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Interaction of Ca\textsuperscript{2+}-dependent activator protein for secretion 1 (CAPS1) with septin family proteins in mouse brain

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Highlights

- We showed a novel interaction between CAPS1 and septin family proteins.
- CAPS1 specifically binds to septins 1, 2, 4, 6 and 8–11.
- The C-terminal of CAPS1 binds to part of the deduced GTP-binding domain of septin.

Abstract

The Ca^{2+}-dependent activator protein for secretion 1 (CAPS1) protein plays a regulatory role in the dense-core vesicle exocytosis pathway. To clarify the functions of this protein in the brain, we searched for novel interaction partners of CAPS1 by mass spectrometry. We identified a specific interaction of CAPS1 with septin family proteins. We also demonstrated that the C-terminal region of the CAPS1 protein binds to part of the deduced GTP-binding domain of septin proteins. It is possible that a tertiary complex of septin, CAPS, and syntaxin contributes to dense-core vesicle trafficking and exocytosis in neurons.

Abbreviations: Ca^{2+}-dependent activator protein for secretion 1 (CAPS1); ADP ribosylation factor (ARF); Mass spectrometry (MS); Soluble NSF attachment protein receptor (SNARE)

Keywords: Exocytosis; Dense-core vesicle; Protein–protein interaction; Mass spectrometry; Immunoprecipitation


1. Introduction

The Ca\(^{2+}\)-dependent activator protein for secretion (CAPS) family is involved in dense-core vesicle (DCV) release [2, 5, 9, 13, 18, 24, 29, 31]. In mammals, there are two family members: CAPS1 [5, 20] and CAPS2 [19, 21, 23, 25, 27]. Previous studies have shown that CAPS1 deficiency affects the secretion of catecholamines (e.g., norepinephrine [5, 29]), neuropeptides (e.g., neuropeptide Y [8]), and peptide hormones (e.g., insulin [26]).

Our previous studies showed that CAPS1 interacts with the Golgi membrane and the class II ADP ribosylation factor (ARF) small GTPases (ARF4 and ARF5), and that CAPS1 knockdown and mutations in the ARF-binding region result in abnormal morphology of the trans-Golgi network and a decrease in the number of DCVs. This suggests that CAPS1 plays a role in the synthesis of DCVs at the Golgi membrane [24]. However, the cellular and biochemical functions of CAPS1 in the brain remain unclear. To clarify the functions of this protein in the brain, we searched for novel interaction partners of CAPS1 by mass spectrometry (MS).

2. Materials and methods

2.1. Antibodies

Rat monoclonal anti-HA (cat. no. 1867423; Roche, Mannheim, Germany) and rabbit anti-CAPS1 (amino acid 18–107) [21] antibodies was used for immunoprecipitation. Rabbit anti-CAPS1 (amino acid 266–366) [24], mouse monoclonal anti-FLAG (1:1,000 dilution; cat. no. F1804; Sigma-Aldrich, St. Louis, MO), rat monoclonal anti-HA (1:1,000 dilution; cat. no. 1867423; Roche), rabbit anti-septin 6 (1:100 dilution; cat. no. sc-20180; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-septin 8 (1:100 dilution; cat. no. sc-390105; Santa Cruz) antibodies were used for western blotting. Rat monoclonal anti-HA (cat. no. 1867423; Roche) and mouse monoclonal anti-FLAG (1:1,000 dilution; cat. no. F1804; Sigma) antibodies were used for immunocytochemistry.

2.2. Immunoprecipitation and Mass Spectrometry

A mouse CAPS1 cDNA that lacks the C2 domain was subcloned into the pEF-BOS plasmid vector containing the EF-1\(\alpha\) promoter [14] with a C-terminal HA primer to create pEF-BOS-CAPS1(\(\Delta\)C2)-HA. At 24 h after transfection, Neuro2a cells were harvested and lysed in 1.3 ml of buffer A (5 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton-X, and
0.01% 2-mercaptoethanol) supplemented with a cocktail of protease inhibitors. The supernatants, after preabsorption with protein A-sepharose, were incubated with 1 µg rat normal IgG or anti-HA antibody, and the immunocomplexes were then associated with protein A-sepharose. The resins were washed twice with buffer A, three times with buffer B (5 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% n-Octyl-β-D-glucopyranoside, and 0.01% 2-mercaptoethanol), and twice with buffer C (5 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 0.01% 2-mercaptoethanol). The bound proteins were transferred to 16.25 µl of urea solution (8 M urea, 0.5 M Tris, HEPES, 1 mM EDTA, pH 8.5). Samples were reduced (final concentration 35 mM dithiothreitol, 37°C, 90 min), and alkylated (final concentration 55 mM iodoacetamide, 37°C, 30 min in darkness). The samples were diluted to lower the urea concentration below 2 M, and then further digested for 16 h with 0.5 µg modified trypsin (cat. no. V5280; Promega, Madison, WI). Trypsin digestion was quenched by lowering the reaction mixture to pH ~2 with formic acid.

