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The Ca^{2+}-dependent Activator Protein for Secretion CAPS: Do I Dock or do I Prime?

David R. Stevens · Jens Rettig

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Abstract The "Ca^{2+}-dependent activator protein for secretion" (CAPS) is a protein which reconstitutes regulated secretion in permeabilized neuroendocrine cells. It is generally accepted that CAPS plays an important role in the release of the contents of dense core vesicles in the nervous system as well as in a variety of other secretory tissues. At which step in the exocytotic process CAPS functions as well as its role in the fusion of synaptic vesicles is still under dispute. A recent growth spurt in the CAPS field has been fueled by genetic approaches in *Caenorhabditis elegans* and *Drosophila* as well as the application of knockout and knockdown approaches in mouse cells and in cell lines, respectively. We have attempted to review the body of work that established CAPS as an important regulator of secretion and to describe new information that has furthered our understanding of how CAPS may function. We discuss the conclusions, point out areas where controversy remains, and suggest directions for future experiments.

Keywords Exocytosis · Synaptic transmission · Neurotransmitter release · Docking · Priming · Vesicle pools · Large dense core vesicles

CAPS: A Cytosolic Factor that Enhances Regulated Neurosecretion

Ca^{2+}-dependent activator protein for secretion (CAPS) was discovered by the group of Tom Martin as a soluble factor which reconstitutes calcium-dependent secretion in permeabilized and cytosol-deprived PC12 cells [1, 2]. The approach during this initial characterization was to permeabilize PC12 cells, wash them extensively, and then determine what must be added to reconstitute calcium-dependent secretion. In earlier studies using neuroendocrine cells, it was reported that cytosolic factors were required to reconstitute regulated (calcium-dependent) release [3]. A 145-kDa protein was identified which was required for release. This protein (p145) also supported secretion in GH3 cell ghosts [1]. Using a rabbit anti-p145 serum, this protein was shown to be present in the cytosolic fraction from brain, pituitary, pancreas, and adrenal medulla but not present in adrenal cortex, heart, muscle, kidney, or lung. This distribution indicated that p145 was a protein likely involved in regulated neuronal and endocrine secretion. When the anti-p145 serum was added to brain cytosol, it reduced the reconstituting activity of p145 by about 60%. Subsequent experiments indicated that p145 was a substrate of protein kinase C (PKC) [4].

p145 was then sequenced and found to be similar to the protein UNC-31 of *Caenorhabditis elegans* [5]. UNC-31 is a nervous system protein whose mutation leads to motor and nervous system defects that result in uncoordinated movement and diverse defects in behavior [6, 7], hence the UNC label. Expressed full-length p145 reacted with an antibody to native rat brain p145. p145 was also shown to bind calcium with moderate affinity (K_d=270 μM), indicating a calcium-dependent function and resulting in the designation "Ca^{2+}-dependent activator protein for secretion (CAPS)".

The Domain Structure of CAPS Proteins

The domain structure of CAPS proteins is shown in Fig. 1a. There is one CAPS (unc-31) gene in *C. elegans* and in

D. R. Stevens · J. Rettig (✉)
Physiologisches Institut, Universität des Saarlandes,
Gebäude 59, Kirrberger Str. 8,
66421 Homburg/Saar, Germany
e-mail: jrettig@uks.eu

