27), suggesting that CePPEF may be membrane-associated via an N-terminal lipid modification. A membrane association of this type could play an important role in localizing CePPEF to axons, dendrites, or cilia. As an initial step in assessing this possibility, we generated transgenic lines in which 1, 5, 8, 11, or 14 N-terminal amino acids from CePPEF were fused to an NLS-tagged GFP(aa-(1)-NLS-GFP, aa-(1–5)-NLS-GFP, etc.) (Fig. 1). The NLS-GFP reporter showed little nuclear retention, presumably due to its small size. The aa-(1)-NLS-GFP and aa-(1–5)-NLS-GFP transgenic lines showed localization primarily in the nucleus and cell body and weak or no localization in dendrites and cilia (Fig. 4, A and B). In contrast, the aa-(1–11)-NLS-GFP and aa-(1–14)-NLS-GFP transgenic lines showed GFP localization primarily in axons, dendrites, and cilia, but not in the cell soma (Fig. 4, D and E). The aa-(1–8)-NLS-GFP fusion showed an intermediate pattern, with localization in axons, dendrites and cilia as well as in the cell body and nucleus (Fig. 4C).

To test whether the myristoylation and/or palmitoylation consensus sites are required for localization outside of the cell soma, site-directed mutants were constructed that disrupted either one or both fatty acylation sites. In one set of experiments, Gly³ was substituted with alanine in the context of aa-(1–14)-NLS-GFP or FL-GFP to simultaneously disrupt the myristoylation and palmitoylation consensus sequences. In both cases, the G2A mutant proteins localized to the cytoplasm and nucleus, suggesting that myristoylation, palmitoylation, or both modifications together confer membrane attachment and concomitant localization to axons, dendrites, and cilia (Fig. 4). To further refine this analysis, Cys³ was substituted with serine in the context of FL-GFP to eliminate only the palmitoylation site. This mutant protein showed low levels of axonal, dendritic, and ciliary staining (Fig. 4, compare F with E and H with G), suggesting that palmitoylation in particular is critical for membrane attachment.

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to either a CePPEF degradation product or a heretofore unrecognized splice variant (Fig. 3B). Immunoblots of lysates from FL-GFP transgenic animals showed a higher molecular mass band at ~120 kDa derived from the CePPEF-GFP fusion protein in addition to the endogenous CePPEF band. These data strongly imply that the anti-CePPEF antibodies specifically recognize CePPEF in C. elegans lysates. Immunostaining of wild-type L1 larvae with the anti-CePPEF antibodies demonstrated CePPEF in several cell bodies in the region of the nerve ring, in dendritic processes leading from these cells to the anterior end of the animal, and in sensory cilia at the extreme anterior end of the animal (Fig. 3C). This expression pattern closely resembles that observed with the GFP transgenic animals, indicating that the CePPEF-GFP transgenes accurately reproduce the endogenous CePPEF expression pattern.
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G2A or C3S mutation were analyzed by both subcellular fractionation and confocal microscopy. Subcellular fractionation showed that all of the wild-type protein, but little of the G2A or C3S mutant protein, was associated with the membrane fraction following high speed centrifugation (Fig. 5). Attempts to perform an analogous experiments using C. elegans failed because the tough C. elegans cuticle protects the animal from rupture by methods compatible with subcellular fractionation (data not shown). Confocal microscopy of transfected 293 cells immunostained with anti-CePPEF antibodies revealed wild-type and mutant proteins accumulated to comparable levels in the cytosol (Fig. 6). As observed in transgenic animals, the N-terminal 14 amino acids of CePPEF were sufficient to confer plasma membrane association when joined to GFP, and mutation of Gly² or Cys³ resulted in cytosolic localization (Fig. 7).

To define the presumptive N-terminal lipid modifications of CePPEF, wild-type and mutant CePPEFs tagged with triple-Myc epitopes at their C termini were expressed in 293 cells, metabolically labeled with [³H]myristic acid or [³H]palmitic acid, and immunoprecipitated. The wild-type protein incorporated both myristate and palmitate. By contrast, the G2A mutant, which affected only the palmitoylation consensus site, incorporated neither label, and the C3S mutant, which affected both the myristoylation and palmitoylation consensus sites, incorporated ⁴⁵Ca²⁺ and ⁵⁸⁵⁶g for 30 min to yield supernatant (S) and pellet (P) fractions. Equal fractions of each were subjected to SDS-PAGE and immunoblotting using affinity-purified antibodies directed against the CePPEF C terminus.

