



Evolution and functional diversity of the Calcium Binding Proteins (CaBPs)

Lee P. Haynes*, Hannah V. McCue and Robert D. Burgoyne

The Physiological Laboratory, Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool, UK

Edited by:

Karl-Wilhelm Koch, Carl von Ossietzky University Oldenburg, Germany

Reviewed by:

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*Correspondence:

Lee P. Haynes, The Physiological Laboratory, Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Crown Street, Liverpool L69 3BX, UK.
e-mail: leeh@liv.ac.uk

The mammalian central nervous system (CNS) exhibits a remarkable ability to process, store, and transfer information. Key to these activities is the use of highly regulated and unique patterns of calcium signals encoded by calcium channels and decoded by families of specific calcium-sensing proteins. The largest family of eukaryotic calcium sensors is those related to the small EF-hand containing protein calmodulin (CaM). In order to maximize the usefulness of calcium as a signaling species and to permit the evolution and fine tuning of the mammalian CNS, families of related proteins have arisen that exhibit characteristic calcium binding properties and tissue-, cellular-, and sub-cellular distribution profiles. The Calcium Binding Proteins (CaBPs) represent one such family of vertebrate specific CaM like proteins that have emerged in recent years as important regulators of essential neuronal target proteins. Bioinformatic analyses indicate that the CaBPs consist of two subfamilies and that the ancestral members of these are CaBP1 and CaBP8. The CaBPs have distinct intracellular localizations based on different targeting mechanisms including a novel type-II transmembrane domain in CaBPs 7 and 8 (otherwise known as calneuron II and calneuron I, respectively). Recent work has led to the identification of new target interactions and possible functions for the CaBPs suggesting that they have multiple physiological roles with relevance for the normal functioning of the CNS.

Keywords: calcium, Calcium Binding Protein, bioinformatics, protein evolution, protein targeting, protein-protein interaction

INTRODUCTION

Communication between neurons lies at the heart of abstract higher level cognitive processes including memory acquisition, learning, and complex reasoning. Fundamental to the mechanism by which all mammalian neurons communicate is intracellular calcium signaling (Berridge, 1998; Lohmann, 2009). Complex patterns of spatial and temporal calcium signals drive alterations in synaptic plasticity and neuronal gene expression which in turn affect neuronal architecture and connectivity to influence higher brain function (Catterall and Few, 2008; Greer and Greenberg, 2008). At the single neuron level, unique pre- and post-synaptic calcium signals generated by the opening of plasma membrane (PM) voltage sensitive- or ligand gated-ion channels are initially decoded by families of small calcium sensing proteins that exhibit distinct calcium binding characteristics in combination with specific patterns of cellular expression and sub-cellular localization (Haeseleer et al., 2000; Burgoyne and Weiss, 2001; Burgoyne et al., 2004; Burgoyne, 2007). Calcium binding typically elicits a conformational switch in the sensor (Haynes and Burgoyne, 2008; Ames and Lim, 2011) which in turn permits association with specific downstream effectors to modulate intracellular signaling cascades and ultimately neuronal activity and local synaptic structure.

In mammals, the largest class of calcium sensing proteins are those belonging to the calmodulin (CaM) superfamily that is defined by the EF-hand calcium binding motif (Kawasaki et al., 1998). CaM is expressed in all plants and animals and exerts essential functions in many aspects of normal cellular physiology

(Klee et al., 1980). One CaM-related sub-family of calcium sensors, the Calcium Binding Proteins or CaBPs, has recently been shown to have co-evolved with vertebrate animals (McCue et al., 2010a). The CaBPs share a similar domain organization with CaM and have four EF-hand motifs (Haeseleer et al., 2000; Mikhaylova et al., 2006) however, they exhibit significant sequence divergence from their common ancestor and this is reflected in unique structural and functional properties (McCue et al., 2010b; Mikhaylova et al., 2011). The CaBPs are enriched in neuronal tissues where they have been shown to act as important regulators of key calcium influx channels. Coupled to their vertebrate specific expression profile the available evidence implicates CaBPs specifically as mediators of central nervous system (CNS) behavior in higher animals. Perhaps not unexpectedly members of the CaBP family regulate target effectors in common with CaM however, in every instance thus far examined there has been no detectable redundancy and CaBP target regulation appears distinct to that exerted by CaM (Lee et al., 2002; Haynes et al., 2004; Kinoshita-Kawada et al., 2005; Zhou et al., 2005; Tang et al., 2007; Li et al., 2009; Findeisen and Minor, 2010; Minor and Findeisen, 2010; Few et al., 2011; Oz et al., 2011). The importance and specificity of CaBP function is further highlighted by the distinct phenotypes exhibited by CaBP4 and CaBP5 knock-out mice and the visual impairment observed in human patients carrying mutations in the CaBP4 gene (Jeziorski et al., 2000; Williams, 2006; Zeitz et al., 2006; Rieke et al., 2008; Littink et al., 2009; Aldahmesh et al., 2010). It has also been discovered that the CaBPs exhibit specific

target interactions independent of CaM and intriguingly some of these appear to have co-evolved with the CaBP family during the emergence of vertebrates (McCue et al., 2010a).

A picture is now emerging whereby the CaBP family can be viewed as providing an expansion in CaM functionality by exerting additional levels of target regulation for shared effectors in addition to executing novel functions through unique effector interactions. This increase in signaling complexity maximizes the range of physiological calcium signals that can be utilized and may have been instrumental in the evolution of complex vertebrate nervous systems that we observe today (Williams, 2006). This paper will focus on the evolution, mechanisms of targeting, and emerging roles of the CaBP protein family and discuss the importance of recent experimental findings in the context of mammalian CNS function.

METHODS

Hela cells were cultured in 75 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells for transfection were seeded onto coverslips in a 24-well tray at a density of ~4 × 10⁵ cells/well. 1 μg of mCherry-TM7 [residues 188–215 of human CaBP7 (transmembrane domain: residues 189–205)] or mCherry-CaBP7 plasmid was transfected per well using Genejuice transfection reagent (Novagen) according to manufacturer's instructions. Cells were maintained for 24 h post-transfection before fixation. Cells were washed three times in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 2 mM NaH₂PO₄, pH 7.4) then fixed in 4% formaldehyde in PBS at room temperature for 10 min. Cells were then washed a further three times in PBS then permeabilized using 0.2% triton X-100 in PBS for 6 min at room temperature. Cells were washed three times in PBS to remove detergent then twice in PBS containing 5% (w/v) BSA (PBSB). Cells were incubated with anti-TOM20 (1:1000, BD Biosciences) or anti-Calnexin (1:200, Sigma) primary antibodies for 1 h at room temperature in PBSB. Cells were washed three times in PBS and twice in PBSB prior to incubation with the relevant species specific fluorophore conjugated secondary antibody for 1 h at room temperature. Cells were washed again three times in PBS, rinsed with deionized H₂O, dried, and mounted onto glass slides using ProLong Gold antifade reagent (Invitrogen). Imaging of transfected and immunostained cells was carried out using a Leica AOBSP2 microscope (Leica microsystems, Heidelberg, Germany) using a 63x oil immersion objective with a 1.4 numerical aperture. In most cases the pinhole was set to Airy1 to give the optimum signal to noise ratio and hence minimum thickness confocal section for the excitation laser used.

