Calcium-dependent Activator Protein for Secretion 1 (CAPS1) Binds to Syntaxin-1 in a Distinct Mode from Munc13-1*

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Calcium-dependent activator protein for secretion 1 (CAPS1) is a multidomain protein containing a Munc13 homology domain 1 (MHD1). Although CAPS1 and Munc13-1 play crucial roles in the priming stage of secretion, their functions are non-redundant. Similar to Munc13-1, CAPS1 binds to syntaxin-1, a key t-SNARE protein in neurosecretion. However, whether CAPS1 interacts with syntaxin-1 in a similar mode to Munc13-1 remains unclear. Here, using yeast two-hybrid assays followed by biochemical binding experiments, we show that the region in CAPS1 consisting of the C-terminal half of the MHD1 with the corresponding C-terminal region can bind to syntaxin-1. Importantly, the binding mode of CAPS1 to syntaxin-1 is distinct from that of Munc13-1; CAPS1 binds to the full-length of cytoplasmic syntaxin-1 with preference to its “open” conformation, whereas Munc13-1 binds to the N terminus of syntaxin-1. Unexpectedly, the majority of the MHD1 of CAPS1 is dispensable, whereas the C-terminal 69 residues are crucial for the binding to syntaxin-1. Functionally, a C-terminal truncation of 69 or 134 residues in CAPS1 abolishes its ability to reconstitute secretion in permeabilized PC12 cells. Our results reveal a novel mode of binding between CAPS1 and syntaxin-1, which play a crucial role in neurosecretion. We suggest that the distinct binding modes between CAPS1 and Munc13-1 can account for their non-redundant functions in neurosecretion. We also propose that the preferential binding of CAPS1 to open syntaxin-1 can contribute to the stabilization of the open state of syntaxin-1 during its transition from “closed” state to the SNARE complex formation.

Calcium-dependent activator protein for secretion 1 (CAPS1) is highly expressed in neurons and neuroendocrine cells. It was originally purified from the brain as a critical cytosolic factor that can reconstitute secretion of dense-core vesicles of permeabilized neuroendocrine pheochromocytoma 12 (PC12) cells (1). More recently, its close isoform with distinct expression patterns was identified and termed CAPS2 (2–4). Both CAPS1 and CAPS2 are large proteins (~135–145 kDa), each consisting of an uncharacterized C2-like domain, central pleckstrin homology (PH) domain, Munc-13 homology domain 1 (MHD1), and C-terminal domain, which may mediate binding to dense-core vesicles (Fig. 1A) (5, 6). Interestingly, CAPS1 and CAPS2 are also subject to alternative splicing (7).

The physiological functions of CAPS1 and CAPS2 in dense-core vesicle secretion from mammalian cells have been demonstrated in recent years through the analysis of CAPS1, CAPS2 single knock-out, and CAPS1/CAPS2 double knock-out mice as well as CAPS1 knockdown PC12 cells (8–10). Because CAPS1 knock-out results in prenatal death, embryonic (E18) adrenal chromaffin cells from the knock-out mice have been used for the analysis of neurosecretory phenotype of these mice (3, 10). CAPS1 single knock-out did not show strong catecholamine secretion defects (9) because of the presence and compensatory up-regulation of CAPS2. On the other hand, CAPS1/CAPS2-deficient chromaffin cells showed strong secretory phenotypes including (10) a reduction in the pool of rapidly releasable chromaffin granules and of sustained release during ongoing stimulation. A similar secretory defect was observed in CAPS1 knockdown PC12 cells (8). These results suggest that CAPS1 (and CAPS2) are required for the refilling and/or maintenance of a rapidly releasable secretory granule pool (8, 10). CAPS1 knock-out mice as well as CAPS1/CAPS2 double knock-out mice also exhibited deficits in insulin secretion from pancreatic β-cells (11), which indicates that CAPS1 is also critical for insulin release. Unlike CAPS1, CAPS2 single knock-out mice survived to adulthood. However, these mice

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The abbreviations used are: CAPS1, calcium-dependent activator protein for secretion 1; PC12, pheochromocytoma 12; MHD1, Munc-13 homology domain 1; SNM, silent nucleotide mutation; NA, noradrenaline; TMR, transmembrane region; EmGFP, Emerald GFP.
were deficient in the release of netrotrofin-3 (NT-3) and BDNF in the cerebellum and exhibited pronounced impairments in cerebellar development and functions, including neuronal survival, differentiation and migration of postmitotic granule cells, and dendritogenesis of Purkinje cells (12). Altogether these results support the roles of CAPS1 and CAPS2 in the release of catecholamines, insulin, and neurotrophic factors from dense-core vesicles.

Although the majority of previous studies emphasized the functions of CAPS1 and CAPS2 in dense-core vesicle secretion, a more recent study has revealed the crucial roles of these proteins in synaptic vesicle release as well. CAPS1 knock-out as well as CAPS1/CAPS2 double knock-out resulted in severe reductions in priming of synaptic glutamate release in pyramidal neurons (13). Null mutation of the CAPS homologue in Drosophila also resulted in a 50% reduction in glutamate release in neuromuscular junction (14). These results indicate the conserved function of CAPS proteins in synaptic vesicle exocytosis, probably at the stage of priming.