Each CePPEF C-terminal fusion protein was assayed for calcium binding based on its ability to bind and retain ⁴⁵Ca²⁺ during rapid passage through a small Sephadex G-50 spin column at 4 °C. This method has been used previously to measure the binding of radioligands to solubilized β-adrenergic (29) and γ-aminobutyric acid (30) receptors and of ⁴⁵Ca²⁺ to the solubilized mitochondrial F₄₅-ATPase (31). For ligands with dissociation rates of greater than several seconds, this method appears to give an accurate estimate of equilibrium binding. The assay consists of two steps: first, a binding step in which the fusion protein is incubated with ⁴⁵Ca²⁺, and second, a separation step in which free and bound Ca²⁺ are separated by passing the binding reaction over a Sephadex G-50 spin column. Centrifugation of the spin column leads to the retention of >97% of the free Ca²⁺ in the column and passage of 80–90% of the fusion protein together with bound Ca²⁺ into the void volume. In this assay, ²⁵Ca²⁺ was used as a probe of the increased ciliary concentration of CePPEF-GFP characteristic of the wild-type FL-GFP transgenic lines (compare with G and Fig. 2B).

**Fig. 4.** The N terminus of CePPEF is necessary and sufficient for localizing transgenic GFP fusion protein to axons, dendrites, and cilia of AWB, AWC, and BAG. In each panel, anterior is to the right, and the position of the sensory cilia is indicated by an arrow. The bars correspond to 25 μm. aa-(1)-NLS-GFP (A) and aa-(1–5)-NLS-GFP (B) show minimal dendritic and ciliary localization; aa-(1–8)-NLS-GFP (C) show an intermediate level of dendritic and ciliary localization, and aa-(1–11)-NLS-GFP (D) and aa-(1–14)-NLS-GFP (E) show strong dendritic and ciliary localization. The transgenic line in which Gly² was mutated to alanine (G2A) in the context of aa-(1–14)-NLS-GFP shows loss of axonal, dendritic, and ciliary localization (F). Also shown is transgenic C. elegans carrying wild-type FL-GFP (G), FL-GFP carrying the G2A mutation (H), or an FL-GFP transgene in which cysteine at position 3 was mutated to serine (C3S) (I). G2A-FL-GFP animals show a dramatic loss of axonal, dendritic, and ciliary localization. C3S-FL-GFP animals show diminished axonal, dendritic, and ciliary localization, with no evidence of the increased ciliary concentration of CePPEF-GFP characteristic of the wild-type FL-GFP transgenic lines (compare with G and Fig. 2B).

**Fig. 5.** CePPEF expressed in 293 cells is associated with the membrane fraction. 293 cells were transiently transfected with wild-type CePPEF cDNA (WT) or with the corresponding G2A or C3S mutants. Twenty-four hours after transfection, the cells were harvested, and a post-nuclear lysate (L) was centrifuged at 58,460 × g for 30 min to yield supernatant (S) and pellet (P) fractions. Equal fractions of each were subjected to SDS-PAGE and immunoblotting using affinity-purified antibodies directed against the CePPEF C terminus.

|        | WT    | G2A   | C3S   |
|--------|-------|-------|-------|
| L      | 97%   | 66%   | 46%   |
| S      | 97%   | 66%   | 46%   |

The CePPEF C Terminus Binds between 1 and 2 mol of Calcium/m mol of Protein—The PPEF family of protein phosphatases are so named because they contain two consensus EF-hand motifs near their C termini; but to date, there is no direct evidence that any PPEF family member binds calcium. Moreover, the loose nature of the EF-hand consensus has led to the suggestion that there may be as many as five EF-hand motifs within a single PPEF sequence (9). To directly assess the calcium-binding capacity of the CePPEF C terminus, a C-terminal segment of 142 amino acids containing the two CePPEF consensus EF-hand motifs was expressed as a C-terminal fusion to MBP in E. coli and purified by amylose affinity chromatography. Additionally, we constructed mutants EF1' and EF2', which would be predicted to impair calcium binding to the consensus EF-hands starting at amino acids 642 and 682, respectively, and the corresponding double mutant, EF1' + 2' (Fig. 9A).

