

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

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**Background:** CAPS1 and Munc13-1 play crucial but non-redundant roles in exocytosis.

**Results:** CAPS1 binds to the full-length, cytoplasmic syntaxin with preference to its open conformation, whereas Munc13-1 binds to the N terminus of syntaxin-1.

**Conclusion:** The distinct binding modes of CAPS1 and Munc13-1 can account for their non-redundant functions.

**Significance:** Mechanistic insights into how CAPS1 contributes to exocytosis.

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 first 80 N-terminal residues 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)<sup>3</sup> 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). 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  $\beta$ -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

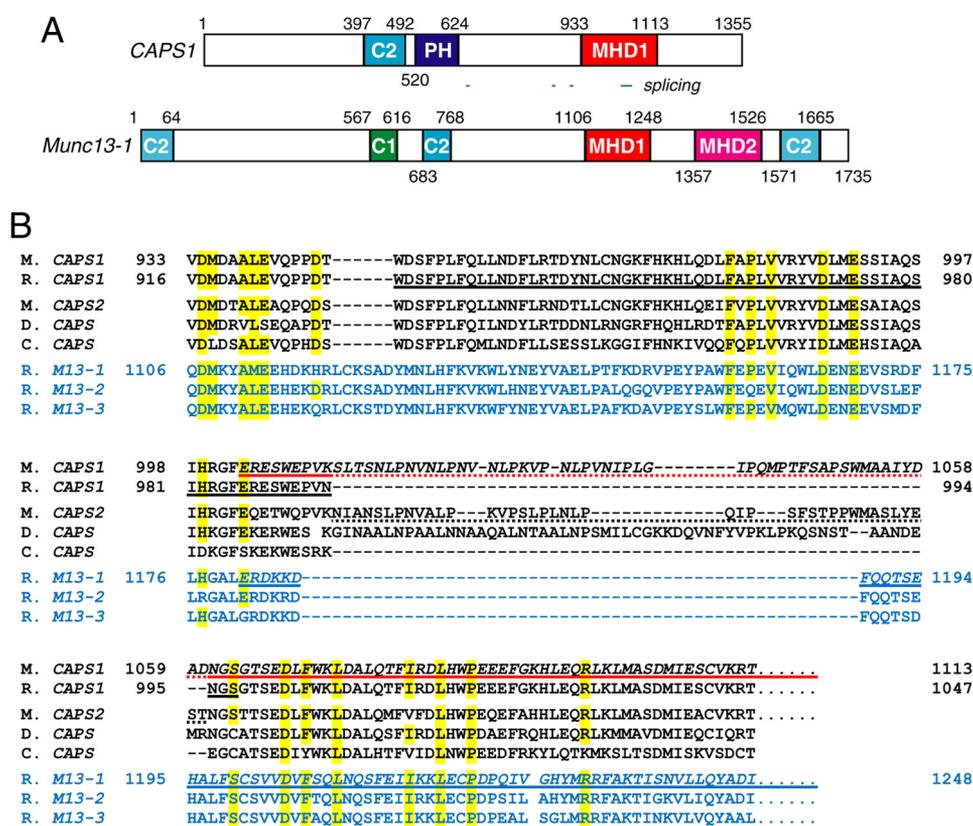
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<sup>3</sup> The abbreviations used are: CAPS1, calcium-dependent activator protein for secretion 1; PC12, pheochromocytoma 12; MHD1, Munc-13 homology domain 1; SNM, silent nucleotide mutation; NA, noradrenaline; TMR, transmembrane region; EmGFP, Emerald GFP.

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



**FIGURE 1. Domain structure and multiple sequence alignment of MHD1 of CAPS homologues and Munc13 isoforms.** A, shown are the domain structures of CAPS1 and Munc13-1. They share a MHD1. B, the underline in red indicates the residues of mouse CAPS1 that are used to examine the binding to syntaxin-1 in this study, whereas underline in blue indicates the residue of rat Munc13-1 that were found to bind to N-terminal syntaxin-1B (17). The underline in 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.

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 interact with the N-terminal region of syntaxin-1. Importantly, this binding mode is conserved in *Caenorhabditis elegans*, although in this case the exact binding site in the CAPS homologue Unc-31 was MHD2, not MHD1 (21). More recently, NMR and fluorescence experiments revealed another interaction mode; that is, weak ( $K_d \sim 20\text{--}50 \mu\text{M}$ ) interaction of the MUN domain containing both MHD1 and MHD2 of Munc13-1 with the syntaxin-1 SNARE motif (also called H3 domain) as well as the SNARE complex (22). Regarding CAPS1, using liposome flotation assays, the N-terminal half of the MHD1 of CAPS1 was found to bind to syntaxin-1 SNARE motif plus the linker region preceding the transmembrane region (TMR) as well as with the SNARE complex (18–20). These recent results of Munc13-1 and CAPS1 indicate that their binding modes to the syntaxin-1 SNARE motif and/or the SNARE complex are similar.