The function of CAPS1 has been compared with that of Munc13-1, another key protein involved in the priming of synaptic vesicle and dense-core vesicle exocytosis (15, 16). Both proteins share structurally homologous MHD1 domain (Fig. 1A). Furthermore, these proteins were found to bind syntaxin-1 and potentially the SNARE complex containing syntaxin-1 as well (17–20). The binding between Munc13-1 and syntaxin-1 was first discovered using yeast two-hybrid assays and further confirmed by biochemical binding experiments (17). In these studies Munc13-1 MHD1 was found to bind to N-terminal syntaxin-1B (17). The underlined black indicates the residues of mouse CAPS1 that are required for binding to syntaxin-1A (19). The dotted underline indicates the alternative splicing site of 49 residues that are conserved between CAPS1 and CAPS2. Highlighted residues in yellow indicate conserved amino acids between CAPS homologues and Munc13 isoforms. M, R, D, and C indicate mouse, rat, Drosophila, and C. elegans, respectively.

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ties toward syntaxin-1 and reveal striking difference in syntaxin-1 binding modes between these two proteins. We also examine the functional importance of the C-terminal region of CAPS1 that is found to be crucial for the binding to syntaxin-1 in this study.

**EXPERIMENTAL PROCEDURES**

**General Materials**—Mouse monoclonal antibodies against CAPS1 were obtained from BD Biosciences, syntaxin-1 (clone HPC-1) was from Sigma, and SNAP-25 (clone SMI 81) was from Covance (Berkeley, CA); rabbit polyclonal antibodies against N-terminal CAPS1 were from Promokine; rabbit polyclonal antibodies against GFP were from Invitrogen. Monoclonal antibody against synaptobrevin-2 (Cl69.1) was a kind gift from Dr. Reinhard Jahn (Max Planck Institute for Biophysical Chemistry).

**Plasmids for Yeast Two-hybrid Assays**—The mouse CAPS1 sequence in the expression plasmids with silent nucleotide mutations within the knockdown-targeted sequence of 19 residues, pCMV-mCAPS1(SNM)-1 (splicing site positive) and pCMV-mCAPS1(SNM)-2 (splicing site negative), were previously described (8). Mouse CAPS1 truncations were amplified by PCR using pCMV-mCAPS1(SNM)-1 or pCMV-mCAPS1(SNM)-2 as a template, digested with EcoRI and BamHI, and subcloned into the same site of a bait plasmid, pLexN (24–26). A cytoplasmic domain (residues 1–264) of rat syntaxin-1A in pCMV5 (27) was digested with EcoRI and XbaI and subcloned into the same site of a prey vector, pVP16-3 (24, 25, 28). An open conformation mutant (L165A/E166A) of syntaxin-1A (1–264) in pVP16-3 was generated by site-directed mutagenesis. N-terminal syntaxin 1A (residues 1–80) and syntaxin 1B (1–79) were amplified by PCR and subcloned into EcoRI-BglII site of pVP16-3. C-terminal-deleted cytoplasmic regions of syntaxin-1A (1–253, 1–242, 1–220, 1–180) were digested from the respective constructs in pGex-KG (29, 30) with EcoRI and subcloned into the same site of pVP16-3. pLexN-containing residues 1181–1735 of Munc13-1 were constructed by amplifying the corresponding cDNA fragment from pCMV-Munc13-1-EGFP (17) by PCR and subcloning the resulting 1.65-kb fragment into EcoRI-Sall site of pLexN.

**Yeast Two-hybrid Assays**—Yeast strain L40 (26) was transformed with bait and prey vectors by using the lithium acetate method (31). Transformants were plated on selection plates lacking uracil, tryptophan, and leucine and placed in minimal medium lacking uracil, tryptophan, and leucine. After 2 days of incubation at 30 °C, colonies were inoculated into supplemented minimal medium lacking uracil, tryptophan, and leucine and placed in a shaking incubator at 30 °C for 2 days. β-Galactosidase assays were performed as follows. Yeast cells were chilled on ice and harvested by centrifugation (2000 rpm for 5 min). The collected yeast cells were resuspended in 250 μl of breaking buffer (100 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, and 20% glycerol). Then, glass beads (0.45–0.5 mm; Sigma) were added to the yeast suspension to a level just below the meniscus of the liquid followed by 12.5 μl of phenylmethylsulfonyl fluoride stock solution (40 mM in 100% isopropyl alcohol stored at −20 °C). The mixture was then vortexed 6 times at top speed in 15-s bursts. After that, another 250 μl of breaking buffer was added, mixed well, and centrifuged for 1 min. The liquid extract was withdrawn and transferred to new tubes. The extracted liquid was further clarified by centrifuging for 15 min in a microcentrifuge. To perform the assay, 80 μl of the extract was added to 720 μl of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 2.7 ml/l β-mercaptoethanol, pH 7.0). The mixture was then incubated in a water bath at room temperature for 5 min. The reaction was initiated by adding 0.16 ml of stock solution (4 mg/ml α-nitrophenyl-β-D-galactoside in Z buffer; −20 °C), and the reaction mixture was incubated at room temperature. The reaction was precisely terminated at the end of a 7-min incubation by the addition of 0.4 ml of 1 M Na₂CO₃ stock solution in distilled water, and the absorbance of the reaction mixture was measured at 420 nm by using a spectrophotometer. At the same time the protein concentration in the extract was measured using Bradford dye binding assay. A standard curve was prepared using serial dilutions of BSA dissolved in breaking buffer. 10 μl of the extract was added to 1 ml of the Bradford reagent (Bio-Rad), and the formation of the blue color was measured at 595 nm by using a spectrophotometer. The specific activity of β-galactosidase in the extract was calculated according to the formula (A₄₂₀ × 1.36)/(0.0045 × protein concentration (mg/ml) × extract volume (0.08 ml) × 7 min), where A₄₂₀ is the absorbance of the product α-nitrophenol at 420 nm. The factor 1.36 corrects for the reaction volume, and the factor 0.0045 is the absorbance of a 1-nmol/ml solution of α-nitrophenol. The unit of β-galactosidase-specific activity is, therefore, expressed as nmol/mg of protein.

