Ca\textsuperscript{2+}-dependent activator protein for secretion (CAPS) 1 is an essential cytosolic component of the protein machinery involved in large dense-core vesicle (LDCV) exocytosis and in the secretion of a subset of neurotransmitters. In the present study, we report the identification, cloning, and comparative characterization of a second mammalian CAPS isoform, CAPS2. The structure of CAPS2 and its function in LDCV exocytosis from PC12 cells are very similar to those of CAPS1. Both isoforms are strongly expressed in neuroendocrine cells and in the brain. In subcellular fractions of the brain, both CAPS isoforms are enriched in synaptic cytosolic fractions and also present on vesicular fractions. In contrast to CAPS1, which is expressed almost exclusively in brain and neuroendocrine tissues, CAPS2 is also expressed in lung, liver, and testis. Within the brain, CAPS2 expression seems to be restricted to certain brain regions and cell populations, whereas CAPS1 expression is strong in all neurons. During development, CAPS2 expression is constant between embryonic day 10 and postnatal day 60, whereas CAPS1 expression is very low before birth and increases after postnatal day 0 to reach a plateau at postnatal day 21. Light microscopic data indicate that both CAPS isoforms are specifically enriched in synaptic terminals. Ultrastructural analyses show that CAPS1 is specifically localized to glutamatergic nerve terminals. We conclude that at the functional level, CAPS2 is largely redundant with CAPS1. Differences in the spatial and temporal expression patterns of the two CAPS isoforms most likely reflect as yet unidentified subtle functional differences required in particular cell types or during a particular developmental period. The abundance of CAPS proteins in synaptic terminals indicates that they may also be important for neuronal functions that are not exclusively related to LDCV exocytosis.

Regulated secretion of neurotransmitters, hormones, or peptides from neurons and neuroendocrine cells is mediated by the Ca\textsuperscript{2+}-dependent fusion of secretory vesicles with the plasma membrane (1–3). Two main types of secretory vesicles, small clear (SCV)\textsuperscript{1} and large dense-core (LDCV) vesicles, are responsible for the secretion of classical neurotransmitters and peptides/neuromodulators, respectively.

Despite some striking differences between SCVs and LDCVs with respect to their structure, release kinetics, and recycling, the two vesicle types employ a very similar set of proteins for the regulation and execution of their Ca\textsuperscript{2+}-regulated fusion with the plasma membrane. Such conserved components of the secretory machinery of SCVs and LDCVs include (i) the SNARE complex components synaptobrevin, SNAP-25, and syntaxin, which mediate the fusion reaction, (ii) the putative exocytotic Ca\textsuperscript{2+}-sensor synaptotagmin, (iii) the SNARE complex regulators soluble NSF-attachment protein and NSF, or (iv) the syntaxin regulator Munc18 (1, 4–6).

In contrast to the considerable number of proteins that function in both the SCV and LDCV secretory pathways, only very few proteins were proposed to be more or less specifically involved in the secretion of only one type of vesicle. One such protein is CAPS1, which was discovered as an essential cytosolic factor in Ca\textsuperscript{2+}-triggered norepinephrine release from cracked PC12 cells where it is required for a secretory step that follows ATP-dependent priming (7–9). CAPS1 is a 145-kDa protein that contains a central PH domain whose binding to acidic phospholipids in the plasma membrane is essential for CAPS1 function (10), an MH domain of unknown function which is also found in members of the Munc13 family of vesicle-priming proteins (11), and a C-terminal membrane-association domain that mediates LDCV binding (10). In addition, a C\textsubscript{2} domain precedes the central PH domain (10). Not all aspartic acid residues essential for Ca\textsuperscript{2+} binding to C\textsubscript{2} domains are conserved in the CAPS1 C\textsubscript{2} domain, indicating that this domain may not serve as a Ca\textsuperscript{2+} sensor. Genetic studies on the invertebrate orthologues of CAPS1 in Caenorhabditis elegans (12) and Drosophila melanogaster (5) demonstrated an important role of CAPS isoforms in the secretion of a subset of neurotransmitters. Antibody inhibition studies in chromaffin cells (13) and melanotrophs (14) showed that CAPS1 is required for the release of LDCVs that are rapidly releasable and...
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 tightlt coupled to Ca\(^{2+}\) influx. A specific function of CAPS1 for LDCV exocytosis was deduced from the findings that norepinephrine but not glutamate release from permeabilized synaptosomes is dependent on CAPS1 (15) and that membrane-bound CAPS1 in brain homogenates is associated with LDCVs but not with synaptic vesicles (16). Currently, the molecular mechanism of CAPS1 function in LDCV secretion is unknown.

