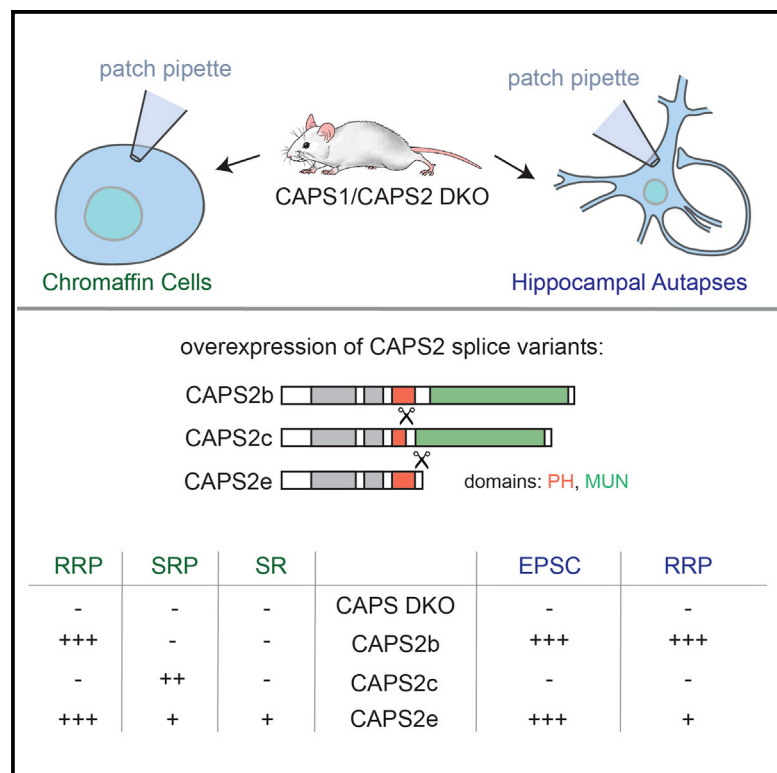# Cell Reports

Secretory Vesicle Priming by CAPS Is Independent of Its SNARE-Binding MUN Domain

Graphical Abstract

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In Brief

Secretory vesicles must be primed before they can fuse in a Ca^{2+}-dependent manner with the plasma membrane. Nguyen Truong et al. show that priming by the CAPS protein is mechanistically different from the priming mediated by Munc13. Whereas Munc13 primes secretory vesicles through syntaxin interaction via the MUN domain, CAPS apparently primes secretory vesicles through phospholipid interaction via its pleckstrin homology domain.

Highlights

CAPSs and Munc13s employ different vesicle priming modes

The MUN domain of CAPS is dispensable for its vesicle priming function

Vesicle priming by CAPSs requires their pleckstrin homology domain

CellPress

Secretory Vesicle Priming by CAPS Is Independent of Its SNARE-Binding MUN Domain

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SUMMARY

Priming of secretory vesicles is a prerequisite for their Ca^{2+}-dependent fusion with the plasma membrane. The key vesicle priming proteins, Munc13s and CAPSs, are thought to mediate vesicle priming by regulating the conformation of the t-SNARE syntaxin, thereby facilitating SNARE complex assembly. Munc13s execute their priming function through their MUN domain. Given that the MUN domain of Ca^{2+}-dependent activator protein for secretion (CAPS) also binds syntaxin, it was assumed that CAPSs prime vesicles through the same mechanism as Munc13s. We studied naturally occurring splice variants of CAPS2 in CAPS1/CAPS2-deficient cells and found that CAPS2 primes vesicles independently of its MUN domain. Instead, the pleckstrin homology domain of CAPS2 seemingly is essential for its priming function. Our findings indicate a priming mode for secretory vesicles. This process apparently requires membrane phospholipids, does not involve the binding or direct conformational regulation of syntaxin by MUN domains of CAPSs, and is therefore not redundant with Munc13 action.

INTRODUCTION

Priming of synaptic vesicles (SVs) and large dense-core vesicles (LDCVs) is a prerequisite for any form of secretory communication by neurons and neuroendocrine cells, as it renders secretory vesicles fusion competent. It is thought that the priming process involves the partial assembly of a trans-SNARE complex between syntaxin and SNAP-25 on the plasma membrane and Synaptobrevin-2 in the vesicle membrane (Wojcik and Brose, 2007; Jahn and Fasshauer, 2012; Südhof, 2012, 2013). The SNARE complex then drives the fusion reaction upon the activation of synaptotagmin by Ca^{2+} (Jahn and Fasshauer, 2012).

Two protein families of bona fide priming factors have been identified thus far, Munc13s and Ca^{2+}-dependent activator proteins for secretion (CAPSs). The mammalian Munc13 family con-sists of five members, Munc13-1 to Munc13-4 (Brose et al., 2000; Koch et al., 2000) and Baiap3 (Shiratsuchi et al., 1998). Munc13s are multidomain proteins containing a phorbol-ester-binding C1 domain and multiple C2 domains, at least one of which binds Ca^{2+}. Importantly, all Munc13 isoforms possess a so-called MUN domain, which represents the minimal binding region for the t-SNARE syntaxin and is sufficient to rescue the deficit in exocytosis in neurons and neuroendocrine cells lacking Munc13s (Basu et al., 2005; Madison et al., 2005; Stevens et al., 2005). The crystal structure of an autonomously folded module within the MUN domain is strikingly similar to that of CATCHR-type (complex associated with tethering containing helical rods) tethering factors, which bind to SNARE proteins through stacked α-helical bundles and tether vesicles to sites of fusion (Li et al., 2011).