Although CAPS1 and Munc13-1 play critical roles in the priming stage of secretory vesicle exocytosis, their functions are non-redundant. This was demonstrated by the observation in which exocytosis deficits of CAPS1/CAPS2 double-knock-out neurons and adrenal chromaffin cells are not rescued by over-expression of Munc13-1 (13, 23). Therefore, we hypothesize that their binding mode is more distinct than currently recognized. In this study we directly compare their binding proper-

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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-SalI 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. 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.  $\beta$ -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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of breaking buffer was added, mixed well, and centrifuged for 1 min. The liquid extract was with-

drawn and transferred to new tubes. The extracted liquid was further clarified by centrifuging for 15 min in a microcentrifuge. To perform the assay, 80  $\mu$ l of the extract was added to 720  $\mu$ l of Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, and 2.7 ml/l  $\beta$ -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 *o*-nitrophenyl- $\beta$ -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 Na<sub>2</sub>CO<sub>3</sub> 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  $\mu$ l 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  $\beta$ -galactosidase in the extract was calculated according to the formula ( $A_{420} \times 1.36$ )/(0.0045  $\times$  protein concentration (mg/ml)  $\times$  extract volume (0.08 ml)  $\times$  7min), where  $A_{420}$  is the absorbance of the product *o*-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 *o*-nitrophenol. The unit of  $\beta$ -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  $\times$  g for 10 min. The supernatant was centrifuged at 12,000  $\times$  g for 20 min, and the pellet was resuspended with 5 ml of KGlu 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  $\times$  g for 30 min, and the supernatant (we call this total brain homogenate) was used for binding. GSH-agarose containing ~10–40  $\mu$ g of GST alone or GST-CAPS1 proteins was incubated with 1 ml of brain homogenate at 4 °C overnight, washed 5 times with KGlu buffer containing 0.3% Triton X-100, and resuspended in 100  $\mu$ l of SDS-PAGE sample buffer. 20- $\mu$ l 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 NdeI-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 MuiI-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) (Sigma), 250 ng/ml amphotericin B (Sigma), puromycin (2.5  $\mu$ g/ml), and G418 (700  $\mu$ g/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  $\mu$ g/ml).

**[<sup>3</sup>H]Noradrenaline Release Assays from Intact PC12 Cells**—PC12 cells were incubated with 0.5  $\mu$ Ci/ml [<sup>3</sup>H]noradrenaline ([<sup>3</sup>H]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 [<sup>3</sup>H]NA. The cells were washed once with physiological saline solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.6 mM glucose, and 15 mM HEPES, pH 7.4, and NA secretion was stimulated with 200  $\mu$ l of physiological saline solution and high K<sup>+</sup>-physiological saline solution (containing 81 mM NaCl and 70 mM KCl). Secretion was terminated after a 15-min (high K<sup>+</sup>) 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  $\mu$ l of KGlu buffer cells and homogenized by multiple passes through a 10- $\mu$ m clearance ball homogenizer. Cytosols were prepared by centrifugation at 21,910  $\times$  g. The relative content of full-length and truncated CAPS1 was estimated by immunoblot analysis and adjusted with cytosol from mock-transfected HEK-293FT

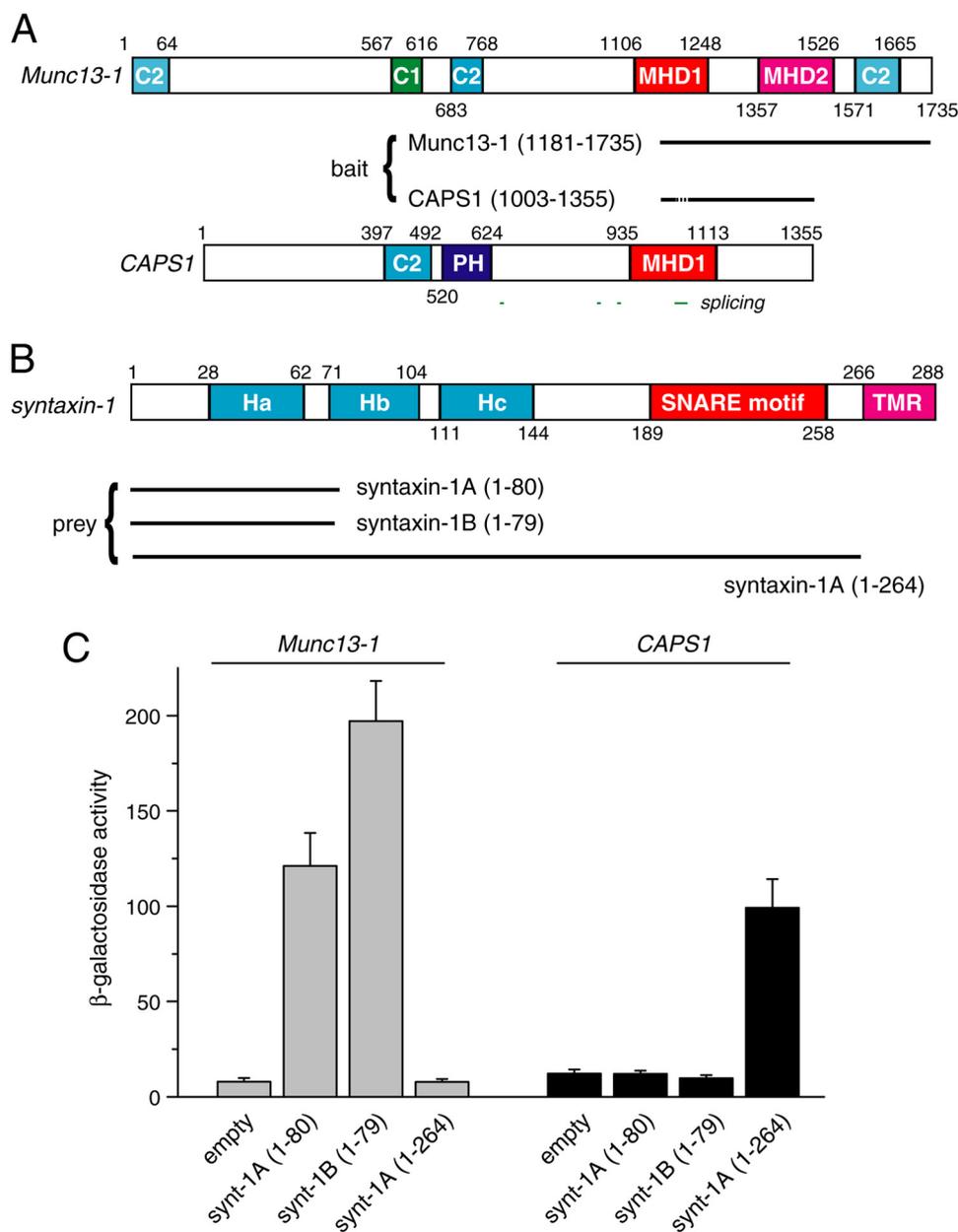
cells to equalize the amount of CAPS1/mg of cytosol protein. For reconstituted two-stage assays, PC12 cells labeled with 0.5  $\mu$ Ci/ml [<sup>3</sup>H]NA were washed, harvested in KGlu buffer with 0.1% bovine serum albumin, permeabilized with a 2.5- $\mu$ m clearance ball homogenizer, and incubated 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 Ca<sup>2+</sup> (1.72 mM; free Ca<sup>2+</sup> concentrations are estimated to be ~1–10  $\mu$ M) 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 *dot-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-1A in the prey vector.