**GST Pulldown Experiments with Recombinant CAPS1 Proteins**—The plasmids to express the C-terminal regions of mouse CAPS1 were generated by amplifying the corresponding cDNA fragments by PCR using pCMV-mCAPS1(SNM)-2: as a template, digesting them with EcoRI and HindIII, and subcloning them into the same site of an expression plasmid, pGex-KG (32–34). GST fusion proteins were expressed in the BL21(DE3) strain and purified with glutathione (GSH)-agarose (Sigma). To prepare the brain homogenate, 1 frozen rat brain was homogenized with 10 ml of homogenization buffer (10 mM HEPES-NaOH, pH 7.4, 320 mM sucrose) and centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 12,000 × g for 20 min, and the pellet was resuspended with 5 ml of KGl buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA) containing 0.3% Triton X-100. The resuspended material was centrifuged at 100,000 × g for 30 min, and the supernatant (we call this total brain homogenate) was used for binding. GSH-agarose containing ~10–40 μg of GST alone or GST-CAPS1 proteins was incubated with 1 ml of brain homogenate at 4 °C overnight, washed 5 times with KGl buffer containing 0.3% Triton X-100, and resuspended in 100 μl of SDS-PAGE sample buffer. 20–μl samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The presence of syntaxin-1, SNAP-25, and synaptobrevin-2 was probed with the monoclonal antibodies and detected by chemiluminescence (Novex ECL; Invitrogen).

**Expression Construct for the Rescue Experiments**—A 4.1-kb EcoRI-BamHI fragment from pCMV-mCAPS1(SNM)-2 was subcloned into the same site of pLVX-IRES-blast (25, 35) to generate pLVX-IB-mCAPS1(SNM)-2. To improve the stable
expression of CAPS1 in the CAPS1 knockdown PC12 cells, the CMV promoter in pLVX-IB-mCAPS1(SNM)-2 was replaced with a CAG promoter using a Ndel-EcoRI fragment from pCAG-GFP (36), which resulted in pLVX-CAG-IB-mCAPS1(SNM)-2. Subsequent expression constructs of CAPS1 truncations were generated by replacing the SpeI-BamHI fragment of pLVX-CAG-IB-mCAPS1(SNM)-2 with the PCR fragment digested with the same restriction enzymes.

To express the C-terminal region (residues 1076–1355) of CAPS1 as a fusion protein with N-terminal EmGFP in mammalian cells, we first subcloned a PCR fragment encoding EmGFP without a stop codon digested with MunI-EcoRI into the EcoRI site of pLVX-IB, resulting in the generation of pLVX-IB-EmGFP-2. We also subcloned a EcoRI-HindIII fragment from pGex-mCAPS1-(1076–1355) into pCMV5, resulting in pCMV-mCAPS1-(1076–1355). A EcoRI-XbaI fragment from pCMV-mCAPS1-(1076–1355) was subcloned into pLVX-IB-EmGFP-2, resulting in pLVX-IB-EmGFP-mCAPS1-(1076–1355).

**Lentivirus-mediated Expression of mCAPS1 Wild-type and Variants in Knockdown Cells**—Clonal lines of CAPS1 knockdown cells (KD5) (8) were maintained in DMEM (Invitrogen) containing 5% calf serum, 5% horse serum (both from HyClone Laboratories, Logan, UT), penicillin (100 units/ml)/streptomycin (0.1 mg/ml), 250 ng/ml amphotericin B (Sigma), puromycin (2.5 µg/ml), and G418 (700 µg/ml). The CAPS1 expression plasmid was cotransfected with pSPAX2 and pMD.G into HEK-293FT cells to generate recombinant lentiviruses that express CAPS1 wild type or its variant. The KD5 cells that were infected with lentiviruses expressing rescue proteins were selected with blasticidin (5 µg/ml).