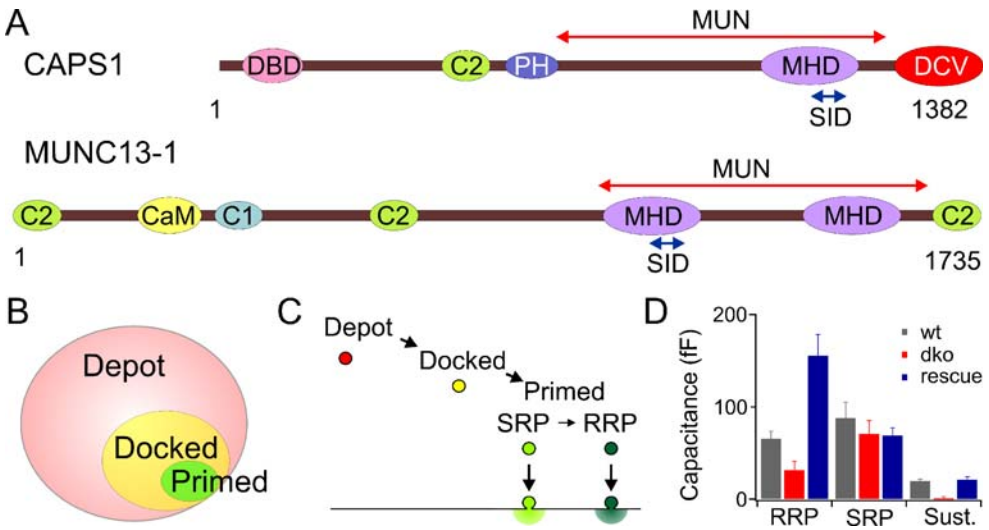

Fig. 1 CAPS function in exocytosis. **a** The domain structure of CAPS and that of the priming factor Munc13-1. CAPS contains a N-terminal dynactin 1 binding domain (*DBD*). The central portion of CAPS contains two lipid interacting domains, the C2 domain whose function is unclear, and the PH domain which is necessary for an interaction with PIP_2 rich areas in the inner leaflet of the plasma membrane. Distal to these domains are a Munc-13 homology domain including the syntaxin interaction domain (*SID*) required for priming in SVs and a C-terminal domain required for association with DCVs. Thus CAPS contains a MHD domain which in Munc13 is part of the minimal structure required to carry out priming (MUN domain, see text). **b**, **c** Current models suggest the primed vesicle can exist in two states, a slowly releasable and a rapidly releasable state. Priming is likely a sequential process with docked vesicles being primed to the slowly releasable pool (SRP) and then proceeding to the rapidly releasable pool (RRP). **d** High time resolution experiments in mouse chromaffin cells indicate that CAPS acts in conversion of SRP vesicles to rapidly releasable vesicles since the RRP and sustained release observed in wild-type mice (*wt*) are strongly reduced in CAPS knock out mice (*dko*) and this effect can be rescued by ectopic CAPS1 expression (*rescue*)

Drosophila. A second CAPS gene (CAPS2) with multiple splice variants has recently been found in mouse and both CAPS1 and CAPS2 are present in humans as well [8, 9]. CAPS1 and CAPS2 have a high degree of homology and appear functionally similar. Both CAPS isoforms are present in brain and adrenal glands. CAPS1 is also found in mouse pancreas, spleen, and stomach, while CAPS2 is detected in liver and testis [8, 9]. CAPS1 and CAPS2 are expressed in the pars nervosa and the pars intermedia of the pituitary gland, whereas CAPS2 is also present in bronchial epithelial cells, thyroid parafollicular cells, chief cells of the stomach, ductal epithelium of the salivary gland, kidney proximal tubules, thymus, spleen, and colon. Thus, CAPS may be involved in secretion in a variety of non-neural cells as well [10].

The domain structure of CAPS is conserved across species. An N-terminal dynactin 1 binding domain (DBD) that may be required for sorting has recently been described [11]. This is followed by a C2 domain [12], which likely interacts with membranes in a calcium-dependent manner [13] allowing the C2 domains to act as calcium sensors. Adjacent to the C2 domain, there is a pleckstrin homology (PH) domain [12]. PH domains associate with acidic phospholipids and thus may mediate interactions with membranes as well [14]. CAPS contains a Munc-13 homology domain (MHD) [15] that contains a syntaxin interacting domain (SID) [16] and is required for priming activity in unc-13 homologs in mouse and *C. elegans* [17–19]. Munc-13s are priming factors which are thought to function by interacting with syntaxin bound to Munc18, promoting a conformational change in syntaxin to its open form which then can interact with the SNARE complex [20], producing the primed state. Munc-13s have two MHDs in series and the required priming region (MUN domain) spans both of these domains [17–19]. The C-terminal end of the CAPS protein is required for association with dense core vesicles (DCVs) and is referred to as the DCV binding domain [12]. Speese et al. [21] suggest that the sequence spanning the end of the PH domain and the beginning of the DCV binding domain of CAPS is a MUN domain.

The domain structure gives hints as to how CAPS might function. Full-length CAPS binds to phosphatidyl inositol 4,5 bisphosphate ($PI(4,5)P_2$) [22]. Liposome binding assays using GST–CAPS–PH domain proteins indicated that the PH domain of CAPS interacted with liposomes containing phosphatidylserine and that binding was enhanced by the presence of $PI(4,5)P_2$ [12]. Mutation analysis of the role of the PH domains in CAPS binding to liposomes and to lipids applied to nitrocellulose arrays indicated that while binding was selectively enhanced by $PI(4,5)P_2$ and $PI(3,5)P_2$, mutations selectively reducing PIP_2 binding had little effect on cellular localization or on reconstitution of function [12].

Full-length CAPS with mutations which block the function of the PH domain was still able to reconstitute secretion in cracked PC12 cells, albeit at a much higher protein concentration. Interestingly, mutation of the PIP_2 binding pocket of the PH domain of CAPS did not prevent PIP_2 binding, indicating that the PH domain is not the only PIP_2 binding domain in CAPS. The PH domain of CAPS bound to plasma membranes but failed to bind to DCV membranes. Thus, it appears that the PH domain may mediate plasma membrane association but this is not dependent on PIP_2 binding to the PH domain.

PIP_2 binding to CAPS does, however, appear to be a requirement for CAPS function [23]. Using the fluorescence intensity of phospholipase C-δ_1-PH-GFP as an assay for PI(4,5)P_2, James et al. [24] confirmed, in PC12 cell membrane sheets, the presence of PIP_2 rich microdomains which associated with sites of exocytosis [25, 26]. They report that about half of CAPS binding coincides with these areas and that there is a relatively high probability DCVs will dock at these areas. CAPS accelerated secretion in a calcium- and PIP_2-dependent manner in liposomes containing phosphatidyl choline/PI(4,5)P_2 but not in liposomes containing phosphatidyl choline/phosphatidyl serine, indicating that PI(4,5)P_2 is necessary for CAPS-mediated acceleration of secretion. CAPS with PIP_2 binding mutations in the PH domain did not reconstitute secretion, indicating that in full-length CAPS the PH domain is indeed a PIP_2 receptor in CAPS-mediated stimulation of secretion.