We first confirmed that the C-terminal region containing MHD1 of Munc13-1 binds to the N-terminal domain of syntaxin-1B as reported before (17) and extended the finding such that this interaction is also conserved in syntaxin-1A (Fig. 2C). Unexpectedly, the Munc13-1 C-terminal region did not interact with the whole cytoplasmic domain (1–264) of syntaxin-1A in this assay, implying that the Munc13-1 interaction site in syntaxin-1A is hidden once syntaxin-1A Habc domain folds

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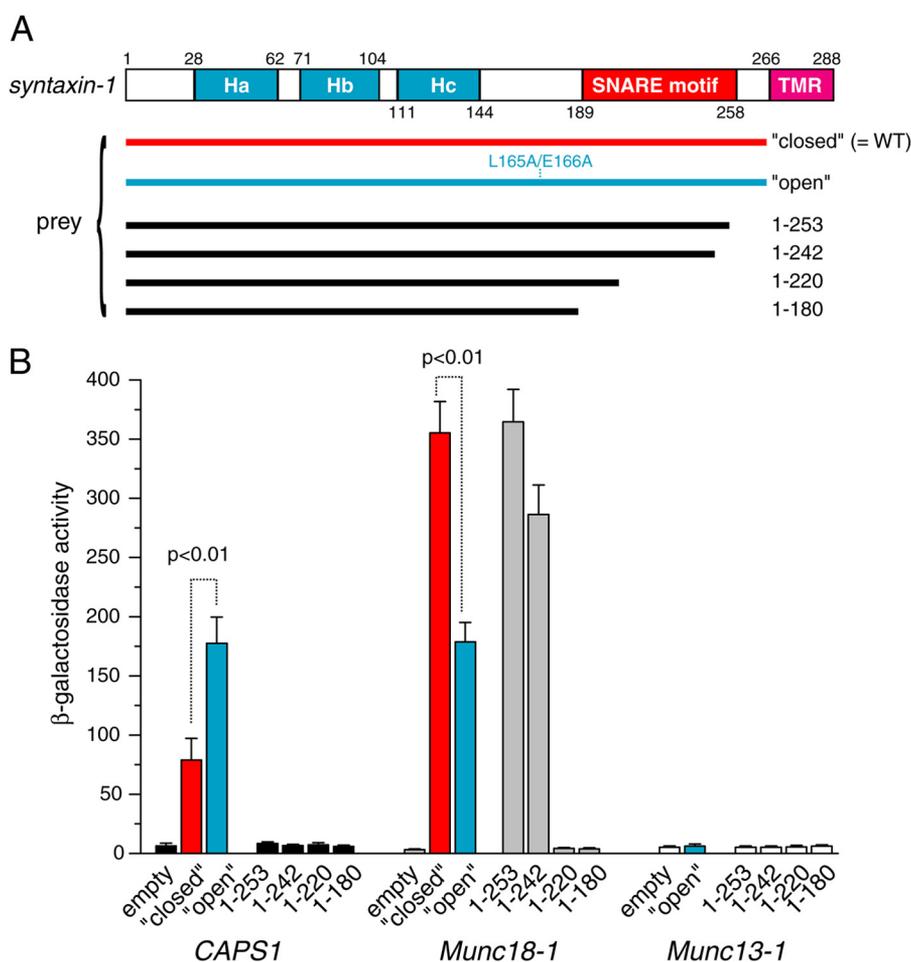


**FIGURE 2. Munc13-1 C-terminal region binds to the N-terminal region of syntaxin-1, whereas CAPS1 C-terminal region binds to the entire cytoplasmic domain of syntaxin-1.** *A* and *B*, the C-terminal region of Munc13-1 (residues 1181–1735) and CAPS1 (1003–1355) were tested on their abilities to bind 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  $\pm$ S.E. ( $n = 8$ ).