Reverse-phase liquid chromatography/tandem MS analysis was performed on a NanoFrontier eLD (Hitachi High-Technologies, Tokyo, Japan). The digested peptides were eluted through a MonoCap for Fast-flow (0.05 mm i.d. × 150 mm) C18 monolith column using a linear gradient of mobile phase A/B = 98/2 to 60/40 (where mobile phase A consisted of H₂O/acetonitrile/formic acid at 98/2/0.1 (v/v/v) and B consisted of H₂O/acetonitrile/formic acid at 2/98/0.1 (v/v/v)) for 60 min at a flow rate of 200 nL/min, then subjected to online MS by electrospray ionization. MS/MS spectra were acquired using positive ion mode and the collision-induced dissociation method. Proteins were identified using Mascot (www.matrixscience.com) with the SwissProt database.

2.3. Immunocytochemistry

Immunocytochemistry was performed as previously described [24].

3. Results and discussion

We searched for novel interaction partners of CAPS1 by MS. The C2 domain of CAPS1 tends to interact with various proteins nonspecifically. Therefore, we constructed a vector (pEF-BOS-CAPS1(ΔC2)-HA) that expressed a C2-domain-skipped CAPS1 protein with a C-terminal HA epitope tag. Neuro2a, a mouse neuroblastoma cell line, was chosen for this purpose because it expresses CAPS1 protein weakly and can be transfected effectively. The vector, pEF-BOS-CAPS1(ΔC2)-HA, drove high CAPS1 expression in Neuro2a cells. Forty-eight hours after transfection, we lysed the cells, immunoprecipitated CAPS1-associated
molecules using an anti-CAPS1 antibody, and analyzed them by MS. Out of eight proteins specifically identified in the CAPS1-bound fraction, we focused on septin 10 (Sept10) because septin scaffolds are associated with vesicle fusion events [1, 3, 4, 11, 30]. The septins are a family of GTPases that polymerize into non-polar filamentous structures, which form scaffolds and restrict protein localization [28]. There are 13 septin family members in mouse (septins 1–12 and septin 14; septin 13 is a pseudogene). To confirm the results of MS, we expressed CAPS1-HA and FLAG-septin and conducted a series of co-immunoprecipitation experiments. We examined all the septin family members and found a specific interaction of CAPS1 with septins 1, 2, 4, 6, and 8–11 (Fig. 1).

We also examined the structural properties of the CAPS1–septin interaction. Septins consist of four conserved domains: an N-terminal proline-rich domain, a phosphoinositide-binding polybasic region, a GTP-binding domain, and the septin unique element [15]. Septin 1, like the best-characterized septin 2, is classified into group 2B of the septin family. Immunoprecipitation experiments indicated that septin 1 interacts with CAPS1 via residues 90 to 149, which correspond to part of the GTP-binding domain deduced from septin 2 (Fig. 2).

In our MS experiments, we used CAPS1 lacking a C2 domain. Therefore, we examined the septin-interacting domain of CAPS1 excluding the C2 domain. We found that the C-terminal domain (amino acids 1067–1358) interacts with septin 1 (Fig. 3).

We examined whether CAPS1 interacts with septin at the endogenous level. Septin 1 strongly interacts with CAPS1 but is not expressed in mouse brain. Therefore, we examined the interaction with septins 6 and 8, for which antibodies are commercially available. We immunoprecipitated CAPS1 from mouse whole brain, and confirmed the existence of septin 6 and septin 8 in the immunoprecipitates by western blotting (Fig. 4A). Moreover, when we co-expressed CAPS1 and septin 6 in COS7 cells, CAPS1 co-localized with septin 6 fiber structures (Fig. 4B–D). We obtained similar results with septin 8 (Fig. 4E–G).