CaBP EVOLUTION

The CaBP family in humans comprises six proteins: Caldendrin/CaBP1, 2, 4, 5, 7, and 8 (Haeseleer et al., 2000; Wu et al., 2001; Mikhaylova et al., 2006). The CaBP3 that was originally identified is likely to be a pseudogene and no CaBP6 gene exists. CaBP7 and CaBP8 have been referred to by the alternative names calneuron II and calneuron I, respectively, in other studies (Wu et al., 2001; Mikhaylova et al., 2006, 2009;

Hradsky et al., 2011) but will henceforth be referred to as CaBP7 and CaBP8 in this paper. We have taken this approach for self-consistency (our previously published work has used these protein identifiers) and also because bioinformatics analyses links CaBP 7 and 8 to CaBPs 1–5 more than to any other small EF-hand Ca²⁺-sensors (McCue et al., 2010a). Alternative splicing of the CaBP1 and 2 genes generates additional novel transcripts so that the final complement of CaBPs numbers nine distinct proteins in humans (McCue et al., 2010a). These proteins share a core domain comprised of four EF-hand motifs but differ in unique regions located at the extreme N- or C-termini (McCue et al., 2010b). The EF-hand core represents the region with greatest similarity to CaM however, the most closely related family member, CaBP2-Short, only shares 37.8% total sequence identity with its primordial ancestor suggesting that the unique N- and C-terminal domains of the CaBPs are highly divergent and likely confer specific cellular functions and target regulation distinct from that exerted by CaM (McCue et al., 2010a). The EF-hand core additionally exhibits divergence within the CaBP family and there are unique patterns of EF-hand inactivation coupled to sequence substitutions conferring binding specificity preferentially toward either Ca²⁺ or Mg²⁺ ions (McCue et al., 2010a). The unique cation binding properties of individual CaBP proteins will further influence their biological activity and increase the specificity of cellular Ca²⁺-signals to which they are able to respond.

How these proteins evolved from their CaM ancestor was unclear until a recent bioinformatic analyses examined available invertebrate and vertebrate genome sequence databases in an effort to locate CaBP-related transcripts (McCue et al., 2010a). From these analyses it was determined that the oldest species harboring CaBP-related genomic DNA sequences was the cartilaginous fish, the elephant shark (*Callorhincus milli*). No invertebrate genomes analyzed, including the widely used experimental model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, where genome coverage is >10X, contained CaBP like sequences (C.elegans, 1998; Adams et al., 2000; McCue et al., 2010a). The elephant shark genome sequencing project currently stands at 1.4X coverage however, sequences with homology to CaBPs 1, 2, 5, 7, and 8 are clearly present based on BLAST searches against the human coding sequences [(McCue et al., 2010a) and **Table 1**].

Further detailed analysis of protein alignment data showed that the elephant shark genome contains a CaBP1 like sequence having 46% sequence coverage of the human orthologue and 90% identity (**Figure 1A**). The coverage for elephant shark CaBP7 and CaBP8 sequences was found to be significantly greater and these again shared considerable identity with the corresponding human sequences (**Figure 1B**). In the study of McCue et al. (McCue et al., 2010a) no CaBP like sequences were discovered in the jawless fish, lamprey which is thought to represent the oldest living organism related to the human vertebrate ancestor. Coverage of the Lamprey genome has since improved and a new series of specific BLAT and BLAST searches performed here has uncovered two partial CaBP like sequences with highest homology to human CaBP1 and CaBP8 (**Figures 1A,B**). It would appear, therefore, that there were two ancestral members of the CaBP family, CaBP1 and CaBP8. This observation is consistent with the

Table 1 | Protein sequence fragments identified in BLAST searches against the elephant shark genome (left column) along with the sequence of the corresponding CaBP exon in the human genome (right column) and the percentage identity between the two sequences.

Contig	Sequence	Homology	Exon	Human exon sequence	%Identity
AAVX01573327.1	FDRDRDGLISCRDLGNLMRTMG YMPTEMELIELSQINMN	CaBP5	3	ELREAFLEFDKDRDGFISCKDL GNLMRTMGYMPTEMELI ELGQQIRMN	87
AAVX01478821.1	VGGHVDFDDFVELMGPKLLAETADM IGVKELRDAFKE	CaBP1	4	LGGHVDFDDFVELMGPKLLAETADMIGVK ELRDAFRE	94
AAV01407556.1	VGGRVNFEDFVE*MAPKLLAETADMIGIK	CaBP2	5	SGGKVDFFDFVELMGPKLLAETADM IGVRELDAFRE	78
AAVX01108357.1	EIRKAFKVFDRDNGFISKQELG MAMRSLGYMPNEVELEVIQRLDMD	CaBP7	2	EIREAFKVFDRDNGFISKQOE LGTAM'RSLGYMPN EVELEVIQRLDMD	95
AAVX01059912.1	GDGQVDFEEFVSLGPRLSSAA IPEKFGHTEFDNVFWK	CaBP7	3	GDGQVDFEEFVTLGPKLSTSGIPEKFHGT DFDTVFWK	81
AAVX01059912.1	CDMQRMTVEELKRLLYEAFCEHLSMKDEI NIIMTEEGHVDNPDECPVDIDSK	CaBP7	4	CDMQKLTVDLKRLLYDT FCEHL SMKDIENIIMTEESHGTAEECPVDVET	71
AAVX01124961.1	STQIQKQTCRLKSLICAFIAFIIS VMLIAANQVLRSGMK	CaBP7	5	CSNQQIRQTCVRKSLICAFIAFII SVMLIAANQVLRSGMK	80
AAVX01563725.1	MPFHPVHGSLLYKGSFLESLSDTSET EQLANISEEELD	CaBP8	1	MPFHHTAGLLYKGNLYLNRSLSA GSDSEQLANISVEELD	66
AAVX01190659.1	EIREAFRVLDRDNGFISKQELGMAMRSL GYMPSEVELAIIMQRLDMDG	CaBP8	2	EIREAFRVLDRDNGFISKQELGMA MRSLGY MPSEVELAIIMQRLDMDG	98
AAVX01097552.1	DGQVDFEEFMTILGPKLLTSEV REGFHGSAIDSIFW	CaBP8	3	DGQVDFDEFMTILGPKLVSEGR DGFLGNTIDSIFWQ	77
AAVX01062942.1	QFDMQRITLLEELKHILFHAFRDHLMKDD IENIIINEEESLNENSNSCQTEFEG	CaBP8	4	FDMQRITLLEELKHILYHAFRDHLT MKDIENIIINEEESLNENSGNCQTEFEG	90
AAVX01282372.1	VHSQKQNRQTCVRKSLICAFGVA FIISVMLIAANQILRNGME	CaBP8	5	VHSQKQNRQTCVRKSLICAFAMAFIIS VMLIAA NQILRSGME	92

CaBP family forming two distinct sub-families based on unique sequence characteristics, the first comprising CaBPs1–5 and the second consisting of CaBP7 and CaBP8 (McCue et al., 2010a). Collectively, these results suggest that the CaBP protein family arose specifically with the emergence of vertebrate species and that whole genome duplication event, acting initially on CaBP1- and CaBP8-like genes present in the jawless fish, likely gave rise to the full complement of CaBP proteins found from the bony fish onwards through vertebrate evolution (McCue et al., 2010a).