The C-terminal DCV binding domain binds to DCV membranes but not to plasma membrane. Deletion of C-terminal portions containing this domain reduced the activity of CAPS in secretion assays. A distal acidic cluster consisting of MKDSDEEDEEDD is necessary for DCV targeting and is required for normal function [23]. These authors suggested that CAPS may bind to DCVs and bridge the gap between the plasma membrane and DCV, transiently enhancing the association of DCVs with PI(4,5)P_2-rich regions of the plasma membrane.

CAPS Functions in DCV Secretion

A number of approaches that vastly differ in their specificity have been used to demonstrate a role of CAPS in neurosecretion. They can be grouped broadly into three classes: The first, mostly biochemical approach used sonicated cell ghosts (e.g., cracked PC12 cells) and re-established secretion by adding cytosolic components. The second, more physiological approach applied substances (e.g., anti-CAPS antibodies) through the patch pipette and measured the resulting effects of this manipulation by electrophysiological methods. The third approach used genetic deletions of the corresponding genes (e.g., CAPS1) and compared the morphological, biochemical, and electrophysiological phenotype with that of wild-type littermates. While all approaches have their limitations, the third approach, particularly in conjunction with rescue experiments, clearly yields the most robust and reliable results.

Tandon et al. [27] have examined the involvement of CAPS in transmitter release from rat brain synaptosomes which had been permeabilized with streptolysin. These experiments are analogous to the initial experiments on cracked PC12 cells, and showed that rat brain cytosol but not CAPS could reconstitute secretion of glutamate in a calcium-dependent manner. When the synaptosomes were incubated with tritiated norepinephrine (NE) prior to stimulation, CAPS as well as brain cytosol could reconstitute NE release. The calcium requirement for NE release was much lower than that for glutamate release, indicating differing release mechanisms for synaptic vesicles (SVs, glutamate release) and DCVs (NE release). Although the conditions were highly artificial, the results are consistent with the author's interpretation that CAPS is selectively involved in DCV secretion.

Subcellular fractionation showed that while most CAPS was cytosolic, 37% was associated with the membrane fraction of brain homogenates [28]. Membrane-associated CAPS could be solubilized in synaptosomes by treatment with Triton-X100 indicating that much of the CAPS is associated with lipid membranes rather than interacting with other membrane proteins. CAPS was enriched in DCV fractions relative to SV fractions and was essential for calcium-dependent, botulinum toxin-sensitive release of tritiated NE from lysed synaptosomes, supporting the idea that CAPS functions preferentially in DCV exocytosis.

The role of CAPS in the release of the contents of DCVs in rat melanotrophs has also been examined [29]. CAPS antibody produced a punctate staining in immunofluorescence. Using immunogold labelling in EM, staining of dense core vesicles was demonstrated. Synaptotagmin I was present on all vesicles but CAPS was present only on a subset of DCVs. In electrophysiological experiments, photolysis of caged calcium led to biphasic secretion. Injection of CAPS antibody blocked only the rapid phase of secretion though both phases were blocked by botulinum toxin B and E, indicating that both phases were mediated by SNARE complexes. The observation that only the rapid phase of secretion was sensitive to CAPS antibody led to the conclusion that rapid and slower phases of secretion from DCVs utilize different pathways.

The ability of CAPS to support secretion was examined in bovine chromaffin cells [30] using the IgG fractions of anti-CAPS antibodies applied via the pipette solution. Secretion was stimulated with action potential trains with a frequency of 7 Hz and DCV fusion was monitored using

carbon fiber electrodes for amperometric detection of catecholamine release. CAPS antibodies progressively inhibited catecholamine release. There was no observable effect of antibody application on whole cell calcium currents, so the calcium stimulus was assumed to be the same in controls and in antibody-treated cells. There was a preferential effect on events that were tightly coupled in time with the action potential stimulus, likely corresponding to a readily releasable pool. The number of amperometric events was reduced by up to 80%, event amplitudes were strongly reduced, and event duration increased 5-fold, leading to the conclusion that CAPS was involved in the fusion event.

CAPS Affects the Size of Releasable Vesicle Pools

The early biochemical assays identified CAPS as a cytosolic factor necessary for DCV release but lacked the time resolution to address questions as to where in the secretory pathway CAPS functions. Vesicles exist in varying stages of maturity (see Fig. 1b). Most vesicles reside in a depot pool which is not releasable. In order to become release ready, vesicles must dock with the plasma membrane. This state is morphologically defined by vesicle proximity to the membrane. After the docking process, the vesicles undergo maturation and then enter the primed state (see Fig. 1c). Releasable (primed) vesicles exist in two pools with different release kinetics, a slowly releasable pool (SRP) and a rapidly releasable pool (RRP). Changes in release can result from changes in numbers of available vesicles at the plasma membrane (docking) or from changes in numbers of vesicles in releasable pools (priming) or from changes in the fusion event.