The mammalian CAPS family contains only two members, CAPS1 and CAPS2 (Ann et al., 1997; Speidel et al., 2003). CAPS1 was originally discovered as an essential component for Ca^{2+}-dependent exocytosis in neuroendocrine cells (Walent et al., 1992). While initially thought to be specifically required for LDCV fusion, it was later shown that the absence of CAPS proteins also leads to a complete block of fast phasic transmitter release from hippocampal neurons (Jockusch et al., 2007). Functional CAPS isoforms act as dimers (Nojiri et al., 2009) and contain several domains, including a dynactin interacting domain (DID), a C2 domain, a pleckstrin homology (PH) domain, a MUN domain that is also structurally related to CATCHR proteins, and a C-terminal LDCV-binding site (Stevens and Rettig, 2009; James and Martin, 2013). As with Munc13s (Ma et al., 2013), biochemical studies demonstrated that CAPS interacts with SNARE proteins through this MUN domain (James et al., 2009; Daily et al., 2010; Khodthong et al., 2011; Parsaud et al., 2013) and thereby promotes SNARE-mediated membrane fusion in vitro. This led to the conclusion that CAPSs prime LDCVs and SVs by the same mechanism as Munc13s (Hammarlund et al., 2008; James et al., 2009), although the SNARE binding mode might be slightly different (Parsaud et al., 2013).

Here, we show that CAPSs prime vesicles independently of their MUN domain. Instead, our findings reveal a CAPS-mediated priming mode for secretory vesicles, which requires membrane phospholipids, does not involve the binding or direct conformational regulation of syntaxin by MUN domains of CAPSs, and is therefore not redundant with Munc13 action.
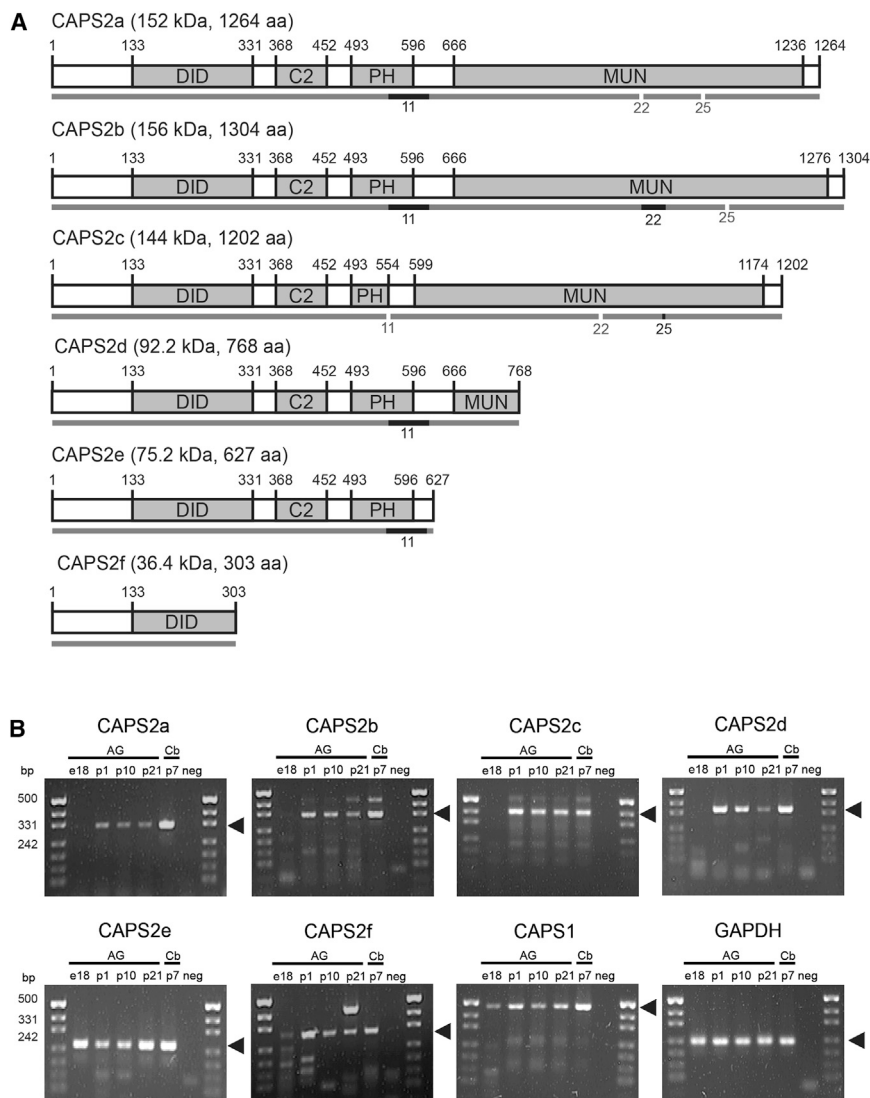
Figure 1. CAPS2 Splice Variants Are Present in Adrenal Glands

(A) Domain structure of CAPS2 splice variants (Sadakata et al., 2007). Domains are as follows: DID, C2 domain, PH domain, MUN domain. Numbers refer to the amino acids defining the respective domains. Alternatively spliced exons are indicated underneath.