In addition to examining the evolution of CaBP proteins by performing searches based on the full-length human coding sequences (McCue et al., 2010a), domains unique to individual CaBPs were also analyzed to examine if there was evolutionary conservation or divergence which might indicate correspondingly conserved or unique cellular functions. It was determined that the novel N-terminal domain of CaBP1-Long (residues 16–75) was relatively well conserved from amphibians to humans which may correlate with an important vertebrate specific activity in the inhibition of Ca²⁺-release from ER resident inositol 1, 4, 5-trisphosphate (IP₃) receptors (IP₃Rs) (Haynes et al., 2004; Kasri et al., 2004; Li et al., 2009; McCue et al., 2010a). In contrast, the variable N-terminal regions of CaBP2 and CaBP4 both exhibited a progressive increase in sequence similarity from the bony fish to humans. As both of these proteins are expressed in sensory neurons these observations may correspond to an increase in the complexity of the audio-visual sensory system during vertebrate evolution. Specific domains of CaBP7 and

CaBP8 were not included in this analysis but recent evidence characterizing the unique functional properties of the hydrophobic C-termini of both of these family members (McCue et al., 2011) led us to evaluate the evolutionary conservation of these regions (**Figure 1B**). The hydrophobic C-terminal 38 residues of both CaBP7 (residues 177–215) and CaBP8 (residues 181–219) exhibit significant sequence conservation across vertebrate evolution. There is 92% sequence similarity between lamprey and human CaBP8 sequences in the C-terminal region and 94% similarity between elephant shark and human CaBP7 in the corresponding region. This high degree of domain conservation is indicative of a conserved function and is consistent with this region of CaBP7 and CaBP8 controlling the normal sub-cellular targeting of both proteins to the trans-Golgi network (TGN) and post-TGN vesicles (McCue et al., 2009; Mikhaylova et al., 2009; Hradsky et al., 2011).

The conservation of CaBP family members throughout vertebrate evolution raised a further interesting question regarding the potential co-evolution of specific interacting proteins (McCue et al., 2010a) which might shed light on vertebrate specific functions for this protein family. There are numerous documented interactions between CaBPs and various classes of cellular calcium channels including IP₃Rs (Haynes et al., 2004; Kasri et al., 2004) and voltage gated calcium channels (VGCCs) (Lee et al., 2002; Haeseleer et al., 2004; Zhou et al., 2004, 2005; Cui et al., 2007; Lee et al., 2007b; Tippens and Lee, 2007; Few et al., 2011). These channels are also targets of CaM, however, in mammalian

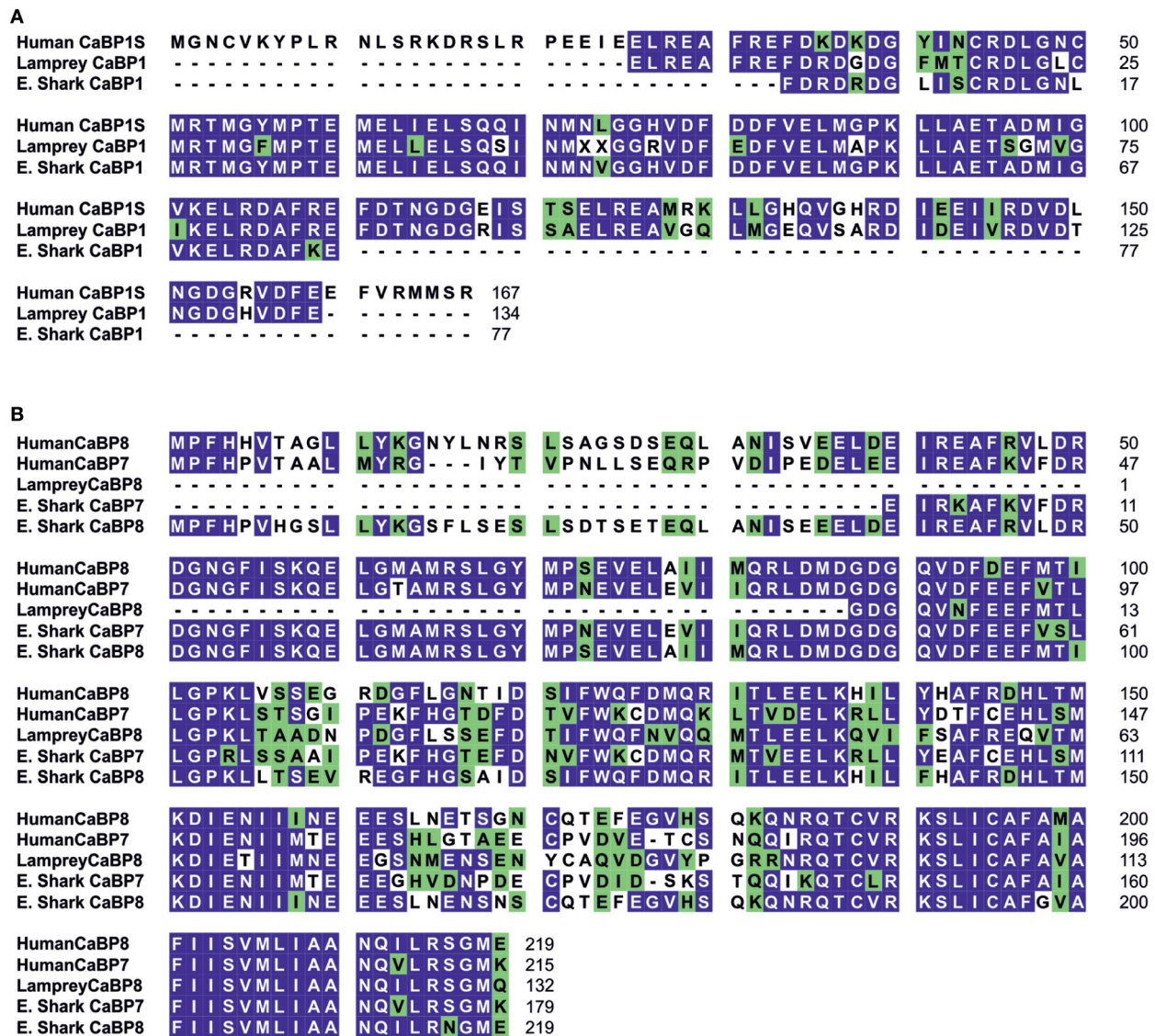


FIGURE 1 | Bioinformatic analysis of CaBP1, 7, and 8 proteins during vertebrate evolution. (A) Protein sequence alignment for human CaBP1-Short (Genbank accession: NM_004276) with related protein sequences identified from BLAT/BLAST searches of Lamprey and Elephant Shark (E. Shark) genomes. Identical residues between = 50% aligned sequences are highlighted in blue, similar residues are highlighted in green. Lamprey CaBP1 shares 80% coverage and 76% identity with human CaBP1-Short. Elephant Shark CaBP1 shares 46% coverage and 90% identity with human CaBP1-Short. **(B)** Protein sequence alignment for human CaBP7 (Genbank accession: NM_182527) and CaBP8 (Genbank accession:

AY007302) with BLAT/BLAST identified related sequences from Lamprey (CaBP8) and Elephant Shark (CaBP7 and CaBP8). Identical residues between = 50% aligned sequences are highlighted in blue, similar residues are highlighted in green. Lamprey CaBP8 exhibited 61% and 60% coverage and 56% and 65% identity with human CaBP7 and human CaBP8, respectively. Elephant Shark CaBP7 exhibited 83% and 82% coverage and 84% and 66% identity with human CaBP7 and human CaBP8, respectively. Elephant Shark CaBP8 exhibited 102% and 100% coverage and 63% and 87% identity with human CaBP7 and human CaBP8, respectively.

systems it would appear that the CaBPs and CaM have the capacity to differentially regulate common channel targets (Lee et al., 2002; Haynes et al., 2004; Kasri et al., 2006; Minor and Findeisen, 2010). Within the CaBP family further examples of regulatory diversity exist and both Caldendrin and CaBP1-Short modulate L-type VGCCs in distinct ways even though they share a common C-terminal EF-hand domain (Tippens and Lee, 2007). VGCCs and IP₃Rs are present in invertebrates, however, the

number of specific isoforms has increased with the appearance of vertebrates (Jeziorski et al., 2000; Iwasaki et al., 2002; Zhang et al., 2007; McCue et al., 2010a). It seems likely that the co-evolution of key calcium channels tailored to discrete functions within the mammalian CNS in conjunction with new types of calcium sensor proteins including the CaBPs has been instrumental in permitting vertebrates to generate and utilize an extended repertoire of spatio-temporal calcium signals. This in turn has

likely influenced the evolution of increasingly complex modes of neuronal communication and enhanced CNS processing power.

CaBP TARGETING

CaM is a cytosolic protein that lacks primary sequence information or post-translational modifications which would mediate its specific localization to subcellular organelles (Mikhaylova et al., 2006). This feature of CaM perhaps underlies its versatility as a Ca^{2+} -sensor and helps explain its ability to interact with a wide spectrum of cellular effectors, only becoming recruited to specific cellular domains at specific time points through formation of multiple distinct complexes. The cellular activity of CaM consequently often relies on Ca^{2+} increases that are generated globally throughout the cytoplasm (Parekh, 2011). In order to maximize the usefulness of Ca^{2+} as a second messenger, mechanisms have evolved for the generation Ca^{2+} -signals that are highly restricted in both space and time to ensure that only specific cell signaling pathways are activated or inhibited without perturbing other Ca^{2+} -sensitive events within the cell. For Ca^{2+} to be used in this manner it follows that calcium sensing proteins dedicated to the detection of such specific signals must be similarly localized or harbor the ability to become localized in response to the signal. A variety of mechanisms are employed by the CaBPs to restrict their localization to particular cellular organelles and domains. Like CaM, a number of the CaBP proteins (Caldendrin, CaBP4 and CaBP5) contain no inherent primary sequence targeting information or consensus sequences for post translational modification that might influence their retention at specific subcellular locations (Haeseleer et al., 2000). The strategy employed in these instances relies on interactions with target effectors that in turn are specifically localized (Haeseleer et al., 2004; Rieke et al., 2008).

Caldendrin is enriched in post-synaptic density protein fractions (Seidenbecher et al., 1998) and specifically interacts with a light chain of the microtubule associated protein MAP1A/B (Seidenbecher et al., 2004). More recently a novel interaction between Caldendrin and a retinal specific small EF-hand containing calcium sensor, recoverin, was reported (Fries et al., 2010). Recoverin is a photoreceptor-enriched, myristoylated protein that exhibits a so called Ca^{2+} -myristoyl switch mechanism (Tanaka et al., 1995; Ames et al., 1997; Ames and Ikura, 2002) whereby the acyl group is sequestered within the protein core in the absence of Ca^{2+} and, on Ca^{2+} -binding and a conformational rearrangement (Yap et al., 1999), is extruded to permit dynamic association with cellular membranes. Caldendrin interacted with recoverin in a calcium dependent manner and this was reported to traffic the normally cytosolic Caldendrin protein to Golgi membranes (Fries et al., 2010). This mechanism of targeting is potentially interesting since many neuronal populations often express multiple different Ca^{2+} -sensing proteins. Further investigations will be required to validate this targeting mechanism for the endogenous proteins, in particular it would be of interest to examine what effect depletion of recoverin has on the ability of Caldendrin to redistribute in response to elevations in cytoplasmic Ca^{2+} concentration. Since the C-terminal EF-hand containing domain of Caldendrin and not its variable N-terminal region was able to bind to recoverin (Fries et al., 2010) and because this domain is identical in the other CaBP1 splice isoforms, CaBP1-Long and CaBP1-Short,

it would also be of interest to test the promiscuity of recoverin binding with respect to other CaBP1 interactions.

Shorter splice variants of the Caldendrin/CaBP1 gene, CaBP1-Long, and CaBP1-Short (Landwehr et al., 2003; Haynes et al., 2004; Kasri et al., 2004) incorporate a distinct exon, not present in the Caldendrin transcript, that encodes for an N-terminal myristoylation consensus site (Landwehr et al., 2003). N-myristoylation of both proteins has been proven essential for localization to the PM and membranes of the Golgi apparatus and for functional inhibition of IP_3Rs (Haynes et al., 2004; Kasri et al., 2004).

The remaining two members of the CaBP family, CaBP7 and CaBP8, like CaM, Caldendrin, CaBP4 and CaBP5 contain no consensus motifs for post-translational modifications that are known to mediate association with cellular membranes (Wu et al., 2001; Mikhaylova et al., 2006). Surprisingly, when the localization of these proteins was examined in mammalian cell lines, they were found to specifically localize to membranes of the TGN and vesicular compartments of the constitutive secretory pathway (McCue et al., 2009; Mikhaylova et al., 2009). Closer inspection of the CaBP7 and CaBP8 primary sequences (McCue et al., 2009) using transmembrane domain prediction tools uncovered the presence of a highly hydrophobic 38 residue C-terminal extension not present in the other CaBPs that was strongly predicted to form a transmembrane domain. Cellular and biochemical characterization of these sequences determined that they do indeed operate as transmembrane domains which are essential for normal CaBP7 and CaBP8 localization (McCue et al., 2009). Furthermore it was demonstrated that chimeric constructs encoding normally cytosolic proteins fused to the CaBP7 and CaBP8 transmembrane domains were efficiently targeted to membranes of the secretory pathway (McCue et al., 2009).