DCV release in neuroendocrine cells indicates the presence of three phases of secretion [31, 32]. There is an initial burst phase of secretion consisting of the RRP and SRP, followed by a sustained phase of secretion in which docked vesicles are primed and then immediately released as long as the intracellular calcium level is high enough to support release. Only a fraction of morphologically docked vesicles, the primed vesicles, are releasable upon Ca^{2+} influx, while the majority of morphologically docked vesicles are not releasable and thus not primed. There is ample evidence that the formation of the SNARE complex represents the molecular event which underlies the priming process [31, 33, 34]. Changes in priming will lead to changes in the releasable pools as well as in the sustained component of release.

In adrenal chromaffin cells [35], flash photolysis whereby a uniform intracellular calcium concentration of about 20 μM is achieved, leads to a burst of secretion which can be resolved into a rapid and a slow burst phase followed by a sustained phase. In CAPS1 knockout mouse chromaffin cells in primary culture, there was no difference in secretion between wild-type cells and cells from which CAPS1 had been deleted when cultured at embryonic day 18–19 release [36] though heterozygous CAPS1 deletions cultured at P30 exhibited a 30–35% decrease in catecholamine release when compared to wild-type littermates, with no preferential effect on a single phase. When single amperometric events were examined, CAPS1 knockout E19 chromaffin cells exhibited 60% fewer amperometric events than wild-type littermates. This was surprising considering that there was no reduction in secretion in E19 cells when measured by capacitance change.

In amperometric events, the distribution of charge per vesicle, event amplitude, event rise time, half width, and foot duration were unchanged. Only the frequency of release events was decreased, indicating that there was no shift in vesicle content or change in the vesicle fusion event. A strong reduction in release of catecholamines in the absence of reductions of single spike charge or capacitance changes indicate that in CAPS1 knockout cells, many fused vesicles contained no catecholamines (empty vesicles will not be detected by amperometry). When vesicle release was visually detected using TIRF microscopy and amperometric recordings were carried out simultaneously, a similar deficit in amperometric events was observed. These results indicate a role for CAPS in exocytosis and an additional role in loading of catecholamines into DCVs in chromaffin cells.

Knockdown of CAPS1 in PC12 cells resulted in a decrease in tritiated NE secretion as well as a decrease in neuropeptide Y (NPY) secretion when NPY-Venus was transfected into these cells in a cracked cell assay [37]. CAPS knockdown strongly reduced tritiated NE secretion induced by KCl, calcium, or alpha-Latrotoxin, and tritiated NE release was reconstituted by addition of brain cytosol from wild-type cells. Capacitance measurements following flash photolysis of caged calcium indicated that knockdown of CAPS1 (CAPS2 does not appear to be expressed in PC12 cells) reduced the secretory burst by about 30% and the sustained phase by about 50%. The fast burst (RRP) was not changed. Determination of the distribution of DCVs using electron microscopy led to the conclusion that docking proceeded normally. The authors concluded that there is a deficit in catecholamine secretion and that it is due to decreased priming.

Since secretion of NPY was also suppressed, the authors reasoned that a deficit in loading of vesicles was unlikely to be the cause of the deficit since peptides and catecholamines use very different mechanisms for entering DCVs. There was an increase in the accumulation of both tritiated NE and dopamine (DA) in the CAPS1 knockdown cells, which was attributed to a decrease in constitutive secretion.

This conclusion is weakened by the fact that, while there was a very strong correlation between the lack of expression of CAPS and decreased secretion, there was a poor correlation between the levels of NA and DA in the various knockdown clones.

The result of CAPS1 deletion in chromaffin cells [36] was likely mitigated by the presence of CAPS2 in E19 mice. The effect of deletion of both CAPS genes in mice has therefore been examined [38]. Chromaffin cells from CAPS1/CAPS2 double knockout (DKO) mice exhibited a strong reduction in the burst phase of secretion, due to a reduction in the RRP, as well as a virtual absence of the sustained phase (see Fig. 1d). There was no sign of a change in the spatial distribution of vesicles that would indicate a docking effect in these cells. Rescue using CAPS1-GFP via a Semliki forest virus vector reversed the deficit in the burst phase and to a lesser extent the deficit in sustained release. There was a deficit in released catecholamines relative to the observed capacitance change similar to that observed in CAPS1 deletions, consistent with the above described effect of CAPS in catecholamine loading [36]. There were no changes in amperometric spike parameters, including spike amplitude distribution, in these cells. Thus, genetic deletion of both CAPS isoforms does not lead to the reduction in amplitude and broadening of amperometric events reported in permeabilized chromaffin cells perfused with CAPS antibodies [30], so it is unlikely that CAPS is involved in the fusion event.