**[3H]Noradrenaline Release Assays from Intact PC12 Cells**—PC12 cells were incubated with 0.5 µCi/ml [3H]noradrenaline ([3H]NA) in the presence of 0.5 mM ascorbic acid for 12–16 h. The labeled PC12 cells were incubated with the fresh complete Dulbecco’s modified Eagle’s medium for 1–5 h to remove unincorporated [3H]NA. The cells were washed once with physiologically saline solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 15 mM HEPES, pH 7.4, and NA secretion was stimulated with 200 µl of physiological saline solution and high K+—physiological saline solution (containing 81 mM NaCl and 70 mM KCl). Secretion was terminated after a 15-min (high K+) incubation at 37 °C by chilling to 0 °C, and samples were centrifuged at 4 °C for 3 min. Supernatants were removed, and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

**Reconstituted Secretion Assays Using Permeabilized PC12 Cells**—HEK-293FT cells (Invitrogen) in a 10-cm dish were transfected with pLVX-IB plasmid encoding full-length or C-terminal-truncated CAPS1 proteins. 96 h after transfection, cells were harvested using phosphate-buffered saline with 1 mM EDTA and washed twice with KGlu buffer (50 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA). Cell pellets were resuspended in 500 µl of KGlu buffer cells and homogenized by multiple passes through a 10-µm clearance ball homogenizer. Cytosols were prepared by centrifugation at 21,910 × g. The relative content of full-length and truncated CAPS1 was estimated by immunoblot analysis and adjusted with cytosol from mock-transfected HEK-293FT cells to equalize the amount of CAPS1/mg of cytosol protein. For reconstituted two-stage assays, PC12 cells labeled with 0.5 µCi/ml [3H]NA were washed, harvested in KGlu buffer with 0.1% bovine serum albumin, permeabilized with a 2.5-µm clearance ball homogenizer, and incubated for 1–3 h on ice in the presence of 10 mM EGTA to extract the cytosolic proteins (5, 37). Thirty-minute priming incubations at 30 °C contained permeabilized PC12 cells, 2 mM MgATP, and 0.2 mg/ml rat brain cytosol. The cells were recovered by centrifugation, washed once with KGlu buffer with 0.1% bovine serum albumin, and used for 7-min triggering incubations at 30 °C that contained Ca2+ (1.72 mM; free Ca2+ concentrations are estimated to be ~1–10 µM) and cytosols prepared from HEK-293FT cells that overexpress CAPS1 variants. Supernatants were removed, and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

**RESULTS**

**C-terminal Region of CAPS1 Binds to the Cytoplasmic Domain of Syntaxin-1 in a Distinct Mode from Munc13-1**—The original interaction between the Munc13-1 C-terminal region (residues 1181–1736) containing the MHD1 and syntaxin-1 N-terminal region was discovered via yeast two-hybrid screening (17). This interaction mode was further found to be conserved in C. elegans; Unc13 C-terminal region was found to bind to the N-terminal region of Unc-64 using the same assay (21). We hypothesized that a similar approach can be employed to elucidate the interaction between CAPS1 and syntaxin-1. We first cloned the C-terminal regions of mouse CAPS1 as well as rat Munc13 in a bait vector (pLexN) and generated pLexN-CAPS1 (1003–1355) and pLexN-Munc13-1 (1181–1735), respectively (Fig. 2A). Our Munc13-1 bait construct is very similar to the one used by Betz et al. (17), in which LexA is fused with Munc13-1 starting at Glu-1181 in the middle of MHD1. Glu-1003 of CAPS1 corresponds to the residue Glu-1181 of Munc13-1 (Fig. 1). In the MHD1 of CAPS1, there is an alternative splicing site of 49 residues (residues 1012–1060) that is conserved between CAPS1 and CAPS2 (shown by the dotted underline in Fig. 1B). Because a well characterized rat CAPS1 does not contain this splicing sequence, we primarily used a splicing-site negative bait construct (residues 1003–1011 plus 1061–1355) for our binding analysis. However, as demonstrated below, we found that this alternative splicing did not affect the binding between CAPS1 and syntaxin-1 (see Fig. 5). To examine the binding to the N-terminal region of syntaxin-1, we cloned the first 80 residues of syntaxin-1A and 79 residues of syntaxin-1B in a prey vector pVP16-3 (Fig. 2B). To examine the binding to the whole cytoplasmic region of syntaxin-1, we cloned the residue 1–264 of syntaxin-1 (Fig. 5). To examine the binding to the N-terminal region of syntaxin-1, we cloned the first 80 residues of syntaxin-1A and 79 residues of syntaxin-1B in a prey vector pVP16-3 (Fig. 2B). To examine the binding to the whole cytoplasmic region of syntaxin-1, we cloned the residue 1–264 of syntaxin-1A (see Fig. 5). To examine the binding to the N-terminal region of syntaxin-1, we cloned the first 80 residues of syntaxin-1A and 79 residues of syntaxin-1B in a prey vector pVP16-3 (Fig. 2B). To examine the binding to the whole cytoplasmic region of syntaxin-1, we cloned the residue 1–264 of syntaxin-1A (see Fig. 5).
and/or the Habc domain folds back onto the SNARE motif. Because of the homology in the MHD1 domain between CAPS1 and Munc13-1 (Fig. 1), we anticipated a similar binding mode between CAPS1 C-terminal region and syntaxin-1A. Surprisingly, the C-terminal region of CAPS1 did not bind the N-terminal region of syntaxin-1A (1-80) and -1B (1-79) as well the cytoplasmic region (1–264) of syntaxin-1A in yeast two-hybrid assays. CAPS1 on the other hand required the entire cytoplasmic region of syntaxin-1 to bind. Error bars indicate ±S.E. (n = 8).

