INTERACTIONS WITH MEMBRANE LIPIDS AND SNARE COMPLEX UNDERLIE DUAL ROLES FOR CALCIUM-DEPENDENT ACTIVATOR PROTEIN FOR SECRETION (CAPS) IN LARGE DENSE-CORE VESICLES (LDCVS) PRIMING

David R. Stevens[1], Olga Ratai[1] and Jens Rettig[1,*]

[1]Cellular Neurophysiology, Center for Integrative Physiology and Molecular Medicine (CIPMM), Saarland University, Building 48, 66421 Homburg, Germany

***Correspondence to:**
Dr. J. Rettig (jens.rettig@uniklinikum-saarland.de)

ABSTRACT

CAPS (Calcium-dependent activator protein for secretion), was discovered as a soluble factor required for catecholamine secretion from PC12 cells. CAPS facilitates release of dense-core vesicles and release of neurotransmitters. In vertebrates CAPS is required for priming of synaptic vesicles and dense-core vesicles in neurons and in catecholamine release from the adrenal medulla. The pleckstrin homology domain of CAPS has been shown to mediate an interaction with the plasma membrane. An interaction with SNARE proteins and assembled SNARE complexes requires the Munc13 homology domain. The interaction with SNARE components facilitates SNARE complex formation, the molecular basis of the priming reaction, while interactions with the phosphoinositol 4,5 bisphosphate (PI(4,5)P2) rich areas of the plasma membrane localize CAPS at the release site but may also promote the fusion of primed vesicles. CAPS splice variants lacking the Munc13 homology domain but with an intact pleckstrin homology domain promote priming to the readily releasable pool while CAPS splice variants with a deletion in the pleckstrin homology domain promote catecholamine release, albeit at slower rates. These results confirm a dual action of CAPS dependent on the Munc13 homology- and the pleckstrin homology domains, respectively.

Keywords: Priming; exocytosis; synaptic vesicles; large dense-core vesicles

Glossary:

SNARE complex: SNARE proteins (an acronym derived from "SNAP (Soluble NSF Attachment Protein REceptor") are a large protein superfamily consisting of more than 60 members in yeast and mammalian cells. The primary role of SNARE proteins is to mediate vesicle fusion, that is, the fusion of vesicles with their target membrane bound compartments (such as a lysosome). The best studied SNAREs are those that mediate fusion of synaptic vesicles with the presynaptic membrane in neurons. These SNAREs are the targets of the bacterial neurotoxins responsible for botulism and tetanus.

Dense-core vesicles: Vesicles which occur in nerve terminals or somata of neurons and neuroendocrine cells. Contain either neurotransmitter like epinephrins or neuro-peptides. Called also granular vesicle.

Pleckstrin homology domain (PH domain) is a protein domain of approximately 120 amino acids that occurs in a wide range of proteins involved in intracellular signaling or as constituents of the cytoskeleton. This domain can bind Phosphatidylinositol lipids within biological membranes (such as Phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (4,5)-bisphosphate) and proteins such as the βγ-subunits of heterotrimeric G proteins and protein kinase C. Through these interactions, PH domains play a role in recruiting proteins to different membranes, thus targeting them to appropriate cellular compartments or enabling them to interact with other components of the signal transduction pathways.

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Most nerve cells communicate with each other through synaptic transmission at chemical synapses. The regulated exocytosis of neurotransmitters, hormones, and peptides occurs at specialized membrane areas through Ca^{2+}-triggered fusion of secretory vesicles with the plasma membrane. Prior to fusion, vesicles are docked at the plasma membrane and must then be rendered fusion-competent through a process called priming. The molecular mechanism underlying this priming process is most likely the formation of the SNARE complex consisting of Syntaxin 1, SNAP-25, and Synaptobrevin 2. Members of the Munc13 protein family consisting of Munc13-1, -2, -3, and -4 were found to be absolutely required for this priming process.