Further support for an effect of CAPS on vesicle filling comes from the demonstration that catecholamine uptake into vesicular preparations from embryonic brains of CAPS1 deletion mutants is decreased compared to preparations from wild-type littermates [39]. Anti-CAPS1 or anti-CAPS2 antibodies inhibited monoamine sequestration by vesicles from adult mouse brain, and CAPS1 or CAPS2 overexpression enhances catecholamine uptake into CHO cells stably transfected with the vesicular monoamine transporters VMAT1 or VMAT2. Thus, CAPS promotes monoamine uptake and storage mediated by these monoamine transporters.

CAPS Role in *C. elegans*

CAPS was identified as a homolog of the *C. elegans* unc-31 gene [5] which is one of many genes in *C. elegans* whose mutation leads to an uncoordinated phenotype indicative of defects in motor function [6]. Mutation of unc-31 leads to lethargy, constitutive feeding, egg-laying defects, and to prolongation of a larval state induced by stress and overcrowding [7]. The defects were neuronal rather than myogenic and indicate that either UNC-31 functions in multiple neurons with differing functions or that UNC-31 is necessary for function of one neuron which co-ordinately controls all four behaviors. Since UNC-31 is expressed in virtually all *C. elegans* neurons (D. Livingstone, communicated in [7]), it appears that the former possibility is indeed the case. This is difficult to reconcile with the relatively mild phenotype of unc-31 null mutants. Though UNC-31 functions in many neurons, its loss might lead to a change in activity short of a loss of function, for example, “low level constitutive release of neurotransmitter” [7]. Unc-31 was shortly thereafter identified as a gene generally involved in synaptic transmission [40], and subsequently the UNC-31 protein was localized to synaptic contacts using immunohistochemical methods [41].

Speese et al. [21] have further examined the role of UNC-31 in *C. elegans*. They confirm that UNC-31/CAPS is located in virtually all neurons and in other secretory cells. Unc-31 null mutations could be rescued by expression of UNC-31 under the control of a heat shock promoter, confirming that the deficits were indeed due to the lack of UNC-31. UNC-31 localized to neuronal cell bodies and axonal projections and was colocalized with synaptobrevin, indicating its presence at synaptic contacts. There was no evidence of a developmental defect. GABAergic motor neuron outgrowth, cell body number, and synapse density in unc-31 null mutants were comparable to those observed in wild-type worms. They then used expression of a prepro-atrial natriuretic peptide (ANP)–GFP fusion protein to identify DCVs. ANP–GFP was localized in the nerve ring and dorsal and ventral nerve cords, and was transported in an UNC-104 (kinesin)-dependent manner.

Endocytic accumulation of ANP–GFP in coelomocyte cells was used as an index of release of ANP from neurons. UNC-31 mutants exhibited decreased ANP–GFP accumulation *in vivo* and in cultured neurons as compared to wild-type worms. These experiments indicate that UNC-31/CAPS functions in the release of dense core vesicles in *C. elegans*. This conclusion was confirmed in the first-ever capacitance recordings from *C. elegans* neurons [42]. The release of serotonin containing SVs and peptide containing DCVs following flash photolysis of caged calcium was examined in neurons in primary culture. In UNC-31 mutants, peptidergic neurons exhibited strongly reduced fusion (presumably via DCVs). UNC-13 mutants exhibited the expected strong deficit in the burst phase as well as a decrease in sustained serotonin release via SV fusion, with normal DCV fusion. The effects on serotonin release were confirmed using amperometry. The burst phase of serotonin release was depressed, with no effect on sustained release, when serotonergic neurons from UNC-31 null worms were tested. These results indicate that UNC-31 is involved in peptide release with little contribution from UNC-13, while UNC-13 is important in serotonin release with a weaker but apparent role for UNC-31.

Application of forskolin, which increases cAMP levels by activating adenylate cyclase, to UNC-31 mutant neurons led to normal secretion in cells secreting via DCVs. Since increased cAMP leads to increased protein kinase A activity, these authors also examined the role of protein kinase A in secretion. Cells from worms carrying the kin2 (constitutively active PKA mutant) mutant were tested. These cells released peptides from DCVs to a greater extent than wild-type neurons, and when the kin2 mutation and the UNC-31 mutation were both present, the neurons secreted at the wild-type level. Thus, DCV fusion may be modulated by PKA and loss of UNC-31 is rescued by activation of PKA. Phorbol esters, activators of PKC, also led to strongly augmented DCV fusion. The actions of phorbol esters were lost in unc-13 null mutants, supporting the idea that the C1 domain of UNC-13 is a relevant phorbol ester receptor in synaptic plasticity [43]. Quite surprisingly, phorbol ester effects were also absent when unc-31 was deleted, perhaps indicating an interaction between the function of UNC-13s and UNC-31s.

DCV movements observed using TIRF microscopy were interpreted as evidence for a deficit in docking in UNC-31 mutants. This was based on a decrease in the rate of putative docking events and a shorter dwell time of putatively docked vesicles. In an EM study of SVs and DCVs in *C. elegans* [44], docking of DCVs in motor neurons, as defined by visible contact with the plasma membrane, was absent in unc-31 null mutants. Surprisingly, an open form of syntaxin [20] reversed the docking deficit. In this study, unc-13 mutants exhibited strong defects in SV docking but modest effects on DCV docking. unc-31 null mutants had a modest effect on SV docking. Thus, it was concluded that UNC-13 is required for SV docking and UNC-31 is required for DCV docking, though there appears to be some crosstalk.