In the present study, we report the identification, cloning, and comparative characterization of a second mammalian CAPS isoform, CAPS2. Our data demonstrate that CAPS isoforms in higher mammals compose a family of two structurally and functionally highly related proteins with striking differences in their spatial and temporal expression patterns.

MATERIALS AND METHODS

Cloning of Human CAPS2 cDNA—Protein profile screens for proteins containing MHD domains identified a murine cDNA fragment (GenBank\#EBI accession no. AF009689) with high homology to rat CAPS1 (11). Subsequent searches in human and murine genomic database bases (Celera) showed that the initially identified murine cDNA fragment corresponded to an as yet unidentified second CAPS gene, CAPS2. The complete coding sequences of murine and human CAPS2 were assembled from genomic DNA sequence data (Celera; GenBank\#/EBI accession no. AY189262) and mouse CAPS2 cDNA (bp 10–744; GenBank\#EBI accession no. AF009689). In Situ Hybridization—In situ hybridization experiments with mouse brain and adrenal gland sections were performed as described previously (17). Antisense oligonucleotides representing the following sequences were chosen as probes: probe 1896–1740 of mouse CAPS1 cDNA (GenBank\#/EBI accession no. NM_012061) and bp 461–505 of mouse CAPS2 cDNA (GenBank\#EBI accession no. AF009689). Images were captured with a Camedia C-3030 Zoom digital camera (Olympus).

Generation of Antibodies Against CAPS1 and CAPS2—Recombinant GST-rCAPS1 (218–390) fusion protein was generated by using the expression plasmid pGEX-rCAPS1 (218–390), which encodes rat CAPS1 residues 218–390 in frame with GST (18). A polyclonal antibody directed against rat CAPS1 was generated with GST-rCAPS1 (218–390) fusion protein as the antigen. The antiserum was affinity-purified with 5 ml of antiserum and then washed extensively, first with 0.1M phosphate buffer. The antigen-conjugated resin was incubated with 5 ml of antiserum and then washed extensively, first with 0.1M phosphate buffer. The peptide-conjugated resin was incubated with 5 ml of antiserum and then washed extensively, first with 0.1M phosphate buffer. The peptide-conjugated resin was incubated with 5 ml of antiserum and then washed extensively, first with 0.1M phosphate buffer. The peptide-conjugated resin was incubated with 5 ml of antiserum and then washed extensively, first with 0.1M phosphate buffer.

Subcellular Fractionation—Subcellular fractionations were prepared essentially as described previously (23). They were designated as follows: H, homogenate; P1, nuclear pellet; P2, crude synaptosomal pellet; P3, light membrane pellet; S1, cytosolic fraction; L1, lysed synaptosomal membranes; L2, crude synaptic vesicle fraction; L3, cytosolic synaptosomal fraction; S1, supernatant after synaptosome sedimentation; and S1, supernatant after LP1 sedimentation.

Immunocytochemistry—Animals were deeply anesthetized with tri bromoethanol. After a brief rinse with normal saline (50–100 ml), each rat was perfused transcardially with 300 ml of cold 4% paraformaldehyde in PBS (pH 7.4) for 10 min. The brains were quickly removed, placed in 20% sucrose/0.2 M potassium PBS for cryoprotection, and frozen on dry ice. Sagittal 10-μm frozen cryostat sections were collected and blocked in PBS containing 2% normal goat serum and 0.25% Triton X-100 for 30 min at room temperature. Thereafter, sections were incubated with polyclonal antibodies to CAPS1 (1:2000) or CAPS2 (1:2000) and monoclonal antibodies to GAD65 (1:1000, Chemicon) or synaptophysin (1:500) at 4°C for 16 h. The antibodies were diluted in PBS that contained 2% normal goat serum and 0.25% Triton X-100. After 3 rinses in PBS, the sections were incubated with Alexa488- or Alexa568-labeled goat anti-rabbit or anti-mouse IgG secondary antibodies (Vector Laboratories) at room temperature. The sections were then rinsed in PBS and mounted with 1.5% N-propyl gallate. Digital images were captured with a LSM510 laser scanning microscope (Zeiss) and analyzed with the LSM510 software.