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 or 1B (Fig. 2C). On the other hand, CAPS1 bound to the entire cytoplasmic domain of syntaxin-1A. These contrasting results suggest that although the C-terminal region-containing C-terminal half of the conserved MHD1 of CAPS1 and Munc13-1 can bind to syntaxin-1, their binding modes are indeed distinct.

*CAPS1 Binds to the Cytoplasmic Domain of Syntaxin-1 in a Distinct Mode from Munc13-1 and Munc18-1; Preference to*

*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. 3*B*). The



**FIGURE 3. CAPS1 preferentially binds to the open conformation of syntaxin-1, whereas Munc18-1 preferentially binds to the closed syntaxin-1.** A, CAPS1 (residues 1003–1355), full-length Munc18-1, and Munc13-1 (residues 1181–1735) constructs were tested on their ability to interact with cytoplasmic syntaxin-1A (1–264) closed (=wild type) and open variant (L165A/E166A) as well as syntaxin 1A C-terminal truncations of residues 1–253, 1–242, 1–220, and 1–180. TMR, transmembrane region. B, CAPS1 required the entire cytoplasmic region of syntaxin-1 (1–264) to bind and preferentially bound to the open conformation of syntaxin-1. On the other hand, Munc18-1 preferentially bound to the closed syntaxin-1 even with the removal of membrane proximal region of 22 residues (1–242). Munc13-1 C-terminal region did not bind to open syntaxin-1A or the one of the entire Habc domain (1–180) without SNARE motif. Error bars indicate  $\pm$  S.E. ( $n = 8$ –12).

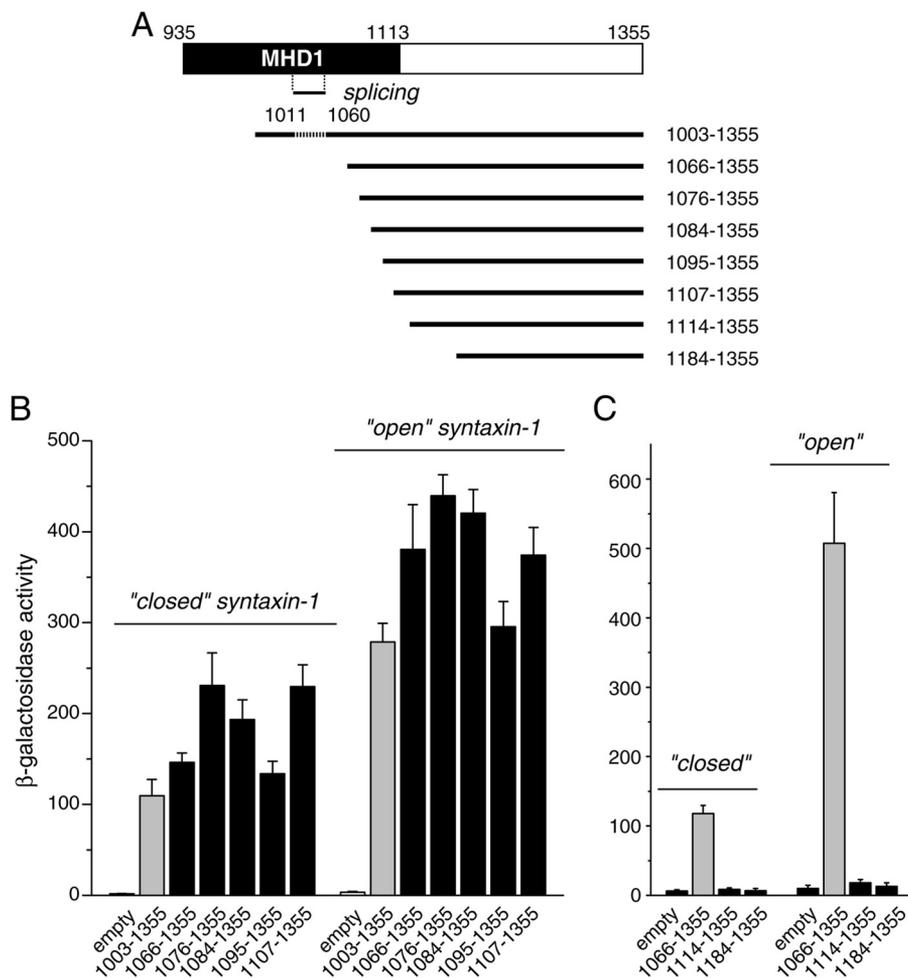
preferential binding of CAPS1 to open syntaxin may explain the recent results of functional studies of CAPS1 in which the open conformation mutant (L165A/E166A) of syntaxin-1 (27) can bypass the requirement of CAPS1 (23). We suggest that CAPS1 may contribute to stabilizing the open conformation of syntaxin-1 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

(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 support 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, which suggests that the Munc13-1 interaction epitope of syntaxin-1A is hidden once the syntaxin-1A Habc domain folds without the presence of 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

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**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. *C*, Additional truncations abolished the binding to syntaxin-1. Error bars indicate  $\pm$ S.E. ( $n = 8$ ).