The CaBP7 and CaBP8 transmembrane domains are located 10 residues from the C-terminus in each protein and this topology is consistent with that of the tail-anchor class of type-II transmembrane proteins that have cytosolically oriented N-terminal functional domains, a transmembrane domain and a short luminal C-terminal tail (Borgese et al., 2003, 2007; Brambillasca et al., 2006; McCue et al., 2011). Tail-anchor proteins are defined by their post-translational insertion into biological membranes through a series of novel pathways, the molecular details of which remain to be fully resolved (Abell et al., 2004; Stefanovic and Hegde, 2007; Borgese and Fasana, 2011). CaBP7 and CaBP8 have recently been shown to adopt the expected tail-anchor protein topology (Hradsky et al., 2011; McCue et al., 2011) and to interact with the ATPase TRC40/Asna1 (Hradsky et al., 2011) (**Figure 3**) a protein implicated in the post-translational membrane insertion of other tail-anchored proteins (Stefanovic and Hegde, 2007; Rabu et al., 2008). Tail-anchor proteins are initially post-translationally inserted either into the endoplasmic reticulum, outer mitochondrial- or peroxisomal-membranes (Borgese et al., 2007; Borgese and Fasana, 2011). Peroxisomes and mitochondria represent terminal destinations, however, ER targeted tail-anchor proteins can subsequently traffic along the secretory pathway and access a number of possible cellular membranes. Analysis of the CaBP7 and CaBP8 transmembrane domains explains why these proteins are observed at the TGN and on transport vesicles (McCue et al., 2009, 2011; Hradsky et al., 2011).

When fused to the normally cytosolic fluorescent tag protein mCherry, a C-terminal domain fragment of CaBP7 incorporating the predicted transmembrane domain [residues 188–215 (transmembrane domain: residues 189–205)] showed no colocalization with the outer mitochondrial membrane tail-anchored protein TOM20 (**Figure 2A**). In contrast, it exhibited a reticular distribution in a proportion of HeLa cells where it colocalized extensively with the ER marker calnexin (**Figure 2B**). This construct is also competent for correct traffic to the TGN in another

proportion of HeLa cells (data not shown) as has been observed in the neuronal like N2A cell line (McCue et al., 2009). This observation may be consistent with differential expression levels between cells: in low to moderate expressing situations the CaBP7 transmembrane domain fusion remains ER-trapped, however, at higher expression levels ER-based retention mechanisms are likely saturated and the construct escapes to latter compartments of the secretory pathway. Collectively these data illustrate that the transmembrane domain of CaBP7 is of the class that

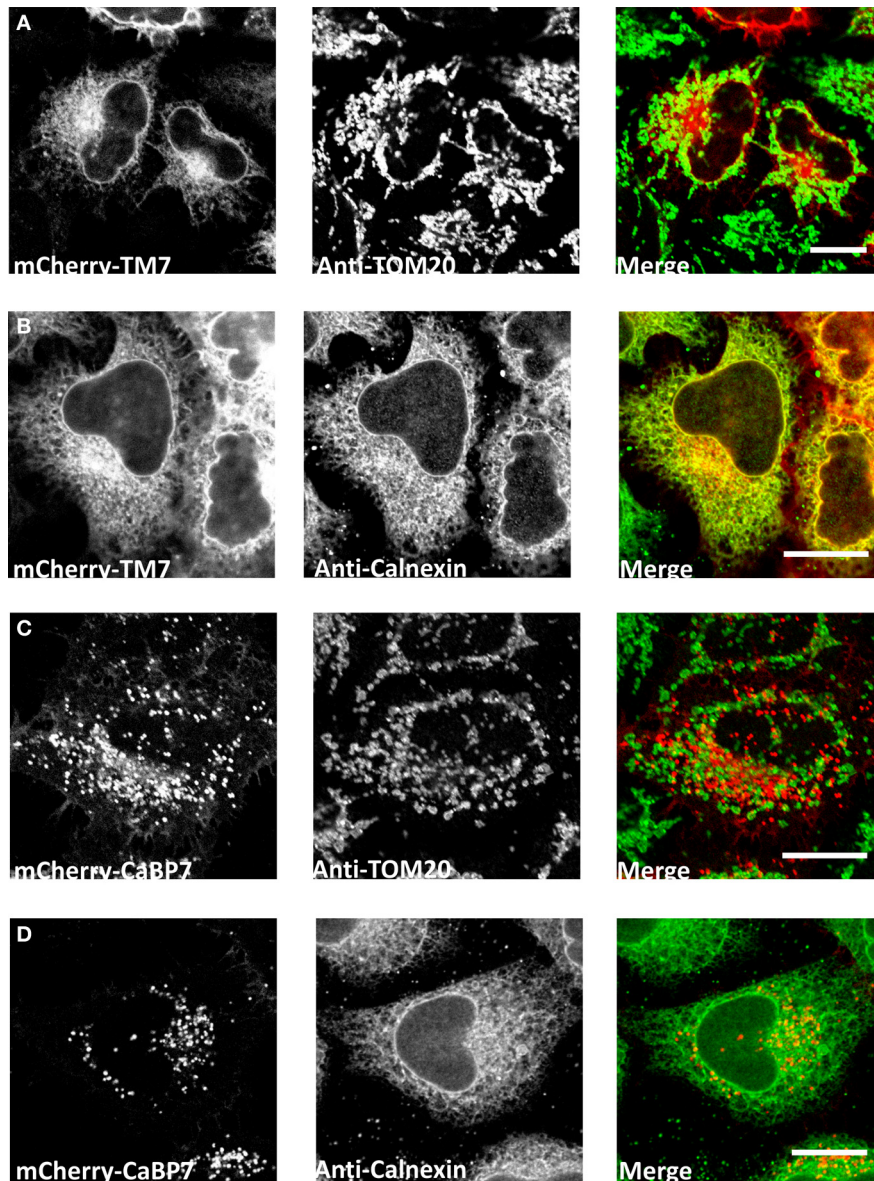
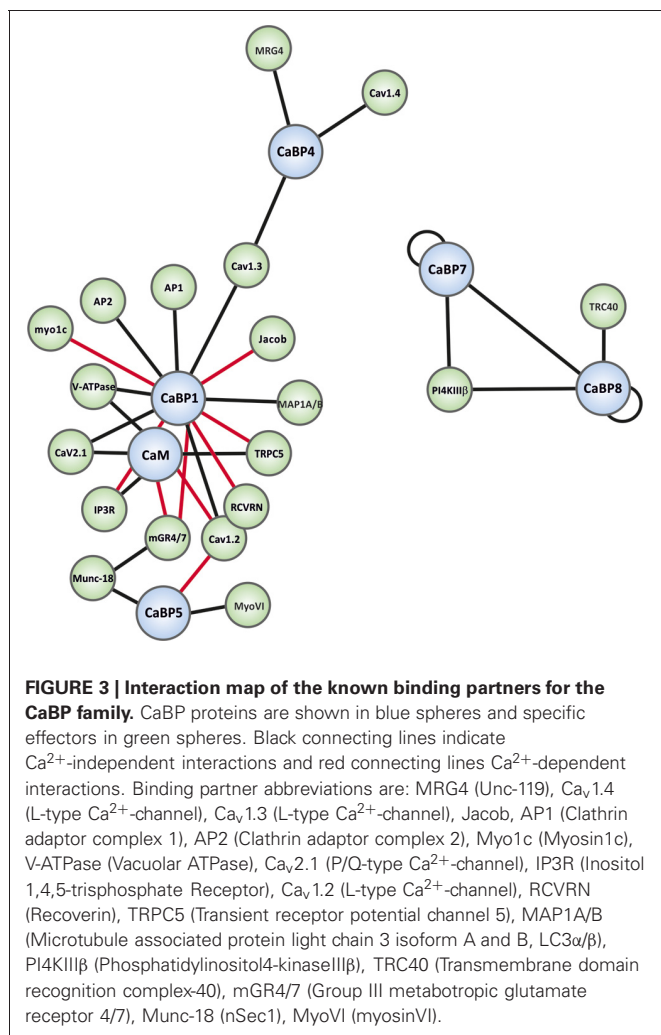