CAPS was discovered as a soluble factor required for calcium-dependent fusion of large dense-core vesicles (LDCVs) in PC12 cells [1,2]. The results leading to its establishment as a priming factor in exocytosis have been reviewed [3]. Isolation and sequencing of the factor, known as p145, indicated that it was a homolog of a protein already described in *C. elegans,* named UNC31 [4]. UNC31 was discovered in screens of mutations in *C. elegans,* and its mutation is associated with nervous system and motor deficits which lead to uncoordinated movements hence the name [5,6]. A homolog is also found in *Drosophila* [7]. P145 was shown to be phosphorylated by protein kinase C and to have a moderate affinity for calcium [8], which results in the designation of calcium-dependent activator protein for secretion.

The domain structure of CAPS has been previously described [9]. CAPS genes contain a Munc13 homology domain (MHD) which was defined by Koch et al. [10]. Two such MHDs are found in the synaptic vesicle priming factor Munc13 and are involved in the interaction of Munc13s with the SNARE complex. Both MHDs are required for the priming function of Munc13 in calcium-dependent fusion of synaptic vesicles [11,12]. The presence of an MHD in CAPS may indicate a role in priming of vesicles.

The structure of CAPS is conserved across species. The domain structure is shown in **Figure 1**. An N-terminal dynactin-interacting domain (DID) that may be required for sorting has recently been described [13]. This is followed by a C2 domain, which may interact with calcium and phospholipids, though its role in CAPS function is unknown. Adjacent to the C2 domain there is a pleckstrin homology (PH) domain. PH domains associate with acidic phospholipids and thus may mediate interactions with membranes [14,15]. Additionally, CAPS contains the above-mentioned Munc-13 homology domain that comprises a Syntaxin interacting domain (SID) [10] that is required for priming activity in Unc-13 homologs.

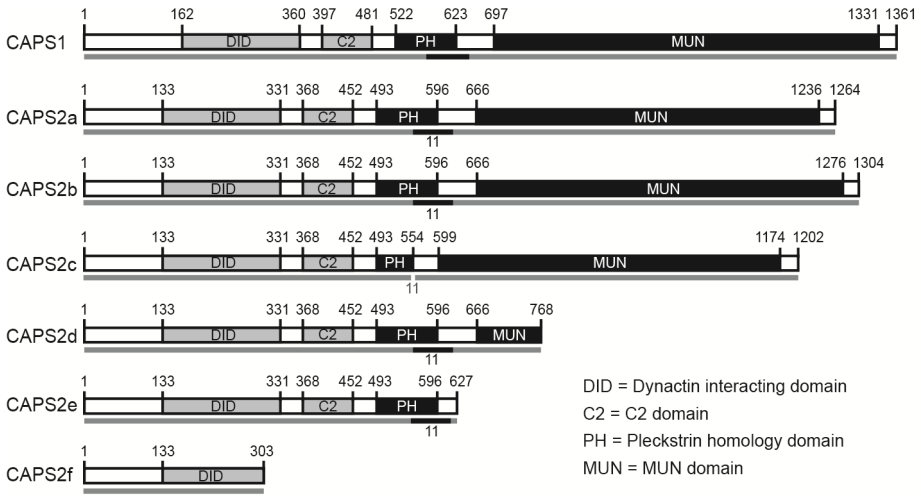

Figure 1. The domain structure of CAPS proteins. Mammalian CAPS genes contain a dynactin-interacting domain followed by a C2 domain, a pleckstrin homology domain and a Munc13 homology domain. The reported splice variants show considerable variability in which domains are expressed, as shown here.

There are two CAPS genes in mammals referred to as CAPS1 and CAPS2 [3]. The genes contain up to 31 exons with a number of splice variants being expressed [9,16].

In *C. elegans*, CAPS has been reported to play a role in docking and in the release of dense-core vesicles and synaptic vesicles [3,6,17]. In *Drosophila*, loss of CAPS leads to motor and nervous system defects in larvae with larva not reaching adulthood [7]. The deficits in muscle function are neurogenic, but do not appear to be cell autonomous. Thus, CAPS has been assigned an important role in calcium-dependent exocytosis of LDCVs and synaptic vesicles in invertebrates.