(B) RT-PCR of adrenal gland (AG) and cerebellum (Cb) using primers specific for CAPS2 splice variants. Lysates from adrenal glands obtained from mouse embryos at e18 and postnatal days 1, 10, and 21 were used as templates for lanes 1–4, and for lane 5, cerebellar lysate from postnatal day 7 mice was used as template. The outer lanes contain size markers.

RT-PCR from mouse adrenal glands derived from different developmental stages detected all six CAPS2 splice variants at postnatal days p1, p10, and p21. In contrast, at embryonic day 18, only CAPS2e and to a lesser extent CAPS2f were amplified. The other CAPS paralog, CAPS1, was detected at all developmental stages (Figure 1B). We cloned the mouse cDNAs of all CAPS2 splice variants as IRES-eGFP constructs into a Semliki Forest virus vector (pSFV1) and generated virus particles via established protocols (Ashery et al., 1999). We verified the correct expression of the splice variants by western blotting of BHK cell lysates with anti-CAPS2 antibodies and obtained single bands of the expected molecular weights for CAPS2a-CAPS2c, CAPS2e, and CAPS2f. For CAPS2d, we failed to generate a full-length protein, most likely due to the high GC content of the unique C terminus of this construct (Figure S1).

Since all CAPS2 splice variants are expressed in adrenal glands, we first tested their ability to rescue the exocytosis deficit in chromaffin cells derived from CAPS1/CAPS2 DKO mice (Liu et al., 2008; Liu et al., 2010). For this purpose, we performed Ca^{2+} uncaging experiments and recorded LDCV fusion by measuring the resulting increase in membrane capacitance. This recording method has a high temporal resolution and allows separation of different kinetic release components that reflect different releasable vesicle pools, a slowly releasable vesicle pool (SRP) and a rapidly releasing vesicle pool (RRP) (Rettig and Neher, 2002). The absence of both CAPS isoforms leads to a selective loss of the RRP, with the SRP remaining unaffected (Liu et al., 2008, 2010), an almost complete loss of the sustained release component, which represents ongoing priming and fusion of LDCVs in the presence of high intracellular Ca^{2+} concentrations, and a deficit in catecholamine loading of LDCVs (Speidel et al., 2005). All of these phenotypic changes are

RESULTS

We examined the functionality of six naturally occurring splice variants of CAPS2 (Sadakata et al., 2007) by assessing their ability to reverse the profound secretory deficits of CAPS1/CAPS2-deficient (CAPS1/CAPS2 DKO) chromaffin cells and hippocampal neurons. Two of these splice variants, CAPS2a and CAPS2b, contain all domains, but differ by the presence or absence of one exon in the MUN domain (exon 22; Figure 1A). A third splice variant, CAPS2c, has an incomplete PH domain due to the absence of exon 11 (Figure 1A). The three remaining splice variants, CAPS2d-CAPS2f, lack one or several domains, with CAPS2d lacking part of the MUN domain, CAPS2e lacking the entire MUN domain, and CAPS2f only containing the DID domain (Figure 1A). The three short splice variants CAPS2d-CAPS2f all contain a unique C terminus, while the N-terminal sequence of all six splice variants is identical.
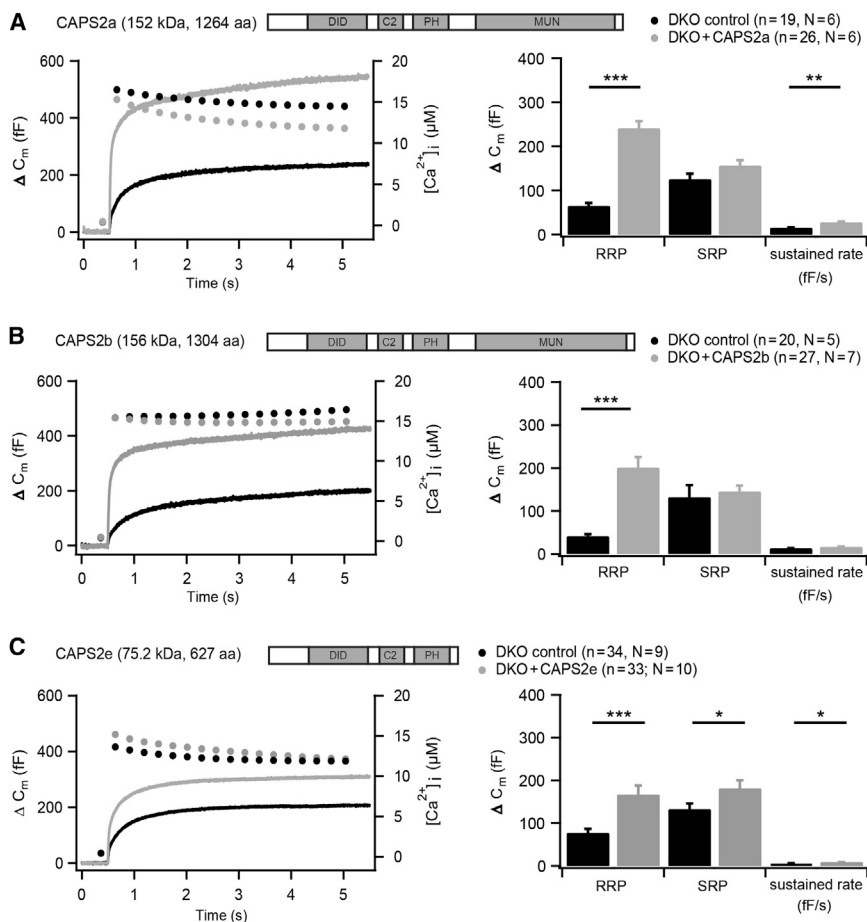
Figure 2. A Short CAPS2 Splice Variant Lacking the Entire MUN Domain Rescues LDCV Exocytosis from CAPS1/CAPS2 DKO Adrenal Chromaffin Cells