**FIGURE 2.** Munc13-1 C-terminal region binds to the N-terminal region of syntaxin-1, whereas CAPS1 C-terminal region binds to the entire cytoplasmic domain of syntaxin-1. A and B, the C-terminal region of Munc13-1 (residues 1181–1735) and CAPS1 (1003–1355) were tested on their abilities to bind to the N terminus of syntaxin-1A (1–80) and -1B (1–79) as well the cytoplasmic region (1–264) of syntaxin-1A in yeast two-hybrid assays. C, Munc13-1 preferentially bound to the N-terminal region of syntaxin 1A/1B but not to the whole cytoplasmic region. CAPS1 on the other hand required the entire cytoplasmic region of syntaxin-1 to bind. Error bars indicate ±S.E. (n = 8).

**Differential Syntaxin-1 Binding of CAPS1 and Munc13-1**

**Bind the Open Conformation Syntaxin-1**—Similar to CAPS1, Munc18-1 also binds to the cytoplasmic domain of syntaxin-1 (38). In addition, Munc18-1 is known to preferentially bind to the closed conformation (i.e. wild type) compared with the L165A/E166A open conformation mutant (27, 39). We examined whether CAPS1 and Munc13-1 show a similar binding modality as Munc18-1. We found that CAPS1 preferentially binds to the open conformation of syntaxin-1 rather than the closed conformation, whereas Munc18-1 showed an opposite binding mode (Fig. 3, A and B). Thus, syntaxin-1 binding mode of CAPS1 is clearly distinct from that of Munc18-1. We also found that Munc13-1 does not bind to the whole cytoplasmic domain even when it is in the open conformation (Fig. 3B).
preferential binding of CAPS1 to open syntaxin may explain the recent results of functional studies of CAPS1 in which the open conformation mutant (L165A/E166A) of syntaxin-1 (27) can bypass the requirement of CAPS1 (23). We suggest that CAPS1 may contribute to stabilizing the open conformation of syntaxin-1 by preferentially binding to this conformation. This function of CAPS1 can be dispensable for the syntaxin-1A mutant of L165A/E166A because it adopts an open conformation without the aid of CAPS1.

Previous biochemical and yeast two-hybrid experiments have demonstrated that Munc18-1 binds to almost the entire cytoplasmic region of syntaxin-1 except for the last C-terminal 20 residues preceding TMR (29). By contrast, CAPS1 binding to syntaxin-1 was disrupted by mutations in a membrane-proximal region of syntaxin-1, indicating the importance of this region for binding (18). Therefore, we compared the ability of CAPS1, Munc13-1, and Munc18-1 to bind to syntaxin-1 in which the membrane-proximal regions are serially deleted (Fig. 3B). We found that a whole cytoplasmic region of syntaxin-1 (residues 1–264) is required for the binding to CAPS1 and that a small deletion of the 11 residues (residues 1–253) abolishes the interaction with CAPS1. By contrast, the residues 1–253 or 1–242 of syntaxin-1 were clearly sufficient for the binding to Munc18-1. Our results suggest that the membrane proximal region of syntaxin-1 is necessary for binding to CAPS1. Munc13-1 C-terminal region did not bind to syntaxin-1A (1–180) containing the entire Habc domain without the SNARE motif. The MHD1 Is Largely Dispensable for Binding to Syntaxin-1—During the course of our study, a minimum region of CAPS1 that binds to the SNARE motif plus linker region of syntaxin-1 was determined by Khodthong et al. (19), which used liposome flotation assays for their binding experiments. The identified region (residues 929–997, underlined in black in Fig. 1B) in rat CAPS1 is the N-terminal half of the MHD1, which corresponds to the residues 947–1011 plus 1061–1063 of mouse CAPS1 (Fig. 1). Because our CAPS1 bait construct that binds syntaxin-1 (Fig. 2) contains the residues 1003–1011 plus 1061–1355, the overlapping sequence between these two results is
limited to only 11 residues. We, therefore, investigated the domain of CAPS1 required for syntaxin-1 binding as well as the relationship between the binding detected in our yeast two-hybrid assays and the one found by Khodthong et al. (19).

For this purpose, we have made a series of N-terminal deletion mutants from the original CAPS1 bait construct (residues 1003–1335) and examined their ability to bind to the whole cytoplasmic syntaxin-1A (both open and closed conformation) in yeast two-hybrid assays (Fig. 4A). To our surprise, a series of N-terminal deletions did not affect the binding, and the CAPS1 construct consisting of residues 1107–1335 could still bind syntaxin-1A with preference to the open conformation (Fig. 4B). However, the constructs in which the entire MHD1 were removed (residues 1114–1355) or the MHD1 plus the following 70 residues were removed (residues 1184–1355) did not bind to syntaxin-1A any longer (Fig. 4C). Our results suggest that almost the entire MHD1 of CAPS1 is dispensable for the interaction.

We also examined whether the inclusion of the N-terminal MHD1 can increase the binding of CAPS1 to syntaxin-1 and if the presence of the long splicing site (49 residues) affects it (Fig. 5). We found that the construct containing the whole MHD1 plus the following C-terminal region (residues 919–1355) can bind to the whole cytoplasmic region of syntaxin-1, but this interaction is weaker than the one with residues 1003–1355, which suggests that the N-terminal half of MHD1 does not seem to contribute to the binding to syntaxin-1. The construct containing the long splicing site can interact with syntaxin-1 as effectively as the one lacking the splicing site, suggesting no role of alternative splicing of CAPS1 in binding to syntaxin-1. Taken together, our results indicate that the N-terminal region of MHD1 has no positive impact on binding to syntaxin-1.