In mouse, CAPS has been studied in central neurons and in chromaffin cells [3]. CAPS plays a critical role in priming of synaptic vesicles in hippocampal neurons [18] and has been shown to function in priming of LDCVs in chromaffin cells [19,20]. Chromaffin cells are ideally suited for such studies, because they are moderate in size and spherical. These features allow high quality voltage-clamp and imaging experiments, thereby enabling the investigation of the molecular mechanisms of release with unparalleled precision [21]. Whole-cell membrane capacitance recording has been used to compare release kinetics in wild type mouse chromaffin cells with that of release from chromaffin cells from mice lacking CAPS1 [22] or both CAPS1 and CAPS2 [19].

Photolytic release of caged-calcium was used to produce stepwise increases in intracellular calcium which were monitored using a combination of Fura4F and MagFura. Such stepwise increases in calcium produce a burst of exocytosis (see **Figure 2**) that consists of a rapid phase and a slow phase, referred to as the readily releasable pool (RRP) and the slowly releasable pool (SRP), respectively [21]. These are distinguished by exponential fits of the response (see **Figure 2A**). A sustained phase is also present, which is seen as a linear component.

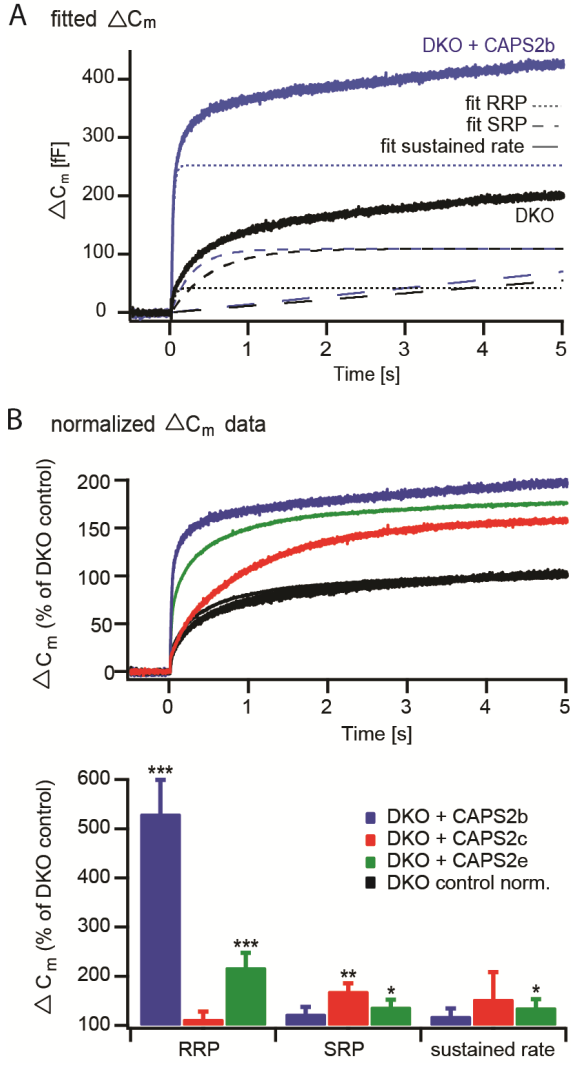

Figure 2. Loss of CAPS proteins leads to significant changes in releasable pools. A. Averaged traces of responses in chromaffin cells in which CAPS1 and CAPS2 are deleted (DKO) and in DKO cells expressing CAPS2b, which rescues secretion to normal levels. Analysis of the kinetic components using a double exponential plus a linear component fit are shown as broken lines (black - DKO, blue - rescue with CAPS2b, see legend). Loss of CAPS leads to a strong reduction of the RRP and the sustained release component. B. Expression of the CAPS 2c splice variant in which the pleckstrin homology domain is partially deleted and the CAPS2e splice variant in which the MH domain is deleted (but the PH domain is intact), lead to enhanced release. Expression of the CAPS2c protein produces a slow, enhanced release while expression of the CAPS2e protein produces enhanced release with partial rescue of rapid release. The bar diagrams show the effects of the tested splice variants on RRP, SRP and sustained components (significance ***, $p<0.001$, **, $p<0.01$, *, $p<0.05$).