Ultrastructural Analysis—Adult male rats were perfused with saline, followed by 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer. The brains were taken out, and 40-μm-thick vibratome sections were collected and stained for CAPS1 as indicated above, except that a biotinylated secondary antibody was used (1:300, Vector Laboratories), followed by an avidin-biotin-peroxidase amplification (Vectastain ABC, Vector Laboratories), a 3,3'-diaminobenzidine labeling reaction, and silver intensification (24). Sections were extensively washed, osmicated for 1 h (1% OsO\(_4\) in phosphate buffer), dehydrated through a graded series of ethanol and propylene oxide, and embedded in Durcupan (Durcupan ACM, Fluka) by a 48-h polymerization at 4°C. Light-gold-uranyl-thin sections were mounted with uranyl acetate and lead citrate, and observed in a LEO 912AB transmission electron microscope (Zeiss). Digital images were captured with a ProScan CCD camera and analyzed with the Analysis version 3.2 software (Soft Imaging System).

Preparation of PC12 Cell Ghosts and Measurement of \([HNE]Release—The preparation of PC12 cell ghosts and the measurement of \([HNE]Release were performed as described (8). The techniques and protocols involved in overexpression of CAPS proteins in COS-1 cells, preparation of cytosol fractions from these cells, and \([HNE]Release assays were published previously (10). Eukaryotic expression vectors encoding full-length rat CAPS1 and mutant CAPS1-bearing mutations in the PH domain (R558D/K566E/K561E) were generated in AML. A full-length human CAPS1 expression vector was generated in pEF6/myc-His C (Invitrogen). All recombinant proteins were expressed in COS-1 cells, purified by nickel-nitritotriacetic acid agarose (Qiagen), analyzed by SDS-PAGE and Coomassie Blue staining, and quantified with known amounts of CAPS1.

RESULTS

Structure and Conservation of CAPS Isoforms—By using MH domain profiles, we identified CAPS1 and a closely related murine protein fragment (mCPD2; GenBank\#/EBI accession no. AF009689) as distant relatives of members of the Munc13 family of synaptic vesicle priming proteins (11). Detailed analysis of the murine and human genomic Celera databases revealed that mCPD2 is part of a novel CAPS gene product,
CAPS2. No evidence for additional CAPS genes in the current murine and human genomic databases was obtained.

We assembled the complete murine and human CAPS2 coding sequences from genomic sequence data (Celera: GA_x6K0272P3E9, murine; GA_x5YV32W188, human) and then used the deduced coding sequences to design specific PCR primers and amplify the full-length human CAPS2 cDNA from a brain cDNA preparation. Three types of PCR amplicons were obtained, subcloned, and sequenced. They differed by the absence or presence of a 333-bp fragment in the 5’ half and/or of a 120-bp fragment in the 3’ half of the cDNA (Fig. 1). Because these regions correspond to individual exons in the murine and human genomic sequences, they are likely to represent alternatively spliced sequences. In the course of the present study, a murine CAPS2 cDNA sequence was deposited in GenBankTM/EBI under accession no. AJ072800. This sequence is almost identical to the sequence we deduced from genomic data and was used for subsequent comparative sequence analyses.