We next examined whether our results in yeast two-hybrid assays are reproducible in biochemical binding experiments and whether CAPS1 binds to monomeric syntaxin-1 or the SNARE complex as a whole. Although previous biochemical experiments have shown the interaction between recombinant CAPS1 and recombinant syntaxin-1, binding of recombinant CAPS1 to native brain syntaxin-1 has not been achieved. On the other hand, recombinant Munc13-1 (residues 1181–1345) can pull down native brain SNARE complexes in GST pulldown experiments (17). Therefore, we expressed the recombinant GST-CAPS1 containing the residues of 993–1355, 1003–1355, or 1076–1355 and examined whether they can pull down syn-

FIGURE 4. CAPS1 minimum interacting domain with syntaxin-1 excludes the majority of MHD1. A, shown is a series of N-terminal truncation constructs of MHD1 from the original CAPS1 bait construct (residues 1003–1335) that were tested on their ability to bind to the whole cytoplasmic syntaxin-1A (both open and closed conformation) in yeast two-hybrid assays. B, shown is the minimum region of CAPS1 that was tested so far and found to be sufficient to interact with syntaxin consisting of residues 1107–1355. This region excluded the majority of the MHD1. Error bars indicate ± S.E. (n = 8). C, Additional truncations abolished the binding to syntaxin-1. Error bars indicate ± S.E. (n = 8).
taxin-1 extracted from the brain homogenate. We found that all of them can interact with native brain syntaxin-1, with the shortest CAPS1 (residues 1076–1355) showing the strongest binding and GST alone showing no binding (Fig. 6). These results agree with our yeast two-hybrid assays in that the N-terminal half of MHD1 of CAPS1 is not necessary for the binding to syntaxin-1. Interestingly, the presence of VAMP2 was not detected, whereas a trace amount of SNAP-25 was detected in our pulldown assays by CAPS1. In contrast with this result, previous work by others showed that Munc13-1 can pull down the whole SNARE complex (17). Our results indicate that the C-terminal region of CAPS1 preferentially binds to the monomeric state of syntaxin-1 or the one complexed with SNAP-25 (t-SNARE complex) but not the SNARE complex as a whole. Therefore, the binding mode of CAPS1 to the SNARE complex seems also to be different from that of Munc13-1 (17).

The C-terminal 69 Residues of CAPS1 Are Crucial for Synapsin-1 Binding—To address whether the C-terminal region of CAPS1 is important for binding to synapsin-1, we made a series of C-terminal-truncated constructs and tested their ability to bind synapsin-1A using yeast two-hybrid assays (Fig. 7). We found that a small deletion (15 and 25 residues) of the C-terminal region strongly reduces but does not abolish its binding ability to synapsin-1 in both conformational states. When the last 69 or 134 residues were removed, the interaction was abolished, which suggests the critical role of the C-terminal region for binding to CAPS1.

We also examined the effects of C-terminal deletion on the binding to synapsin-1 and other SNARE proteins in the brain homogenate using GST pulldown experiments (Fig. 8). We used two GST fusion constructs of CAPS1 (919–1355, 1076–1355) as initial templates to generate the C-terminal deletion constructs (Fig. 8A). Both can bind to synapsin-1 as demonstrated in Fig. 6. We found that serial deletions of C-terminal region cause these CAPS1 proteins to lose their ability to bind to synapsin-1. However, unlike the yeast two-hybrid assays, the loss of binding is milder. Even after the deletion of 69 and 134 residues, significant binding over GST alone was observed in both cases (Fig. 8B).

The C-terminal 69 Residues of CAPS1 Are Crucial for Secretion—As the first step to elucidate the potential functional importance of the binding between C-terminal CAPS1 and synapsin-1, we performed rescue experiments using previously generated stable CAPS1 knockdown (KD5) PC12 cells (8). We engineered the KD5 cells to stably express wild-type CAPS1 proteins and their variants with deletion of C-terminal residues. Although wild-type CAPS1 as well as those with small deletions of 15 and 25 residues can express in the KD5 cells, CAPS1 with a deletion of 69 or 134 residues did not stably express in them (Fig. 9A). These results indicate the critical roles of the C-ter-
minal residues in stabilizing the CAPS1 proteins. We also examined the ability of these CAPS1 variants to rescue NA secretion in comparison with the KD5 cells rescued with EmGFP (control). We found that the expression of wild-type CAPS1 enhances secretion of the KD5 cells more than twice compared with the EmGFP alone. Considering that the knock-down of CAPS1 leads to ~50 – 60% reductions in NA release (8), these results suggest that the recovery of secretion by wild-type CAPS1 is highly significant if not complete. We also found that CAPS1 with a deletion of 25 residues can still rescue secretion but in a significantly reduced manner (n = 6, paired t test, p < 0.05) (Fig. 9B). However, this modest reduction in the rescue can be attributable to its slightly reduced expression level (Fig. 9A). Thus, our results suggest that the C-terminal 25 residues are important, but not essential, for binding syntaxin-1 and supporting secretion. These results can be explained by the remaining syntaxin-1 binding ability of CAPS1 with deletion of the C-terminal 25 residues in both yeast two-hybrid assays and biochemical binding experiments (Figs. 7 and 8).