Each CePPEF C-terminal fusion protein was assayed for calcium binding based on its ability to bind and retain ⁴⁵Ca²⁺ during rapid passage through a small Sephadex G-50 spin column at 4 °C. This method has been used previously to measure the binding of radioligands to solubilized β-adrenergic (29) and γ-aminobutyric acid (30) receptors and of ⁴⁵Ca²⁺ to the solubilized mitochondrial F₄₅-ATPase (31). For ligands with dissociation rates of greater than several seconds, this method appears to give an accurate estimate of equilibrium binding. The assay consists of two steps: first, a binding step in which the fusion protein is incubated with ⁴⁵Ca²⁺, and second, a separation step in which free and bound Ca²⁺ are separated by passing the binding reaction over a Sephadex G-50 spin column. Centrifugation of the spin column leads to the retention of >97% of the free Ca²⁺ in the column and passage of 80–90% of the fusion protein together with bound Ca²⁺ into the void volume. In this assay, the wild-type CePPEF C-terminal fusion protein bound 1.2 mol of Ca²⁺/mol of protein...
Localization, Acylation, and Calcium Binding of CePPEF

...WT CePPEF refers to the wild-type protein. ++ indicates strong membrane localization or fatty acid incorporation, whereas +/- indicates low but detectable levels of membrane association.

### Table I

| Membrane localization | WT CePPEF | G2A mutant | C3S mutant |
|------------------------|-----------|------------|------------|
| C. elegans 293 cells    | ++        | ++         | +/−        |
| Myristate               | +         | +          | +/−        |
| Palmitate               | +         | −          | −          |

...tein under equilibrium conditions, a method first described by Hummel and Dreyer (32), the Sephadex G-50 spin columns were extensively pre-equilibrated with a protein-free solution that was otherwise identical to the solution used for the binding reaction. In these experiments, the maximal observed binding for the wild-type C-terminal fusion protein was 1.4 mol of Ca\(^{2+}\)/mol of protein (Fig. 9C). Fitting these data to a two-ligand binding site dissociation curve yielded macroscopic dissociation constants of 36 and 1.0 \(\mu\)M, affinities typical for EF-hands (28).

### DISCUSSION

This work represents the first characterization of CePPEF, the sole member of the PPEF family of serine/threonine protein phosphatases encoded within the C. elegans genome. The results presented above show that (a) CePPEF gene expression is confined to a small subset of neurons, including primary sensory neurons; (b) the CePPEF protein is highly enriched in sensory cilia and, to a lesser extent, in axons and dendrites; (c) there are adjacent N-terminal myristoylation and palmitoylation sites in CePPEF that play an important role in correct membrane and subcellular localization; and (d) the CePPEF C terminus contains two EF-hand motifs that can bind calcium. We discuss below each of these findings in the context of PPEF function.

**EF-hands at the C Terminus of CePPEF Bind Calcium**—We have measured the stoichiometry and affinity of the C terminus of CePPEF for calcium using centrifugation through a small gel filtration column to effect a rapid separation of free and bound calcium. This assay has been used previously for the analysis of ligand-receptor and enzyme-substrate interactions (29–32) and...
may also be generally useful for the analysis of calcium binding to proteins that are difficult to assay by equilibrium dialysis due to protein adsorption to the dialysis membrane or protein denaturation or degradation during the extended incubation required for equilibrium dialysis. Moreover, for proteins with multiple calcium-binding sites, the analysis of binding stoichiometry is likely to be more straightforward with this gel filtration binding assay compared with assays that monitor intrinsic tryptophan fluorescence or competition with fluorescent calcium chelators.

Using this calcium binding assay, we found that the CePPEF C terminus bound between 1 and 2 mol of calcium/mol of protein, with dissociation constants in the low to mid micromolar range. Mutation of 2 amino acids predicted to coordinate calcium in each of the two consensus EF-hand motifs demonstrated that most of the calcium binding activity is referable to these EF-hand sequences. Mutations in the second EF-hand had a relatively greater effect on calcium binding than mutations in the first EF-hand, possibly due to the additional mutation of the glycine critical for permitting a sharp bend in the calcium-binding loop of the second EF-hand (see Fig. 9 A) (33).