FIGURE 2 | Analysis of localization of mCherry-CaBP7 or mCherry-CaBP7 transmembrane containing domain (residues 188–215) fusion (mCherry-TM7) compared with markers of the mitochondria (TOM20) and the ER (Calnexin). (A and B) mCherry-TM7 expression in HeLa cells (red) and co-staining with antibodies against endogenous TOM20 (A, green) or Calnexin (B, green). Cells with low to moderate levels of mCherry-TM7

were analyzed in this experiment, more highly expressing cells exhibit a localization pattern more consistent with the full-length CaBP7 protein (see text for further discussion). (C and D) mCherry-CaBP7 expression in HeLa cells (red) and co-staining with antibodies against endogenous TOM20 (C, green) or Calnexin (D, green). Regions of colocalization appear yellow in overlay images. Scale bars = 10 μ m.



directs initial insertion into the ER membrane. Additional intrinsic targeting information or specific protein-protein interactions must be required for the efficient direction of CaBP7 and CaBP8 for forward traffic through the secretory pathway to the TGN and beyond as can be observed for full-length CaBP7 protein which exhibits a punctate distribution that does not significantly overlap with either calnexin or TOM20 (**Figure 2C,D**).

CaBP7 and CaBP8 represent the only known CaM-related small EF-hand containing calcium sensors that have adopted the use of a tail-anchor targeting strategy. Tail-anchor proteins perform highly conserved and essential functions in mammalian cells and include SNARE proteins required for membrane fusion events throughout the secretory pathway (Borgese and Fasana, 2011). A number of issues remain to be resolved with regards to the precise targeting of CaBP7 and CaBP8. Although CaBP8 was shown to associate with TRC40 (Hradsky et al., 2011) it remains to be demonstrated that this chaperone is capable of post-translationally inserting recombinantly expressed or *in vitro* translated CaBP7 or CaBP8 across endoplasmic reticulum/microsomal membranes. It will also be of interest to understand the exact molecular determinants and protein-protein or protein-lipid interactions that control the final targeting of CaBP7

and CaBP8 to latter compartments of the secretory pathway. This information will further our understanding of the emerging functions of CaBP7 and CaBP8 as regulatory factors of protein trafficking from the TGN (Mikhaylova et al., 2009).

CaBP-EFFECTOR INTERACTIONS

As noted above, CaBPs along with CaM have been found to be able to regulate several types of VGCCs (Lee et al., 2002; Zhou et al., 2005; Cui et al., 2007; Findeisen and Minor, 2010; Minor and Findeisen, 2010). For a detailed overview of these and other well-characterized CaBP-effector interactions readers are directed to the recent comprehensive review articles (McCue et al., 2010b; Mikhaylova et al., 2011). The current article will focus on novel CaBP interactions that have been reported only in the past few years and which hint at further important physiological functions for the CaBP family in the mammalian CNS. A graphical overview of all presently characterized CaBP interactions is presented in network diagram form in **Figure 3**.

CALDENDRIN/CaBP1

GROUP III METABOTROPIC GLUTAMATE RECEPTORS (mGluRs)

Group III mGluRs function presynaptically to modulate synaptic vesicle exocytosis and neurotransmitter release (Pinheiro and Mülle, 2008). A recent study by Nakajima (Nakajima, 2011) reported an *in vitro* Ca²⁺-dependent interaction between rat CaBP1-Long and the membrane proximal portion of the cytoplasmic C-terminal tail of mGluR4 and mGluR7. This interaction was sensitive to phosphorylation of the mGluR fragment by protein kinase C mimicking the characterized binding of CaM to these receptors (Nakajima et al., 1999; O'Connor et al., 1999; Nakajima et al., 2009). There was competitive binding between CaM and CaBP1-Long suggesting that both proteins could regulate this receptor type through interaction with the same target motif. This is reminiscent of CaBP1/CaM dual regulation of VGCCs (Lee et al., 2002; Few et al., 2005, 2011) and it will be fascinating to examine whether, in the case of mGluR regulation, both Ca²⁺-sensors exert identical or differential modulation of these important neuronal receptors. CaM binding to mGluRs is able to displace the regulatory protein Munc-18 (Nakajima et al., 2009) and it will of interest to test if CaBP1 can elicit the same loss of Munc-18 binding.

The study of Nakajima (Nakajima, 2011) was conducted entirely using *in vitro* biochemical techniques and there is currently no information validating these findings in intact cells. The biochemical characteristics reported in this paper are, however, consistent with documented CaM/CaBP1 *in vivo* interactions (Lee et al., 2002; Few et al., 2005, 2011) and this work remains of great potential interest as Ca²⁺-influx downstream of mGluR activation is known to influence short term facilitation and higher level neuronal function (Cosgrove et al., 2011; Fioravante and Regehr, 2011).