To address the effects of additional truncation of the C-terminal residues of CAPS1, we relied on the two-stage secretion assays using permeabilized PC12 cells (5, 37, 40). Previously, Grishanin et al. (5) showed that CAPS expression in COS-1 cells confers reconstituting secretion activity on cytosol in permeabilized PC12 cells. Using this system, they demonstrated that truncation of the C-terminal 135 residues abolishes the ability of CAPS1 to reconstitute secretion (5). We adopted this system and examined whether cytosols of HEK-293FT cells that overexpress CAPS1 or its truncated mutants can reconstitute secretion (Fig. 10). We first confirmed that cytosol containing full-length CAPS1 can reconstitute Ca2+-dependent secretion activity, whereas cytosol containing EmGFP has no stimulating effects. Importantly, although deletion of the C-terminal 25 residues does not inhibit the reconstituting ability of CAPS1, deletion of 69 or 134 residues abolishes it. Thus, our results are consistent with Grishanin et al. (5) and further extends that the deletion of 69 is sufficient to abolish the ability of CAPS1 to stimulate exocytosis. Because deletion of 69 residues completely abolishes the binding to syntaxin-1 in our yeast two-hybrid assays (Fig. 7) and significantly reduces biochemical binding in GST pulldown experiments (Fig. 8), our results indicate that the syntaxin-1 binding region of CAPS1 that we identified in this study is indeed necessary for the ability of CAPS1 to stimulate secretion.

FIGURE 6. N-terminal half of MHD1 of CAPS1 is not necessary for the binding to syntaxin-1 in GST pulldown experiments. A, GST fusion CAPS1 constructs (residues 993–1355, 1003–1355, and 1076–1355) were tested on their ability to pull down syntaxin-1 and other SNARE proteins in the brain homogenate. B, GST-CAPS1 containing the very C-terminal MHD1 and the following C-terminal region pulled down syntaxin-1 and a trace amount of SNAP-25 but not synaptobrevin-2. The bottom figure shows Ponceau S staining of indicated GST-CAPS1 protein expression.
We finally addressed whether stable expression of the C-terminal syntaxin-1 binding region of CAPS1 (residues 1076–1355) alone can rescue secretion defects of KD5 cells (Fig. 11). Because the expression of a small portion of the protein in mammalian cells is likely to be unstable, we expressed the C-terminal residues of CAPS1 as EmGFP fusion proteins with the hope that EmGFP helps to facilitate or stabilize the expression of the C-terminal CAPS1. Although we could detect the stable expression of GFP-CAPS1 (Fig. 11A), this fusion protein did not exhibit significant rescuing activity (Fig. 11B). We also found that cytosol prepared from HEK-293FT cells that over-express GFP-CAPS1 does not reconstitute secretion in permeabilized PC12 cells (data not shown). These results suggest that the C-terminal syntaxin-1 binding region of CAPS1 alone is not sufficient to stimulate secretion. Thus, the syntaxin-1 binding region of CAPS1 needs to operate in concert with other domains of CAPS1 to support secretion.

**DISCUSSION**

In this study we have elucidated the region of CAPS1 that is sufficient for binding to syntaxin-1 using yeast two-hybrid assays and GST pulldown experiments (Figs. 4–8). Importantly, we found that the binding mode of CAPS1 to syntaxin-1 is distinct from that by Munc13-1; CAPS1 binds to the full-length of cytoplasmic syntaxin-1 with preference to its open conformation, whereas Munc13-1 binds to the first N-terminal
FIGURE 8. Serial deletion of the C terminus of CAPS1 progressively loses its ability to bind to syntaxin-1 in GST pulldown experiments. A, GST fusion CAPS1 constructs were tested on their ability to pull down syntaxin-1 and other SNARE proteins in the brain homogenate. B and C, in both GST-CAPS1 proteins starting residues 919 and 1076, respectively, serial deletions of the C-terminal residues resulted in the progressive loss of binding to syntaxin-1. However, there was residual binding even after the deletion of 134 residues. The bottom figure shows Ponceau S staining of the indicated GST-CAPS1 protein expression.

FIGURE 9. Truncation of the C-terminal of 25 residues of CAPS1 has a modest impact on the ability of CAPS1 to rescue catecholamine secretion from CAPS1 knockdown cells. A, CAPS1 knockdown cells (KDS) were rescued with EmGFP (control), wild-type CAPS1, a 15-amino-acid C-terminal truncation (Δ15), and a 25-amino-acid C-terminal truncation (Δ25). The expression of syntaxin-1 was also tested with the expression being similar to that of wild-type PC12 cells. Moreover, a GAPDH loading control was also used to test for even loading. B, NA release was stimulated by 70 mM KCl for 15 min. Wild-type CAPS1 increased KCl-induced NA release more than 2-fold in comparison with EmGFP control. The 25-amino-acid C-terminal truncation (Δ25) of CAPS1 significantly reduced NA secretion in comparison with the wild type. Error bars indicate ± S.E. (n = 8).
80 residues of syntaxin-1 (Figs. 2 and 3). This difference may account for the non-redundant functions of CAPS1 and Munc13-1 in neurosecretion (13, 23).