Comparison of CAPS sequences between species showed that the respective murine and human CAPS1 and CAPS2 orthologues are very similar to each other, with over 95% identity at the amino acid level. The corresponding sequence identity between the homologous isoforms was about 80% (Fig. 1). All CAPS proteins are large multidomain proteins containing a central PH domain and an MH domain in their C-terminal half. The novel mouse and human CAPS2 isoforms contain 1275 residues (146 kDa) and 1296 residues (148 kDa), respectively, and are slightly smaller than their CAPS1 counterparts which contain 1382 residues (156 kDa) and 1353 residues (153 kDa), respectively. Together, these findings show that CAPS isoforms in mammals form a small family of two highly homologous proteins with identical domain structure.

Expression Patterns of CAPS mRNAs—To determine the expression patterns of CAPS mRNAs in different tissues, we hybridized RNA blots loaded with poly(A)⁺-enriched RNA from different rat tissues at high stringency with probes representing rat CAPS1 (bp 152–997; GenBankTM/EBI accession no. U16802) and mouse CAPS2 (bp 10–744; GenBankTM/EBI accession no. AF000969) cDNA fragments. CAPS1 mRNA was detected almost exclusively in brain. In contrast, CAPS2 mRNA was expressed most strongly in brain but was also detectable in lung, liver, kidney, and testis (Fig. 2). The sizes of the CAPS1 and CAPS2 mRNAs were very similar and in the range of 5.0 kb. Thus, the CAPS protein family, like most other protein families involved in regulated exocytosis, is composed of brain-specific and more ubiquitously expressed isoforms.

Cellular Expression Patterns of CAPS mRNAs in the Central Nervous System—So far, our data demonstrated that the two CAPS isoforms are most strongly expressed in the central nervous system. On the other hand, CAPS1 was initially discovered in the context of LDCV secretion from PC12 cells (7–9), indicating a role in adrenal chromaffin granule secretion in vivo. To determine the cellular expression pattern of CAPS mRNAs in the brain and adrenal, we performed in situ hybridization experiments using representative mouse organs.

We found that in the adult mouse brain, CAPS1 mRNA is strongly expressed in almost all nerve cells of the brain, although it is absent from glial cells (Fig. 3). In contrast, CAPS2 mRNA expression in the adult brain is less uniform, with high mRNA levels in cerebellum, cortex, olfactory bulb, CA1/CA2 regions of the hippocampus, and dentate gyrus, and levels below the detection limit in the CA3 regions of the hippocampus, striatum, thalamus, superior and inferior colliculi, and brain stem (Fig. 3). A similar picture emerged with respect to the brains of newborn mice, where CAPS1 mRNA was strongly expressed in almost all neuron-rich areas, whereas CAPS2 mRNA expression was less uniform and strongest in the developing hippocampus and cerebellum (Fig. 3). In the adult adrenal gland, CAPS1 mRNA expression was restricted to the chromaffin cells of the medulla (Fig. 3). CAPS2 mRNA levels in the adrenal were at the detection limit of our in situ hybridization assays (Fig. 3). These findings demonstrate that the two CAPS isoforms are differentially expressed in the central nervous system and neuroendocrine cells. Thus, despite their high degree of homology, the two CAPS variants may only be partially redundant in mammals.

Expression Patterns of CAPS Proteins—For the isoform-specific detection of CAPS proteins, we generated polyclonal antisera to CAPS1 and CAPS2 using a GST fusion protein with a highly conserved CAPS1 sequence and a highly conserved CAPS2 peptide sequence as antigens (see “Materials and Methods”). The antibodies were found to be isoform-specific, as determined by Western blots of HEK293 cells overexpressing full-length rat CAPS1 or human CAPS2, where single bands of 150 kDa (CAPS1) and 140 kDa (CAPS2) were detected (data not shown). Moreover, the antibody to CAPS1 readily cross-reacted with mouse CAPS1, as determined in Western blot analyses of wild-type and CAPS1-deficient brain samples (data not shown). Like-wise, the antibody to CAPS2, which was raised against a completely conserved mouse/human peptide, cross-reacted with mouse and rat CAPS2, as illustrated by the specific detection of a 140-kDa protein and a slightly smaller band in mouse and rat tissue samples (Fig. 4). The smaller bands at ~120 kDa that were detected in several tissues by our CAPS1 and CAPS2 antibodies do not originate from degradation (not shown) and most likely represent splice variants (Fig. 1).