CAPS Role in *Drosophila*

Using probes containing sequences from rat CAPS, the group of Broadie successfully cloned CAPS from *Drosophila* (dCAPS) [45]. dCAPS has a predicted mass of 153 kDa with an open reading frame 59% identical to rat CAPS. *In situ* hybridization in embryo whole mounts showed that dCAPS is strongly neural specific, being present in all neuronal cells in the brain and ventral nerve cord but absent in the peripheral nervous system. dCAPS protein was also restricted to the nervous system and was highly enriched in neuropil not containing somata, where it was localized presynaptically. At neuromuscular junctions (NMJs), dCAPS was present and clearly presynaptic though its presence was not correlated to the DCV content of the terminals. In dCAPS null mutants, CAPS staining was absent and these flies die at the late embryo/early first instar larva junction. Null dCAPS mutants could be rescued by expression of rat CAPS, suggesting a conserved function.

Null mutant flies showed no gross morphological abnormalities, with a normal body plan and the internal tissues appeared fully differentiated. Mutant neuromuscular junctions appeared normal with a normal punctate distribution of presynaptic proteins. The surviving larvae were very sluggish, exhibited little locomotion, and failed to respond to tactile stimuli. Synaptic transmission was assayed at embryonic NMJs. Excitatory junctional currents were reduced by about 50% at all frequencies tested, though the degree of synaptic depression was unchanged. There was no difference in the coefficient of variation and no difference during prolonged stimulation, suggesting a defect in vesicle release rather than recycling. Miniature synaptic currents at the NMJ had similar amplitude and frequency in wild-type and dCAPS null mutant fly larvae, indicating a presynaptic site of action. These results point to a role for dCAPS in calcium-dependent synaptic vesicle fusion.

Examination of vesicles in *Drosophila* using electron microscopy indicated accumulation of SVs (1.4-fold) and DCVs (3-fold) in presynaptic terminals at the NMJ. For comparison, in DUNC-13 null flies, in which there is a virtual arrest of glutamate release at the NMJ, there is a 1.55-fold accumulation of SVs and no accumulation of DCVs [46]. These results are consistent with an important role of dCAPS in DCV and SV release. However, when dCAPS was introduced into identified motor neurons on the dCAPS null background, using neuron type-specific promoters, it failed to rescue glutamatergic synaptic transmission. This was taken as evidence that dCAPS is not directly responsible for SV release and that the effects on SV release are secondary to dCAPS functions in other cells. The reversal of DCV accumulation by dCAPS expression under this promoter in identified motor neurons was not addressed.

CAPS Role in Mouse Brain

The view that CAPS functions selectively in DCV exocytosis is inconsistent with its abundance and the presence of strong CAPS staining at synapses at which dense core vesicles are quite rare in mouse brain [8, 47]. In *C. elegans* [21] and in *Drosophila* [45], CAPS staining is also often not proportional to dense core vesicle content.

Speidel et al. [8] reported that CAPS1 is distributed over much of the brain while CAPS2 distribution was limited with highest expression in cerebellar granule cells. CAPS1 and CAPS2 are complementarily expressed. CAPS2 is located not only in the granule cell layer but also in the termination zone of developing granule cells, in parallel

fiber presynaptic terminals where it is expressed transiently during the period of cerebellar development and cell migration [48]. CAPS2 expression promoted stimulated release of neurotrophins and brain-derived neurotrophic factor (BDNF) from PC12 cells and promoted stimulated release of neurotrophin from cultured granule cells. CAPS2 overexpression also promoted survival of cultured cerebellar Purkinje cells. Thus, it appears that CAPS2 plays a role in cerebellar development and is associated with release of neurotrophins and BDNF, while CAPS1 may play a broader role in CNS that goes beyond development.

The distribution of CAPS isoforms in mouse CNS has been further examined [47]. The distribution of CAPS2 correlates strongly with BDNF and neurotrophin 3 (NT-3) expression in many areas in the mouse brain. In CAPS2 knockout mice, CAPS1 is detected on dendrites and soma of Purkinje cells. These mice exhibit no overt abnormalities in brain anatomy except for the lack of the development of the intercrural fissure between lobules VI and VII of the cerebellum. There were a number of cytoarchitectonic deficits including reduced arborization of Purkinje cell dendrites, delayed granule cell migration, and subtle changes in presynaptic terminals. There were also behavioral changes and changes in short-term synaptic plasticity. These authors argued that the observed changes were likely the result of reduced release of BDNF and NT-3. Support for this position comes from an observed reduction in BDNF release in cerebellar cultures in CAPS2 knockouts (KO) and the similarity in the CAPS2 KO phenotype and that observed in BDNF KO or NT-3 KO mice [49–52]. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development [51]. NT-3 is required for proper cerebellar development [52].