Deletion of both CAPS genes leads to a reduction in the RRP and an even stronger reduction of the sustained phase [19,20]. The RRP is indicative of a preformed pool of primed granules which fuses when the intracellular calcium increases. There is disagreement as to whether the SRP is also a primed pool which fuses more slowly or an intermediate step in the priming process which must undergo a final priming step, resulting in its slower kinetics. The sustained phase is thought to be the product of priming of unprimed vesicles under elevated calcium and thus an indicator of priming function *per se*. Since in EM studies there is no apparent docking effect [20], these results are consistent with a role in vesicle priming.

Thus, in both LDCV and synaptic vesicle exocytosis it appears that CAPS plays a role in priming. This result is consistent with the fact that an MHD domain is present in CAPS. As stated above, Munc13 has two MHD domains which are in tandem and both are found in the minimal priming structure of Munc13 [11,12]. The tandem MHDs have been included in a structure referred to as the MUN domain which is required for priming by Munc13 [12]. In *C. elegans*, a MUN domain-like structure has been proposed which includes coiled-coil domains in the stretch between the MHD of CAPS and the pleckstrin homology domain [17].

CAPS has been reported to interact with heterodimers of the SNARE proteins Syntaxin and SNAP-25 and with the assembled SNARE complex [23]. SNARE binding in CAPS has been localized to an area containing the MHD and an adjacent more N-terminal portion of the protein containing a domain with no known function [24]. A segment containing these two domains bound SNAREs and was displaced by CAPS. Mutation of the MHD in CAPS results in a loss of priming function and leads to a loss of SNARE binding and to a suppression of LDCV exocytosis. An interaction with Syntaxin appears to play a central role in CAPS priming function [25].

Since CAPS appears to promote priming, it was intuitive to consider whether its priming function entails the unfolding of Syntaxin as has been suggested for Munc13 [26]. In mouse chromaffin cells lacking both CAPS1 and CAPS2 the deficit in the RRP can be partially rescued by expressing "open-Syntaxin" [20], an unfolded form of Syntaxin in which the Habc domain does not hinder priming. This conformation does not require Munc13 for priming [26]. This is consistent with a role for CAPS in priming which is mechanistically similar to that of Munc13. Open-Syntaxin expression enhanced the RRP, but did not rescue sustained release, considered an indicator of active priming. This may indicate that CAPS effects on RRP size and on priming rate are separable. Although an alternative possibility is that open-Syntaxin has a negative effect on docking [27]. In spite of this possibility, these results are consistent with two effects of CAPS, a stabilization of the RRP and a promotion of priming per se.

Munc13 overexpression leads to an increase in both the slowly releasing and rapidly releasing pools [28]. Thus Munc13 may function upstream of CAPS, consistent with two priming steps. Both CAPS and Munc13 have similarities to the CATCHR tethering proteins [29,30] and it has been suggested that CAPS may function via concurrent binding at the membrane via its PH domain and to LDCVs via a C-terminal domain at a tethering step but this is unlikely since CAPS has been reported to promote fusion of LDCVs which are stationary and thus already tethered or docked [31].

The PH domain plays a role in CAPS priming function. Full-length CAPS binds to liposomes containing phosphatidylserine [32], and the presence of PI(4,5)P2 enhances this binding. PI(4,5)P2 contributes to clustering of SNARE proteins at the plasma membrane [33], and PI(4,5)P2 levels have been reported to regulate the readily releasable pool in chromaffin cells [34]. It has been reported that PI(4,5)P2 binding is required for CAPS function [35]. The results indicate that CAPS binds selectively to membranes rich in PI(4,5)P2 and that these areas coincide with LDCV docking [36] such that there are areas of co-localization of CAPS, PI(4,5)P2 and SNAREs. The role of PI(4,5)P2 in vesicle exocytosis has recently been reviewed [37]. CAPS binds at PI (4,5) P2-rich microdomains in the plasma membrane, via its PH domain, and there drives SNARE complex