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. 4*A*). To our surprise, a series of N-terminal deletions did not affect the binding, and the CAPS1 construct consisting of residues of 1107–1335 could still bind syntaxin-1A with preference to the open conformation (Fig. 4*B*). 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. 4*C*). 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 pull-down 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-

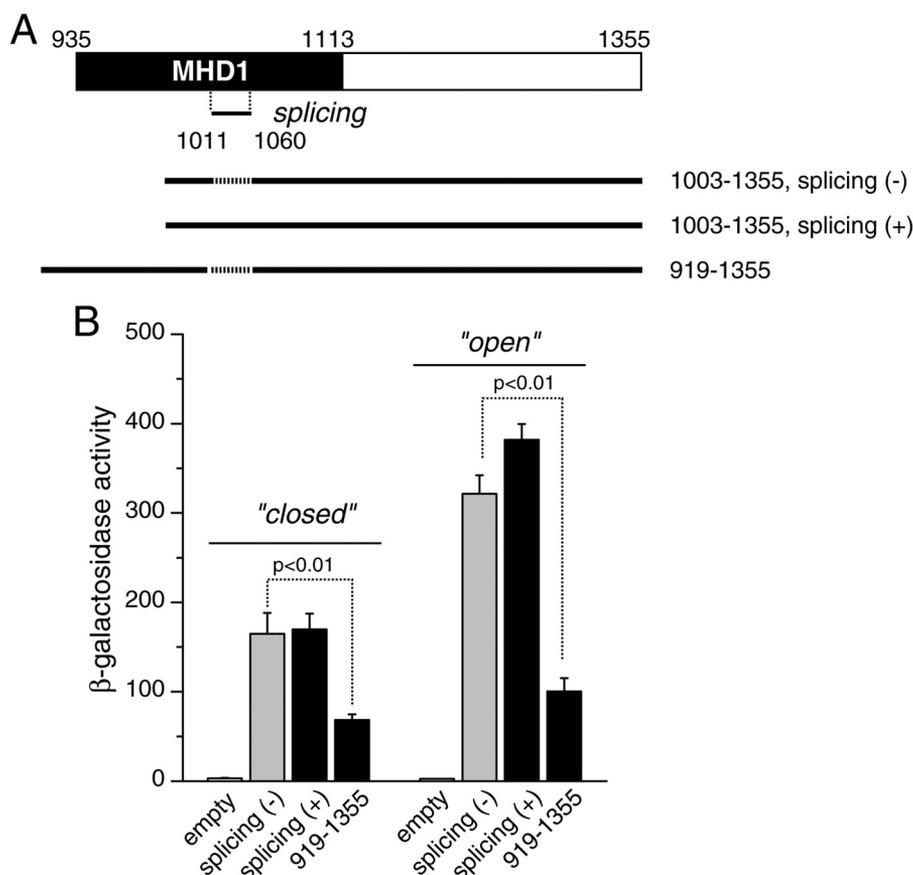


FIGURE 5. **The inclusion of the N-terminal MHD1 but not long alternative splicing site weakens the interaction of CAPS1 with syntaxin-1.** *A*, a long splicing site variant of CAPS1 (1003–1355) containing residues (1011–1060) as well as a CAPS construct containing N-terminal MHD1 (919–1355) were tested on their ability to interact with closed and open syntaxin-1. *B*, the inclusion of the N-terminal half of MHD1 significantly weakened the interaction of CAPS1 with syntaxin-1; however, the alternative splicing site did not have a significant impact on syntaxin-1 binding. Error bars indicate  $\pm$  S.E. ( $n = 6$ ).

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 pull-down 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).