In agreement with the data obtained in RNA blot experiments, CAPS1 protein was found to be predominantly expressed in brain and, at lower levels, in pancreas and adrenal gland. No CAPS1 was detected in liver, kidney, testis, lung, heart muscle, spleen, and skeletal muscle (Fig. 4A). In contrast, high CAPS2 protein expression was not only detected in brain but also in liver and testis. CAPS2 levels in adult adrenal gland and lung were just above the detection limit of the Western blot assay, whereas no CAPS2 was detected in pancreas, kidney, heart muscle, spleen, and skeletal muscle (Fig. 4A).

Within the central nervous system, CAPS1 protein expression was found to be uniformly strong in all brain regions except the spinal cord, where only low amounts were detected. CAPS2 protein expression, on the other hand, was strongest in the cerebellum, moderate in brain stem and thalamus, weak in hippocampus, hypothalamus, and superior/inferior colliculi, and at or below the detection limit in homogenates from all other brain regions tested (Fig. 4B). Analysis of cortical subcellular fractions showed a very similar subcellular distribution of CAPS1 and CAPS2 protein. Both isoforms are largely soluble, with a membrane-bound pool associated mainly with vesicular (LP2) and less with synaptic plasma membrane fractions (LP1) (Fig. 4C). Interestingly, the soluble pools of CAPS1 and CAPS2 were strongly enriched in synaptic fractions (LS2) (Fig. 4C).

During brain development, CAPS1 protein expression is similar to that of synaptic markers. Expression is first detectable late in embryogenesis (embryonic day 14) and increases to reach a plateau about 20 days after birth, when most synapses have been formed. A smaller CAPS1 immunoreactive band, most likely representing a splice variant, is detectable only in late phases of development, at and after postnatal day 21 (Fig. 4D). In contrast, CAPS2 protein expression levels are more stable during development and somewhat higher in the embry-
Primary structures of CAPS1 and CAPS2.

The amino acid sequences of rat (r), mouse (m), and human (h) CAPS1, and mouse (m) and human (h) CAPS2 are shown in single letter amino acid code and are aligned for maximal homology. Residues that are identical in the majority of sequences are boxed. Pairs of arrows indicate alternatively spliced regions. The black bar indicates the C2 domain, the hatched bar indicates the PH domain, and the blank bar indicates the MHD. GenBank™/EBI accession numbers are: U16802/NM_013219, r-CAPS1; NM_012061, m-CAPS1; XM_036915, h-CAPS1; AY072800, m-CAPS2; and AY264289, h-CAPS2.
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Distribution of CAPS Proteins in Neural and Neuroendocrine Tissue—To compare the localization of CAPS1 and CAPS2 in the brain and adrenal gland, we performed immunocytochemical-labeling experiments on rat tissue sections using isoform-specific primary and fluorescently labeled secondary antibodies. CAPS1 distribution in hippocampus was very similar to that of the presynaptic marker synaptophysin. CAPS1 immunoreactivity was absent from the cell body layers of the hippocampus, indicating that different synapses employ eratively complementary synaptic distribution of CAPS1 and CAPS2 mRNAs (Fig. 3). Here, the predominant expression of CAPS2 mRNA in granule cells (Fig. 3) is the reason for the high CAPS2 protein levels in the molecular layer where granule cell axons terminate (Fig. 5B), whereas low levels of CAPS1 mRNA in the granule cell layer (Fig. 3) are paralleled by low CAPS1 protein levels in the molecular layer (Fig. 5B). In the olfactory bulb, CAPS1 was detected mainly in the glomeruli, colocalized with synaptophysin (Fig. 5C), whereas CAPS2 was not detectable (data not shown). Similarly, with our staining and imaging methods, only CAPS1 but not CAPS2 immunoreactivity was detectable in the adult adrenal gland, where it was restricted to the medulla (Fig. 5D). This apparent lack of CAPS2 in the adult adrenal gland, as determined by immunostaining, correlates well with the barely detectable levels of the corresponding mRNA, as determined by in situ hybridization assays (Fig. 3). Only by the extremely sensitive Western blotting technique was CAPS2 detectable in homogenates of adult adrenal gland (Fig. 4A), indicating that CAPS2 protein is indeed expressed in this tissue during adulthood, albeit at extremely low levels. Taken together, our light microscope data demonstrate a strikingly complementary synaptic distribution of CAPS1 and CAPS2 in certain brain regions such as the hippocampus and cerebellum, indicating that different types of synapses employ different CAPS isoforms.