UNCONVENTIONAL MYOSIN 1c

Class I unconventional myosins organize the cortical actin cytoskeleton and are involved in processes encompassing membrane trafficking and cell migration (Soldati, 2003). Myosin1c (myo1c), a protein essential for inner hair cell (IHC) adaptation

in the inner ear (Gillespie and Cyr, 2004), has been shown to interact with CaBP1 through its IQ motif containing regulatory domain (Tang et al., 2007). CaBP1 binding was found to be competitive with that of CaM suggesting that myo1c may represent yet another example of a target protein regulated by multiple small EF-hand Ca^{2+} -sensors. No functional data was presented in this study to confirm a physiological role for the CaBP1/myo1c interaction and further work is required to clarify this issue. Intriguingly, CaBP1 expression in rat and chicken auditory IHCs has been confirmed by both reverse transcription PCR and immunostaining (Yang et al., 2006; Lee et al., 2007b) and these may represent prime cellular models to study the functional implications of a putative CaBP1/myo1c interaction. IHCs have also been demonstrated to express CaBP4 (Yang et al., 2006; Lee et al., 2007b and CaBP7 Lee et al., 2007b) and, therefore, an analysis of myo1c binding by these family members might also further our understanding of potential mechanisms of cytoskeleton organization and regulation by multiple CaBPs in a key special sensory tissue.

JACOB

The interplay of calcium signaling through synaptic and extra-synaptic NMDA receptors influences complex aspects of neuronal function including synaptic connectivity and survival (Hardingham and Bading, 2003). These changes are brought about by alterations in neuronal gene expression (Greer and Greenberg, 2008; Lyons and West, 2011) and one pathway involved in such regulation in mammalian neurons is controlled by Caldendrin (Dieterich et al., 2008). This detailed study isolated and characterized a brain specific Caldendrin binding protein, Jacob, and demonstrated a role for this interaction in modulating activity of the CREB transcription factor to ultimately influence neuronal gene expression. Activation of synaptic NMDA receptors and subsequent Ca^{2+} -binding by Caldendrin localized at the post-synaptic density was shown to drive its interaction with the Jacob nuclear localization signal thereby preventing import of Jacob into the nucleus. Conversely, activation of extra-synaptic NMDA receptors was shown to induce Jacob redistribution to the nucleus where it was able to induce CREB dephosphorylation and inactivation (Dieterich et al., 2008). Jacob mediated inhibition of CREB dependent gene expression elicited a loss of synaptic contacts and extensive simplification of dendritic architecture. This mechanism, therefore, couples synaptic activity to maintenance/loss of synaptic contacts through Ca^{2+} -signaling via Caldendrin. Intriguingly, Jacob is a vertebrate specific protein (McCue et al., 2010a) that may have co-evolved specifically with the CaBP family to regulate this important aspect of synaptic plasticity.

CaBP4

Unc-119 (MRG4)

CaBP4 is the only CaBP member thus far characterized that has a proven role in human disease and mutations in the gene lead to rod and cone dysfunction and visual impairments of varying severity (Zeitze et al., 2006; Litink et al., 2009; Aldahmesh et al., 2010). CaBP4 regulates $Ca_v1.4$ channels in the retina (Haeseleer et al., 2004) and $Ca_v1.3$ channels in auditory IHCs (Yang et al.,

2006; Cui et al., 2007; Lee et al., 2007b), however, non-channel binding partners are also emerging (Lee et al., 2007a).

Vertebrate orthologues of the *C. elegans* Unc-119 protein (also known as MRG4), like CaBP4, exhibit retinal specific expression profiles (Higashide et al., 1998; Higashide and Inana, 1999). Functional deficits due to mutations in MRG4 mirror those observed for CaBP4 mutations and have been found to lead to rod/cone dystrophy in animal models and humans (Kobayashi et al., 2000). Unc-119 orthologues are expressed from invertebrates onwards (Maduro et al., 2000) and appear to have a nervous system specific function in *C. elegans* and *D. melanogaster* (Maduro and Pilgrim, 1995, 1996) which has apparently become restricted to the visual system in vertebrate animals.

MRG4 was found to interact with CaBP4 in a calcium independent manner consistent with binding to the variable N-terminal domain of CaBP4 (Haeseleer, 2008). This observation itself is intriguing as the vast majority of CaBP target effectors characterized to date have been shown to bind to the conserved C-terminal EF-hand containing core of the CaBP under investigation. An interaction with the novel N-terminus of CaBP4 seems to be important for stabilizing MRG4 protein (Haeseleer, 2008) and further hints at a co-evolution of tissue expression and cellular function between these proteins which has likely been important for the development and normal function of vertebrate visual systems. Further investigations into the molecular pathway linking CaBP4 and MRG4 to normal retinal activity are required to gain a complete understanding regarding the roles that both proteins play in human disease pathologies and to ascertain whether this retinal signaling system might provide a useful therapeutic target in the future treatment of some visual impairments.

CaBP5

Munc-18 (nSec-1) AND MYOSIN VI

CaBP5 has been shown to be expressed in IHCs, however, the available data suggests that, in this particular cell type, it is CaBP1 and CaBP4 that functionally regulate $Ca_v1.3$ channels (Cui et al., 2007). CaBP5 is able to modulate the activity of both $Ca_v1.2$ and $Ca_v1.3$ channels when co-expressed in HEK293 cells (Cui et al., 2007; Rieke et al., 2008) and CaBP5 knock-out mice exhibit a reduction in light sensitivity of their retinal ganglion cells suggesting that CaBP5 may instead perform an important modulatory function in the visual system (Rieke et al., 2008).

Munc-18 is a highly conserved protein related to yeast Sec1 that is critical for neurotransmission throughout the mammalian CNS where it interacts with multiple effectors to control membrane fusion. Originally isolated as a syntaxin1 binding partner, Munc-18 has since been shown to interact with the synaptic SNARE complex in multiple unique “modes” to regulate synaptic vesicle fusion with the presynaptic PM (Burgoyne et al., 2009; Sudhof and Rothman, 2009). New, SNARE independent, Munc-18 binding proteins have been discovered suggesting that this protein itself is tightly regulated through multiple routes perhaps to further modulate SNARE complex activity and ultimately neurotransmission (Okamoto and Sudhof, 1997; Verhage et al., 1997; Graham et al., 2008; Hikita et al., 2009; Nakajima et al., 2009; Huang et al., 2011). Recently, a calcium independent

interaction has been described between Munc-18 and CaBP5 in the retina establishing a potential new mechanism of linking calcium sensor function to SNARE activity in the visual system (Sokal and Haeseleer, 2011). CaBP5 was also shown to influence neurotransmitter release when overexpressed in a model neuroendocrine cell line, further implicating it as a regulator of secretion in selected regions of the nervous system (Sokal and Haeseleer, 2011).

In the same study, myosin VI was identified as a second specific binding partner for CaBP5 (Sokal and Haeseleer, 2011). Myosin VI has been implicated in endocytic processes. The related myosin V, a protein associated with synaptic vesicles and involved in their movement to the PM, was also detected in CaBP5 affinity chromatography pull-downs. For technical reasons a myosin V interaction was not characterized further in this study, however, these observations hint at a possible role for CaBP5 in synaptic vesicle recruitment to the presynaptic membrane of retinal neurons, a potentially important role which warrants further investigation. The CaBP5 knock-out mouse is available and it would be interesting to examine if there are defects in synaptic vesicle recruitment to the presynaptic active zone (myosin V pathway) or retrieval of exocytosed synaptic membrane components (myosin VI pathway) in retinal neurons from these animals. It would also be of interest to examine whether CaBP5 mediated stimulation of neurotransmitter release and neurite outgrowth (Sokal and Haeseleer, 2011) can be supported by a CaBP5 EF-hand mutant incapable of binding to Ca^{2+} .