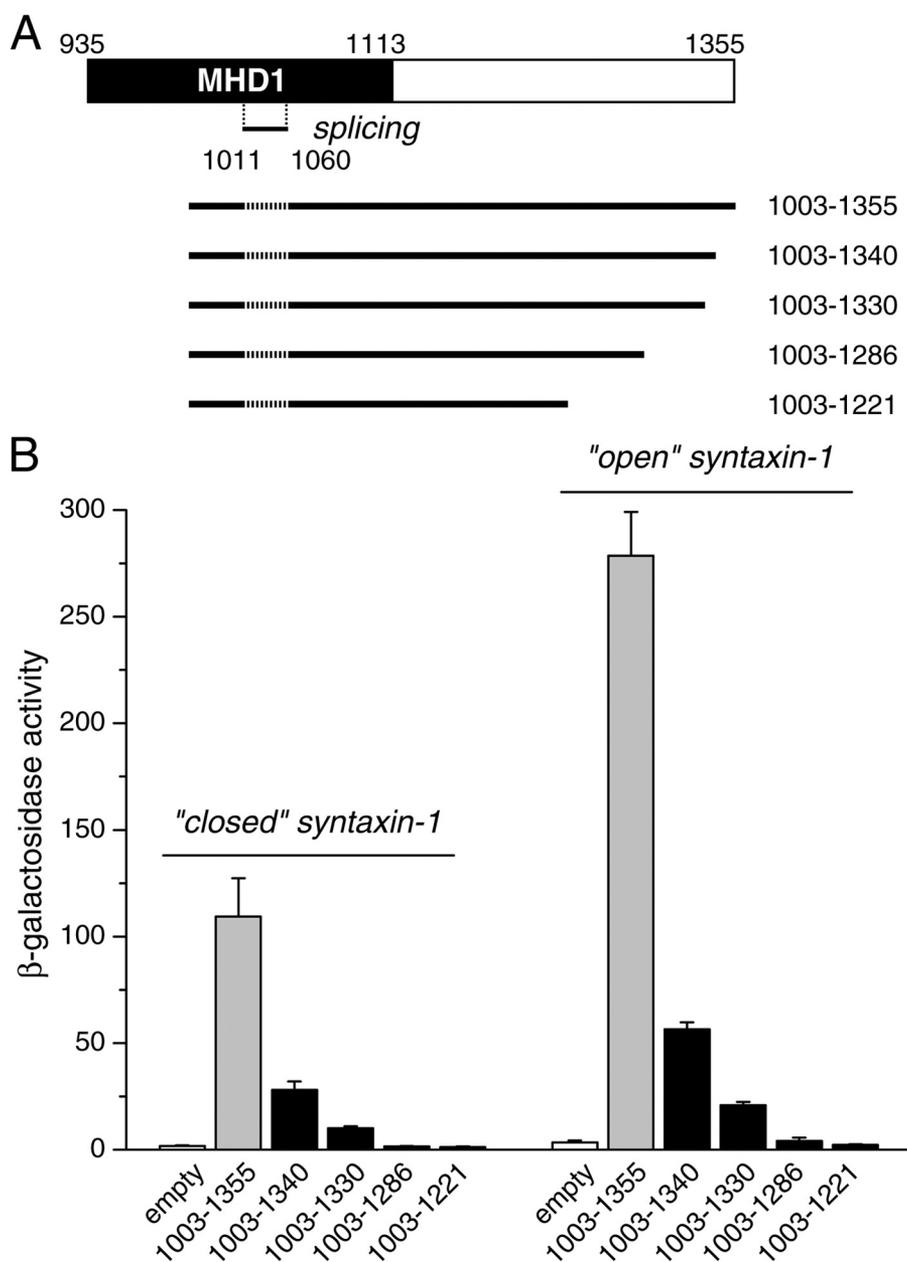
**The C-terminal 69 Residues of CAPS1 Are Crucial for Syntaxin-1 Binding**—To address whether the C-terminal region of CAPS1 is important for binding to syntaxin-1, we made a series of C-terminal-truncated constructs and tested their ability to bind syntaxin-1A using yeast two-hybrid assays (Fig. 7). 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 syntaxin-1 in both conformational states. When the last 69 or 134 residues were removed, the interaction was abol-

ished, 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 syntaxin-1 and other SNARE proteins in the brain homogenate using GST pull-down 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 syntaxin-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 syntaxin-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 syntaxin-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-





**FIGURE 7. C-terminal region of CAPS1 is important for syntaxin-1 binding in yeast two-hybrid assays.** *A*, shown is a list of C-terminal truncations from the original CAPS1 (1003–1355),  $\Delta 15$  (1003–1340),  $\Delta 25$  (1003–1330),  $\Delta 69$  (1003–1286), and  $\Delta 134$  (1003–1221), which were tested on their ability to interact with closed and open syntaxin-1 (1–264) in yeast two-hybrid assays. *B*, larger C-terminal truncations  $\Delta 69$  (1003–1286) and  $\Delta 134$  (1003–1221) abolished the syntaxin-1 interaction. The smaller C-terminal  $\Delta 15$  (1003–1340) and  $\Delta 25$  (1003–1330) severely but not completely reduced the interaction with syntaxin-1. Error bars indicate  $\pm$ S.E. ( $n = 8$ ).