Mammalian septins have been classified into four groups based on their amino acid sequence [15]. The septin 2 group contains septins 1, 2, 4, and 5. The septin 3 group consists of septins 3, 9, and 12. The septin 6 group contains septins 6, 8, 10, 11, and 14. Septin 7 is the only member of the septin 7 group. By interacting as hetero-oligomers, mammalian septins form rod-shaped complexes, which can then form filaments and other higher-order structures, such as rings. Complexes contain three (septin 2–septin 6–septin 7) different septin groups, each of which is present in two copies, thereby generating hexameric (7–6–2–2–6–7) complexes [15]. In this report, CAPS1 bound to septins 1, 2, 4, 6, and 8–11. These septins are not restricted to a specific group; that is, CAPS1 binds to all three groups except the septin 7 group. Similarly, other reports have demonstrated proteins that bind to several different septin groups (e.g.,
UBE2I, SUMO, or PIAS), but the common properties of the bound septins have not been elucidated [16]. Septins 2, 4, 6, and 8–11 are highly expressed in the central nervous system [10]. It is possible that septins highly expressed in neurons have a similar structural domain that binds to CAPS1.

It has been suggested that septin scaffolds regulate vesicle fusion events: septin filaments seem to direct the exocyst complex to the appropriate location at the plasma membrane and to regulate the availability of SNAREs for membrane fusion [1, 3, 4, 7, 11, 30]. On the other hand, it has been indicated that the CAPS family is involved in the synthesis of DCVs at the Golgi membrane [22, 24] and DCV release at the plasma membrane [2, 5, 9, 13, 18, 24, 29, 31]. One of the common properties of septins and CAPS is binding activity with syntaxin protein: septins 2 and 5 were reported to bind directly to syntaxin 1 [4], as was CAPS1 [6, 12, 17]. The relationships among septins, CAPS, and SNARE proteins as regards DCV trafficking and exocytosis remain to be elucidated.

4. Conclusions

To clarify the functions of this protein in the brain, we here searched for novel interaction partners of CAPS1 in the Neuro2a cell line. We demonstrated a specific interaction of CAPS1 with septins 1, 2, 4, 6, and 8–11. We also demonstrated that the C-terminal region of the CAPS1 protein binds to part of the deduced GTP-binding domain of septin proteins. It is possible that a tertiary complex of septin, CAPS, and syntaxin contributes to dense-core vesicle trafficking and exocytosis in neurons.

Acknowledgments

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References


Figure legends

**Fig. 1.** CAPS1 interacts with septin (Sept) family proteins: septins 1, 2, 4, 6, and 8–11. Protein–protein interaction between CAPS1-HA constructs and septin-FLAG constructs co-expressed in COS-7 cells was analyzed by co-immunoprecipitation (co-IP) with anti-HA antibody followed by immunoblotting with anti-FLAG and anti-HA antibodies.

**Fig. 2.** CAPS1 interacts with amino acids 90–149 of septin 1. The protein–protein interaction between CAPS1-HA constructs and septin 1-FLAG constructs co-expressed in COS-7 cells was analyzed by co-IP with anti-HA antibody followed by immunoblotting with anti-FLAG and anti-HA antibodies.

**Fig. 3.** Septin 1 interacts with amino acids 1067–1358 of CAPS1. The protein–protein interaction between CAPS1-HA constructs and septin 1-FLAG constructs co-expressed in COS-7 cells was analyzed by co-IP with anti-HA antibody followed by immunoblotting with anti-FLAG and anti-HA antibodies. Schematic depiction of the predicted CAPS1 protein (amino acids 1–1264). The C2- and PH-like domain, and the Munc13-1-homologous domain 1 (MHD1) are represented at amino acids 397–480, 521–621, and 936–1016, respectively.

**Fig. 4.** CAPS1 interacts with septin 6 and septin 8 *in vivo*. (A) Protein–protein interaction between CAPS1 and septin in mouse brain. Endogenous septin 6 and septin 8 were co-immunoprecipitated with endogenous CAPS1 by an anti-CAPS1 antibody from brain lysates of P21 mice. The blots were immunostained for septin 6, septin 8, and CAPS1. (B–D) Subcellular localization of septin 6 and CAPS1 in COS7 cells. Immunostaining of septin 6-FLAG and CAPS1-HA with anti-FLAG (B) and anti-HA (C) antibodies, respectively, and the merged image (D). (E–G) Subcellular localization of septin 6 and CAPS1 in COS7 cells. Immunostaining of septin 6-FLAG and CAPS1-HA with anti-FLAG (E) and anti-HA (F) antibodies, respectively, and the merged image (G). Scale bar, 10 µm.
Fig 1

Fig 2
**CAPS1-HA**
Sept1(90-177)-FLAG
IP: HA

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Fig 3

A

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Fig 4

**Fig 3**

**Fig 4**