CaBP7 AND CaBP8

PHOSPHATIDYLINOSITOL 4-KINASEIII β (PI4K β)

PI4K β is a lipid modifying enzyme that associates with the TGN to generate phosphatidylinositol 4-phosphate (PI4P) from phosphatidylinositol (Graham and Burd, 2011). PI4P is an essential lipid for budding of transport vesicles from the TGN and, therefore, regulation of PI4K β is a key control checkpoint of the secretory pathway. Known regulators of PI4K β activity include the small GTPase ARF1 and the small calcium sensing protein NCS-1 (Hendricks et al., 1999; Audhya et al., 2000; Haynes et al., 2005, 2007; Burgoyne and Haynes, 2010). NCS-1 modulation of PI4K β has been reported to be both Ca^{2+} -independent (Zhao et al., 2001) and Ca^{2+} -dependent (Haynes et al., 2005). It is known, however, that the Golgi complex stores luminal Ca^{2+} and uses this to generate local Ca^{2+} -signals suggesting a link between Ca^{2+} -release and membrane trafficking events (Dolman and Tepikin, 2006). A further level of regulation in this system was reported with the finding that both CaBP7 and CaBP8 could also interact with and inhibit the activity of PI4K β in mammalian cells to influence trafficking of specific secretory cargo to the PM (Mikhaylova et al., 2009).

The study of Mikhaylova et al. (Mikhaylova et al., 2009) uncovered an inhibitory interaction of CaBP7 and CaBP8 with PI4K β at resting (~ 100 nM) cytosolic calcium concentrations $[(\text{Ca}^{2+})_i]$ that was competitively displaced by NCS-1 at increasing free $[\text{Ca}^{2+}]_i$ to elicit stimulation of kinase activity. The interplay between NCS-1 and CaBP7 and CaBP8, therefore, permits dual control of TGN transport events over a range of physiological $[\text{Ca}^{2+}]_i$ and importantly prevents activation of the enzyme in

the absence of a threshold Ca^{2+} -signal. The interaction of CaBP7 and CaBP8 with PI4K β was specific and was not observed with Caldendrin, however, it would be of interest to test PI4K β regulation with CaBP5, the family member exhibiting highest homology to CaBP7 and CaBP8 (McCue et al., 2010a).

Crosstalk between ARF1 and NCS-1/CaBP7 and CaBP8 was not examined in this study and since the NCS-1-PI4K-ARF1 network has been implicated in a developmental setting (Petko et al., 2009) it would be of interest to examine if CaBP7 and CaBP8 repression of PI4K activity can be linked to a similar physiological model.

VGCCs?

Until recently, the only documented regulatory role for CaBP7 and CaBP8 was that involving the interaction with PI4K discussed in the previous section. As small EF-hand Ca^{2+} -sensing proteins often display promiscuity in their target interactions (Haynes et al., 2006; Burgoyne, 2007) it seems reasonable to expect further binding partners for CaBP7 and CaBP8 to appear in future studies. A new potential interaction between CaBP8 and various VGCCs has been reported which may provide the first clues regarding additional CaBP7 and CaBP8 cellular functions (Shih et al., 2009). This study examined the effect of CaBP8 over-expression in bovine adrenal chromaffin cells on PM Ca^{2+} -channel activity and found that wild-type protein or a mutant deficient in Ca^{2+} -binding both inhibited currents generated through N-, L-, and P/Q-type channels (Shih et al., 2009). A mutant CaBP8 lacking the hydrophobic C-terminal domain mislocalized in this cell type and did not support inhibition of channel currents reinforcing the idea that correct sub-cellular targeting on this Ca^{2+} -sensor is critical for its normal cellular function.

The most profound inhibitory phenotype was observed on N-type currents and N-type channels are widely expressed in the mammalian CNS where they operate presynaptically to regulate neurotransmitter release (Delcour et al., 1993). No direct interaction between CaBP8 and the various Ca^{2+} -channels analyzed was reported in the study of Shih et al. (Shih et al., 2009), however, this merits examination in future investigations in view of the importance of N-type channels, coupled to the widespread expression of CaBP7 and CaBP8, in the mammalian CNS.

Since CaBP7 and CaBP8 regulate PI4K, the possibility exists that these Ca^{2+} -sensors are able to inhibit Ca^{2+} -currents not by direct channel gating but rather by restricting the traffic of Ca^{2+} -channels to the PM (Shih et al., 2009). Other small EF-hand Ca^{2+} -sensors are known to modulate channel activity in this manner, the most well characterized example being the regulation of Kv4 potassium channel traffic to the PM by the KChIP family of Ca^{2+} -sensors (Burgoyne, 2007; Flowerdew and Burgoyne, 2009). Since a mutant CaBP8 incapable of Ca^{2+} -binding was able to inhibit N-type currents and since PI4K activity is inhibited at resting $[\text{Ca}^{2+}]_i$, these observations are consistent with this regulatory model. Distinguishing between channel traffic and direct channel modulation mechanisms should prove straightforward and will provide further insights into the role of CaBP7 and CaBP8 in the normal function of the mammalian CNS.

DISCUSSION

Calcium sensing in the vertebrate CNS is of fundamental importance in establishing and maintaining normal neuronal activity. The complexity of Ca^{2+} -signals generated in the mammalian brain is only just beginning to be understood, however, the identity of many of the protein factors involved in decoding them is already known. The CaM superfamily of small EF-hand containing Ca^{2+} -sensors have emerged as key regulators of multiple important neuronal Ca^{2+} -channels that influence all aspects of neuronal plasticity. Sub-groups of vertebrate specific CaM-related proteins including the CaBPs have seemingly evolved in parallel with increasing levels of CNS complexity in higher animals. These proteins exhibit diversity in patterns of expression, sub-cellular localization and Ca^{2+} -sensing dynamics and are, therefore, well suited to functioning as transducers of the highly specific Ca^{2+} -signals that underpin complex neuronal activity.

We are now aware that the CaBPs not only regulate neuronal specific Ca^{2+} -channels but also targets involved in membrane

trafficking, the organization of the cytoskeleton and proteins that influence neuronal gene expression and synaptic connectivity. The list of CaBP effector interactions is steadily increasing as is our appreciation of CaBP functionality. Testing the importance of CaBP activity in the mammalian CNS is largely restricted to studies utilizing cell lines and would benefit greatly from the generation of transgenic animal models. In particular, the application of Cre-Lox approaches (Sauer, 1998; Nickerson et al., 2011) to test loss of function of CaBPs in particular tissues and cell types during both development and in the adult animal will significantly enhance our understanding of the key physiological functions performed by these multifunctional signaling molecules.

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