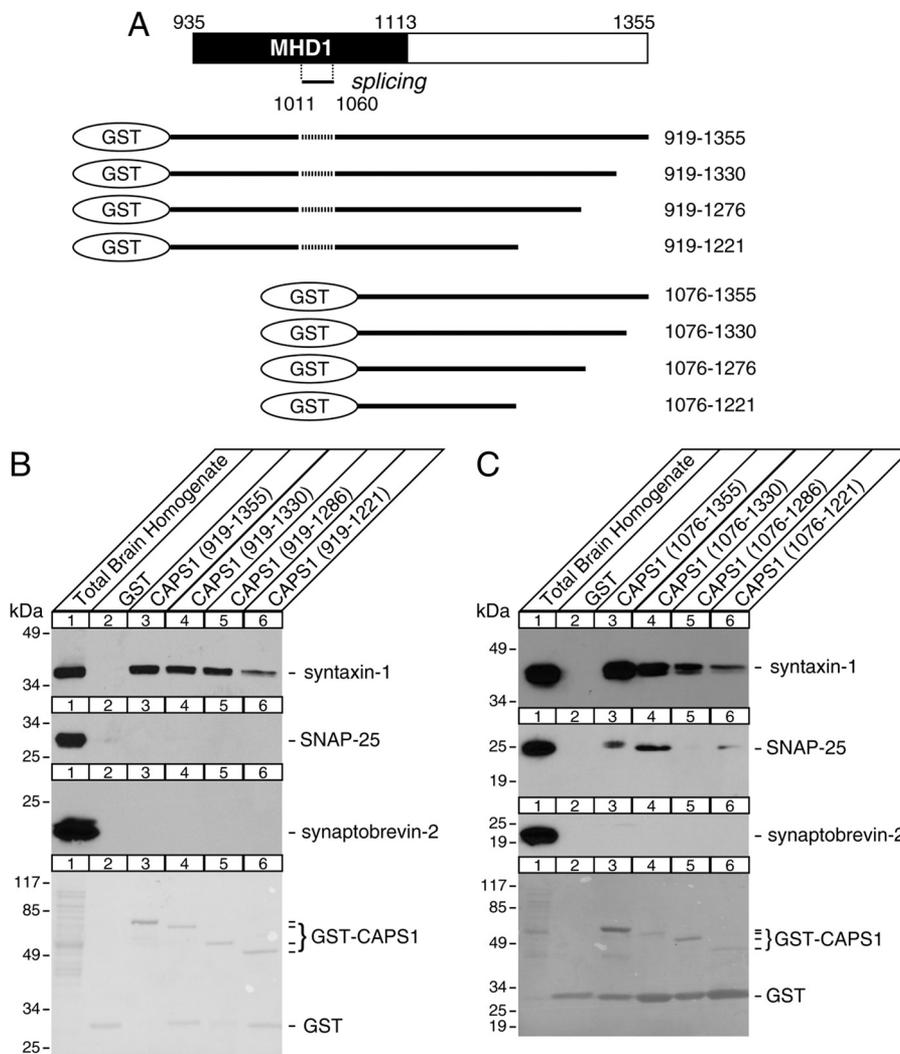
We finally addressed whether stable expression of the C-terminal syntaxin-1 binding region of CAPS1 (residues 1076–1355) alone can rescue secretion defects of KD5 cells (Fig. 11). Because the expression of a small portion of the protein in mammalian cells is likely to be unstable, we expressed the C-terminal 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.

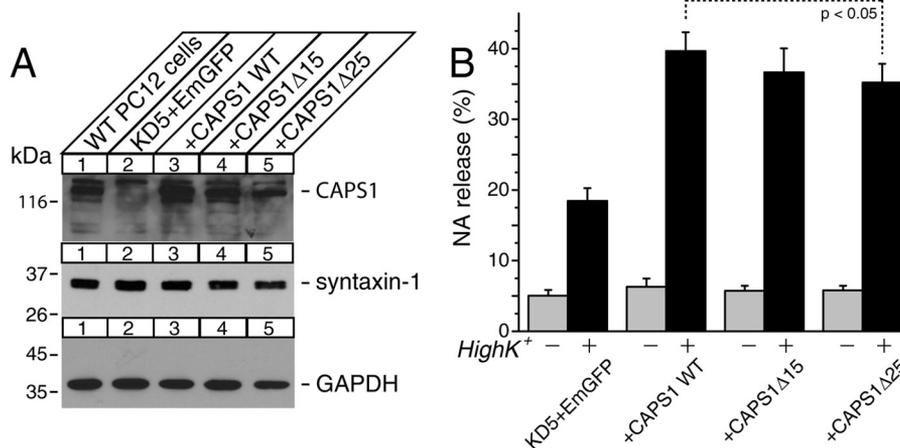
## DISCUSSION

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 pull-down 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

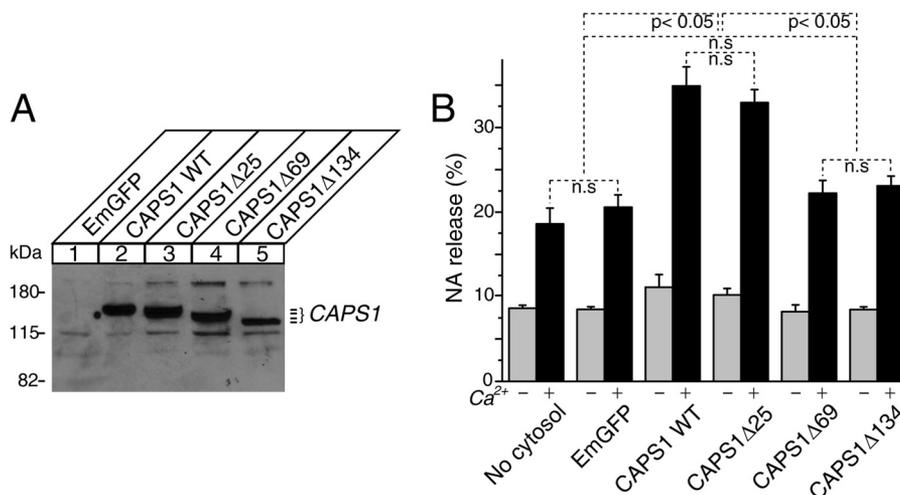
## Differential Syntaxin-1 Binding of CAPS1 and Munc13-1