(Left) Whole-cell capacitance response and free intracellular Ca^{2+} concentration before and during photolysis of caged Ca^{2+} (dots) in CAPS1/CAPS2 DKO chromaffin cells (black trace) and DKO cells expressing the CAPS2a (A), CAPS2b (B), or CAPS2e (C) splice variants (gray traces). The corresponding domain structure is shown above the data traces.

(Right) Bar graphs showing the values of the RRP, SRP, and sustained release (mean ± SEM) of the responses shown on the left.

***p < 0.001, **p < 0.01, *p < 0.05 (Mann Whitney U test). n = number of cells, and N = number of animals.

cells (Figure 2C). Both the SRP and the sustained release component were also increased, although the increase in these parameters was less pronounced than the RRP increase. These data led to the unexpected conclusion that the MUN domain of CAPS2 is dispensable for LDCV priming in chromaffin cells, that CAPS2 primes LDCVs by a different mechanism than Munc13s, and that the unusual priming function of CAPS2 must be mediated by its N terminus, which includes the DID, C2, and PH domains (Figure 1A).

We next attempted to rescue CAPS1/CAPS2 DKO cells with the shortest of the CAPS2 splice variants, CAPS2f, which only contains the DID domain (Figure 1A). We found that CAPS2f cannot rescue the secretory defect in CAPS1/CAPS2 DKO chromaffin cells (Figure 3A), indicating that the priming function of the CAPS2e splice variant is not mediated by the DID domain alone, but requires the C2 domain and/or the PH domain. We then took advantage of the fact that one of the three long CAPS2 splice variants, CAPS2c, contains an incomplete PH domain (Figure 1A) and is therefore ideally suited to test whether the phospholipid binding of the PH domain (James et al., 2008, 2009, 2010) is required for the CAPS2 priming activity. When expressed in CAPS1/CAPS2 DKO chromaffin cells, CAPS2c reversed the defect in the sustained release component of CAPS1/CAPS2 DKO cells and primed vesicles into the SRP, but did not ameliorate the RRP defect (Figure 3B). The effect of all five investigated CAPS2 splice variants on the different components of catecholamine secretion from chromaffin cells is summarized in Figure 3C. Comparison of the responses of splice variant-expressing cells to their respective normalized controls clearly shows a rescue of the RRP by CAPS2a, CAPS2b and CAPS2e, while the CAPS2c mutant increases the SRP, but not the RRP. These data led to the conclusion that the LDCV priming function of CAPS2 (and presumably also of CAPS1) does not depend on the MUN domain, but rather appears to involve a functional PH domain of the protein.

rescued by viral re-expression of CAPS1 (Liu et al., 2008) or rat CAPS2b (Liu et al., 2010).

As expected, both mouse CAPS2a and mouse CAPS2b led to a full rescue of secretory defects in CAPS1/CAPS2 DKO chromaffin cells (Figures 2A and 2B). The RRP was increased more than 4-fold in both CAPS2a-expressing CAPS1/CAPS2 DKO cells and CAPS2b-expressing CAPS1/CAPS2 DKO cells. The sustained component in CAPS2a-expressing CAPS1/CAPS2 DKO cells was also increased, while the SRP was slightly, but not significantly increased. The complete rescue of the secretory defects in CAPS1/CAPS2 DKO chromaffin cells by the two longest CAPS2 splice variants, CAPS2a and CAPS2b, verifies the reversibility of the CAPS1/CAPS2 DKO phenotype and was used as a reference for the subsequent analysis of the other CAPS2 splice variants.