Whether the reduction in maximal calcium binding observed in these EF-hand mutants reflects lower equilibrium calcium affinities and/or increased rates of calcium dissociation is an open question. Finally, we noted that the Ca\textsuperscript{2+}-binding affinity of the C-terminal fusion proteins tested here may differ from that of the full-length protein under in vivo conditions as a result of interactions with the more N-terminal PPEF domains or with other proteins.

If calcium binding to the C terminus of PPEF proteins is a general mechanism for directly regulating phosphatase activity as suggested by the calcium stimulation of rhodopsin dephosphorylation in Drosophila photoreceptor extracts (14), it will be of interest to determine the concentration dependence of this regulation, whether one or both calcium ions must bind to shift the enzyme between inactive and active states, and whether calcium binding activates the catalytic activity of some PPEFs and inhibits others. In rough agreement with this model, Huang and Honkanen (34) have reported that recombinant PPEF-1 exhibits modest calcium-stimulated phosphatase activity on the pseudosubstrate p-nitrophenyl phosphate with a half-maximal concentration for calcium stimulation of \(500 \mu M\). We note, however, that this concentration of half-maximal activation is significantly higher than the typical range of EF-hand calcium-binding affinities (28) and therefore may reflect a calcium effect independent of the EF-hands.

**FIG. 9.** The CePPEF C terminus binds between one and two Ca\textsuperscript{2+} ions through two consensus EF-hands. **A,** the EF-hand consensus sequence (28, 29) (\(J\) and \(O\) represent hydrophobic and oxygen-containing amino acid side chains, respectively); putative EF-hand sequences at the C termini of RdgC, the human PPEFs (hPPEF), and CePPEF; and mutations introduced into the first EF-hand (EF1\textsuperscript{1}), the second EF-hand (EF2\textsuperscript{1}), and both EF-hands (EF1\textsuperscript{+2}) of CePPEF in the context of a MBP fusion to the C-terminal 142 amino acids of CePPEF. Dashes indicate the identical amino acid as the wild-type CePPEF (WT). EF1 contains mutations in two predicted Ca\textsuperscript{2+}-liganding residues, and EF2\textsuperscript{1} contains mutations in two predicted Ca\textsuperscript{2+}-liganding residues and a conserved glycine residue. **B,** each fusion protein was expressed in E. coli, purified to near homogeneity on an amylose resin, and analyzed for \(\text{^{45}}\text{Ca}^2\) binding by rapid passage through a gel filtration column in calcium-free buffer. Continuous lines are best fitting second-order polynomials. **C,** the wild-type CePPEF C-terminal fusion protein was also analyzed after rapid passage through a gel filtration column pre-equilibrated in a solution containing the identical concentration and specific activity of Ca\textsuperscript{2+} as the binding mixture. The continuous line represents fitting of the data to the Aldair-Klotz model for two binding sites. First and second macroscopic binding constants are given.
An additional mode of regulation by calcium deserves consideration: calcium-dependent membrane association, as seen, for example, in the “myristoyl-switch” mechanism of the recovering family of EF-hand proteins (35–37). To test this hypothesis, native PPEF-2 from bovine retina and recombinant CePPEF from transfected 293 cells were analyzed by immunoblotting after homogenization in PBS with 1 mM CaCl₂ or 1 mM EGTA, followed by separation of membrane and cytosol fractions via centrifugation. The presence or absence of Ca²⁺ did not alter the membrane versus cytosolic distribution of either protein (data not shown). Although it remains possible that our choice of in vitro conditions inhibited or masked a calcium-regulated membrane association, these data are most consistent with a model in which calcium binding by the C-terminal EF-hands serves simply to regulate phosphatase activity, as seen in the regulation of the catalytic subunit of calcineurin by the EF-hand-containing β-subunit (38, 39).

Function of N-terminal Myristoylation and Palmitoylation—At its N terminus, CePPEF contains myristoylation (MGXXX . . . ) and palmitoylation (MGC . . . ) consensus sites (26, 27); and in transfected 293 cells, the wild-type protein incorporates [³H]myristate and [³H]palmitate and is efficiently localized to the plasma membrane. Mutation of Cys³ in the palmitoylation consensus sequence still allows for myristoylation at a second site (42).