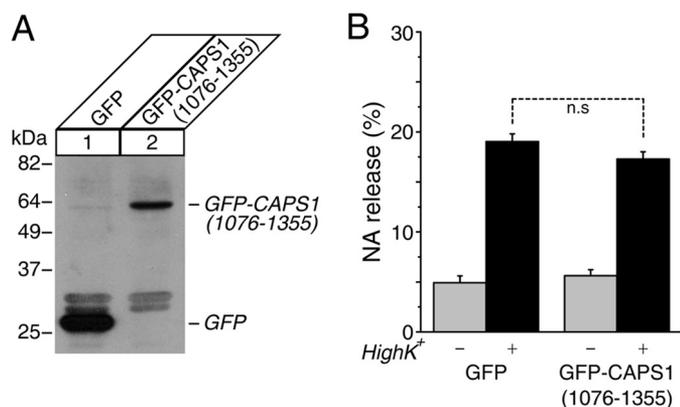
**FIGURE 8. Serial deletion of the C terminus of CAPS1 progressively loses its ability to bind to syntaxin-1 in GST pulldown experiments.** *A*, GST fusion CAPS1 constructs were tested on their ability to pull down syntaxin-1 and other SNARE proteins in the brain homogenate. *B* and *C*, in both GST-CAPS1 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 (KD5) were rescued with EmGFP (control), wild-type CAPS1, a 15-amino acid C-terminal truncation ( $\Delta 15$ ), and a 25-amino acid C-terminal truncation ( $\Delta 25$ ). 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 ( $\Delta 25$ ) of CAPS1 significantly reduced NA secretion in comparison with the wild type. *Error bars* indicate  $\pm$ S.E. ( $n = 8$ ).



**FIGURE 10. Truncation of the C-terminal 69 or 134 residues of CAPS1 abolishes the ability of CAPS1 to reconstitute secretion in permeabilized PC12 cells.** *A*, shown is immunoblot analysis of cytosols (10  $\mu$ g) of HEK-293FT cells that overexpress full-length CAPS1 or its C-terminal-truncated mutants. *B*, PC12 cells loaded with [<sup>3</sup>H]NA were permeabilized and incubated under MgATP-dependent priming conditions as described under "Experimental Procedures." 7-Min incubations for NA release were conducted with cytosols in the presence and absence of Ca<sup>2+</sup>. Error bars indicate  $\pm$ S.E. ( $n = 6$ ). *n.s.*, not significant.



**FIGURE 11. Stable expression of the C-terminal syntaxin-1 binding region of CAPS1 does not rescue secretion defects of KD5 cells.** *A*, shown is immunoblot analysis of KD5 cells (20  $\mu$ g) that were rescued with EmGFP (control) or EmGFP fused with C-terminal residues 1076–1355 of CAPS1. *B*, NA release was stimulated by 70 mM KCl for 15 min. No stimulation was observed by EmGFP-CAPS1 (1076–1355) in comparison with EmGFP alone. Error bars indicate  $\pm$ S.E. ( $n = 6$ ). *n.s.*, not significant.

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).

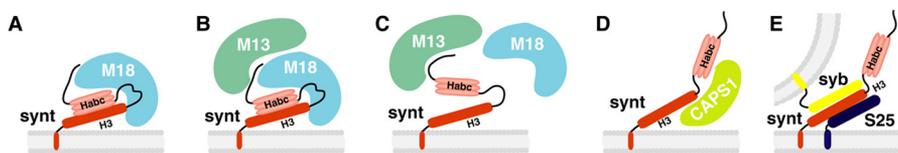
Both Munc13-1 and CAPS1 work at the priming stage of exocytosis. Two schools of thought have existed to explain the functional difference between Munc13-1 and CAPS1 in priming. In one school of thought it is hypothesized that Munc13-1 is primarily involved in priming of synaptic vesicles, whereas CAPS1 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 release. 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 of CAPS1 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 pull-down 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-

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**FIGURE 12. A proposed model for the role of CAPS1 as well as Munc18-1 and Munc13-1 during the priming stage of exocytosis.** The regulation of syntaxin-1, including the regulation of its conformational switch from closed to open leading to the SNARE formation, constitutes one of the key priming steps for exocytosis (A–E). *A*, Munc18-1 binding to closed syntaxin-1 is critical for maintaining syntaxin-1 level and trafficking to the plasma membrane. *B* and *C*, Munc13-1 displaces Munc18-1 from syntaxin-1 by transiently interacting with the N-terminal regions of syntaxin-1. *D*, CAPS1 preferentially binds open syntaxin-1 and stabilizes its open conformational state after the displacement of Munc18-1 by Munc13-1. *E*, the stabilization of the open state of syntaxin-1 by CAPS1 is likely to facilitate the formation of the SNARE complex. *M18*, for Munc18-1; *M13*, Munc13-1; *synt*, syntaxin-1; *S25*, SNAP-25; *syb*, synaptobrevin-2; *H3*, SNARE motif.

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 function downstream of Munc13-1 (23). The stabilization of the open state of syntaxin-1 by CAPS1 is likely to facilitate the formation of the SNARE complex.

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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