We next examined CAPS2e, which lacks the entire MUN domain and is about half the size of CAPS2a and CAPS2b (Figure 1A). In view of previous studies showing that the MUN domain of CAPSs binds SNAREs and promotes SNARE-mediated vesicle fusion in vitro (James et al., 2009; Daily et al., 2010; Khodthong et al., 2011), we expected CAPS2e to be dysfunctional and hence to fail to rescue the secretory defect in CAPS1/CAPS2 DKO chromaffin cells. Surprisingly, however, we found that CAPS2e expression led to a more than 2-fold increase in RRP size in CAPS2e-expressing CAPS1/CAPS2 DKO
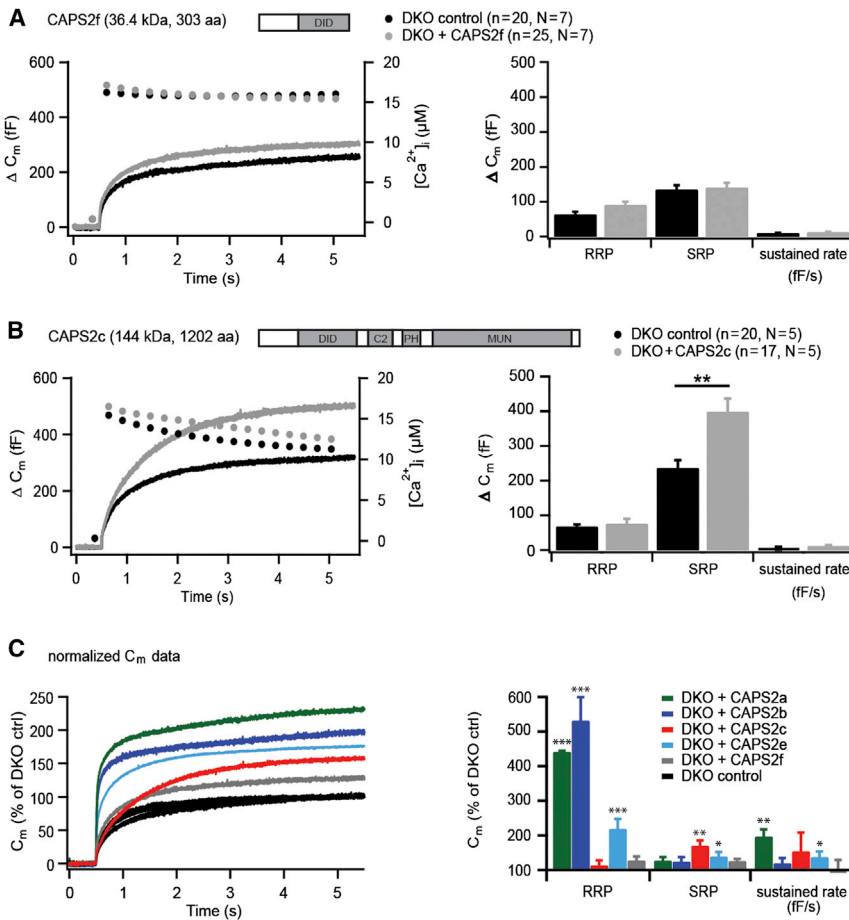
Figure 3. An Intact PH Domain of CAPS2 Is Required for Vesicle Priming

(A and B) Left, whole-cell capacitance response and free intracellular Ca^{2+} concentration before and during photolysis of caged Ca^{2+} (dots) in CAPS1/CAPS2 DKO chromaffin cells (black trace) and DKO cells expressing the CAPS2f (A) or CAPS2c (B) splice variants (gray traces). The corresponding domain structure is shown above the data traces. right, bar graphs showing the values of the RRP, SRP, and sustained release (mean ± SEM) of the responses shown on the left.

(C) Relative capacitance responses of cells expressing CAPS2 splice variants (data from Figures 2A–2C and A and B) as compared with their respective CAPS1/CAPS2 DKO controls (left) and the resulting RRP, SRP, and sustained component values (right).

***p < 0.001, **p < 0.01, *p < 0.05 (Mann Whitney U test). n = number of cells, and N = number of animals.

CAPS2e-expressing CAPS1/CAPS2 DKO neurons to trains of stimuli at 10 and 40 Hz. Expression of CAPS2e in CAPS1/CAPS2 DKO neurons resulted in stronger depression than did CAPS2b expression (Figures 4D and 4E). This result is consistent with the notion that release probability is increased in CAPS2e-expressing DKO neurons.

In contrast to these findings, no rescue of hypertonic sucrose induced EPSC responses was observed in CAPS2c-expressing neurons (Figure 4H). Similar to CAPS1/CAPS2 DKO neurons, CAPS2c-expressing CAPS1/CAPS2 DKO neurons displayed a significantly higher augmentation of transmitter release 2 s after a train of 100 action potentials at 40 Hz when compared with CAPS2b-expressing CAPS1/CAPS2 neurons, which further reflects the inability of CAPS2c to rescue the transmitter release deficiency in CAPS1/CAPS2 DKO cells.