Both Munc13-1 and CAPS1 work at the priming stage of exocytosis. Two schools of thought have existed to explain the functional difference between Munc13-1 and CAPS1 in priming. In one school of thought it is hypothesized that Munc13-1 is primarily involved in priming of synaptic vesicles, whereas CAPS1 is primarily involved in priming of dense-core vesicles (41). Here, their roles are similar, but the types of secretory vesicles that they regulate differ. This hypothesis was supported by functional studies using C. elegans; unc-31 mutant shows a defect on peptide release from dense-core vesicles but not from synaptic vesicle release (41). By contrast, unc-13 mutants show defects on synaptic vesicle exocytosis. Moreover, a recent study from this group has shown that the binding modes of CAPS1 and Munc13-4, a ubiquitous isoform of Munc13-1, toward syntaxin isoforms are similar; both CAPS1 and Munc13-4 bind not only to the SNARE motif of syntaxin isoforms but also to SNAP-25 isoforms and to synaptobrevin-2. As a consequence, they bind to the SNARE complex and accelerate SNARE-mediated liposome fusion (18, 42). In the other school of thought, Munc13-1 and CAPS1 are critical for priming of both synaptic vesicle and dense-core vesicle exocytosis, but their priming functions are different (10, 13). This hypothesis is strongly supported by the finding that CAPS1/2 double knock-out neurons exhibit defects not only in dense-core vesicle exocytosis but also in synaptic vesicle exocytosis (13). Furthermore, priming defects of CAPS1/2 knock-out cannot be rescued by overexpression of Munc13-1 (13, 23). Our results showing a different modality of binding between Munc13-1 and CAPS1 favor the second school of thought. However, it is possible that the functions of CAPS1 and Munc13-1 have changed during the process of evolution, and their functions in humans versus C. elegans could be significantly different.

The region in CAPS1 (residues 1107–1355, the very C-terminal MHD1 with the following C-terminal region) we determined to be sufficient for binding to syntaxin-1 is different from the region (the N-terminal half of MHD1) determined by liposome floatation assays (19). Our results may be the first to demonstrate the direct binding between CAPS1 and syntaxin-1 using approaches other than liposome floatation assays. Yeast two-hybrid assays are unbiased cell biological assays to detect the interaction (26). Combined with the results of GST pull-down experiments (Figs. 6 and 8), we believe that the region we defined contributes to binding between CAPS1 and syntaxin-1. Our results do not exclude the role of N-terminal half of MHD1 in binding to syntaxin-1; however, we did not see any enhancing effects of the N-terminal half of MHD1 on syntaxin-1 binding (Fig. 5). Our GST pulldown experiments also showed that the MHD1 binds to syntaxin-1 but not the whole SNARE complex (Fig. 6, 8), which is another difference from the previous study (18).

In a recent functional study of CAPS1 with syntaxin-1 and Munc13-1, it was shown that the open conformation of syn-
taxin-1 rescues secretion defects of CAPS1/2 double-deficient adrenal chromaffin cells (23). This study also showed that Munc13-1 overexpression cannot enhance secretion in the absence of CAPS1, suggesting that CAPS1 functions downstream of Munc13-1. What would be the potential scenario that can explain these previous functional results of CAPS1 with syntaxin-1 and Munc13-1 based on our binding experiments?

We would like to propose the following model based on our results as well as the results of other previous studies (Fig. 12). Considering the fact that CAPS1 and Munc13-1 as well as Munc18-1 are proteins involved in priming of exocytosis and all bind to syntaxin-1, it is reasonable to speculate that the regulation of syntaxin-1, including the regulation of its conformational switch from closed to open leading to the SNARE formation, would constitute one of the key priming steps for exocytosis (22). Munc18-1 binds to syntaxin-1 using two distinct binding modes (44), one being the binding between the Munc18-1 cavity and closed syntaxin-1 (45) and the other being the binding between the hydrophobic pocket of Munc18-1 and syntaxin-1 N-terminal peptide (residues 1–19) (46). Our previous results and others indicate that these two binding modes work together to chaperone syntaxin-1 to the plasma membrane with the former binding mode playing a major role (24, 25, 44, 47). As has been suggested before, Munc13-1 is involved in displacing Munc18-1 from syntaxin-1 (43) by transiently interacting with the N-terminal regions of syntaxin-1 (17, 21). We propose that CAPS1 then binds syntaxin-1 and stabilizes its open conformational state by preferentially binding to open syntaxin-1 (Fig. 2) after the displacement of Munc18-1 by Munc13-1. This would explain the recent results that CAPS1 seems to regulate exocytosis but not for loading of transmitters into dense core vesicles.

Our functional assays show that the C-terminal 69 residues that are critical for binding to syntaxin-1 are indeed necessary for CAPS1 to support secretion (Fig. 10). However, the C-terminal syntaxin-1 binding region alone is not sufficient to rescue secretion defects of KD5 cells (Fig. 11). Thus, the syntaxin-1 binding region of CAPS1 needs to operate in concert with other domains of CAPS1 to support secretion. Therefore, more work is necessary to refine our working model (Fig. 12), which includes defining the relationship between CAPS1 binding to syntaxin-1 and its functional outcomes in exocytosis. For this purpose, it would become crucial to identify key residues of CAPS1 for binding to syntaxin-1 and their mutations that abolish the binding between these two proteins. Our CAPS1 knockdown cells such as KD5 cells will help to analyze the function of these mutants through the rescue experiments.

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