At higher resolution, the CAPS1 localization within the glomeruli of the cerebellar granule cell layer appeared to overlap only partially with that of synaptophysin. In particular, peripheral regions of glomeruli contained synaptophysin but not CAPS1 (Fig. 6B). Indeed, staining with antibodies to GAD65, a marker of GABA-ergic terminals, revealed very little colocal-
Novel CAPS Isoform

CAPS1 was initially discovered as a cytosolic protein that functions in Ca\(^{2+}\)-dependent exocytosis from permeable PC12 cells at a step that follows ATP-dependent priming (7–9). How this biochemically defined ATP-dependent priming step in the secretory process relates to the LDCV priming steps that are determined by patch-clamp amperometry with high temporal resolution is unclear. Later studies revealed an LDCV-specific role of CAPS1 (15) that correlated well with the fact that membrane-bound CAPS1 in brain homogenates is associated with LDCVs but not with synaptic vesicles (16). However, the molecular basis of CAPS1 function has remained unknown. Its limited homology to Munc13 proteins in a region that is involved in syntaxin binding to Munc13s indicates that CAPS1 might serve a role in LDCV exocytosis analogous to the priming activity of Munc13s, which appears to involve modulation of syntaxin function (25, 26).

In the present paper, we describe a novel CAPS isoform, CAPS2. The same isoform was very recently discovered in an independent study searching for novel interactors of dystrophin. However, proof of an interaction of CAPS2 with dystrophin beyond yeast two-hybrid data was not provided, and CAPS2 was characterized only at the level of its gene structure and mRNA expression using PCR and Northern blotting (27).

CAPS2 is highly homologous to CAPS1 (78–79% identity). Indeed, residues that are essential for CAPS1 function, such as W537, K538, R540, R558, K560, or K561 in the PH domain of rat CAPS1 (10) are conserved in CAPS2 (Fig. 1). As a consequence, CAPS1 was found to be localized specifically to mossy fiber terminals in the cerebellar granule cell layer but absent from neighboring symmetric, presumably GABA-ergic terminals (Fig. 6, C and D). In the molecular layer, CAPS1 was found to be very rare and was occasionally detected in dendritic compartments (Fig. 6E). In the hippocampus, CAPS1 was found mainly in asymmetric, presumably glutamatergic terminals (Fig. 6F). Thus, CAPS1 appears to be specific for glutamatergic terminals. So far, our antibodies to CAPS2 have not provided reliable staining at the ultrastructural level (data not shown).

**Comparison of CAPS1 and CAPS2 Function in Regulated Exocytosis**

To test whether the novel CAPS2 isoform is similar to CAPS1 with respect to its role in the Ca\(^{2+}\)-dependent triggering step of LDCV exocytosis, we examined the ability of proteins purified from COS-1 cells overexpressing wild-type CAPS1, wild-type CAPS2, or an inactive form of CAPS1 (R558D/K560E/K561E) to reconstitute the Ca\(^{2+}\)-dependent triggering step of LDCV exocytosis from permeable PC12 cells. The purified dysfunctional CAPS1 R558D/K560E/K561E mutant (10) showed only background activity (Fig. 7). In contrast, wild-type CAPS1 or wild-type CAPS2 exhibited significantly higher levels of activity that were similar for reconstituting the Ca\(^{2+}\)-dependent triggering step of LDCV exocytosis from permeable PC12 cells. These results indicate that CAPS1 and CAPS2 are functionally equivalent regulators of Ca\(^{2+}\)-dependent LDCV exocytosis.