Since CAPS is also essential for the priming of SVs (Jockusch et al., 2007), we next investigated whether our findings in chromaffin cells, a model system for LDCV secretion, can be extended to SV exocytosis in neurons. For this purpose, we tested the ability of the CAPS2b, CAPS2e, and CAPS2c splice variants to rescue exocytosis in cultured autaptic hippocampal neurons from embryonic day 18 (e18) CAPS1/CAPS2 DKO mice. Expression of CAPS2b in CAPS1/CAPS2 DKO cells led to a striking rescue of action potential-evoked excitatory postsynaptic currents (EPSCs) (Figures 4A and 4G). Importantly, evoked EPSC amplitudes were also rescued in CAPS2e-expressing CAPS1/CAPS2 DKO neurons (Figure 4A), but not in CAPS2c-expressing cells (Figure 4G).

The RRP size, which was assessed by measuring EPSCs triggered by application of hypertonic sucrose solution (Rosenmund and Stevens, 1996), was rescued by expression of CAPS2b in CAPS1/CAPS2 DKO neurons (Figure 4B). A similarly significant rescue of the RRP size was observed in CAPS2e-expressing CAPS1/CAPS2 DKO neurons, although the degree of RRP rescue was less pronounced than with CAPS2b (Figure 4B). The strong rescue of EPSC amplitudes along with the partial rescue of the RRP size in CAPS2e-expressing CAPS1/CAPS2 DKO neurons indicates a higher vesicular release probability as compared with CAPS2b-expressing neurons (Figure 4C). We then examined the responses of the CAPS2b- and

DISCUSSION

We show here that vesicle priming mediated by CAPS proteins does not depend on their syntaxin-binding MUN domain, but rather requires a functional phosphatidylinositol-4,5-bisphosphate(PI 4,5-P$_2$)-binding PH domain. A stimulatory role of PI 4,5-P$_2$ in Ca^{2+}-dependent secretion was demonstrated in chromaffin cells and pancreatic β cells (Olsen et al., 2003; Milosevic et al., 2005). In these cell types, PI 4,5-P$_2$ infusion increases the pool of releasable vesicles as well as the sustained rate of vesicle fusion, while depletion of PI 4,5-P$_2$ levels by phosphatase overexpression or long-term LY294002 application leads to a strong reduction in secretion. PI 4,5-P$_2$ in the plasma membrane is enriched in submicrometer-sized clusters, apparently through juxtamembrane basic residues of syntaxin (Milosevic et al., 2005; James et al., 2008).
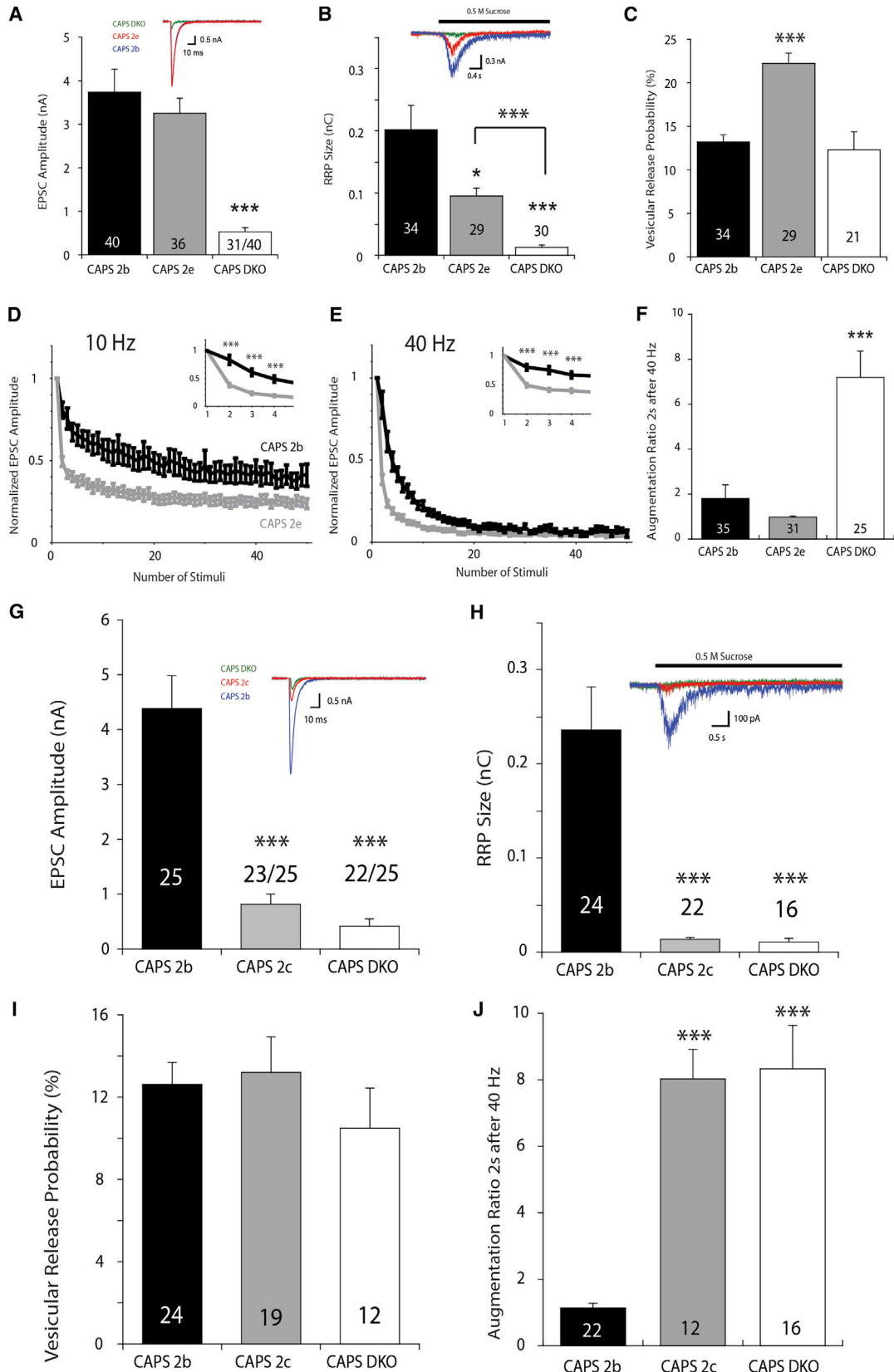
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Already shortly after its discovery as a cytosolic factor required to restore Ca^{2+}-dependent secretion from permeabilized PC12 cells (Ann et al., 1997), CAPS1 was reported to bind PI 4,5-P_2 with high specificity, but moderate affinity (K_D of 50–150 μM) (Loyet et al., 1998). Subsequently, it was shown that CAPSs localizes to PI 4,5-P_2 clusters in the plasma membrane of PC12 cells, and that point mutations in the PH domain of CAPSs abolish this specific localization as well as the stimulatory effect of CAPSs on SNARE-dependent liposome fusion (James et al., 2008). Further in vitro studies revealed that CAPSs drive trans-SNARE complex formation and liposome membrane fusion through syntaxin (James et al., 2009) and that CAPSs bind not only to syntaxin, but also to SNAP-25 and synaptobrevin 2 (Daily et al., 2010).