formation. Clustering of SNAREs in the presence of PI(4,5)P2 may be enhanced via an interaction with vesicle-associated CAPS. How CAPS associates with vesicles, if this occurs at all, is unclear.
In an attempt to better understand the roles of the MHD and PH domains in CAPS function Nguyen-Truong et al. have taken advantage of the naturally occurring splice variants [16] (see **Figure 1**). They used high-resolution membrane capacitance recordings to examine the kinetics of exocytosis in mouse chromaffin cells lacking both CAPS1 and CAPS2 and have tested the ability of various splice variants to rescue secretion [38]. A full-length CAPS (CAPS2b) was tested, as were splice variants with partial deletions of the PH domain (exon 11, CAPS2c) and with a deletion of the MUN domain with an intact PH domain (CAPS2e, **Figure 2A, B**). Expression of CAPS2c resulted in a partial rescue of release with a reduced readily releasable pool. Expression of CAPS2e produced less total secretion than rescue with the full-length CAPS2b but also strongly enhanced the RRP (see **Figure 2B**, lower graph). This is a surprising result which indicates that CAPS promotes the RRP in a manner that does not require the molecular interactions dependent on the MUN domain.

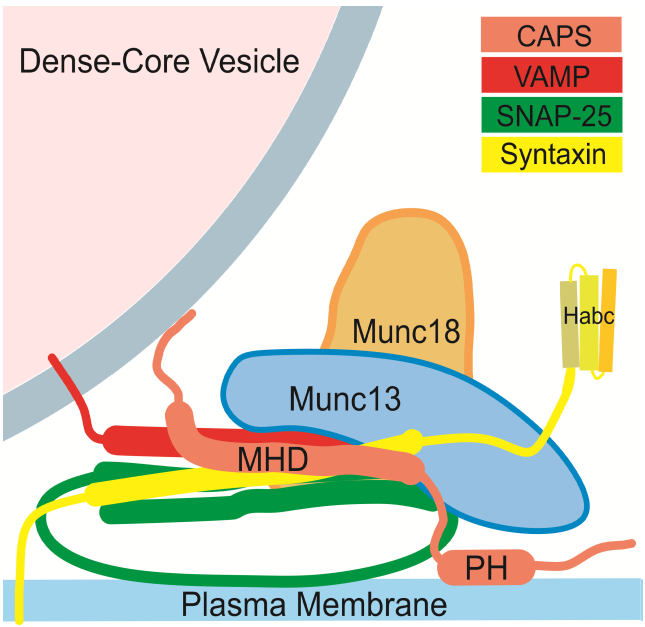

Figure 3. CAPS associates with assembled SNARE complexes as well as with individual SNARE proteins. The initial priming reaction is likely catalyzed by Munc13 which unfolds Syntaxin bound to Munc18, resulting in equal filling of the RRP and SRP. In the presence of CAPS the RRP is enhanced. CAPS appears to support the molecular priming reaction via its MHD domain and to promote or stabilize the RRP via its PH domain.

Conclusions

The results reviewed here support the idea that CAPS has dual functions in the priming process of vesicles, carried out autonomously by different domains (see **Figure 3**). It appears that the MHD allows CAPS to promote assembly of the SNARE complex, likely in cooperation with Munc13s, while the PH domain may stabilize the primed state. The observation that CAPS2 splice variants without a MH domain still cause facilitation of the RRP may indicate that the PH domain functions in an alternative form of priming, e.g. that the interaction with PI(4,5)P2 may promote faster fusion. This could be due to a lowered energy barrier for fusion resulting from the altered lipid composition of the plasma membrane. Apparently, the PH domain has functions other than to simply localize CAPS to areas with SNARE proteins.

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About authors

Jens Rettig is the chairman of the Physiology Department of the medical school of the University of the Saarland. He serves as speaker of the collaborative research group SFB 894, "Calcium Signals: Molecular Mechanisms and Integrative Functions", is a member of the German National Academy of Sciences and is the founding director of the Center for Integrative Physiology and Molecular Medicine where he leads the laboratory for Cellular Neurophysiology.

David R. Stevens leads the adrenal chromaffin cell physiology group in the laboratory for Cellular Neurophysiology of the Physiology Department of the medical school of the University of the Saarland and is actively involved in studies of CAPS function in mouse adrenal chromaffin cells.

Olga Ratai is an advanced doctoral student in the Cellular Neurophysiology laboratory of the Physiology Department of the Medical School of the University of the Saarland where she is studying the mechanism of CAPS function in mouse adrenal chromaffin cells.