In light of the strong presence of CAPS at nerve terminals where dense core vesicles are quite rare, the groups of Brose and Rhee have collaborated to examine the role of CAPS in mouse brain [53] by examining synaptic transmission in autaptic cultures of neurons [54] from mouse hippocampus. Knockout of CAPS1 led to a dramatic reduction of evoked EPSC amplitude in glutamatergic neurons of the hippocampus while CAPS2 knockout had no effect on EPSC amplitude. Deletion of both CAPS1 and CAPS2 genes resulted in a stronger reduction in EPSC amplitude. Examination of release induced by application of hypertonic buffer solutions indicated that the reductions in EPSC amplitude could be accounted for by a reduction in the size of the releasable pool of vesicles. These changes were associated with a uniform reduction in mEPSC amplitude but not associated with changes in release probability, kinetics, or postsynaptic sensitivity to glutamate, kainate, or GABA. Synaptic structure, SV content, and DCV content were unchanged, as were the number of synapses.

While repetitive stimulation at 2.5 and 10 Hz produced synaptic depression in CAPS2 KO neurons, as it does in wild-type cells, repetitive stimulation in CAPS1/CAPS2 DKO neurons resulted in a 5-fold increase in EPSC amplitude in spite of their equal initial release probabilities. Thus, repetitive stimulation shifts secretion in CAPS DKO neurons to wild-type levels. Following high-frequency stimulation, secretion at low frequencies was augmented and also approached that in wild-type neurons for a period after the stimulation. Estimation of the releasable pool indicated that while high-frequency stimulation leads to a reduction in pool size in CAPS2 KOs, it produces a large increase in pool size in CAPS DKO neurons.

Increasing the extracellular calcium concentration produced a greater increase in evoked EPSC amplitude in DKO neurons than in CAPS2 KO neurons, indicating that the facilitation observed in DKO cells is linked to increases in intracellular calcium. When intracellular calcium was uniformly increased by application of the calcium ionophore calcimycin, CAPS2 KO and CAPS DKO neurons exhibited strong secretion, but the release in CAPS DKO neurons was delayed. These findings indicate that the early, weak release observed in DKO is not due to differences in calcium sensitivity of the release process but rather due to a requirement for a relatively slow priming step before release in the CAPS DKO cells. The results were interpreted as evidence for a direct role of CAPS in SV priming. In light of the effects of CAPS2 KO in cerebellum and the observed cytoarchitectonic changes in cerebellum, cortex, and hippocampus, one should be cautious; however, these experiments were carried out in primary culture where developmental and transsynaptic effects are minimized. In addition, expression of CAPS1 rescued synaptic function to wild-type levels in CAPS DKO cells, indicating that the reduced synaptic transmission was indeed due to the absence of CAPS.

How Might CAPS Function?

The above results support a model in which CAPS functions in preparation of vesicles for release. The presence of a MUN domain in CAPS and the ability of an open form of syntaxin to rescue secretion in unc-31 null mutants support the idea that CAPS functions in priming similarly to UNC-13 [44]. This would involve the area of CAPS analogous to the MUN domain of UNC-13s, possibly for binding to syntaxin, promoting the conformational change in syntaxin that allows it to take part in the formation of SNARE complexes [55]. UNC-13 proteins play an important role in priming of SVs in neurons of *Drosophila* [46], *C. elegans* [56], and mouse [57]. Munc13-1 enhances priming of chromaffin granules and plays a role

in insulin secretion [35, 58], though both of these events utilize DCVs and appear to be dependent on CAPS.

The promotion of SNARE complex formation may be the basis of the described docking effects [42, 44] since syntaxin was also required for the docking event. Thus, docking may also involve a molecular state similar to that envisioned as priming in functional assays [31] and would result in very close proximity to the plasma membrane. The lack of agreement in whether CAPS effects docking may well result from differences in the criteria used to define docked vesicles and the methods used to measure them [59].

The pleckstrin homology domain and the DCV binding domain likely function to bridge the gap between DCV and plasma membrane with the PH domain allowing CAPS to interact with PI(4,5)P_2-rich microdomains in the plasma membrane. The C2 domain may also allow a calcium-dependent membrane association although there is no direct evidence for this role.

It has been suggested that Munc13-4, which functions in secretion in leukocytes, is a Rab27 effector [60, 61]. Rab27 is a small GTPase of the Ras family of GTPases which are thought to function in many membrane trafficking events. A recently discovered Ras GTPase, RRP17, functions in DCV exocytosis in cardiomyocytes that secrete ANP [62]. These authors identified CAPS as a potential binding partner of RRP17 in a yeast two-hybrid screen, with the interaction region containing the MUN domain. This is one of the few identified interactions of CAPS, and is consistent with a role for CAPS in ANP secretion. The Ras GTPases Rab3b and Rab27 are thought to function in tethering or docking of vesicles [63] and the Rab3a binding protein RIM interacts with Munc-13 in the docking and priming of SVs [64, 65]. CAPS may localize vesicles to secretory hotspots containing PI(4,5)P_2 rich microdomains, followed by the generation of a transient tethering structure via interaction of CAPS with Ras-like GTPases and syntaxin-like proteins [66], and could facilitate the eventual priming of this tethered intermediary complex and generation of a functional SNARE complex.