Two independent studies showed that the MUN domain of CAPSs is essential for SNARE-binding (Khodthong et al., 2011; Parsaud et al., 2013). According to the model that evolved from these in vitro studies, CAPSs first connect the secretory vesicle membrane and the PI 4,5-P_2-containing plasma membrane via their LDCV-binding site and PH domain, and then bind to SNAREs via their MUN domain (Khodthong et al., 2011; James and Martin, 2013), thereby facilitating SNARE complex assembly, which is thought to be the molecular basis for the vesicle priming process (Jahn and Fasshauer, 2012; Südhof, 2012, 2013).

Our data are incompatible with this model. We show that LDCVs in chromaffin cells are primed into the SRP even in the absence of CAPSs (Figures 2 and 3) and that re-expression of CAPS2 splice variants with an intact N terminus, including the PH domain leads to a rescue of secretory defects in chromaffin cells and neurons, regardless of the presence of the SNARE-binding MUN domain and the C-terminal LDCV-binding site. Thus, the vesicle priming process apparently consists of at least two independent steps, the assembly of the SNARE complex, which likely involves Munc13s, and a CAPS-mediated interaction with PI 4,5-P_2. Such a scenario would also explain the nonredundant functions of Munc13s and CAPSs in secretory vesicle priming (Jockusch et al., 2007; Liu et al., 2010).

The CAPS1 (Cadps1) and CAPS2 (Cadps2) genes contain more than 30 exons each, and the corresponding mRNAs are spliced in a complex manner, resulting in at least four CAPS1 and six CAPS2 splice variants. Our data show that the priming function of CAPS2 resides in the N-terminal part of the protein. This part is present in all known splice variants, except in CAPS2f, which documents the importance of this functionally

essential region of the CAPS2 protein. In chromaffin cells, CAPS2e is the only CAPS2 splice variant whose mRNA is already detectable at embryonic stage e18 (Figure 1B). At this developmental stage, chromaffin cells contain only few LDCVs, which are located in close proximity to the plasma membrane (Liu et al., 2008). It thus seems that priming of the few LDCVs in very young chromaffin cells can be achieved by CAPS2e alone, whereas additional regulatory functions of CAPS2 in more mature chromaffin cells, e.g., in fine tuning LDCV priming, requires the remaining CAPS2 splice variants. Future studies will have to clarify what other functions CAPS proteins might perform (Speidel et al., 2005; Sadakata et al., 2010, 2012) and how alternative splicing contributes to these functions.

On aggregate, our data indicate that CAPSs and Munc13s are nonpromiscuous and nonredundant priming factors with distinct functional roles in the priming process of secretory vesicles. While Munc13s mediate vesicle priming by a direct interaction of their MUN domain with the SNARE protein syntaxin, the priming function of CAPS must involve a different molecular mechanism that does not involve its MUN domain and that depends instead on its PH domain (Figures 3 and 4) (Grishanin et al., 2002), its C2 domain (Grishanin et al., 2004; Speese et al., 2007), and its DID (Speese et al., 2007).