**DISCUSSION**

**CAPS1 was initially discovered as a cytosolic protein that functions in Ca\(^{2+}\)-dependent exocytosis from permeable PC12 cells at a step that follows ATP-dependent priming (7–9).** How this biochemically defined ATP-dependent priming step in the secretory process relates to the LDCV priming steps that are determined by patch-clamp amperometry with high temporal resolution is unclear. Later studies revealed an LDCV-specific role of CAPS1 (15) that correlated well with the fact that membrane-bound CAPS1 in brain homogenates is associated with LDCVs but not with synaptic vesicles (16). However, the molecular basis of CAPS1 function has remained unknown. Its limited homology to Munc13 proteins in a region that is involved in syntaxin binding to Munc13s indicates that CAPS1 might serve a role in LDCV exocytosis analogous to the priming activity of Munc13s, which appears to involve modulation of syntaxin function (25, 26).

In the present paper, we describe a novel CAPS isoform, CAPS2. The same isoform was very recently discovered in an independent study searching for novel interactors of dystrophin. However, proof of an interaction of CAPS2 with dystrophin beyond yeast two-hybrid data was not provided, and CAPS2 was characterized only at the level of its gene structure and mRNA expression using PCR and Northern blotting (27).

CAPS2 is highly homologous to CAPS1 (78–79% identity). Indeed, residues that are essential for CAPS1 function, such as W537, K538, R540, R558, K560, or K561 in the PH domain of rat CAPS1 (10) are conserved in CAPS2 (Fig. 1). As a consequence, stages were designated as follows: En, embryonic day n; Pn, postnatal day n. Note that the subcellular distribution of CAPS1 and CAPS2 is very similar, with a large soluble synaptic pool in LS2 and a small membrane bound pool associated with the vesicle fraction LP2. In contrast, striking differences between CAPS isoforms are apparent with respect to tissue, brain region, and developmental expression patterns.
Sequence, CAPS2 is functionally equivalent to CAPS1 with respect to its role in LDCV exocytosis from PC12 cells (Fig. 7). In addition, the subcellular localization of CAPS1 and CAPS2 within the cortex, as determined biochemically, is very similar (Fig. 4C). Finally, just like CAPS1 (Figs. 5 and 6), CAPS2 appears to be a presynaptic protein in the central nervous system, as illustrated best by the strong mRNA expression in cerebellar granule cells accompanied by a strong protein expression in the cerebellar molecular layer where the granule cell axons terminate (Fig. 5B). Taken together, these data indicate that CAPS1 and CAPS2 have very similar molecular

**Fig. 5. Immunocytochemical localization of CAPS proteins in rat brain sections.** Immunofluorescence double stainings for CAPS1 or CAPS2 and synaptophysin in hippocampus (A), cerebellum (B), and olfactory bulb (C), and immunofluorescence stainings for CAPS1 in adrenal gland at the interface between cortex (top) and medulla (bottom) (D). Large arrowhead in A, top left indicates stratum lacunosum moleculare; large arrowhead in A, bottom left indicates stratum lucidum; small arrowhead in A, bottom left indicates polymorph layer of the dentate gyrus. Bars, 100 μm. Note that despite striking differences in overall distribution, both CAPS isoforms exhibit a presynaptic localization pattern.

**Fig. 6. Localization of CAPS1 protein in rat brain sections at high resolution.** A and B, single-plane confocal images of the cerebellar granule cell layer double-labeled for CAPS1 (green) and GAD65 (red, A) or CAPS1 (green) and synaptophysin (red, B). C–F, electron micrographs of silver-intensified 3,3’-diaminobenzidine stainings for CAPS1 in rat cerebellum. C, mossy fiber terminal in the cerebellar granule cell layer. D, terminals in the cerebellar granule cell layer. E, dendrite with contacting parallel fibers in the cerebellar molecular layer. F, terminal in the pyramidal cell layer of the hippocampus. Bar in B, 1 μm (A and B); bar in F, 500 nm (A) and 250 nm (D–F). The arrow in C indicates a decorated LDCV, the asterisk indicates an unlabeled symmetric/GABA-ergic synapse. Note that CAPS1 appears to be preferentially associated with asymmetric, glutamatergic axon terminals.