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Many classes of signaling molecules, including G protein α-subunits, non-receptor tyrosine kinases, and G protein regulators, have been reported to carry either palmitate or myristate at their N termini (43–47). However, only a few of these molecules are both myristoylated and palmitoylated. Examples of such doubly modified proteins are Goα, Gzα, and Fyn (46–48). The functional consequence of having both modifications is unclear, as palmitoylation alone suffices for efficient membrane association (40, 41, 49). One possibility is that, in C. elegans, palmitoylation of CePPEF may be heterogeneous such that one subset of CePPEF proteins is palmitoylated and strongly membrane-associated, and a second subset of CePPEF proteins carries only a myristoylated group and can therefore shuttle between the membrane and cytosol. A variation on this theme is suggested by the observation that palmitoylation is a dynamic and regulated process (50–53). For example, activating Goα by exposing cells to the β-adrenergic agonist isoproterenol or to cholera toxin accelerates Goα depalmitoylation (54, 55), whereas activating platelets with thrombin leads to rapid palmitoylation of several proteins (50). Our observation that CePPEF immunoreactivity in wild-type nematodes and GFP fluorescence in full-length CePPEF-GFP (FL-GFP) transgenic animals encompass both the cell soma and membrane-rich subcellular compartments such as dendrites and cilia is consistent with the possibility that there may be populations of CePPEF proteins that are differentially targeted.

Another reason for the dual acylation of CePPEF may be to allow the myristoyl group to mediate a function other than membrane attachment. Indeed, fatty acyl groups such as the palmitate groups in Goα and GAP-43 have been shown to activate or inhibit protein-protein interactions (56, 57). It is of interest that other PPEF family members differ with respect to the two fatty acylation consensus sites within their N-terminal sequences: Drosophila RdgC lacks both palmitoylation and myristoylation consensus sites; human PPEF-1 has both sites; and human PPEF-2 has only the myristoylation site. Thus, the degree of membrane association or other characteristics referable to fatty acylation are likely to differ among PPEF family members.

Ciliary Localization of CePPEF—The patterns of immunolocalization for CePPEF and of GFP fluorescence for the various CePPEF-GFP fusion proteins, including those carrying only 14 N-terminal amino acids from CePPEF, show the greatest concentration of signal within the sensory cilia, suggesting that the N terminus of CePPEF carries signals both for membrane targeting and for localizing CePPEF to cilia. These observations are reminiscent of previous work on the neuronal protein GAP-43, which is also palmitoylated at the N terminus. Fusion proteins consisting of the N-terminal 10 amino acids of GAP-43 and choline acetyltransferase localize to growth cones and filopodia when expressed in PC12 cells (58).

The association of CePPEF with membranes and its further enrichment within cilia could confer any of several advantageous properties. First, it could promote more efficient encounters with integral membrane, membrane-associated, or cilium-specific protein substrates; second, it could facilitate calcium regulation by localizing CePPEF to the juxtamembrane zone where the amplitude of calcium transients is greatest; and third, it could allow regulation of CePPEF by membrane-associated regulatory proteins such as kinases.

Localization of PPEFs to Sensory Neurons in Diverse Organisms—The localization of CePPEF described here extends to a new species the pattern of nervous system-specific expression seen for the PPEF family. Like other PPEFs, CePPEF is expressed within a subset of primary sensory neurons; and like RdgC, it is localized to the subcellular compartment in which sensory transduction occurs. This localization reinforces the impression that the PPEF family displays a remarkable degree of tissue-specificity relative to other serine/threonine protein phosphatases. One particularly interesting domain of CePPEF expression is the cilia of the neurons AWB and AWC, as several proteins that are critical for odorant detection and/or adaptation and that are known to be involved in G protein signaling are also strongly concentrated there (24, 25, 59–63). This suggests that CePPEF may also participate in the G protein signaling pathway mediating odorant detection and/or adaptation, possibly by dephosphorylating G protein-coupled receptors much like rdgC dephosphorylates Drosophila rhodopsin. A behavioral analysis of CePPEF mutants utilizing volatile odors recognized by the neurons AWB and AWC would be an important step in addressing these hypotheses.

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