EXPERIMENTAL PROCEDURES

Mice

CAPS1/CAPS2-deficient (DKO) mice (Jockusch et al., 2007) from days e18 and e19, and WT mice from e19, postnatal days p1, p7, p10, and p21 were used for experiments. The breeding strategy for obtaining CAPS1/CAPS2 DKOs involved four breeding steps, the fourth one with male and female CAPS1$^{+/-}$/CAPS2$^{-/-}$ mice. As a result, we typically obtained a Mendelian distribution of 50% CAPS1$^{+/-}$/CAPS2$^{-/-}$, 25% CAPS1$^{+/+}$/CAPS2$^{-/-}$, and 25% CAPS1$^{-/-}$/CAPS2$^{-/-}$ (DKO). Thus, we do not obtain WT littermates that could be used as a proper reference. In view of the typical interlitter and interpreparation variability that we and others have observed in many studies, we always compared DKO cells expressing the respective CAPS2 splice variant with control DKO cells from the same preparation to have optimal (negative) controls. All experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the State of Lower Saxony, and the NIH.

RT-PCR and Western Blot

RNA was isolated from homogenized adrenal glands (e19, p1, p10, 21) and cerebella (p7) by Trizol preparation. Single-strand cDNAs were produced by RT from 10 ng of total RNA using random hexamers. CAPS1 and CAPS2 splice variants were amplified using specific primers by standard PCR. Mouse glyceraldehyde-3-phosphate dehydrogenase was used as internal control. In the

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Figure 4. Expression of CAPS2e Rescues Priming Deficiency in CAPS1/CAPS2 DKO Neurons, whereas CAPS2c Does Not

Evoked EPSC responses from CAPS1/CAPS2 DKO neurons expressing CAPS2b, CAPS2e, and CAPS2c. Nine of 40 CAPS1/CAPS2 DKO cells showed no evoked EPSC responses in experiments with CAPS2e, and 3 of 25 CAPS1/CAPS2 DKO neurons and 2 of 25 CAPS1/CAPS2 DKO neurons expressing CAPS2c showed no evoked EPSCs in experiments with CAPS2c. The inset depicts representative traces for AP-evoked EPSCs.

(B and H) Mean RRP sizes as determined by measuring the charge transfer of EPSCs evoked by application of 0.5 M sucrose solution for 6 s. The inset depicts representative traces for sucrose-evoked EPSCs.

(C and I) Vesicular release probability, calculated by dividing the charge transfer of a single action potential-evoked EPSC by the charge transfer of hypertonic sucrose-induced EPSC.

(D and E) Changes in normalized EPSC amplitude during trains of action potentials at 10 Hz (CAPS2b, n = 23; CAPS2e, n = 18) or 40 Hz (CAPS2b, n = 32; CAPS2e, n = 28), plotted against the number of stimuli.

(F and J) Augmentation ratio as determined by dividing the amplitude of the first EPSC during a train of 100 action potentials at 40 Hz by the EPSC amplitude 2 s after the action potential train. The number of cells analyzed is indicated in histogram bars.

The error bars represent SEM. *p < 0.05, **p < 0.01; ***p < 0.001 (unpaired, two-tailed Student's t test).

negative control, the template was omitted. Western blotting was performed on lysates of transfected BHK cells (pSFV1-CAPS2x-IRES-eGFP) using a polyclonal rabbit antiserum to recombinant CAPS2e protein.

Cell Preparation and Infection

Chromaffin cells and hippocampal neurons from e19 or e18 CAPS1/CAPS2 DKO mice were prepared as described (Jockusch et al., 2007; Liu et al., 2010). For rescue experiments, chromaffin cells were infected for 4–6 hr with 100 μl of activated pSFV1-CAPS2a-IRES-eGFP, pSFV1-CAPS2c-IRES-eGFP, pSFV1-CAPS2e-IRES-eGFP, or pSFV1-CAPS2f-IRES-eGFP virus, following a protocol described previously (Ashery et al., 1999) and resulting in a 3- to 5-fold overexpression as compared with endogenous CAPS2 expression levels. Correspondingly, neurons were infected for 10–14 hr with 30 μl of the activated viruses.

Electrophysiology

Whole-cell recordings and capacitance measurements were performed as described previously (Jockusch et al., 2007; Liu et al., 2010). All experiments were performed at room temperature.

Calcium Measurements and Photolysis of Caged Ca²⁺

Fura-4F and Furaptra were excited by a monochromator-based system (T.I.L.L. Photonics), and the fluorescence signal was measured using a photomultiplier. To convert the ratio R of the fluorescent signals at both wavelengths into $[Ca^{2+}]_i$, an in vivo calibration curve was used (Voets, 2000). To obtain stepwise increases in $[Ca^{2+}]_i$, a short flash of ultraviolet light from a Xenon arc flash lamp (Rapp OptoElectronics) was applied to the entire cell.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.09.050.

AUTHOR CONTRIBUTIONS

C.Q.N.-T. performed the measurements on chromaffin cells. D.N. performed the measurements on hippocampal neurons and assisted in writing of the manuscript. O.R., C.S., and D.R.S. assisted in the RT-PCR and chromaffin cells measurements. N.B. provided KO mouse lines and wrote the manuscript. J.-S.R. assisted in the measurements on hippocampal neurons and writing of the manuscript, and J.R. designed the experiments and wrote the manuscript.

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