**Fig. 7. Functional activity of CAPS proteins in LDCV exocytosis from PC12 cells.** PC12 cells loaded with [3H]norepinephrine were permeabilized and incubated under MgATP-dependent priming conditions as published (10). Incubations for Ca2+-triggered norepinephrine release were conducted for 3 min with the indicated proteins purified from COS-1 cells overexpressing wild-type CAPS1 (●), wild-type CAPS2 (■), or an inactive form of CAPS1 with indicated mutations in the PH domain (▲). Maximal Ca2+-dependent norepinephrine release obtained with 20 nM CAPS1 was set as 100%. Results are representative of two independent experiments, with data shown as the means of duplicate determinations with indicated range.

sequence, CAPS2 is functionally equivalent to CAPS1 with respect to its role in LDCV exocytosis from PC12 cells (Fig. 7). In addition, the subcellular localization of CAPS1 and CAPS2 within the cortex, as determined biochemically, is very similar (Fig. 4C). Finally, just like CAPS1 (Figs. 5 and 6), CAPS2 appears to be a presynaptic protein in the central nervous system, as illustrated best by the strong mRNA expression in cerebellar granule cells accompanied by a strong protein expression in the cerebellar molecular layer where the granule cell axons terminate (Fig. 5B). Taken together, these data indicate that CAPS1 and CAPS2 have very similar molecular
functions in a very similar subcellular compartment. In that context, the striking differences between CAPS1 and CAPS2 with respect to their expression in different tissues (Fig. 4A), different brain regions (Fig. 4B), different synapse populations (Fig. 5, A and B), and different developmental phases (Fig. 4D) most likely reflect as yet unidentified subtle functional differences that are specifically required in the respective tissue or brain area or during a particular developmental period. In view of the apparently specific role at least of CAPS1 in LDCV exocytosis, the possible function of CAPS2 expressed in liver, lung, or testis (Fig. 4A) remains unclear. To address these open questions, we have initiated a detailed genetic study involving ablation of the two CAPS genes in mice.

Presently available data indicate an LDCV-specific function of CAPS1 (15, 16). This is supported by the detection of CAPS1 mRNA and protein in adrenal slices (Figs. 3 and 5D). In view of these published and novel findings, a striking and surprising observation of the present study was the highly specific and strong presynaptic accumulation of CAPS1 and CAPS2 (Fig. 5).

In the case of CAPS1, this presynaptic localization was verified by electron microscopic observations (Fig. 6, C–F) and appeared to be specific for asymmetric glutamatergic synapses (Fig. 6, A–D). Electron micrographs of mossy fiber terminals in the cerebellar granule layer (see example in Fig. 6C) typically showed that entire synapses were filled with CAPS1 immunoreactivity even when no LDCVs were present, and accumulations of immunoreaction product were only sporadically associated with LDCVs (Fig. 6C, arrow). Admittedly, our electron microscopic method did not reach the level of resolution of immunogold labeling, and it does not, in principle, allow one to draw conclusions about the subcellular compartment or secretory vesicle type that CAPS1 is bound to and acts upon. Nevertheless, the abundance of CAPS1 (and CAPS2) in presynaptic terminals is very suggestive of an additional function of these proteins that is not linked exclusively to LDCV exocytosis. Apart from a direct, possibly Munc13-like role of CAPS proteins in classical synaptic transmission, such LDCV-independent functions of CAPS could also involve a role in the delivery of active-zone transport vesicles to synapses (28). Again, detailed genetic studies in vertebrates will be helpful to resolve this problem.

In summary, we have identified a second mammalian CAPS isoform, CAPS2, whose structure and function are very similar to those of CAPS1 and which may function in a very similar subcellular context, i.e., in neuroendocrine cells and presynaptic terminals of the central nervous system. Differences in the spatial and temporal expression patterns of the two CAPS isoforms most likely reflect as yet unidentified subtle functional differences required in particular cell types or during a particular developmental period. The abundance of CAPS proteins in synaptic terminals indicates that they may also be important for neuronal functions that are not related exclusively to LDCV exocytosis.

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