A number of modulatory systems are in place in *C. elegans* that are likely to some degree paralleled in mammals. Protein kinase C-1 is involved in peptide release in motoneurons of *C. elegans* [67]. It has recently been reported that UNC-31 is required to activate the Gα_s pathway that controls synaptic transmission in *C. elegans*. Noting that Gα_s and unc-31 null mutants share similar phenotypes, these authors examined the interaction between Gα_s and UNC-31 [41]. They observed no effect of unc-31 null mutations in the Gα_s null mutation background. In addition, constitutive activation of Gα_s rescued the motor activity in unc-31 null mutations, indicating that Gα_s is downstream from UNC-31 signalling. These results may indicate that CAPS, via fusion of DCVs leads to activation of the Gα_s pathway modulating synaptic transmission in motor neurons and are consistent with the proposal that some effects of UNC-31 mutations on synaptic transmission are the result of a lack of DCV release [21]. This may also relate to the observation that protein kinase A activation also rescues DCV release in unc-31 null mutants in *C. elegans* [42]. The kin-2 mutation, which is a constitutively active form of PKA in *C. elegans*, enhances DCV release, as does treatment with forskolin, which enhances PKA activity. If this is indeed a rescue is not clear since unc-31 null mutations reduced secretion but did not abolish it. Application of forskolin or PMA increased secretion to near wild-type levels, thus "rescuing" the phenotype, but may have enhanced that DCV secretion which was not dependent on UNC-31. In this vein, the observation that PMA enhancement of secretion was absent when either UNC-31 or UNC-13 were mutated, although there was little evidence of a role for UNC-13 in DCV secretion, may be due to an indirect interaction between UNC-13 with the DCV secretory pathway. Zhou et al. [42] suggested that UNC-31 acts downstream in the same pathway as UNC-13. UNC-31 exhibits no C1 domain which could function as a DAG/phorbol ester receptor. Thus, the effects could be due to a role of PKC in an UNC-31-independent pathway for DCV secretion and could explain the observation that unc-13 null mutants abolish secretion of SVs while unc-31 null mutants lead to a reduction in DCV secretion but do not abolish it.

Conclusions

The results reviewed above clearly demonstrate a role for CAPS in DCV secretion. In addition, results from *C. elegans*, *Drosophila*, and in particular experiments in autaptic hippocampal neurons from mouse indicate a role for CAPS in SV secretion [53]. Mechanistically, it is still unclear whether CAPS functions in priming or docking (Fig. 1). Part of the confusion about the seemingly contradictory data clearly arises from the different definitions of these two processes [59]. While the morphological definition stems from the different distances of vesicles from the plasma membrane, the physiological definition uses the kinetics of high-resolution capacitance measurements. Since the physiological definition accounts for dynamic changes over time, this definition should be clearly preferred to define the molecular status of a vesicle at the plasma membrane. There is also hope that TIRF microscopy, which was recently used to correlate molecular states of vesicles with their mobility [33, 68], will remove the uncertainties.

There is ample evidence that CAPS interacts with PIP_2 via the PH domain and an interaction via the C2 domains may occur as well, to facilitate interactions of DCVs with molecules involved in the generation of SNARE com-

plexes. If this occurs via generation of an open form of syntaxin, as has been suggested for the role of UNC-13 [20], is unclear. This is an attractive hypothesis in light of the apparent rescue of the UNC-31 phenotype by open syntaxin in *C. elegans*. Thus, it will be important to address the question as to whether the similarities between CAPS and Munc13 are more than coincidental. Does CAPS cause opening of the syntaxin molecule and thus promote priming in a similar fashion to that attributed to Munc13?

Can Munc13s and CAPS carry out the same function and do they work in separate pathways or in a sequential fashion? That the MHD domain of CAPS shares its function with the two MHD domains (MUN domain) of Munc13 is also an enticing hypothesis but requires rigorous experimental testing. One intriguing possibility is that in DCVs, CAPS is sufficient for priming while in SVs both Munc13s and CAPS collaborate, with Munc13s playing a primary role. Since the priming process including the participating proteins has been meticulously investigated, a vast number of experiments are possible to address this issue.

In addition to its function in priming, some CAPS reports have suggested an additional function in vesicle filling [36, 39]. The effects of CAPS knockout on vesicle filling appear to be specific for catecholamines and appear to be due to interactions of CAPS with the VMAT1 and VMAT2, an effect which is mediated by $G\alpha_o2$ [39]. Such a dual function is well established for other proteins in the synaptic vesicle cycle (e.g., Munc18); [69] and at present cannot be excluded for CAPS.

Whatever the role of CAPS in the secretion of vesicles is, it is likely only facilitatory since CAPS1/2 double knockout mice can generate releasable pools of DCVs, although the amounts of secretion are reduced. It will also be important to learn the basis of the fusion remaining following deletion of CAPS.

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