Ca^{2+}-Independent Syntaxin Binding to the C_2B Effector Region of Synaptotagmin

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Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, 25-kDa synaptosomal-associated protein; mAb, mouse monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium.

ABSTRACT

Although synaptotagmin I, which is a calcium (Ca^{2+})-binding synaptic vesicle protein, may trigger soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated synaptic vesicle exocytosis, the mechanisms underlying the interaction between these proteins remains controversial, especially with respect to the identity of the protein(s) in the SNARE complex that bind(s) to synaptotagmin and whether Ca^{2+} is required for their highly effective binding. To address these questions, native proteins were solubilized, immunoprecipitated from rat brain extracts, and analyzed by immunoblotting. SNARE complexes comprising syntaxin 1, 25-kDa synaptosomal-associated protein (SNAP-25), and synaptobrevin 2 were coprecipitated with synaptotagmin I in the presence of ethylene glycol tetraacetic acid. The amount of coprecipitated proteins was significantly unaltered by the addition of Ca^{2+} to the brain extract. To identify the component of the SNARE complex that bound to synaptotagmin, SNARE was coexpressed with synaptotagmin in HEK293 cells and immunoprecipitated. Syntaxin, but not SNAP-25 and synaptobrevin, bound to synaptotagmin in a Ca^{2+}-independent manner, and the binding was abolished in the presence of 1 M NaCl. Synaptotagmin contains 2 Ca^{2+}-binding domains (C_2A, C_2B). Mutating the positively charged lysine residues in the putative effector-binding region of the C_2B domain, which are critical for transmitter release, markedly inhibited synaptotagmin-syntaxin binding, while similar mutations in the C_2A domain had no effect on binding. Synaptotagmin-syntaxin binding was reduced by mutating multiple negatively charged glutamate residues in the amino-terminal half of the syntaxin SNARE motif. These results indicate that synaptotagmin I binds to syntaxin 1 electrostatically through its C_2B domain effector region in a Ca^{2+}-independent

fashion, providing biochemical evidence that synaptotagmin I binds SNARE complexes before Ca^{2+} influx into presynaptic nerve terminals.

Key words: neurotransmitter release, synaptic vesicle, exocytosis, SNAP-25, synaptobrevin

Introduction

Signal transmission between neurons is mediated by neurotransmitters stored in synaptic vesicles at presynaptic nerve terminals. When an action potential arrives at the nerve terminal, calcium ion (Ca^{2+}) influx through voltage-gated Ca^{2+} channels triggers synaptic vesicle exocytosis. Vesicle exocytosis is caused by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Jahn and Scheller, 2006; Rothman, 1994; Südhof, 2008). SNAREs are classified as follows: v-SNAREs (synaptobrevin 2), which are localized in the vesicular membrane, and t-SNAREs (syntaxin 1 and SNAP-25), which are localized on the target plasma membrane. These SNAREs possess a SNARE motif with a coiled-coil structure in the cytoplasmic regions. Each SNARE motif in synaptobrevin and syntaxin and the 2 motifs in SNAP-25 form a SNARE complex with 4 parallel helix bundles (Sutton et al., 1998), which may bring vesicular and target membranes into close proximity, leading to membrane fusion.

In *in vitro* experiments, v-SNARE- and t-SNARE-reconstituted liposomes fuse with each other (Weber et al., 1998). While SNAREs are constitutively active and show no sensitivities for Ca^{2+} in promoting liposome fusion, synaptic vesicle exocytosis in presynaptic nerve terminals is strictly regulated by Ca^{2+}. Thus, Ca^{2+} binds another protein that serves as a Ca^{2+} sensor, thereby promoting SNARE-mediated vesicle exocytosis. The best candidate for such a Ca^{2+} sensor for neurotransmitter release is the synaptic vesicle membrane protein synaptotagmin I (Augustine, 2001; Chapman, 2008; Südhof, 2008). Synaptotagmin has 2 homologous Ca^{2+}-binding domains (C_2A, C_2B) that are connected by a short linker in the cytoplasmic region. Genetic knockout studies have demonstrated that synaptotagmin I regulates Ca^{2+}-dependent neurotransmitter release in 2 different ways: by triggering synchronous release and by suppressing asynchronous release (Nishiki and Augustine, 2004b; Shin et al., 2003; Yoshihara and Littleton, 2002). Ca^{2+}

binding to the C_2B domain is more important than its binding to the C_2A domain for synaptotagmin to synchronize transmitter release in response to an action potential (Mackler et al., 2002; Nishiki and Augustine, 2004a; Robinson et al., 2002). Many studies have shown that Ca^{2+} promotes synaptotagmin binding to individual t-SNAREs as well as to t-SNARE heterodimers and trimeric SNARE complexes (Bai et al., 2004b; Dai et al., 2007; Fernández-Chacón et al., 2001; Zhang et al., 2002), and these results provide an attractive model in which Ca^{2+} influx can be directly coupled to membrane fusion.

In contrast to the triggering of synchronous release, synaptotagmin suppresses asynchronous release in a Ca^{2+}-independent manner (Kochubey and Schneggenburger, 2011; Nishiki and Augustine, 2004a). Synaptotagmin inhibits SNARE-mediated liposome fusion in the absence of Ca^{2+} *in vitro* (Chicka et al., 2008). These findings raise the possibility that synaptotagmin regulates SNARE function at resting Ca^{2+} levels in nerve terminals. Although some studies have demonstrated significant synaptotagmin-SNARE binding in the absence of Ca^{2+} (Rickman and Davletov, 2003; Weninger et al., 2008), many biochemical studies have shown little binding without Ca^{2+} (Bai et al., 2004b; Dai et al., 2007; Fernández-Chacón et al., 2001; Wang et al., 2003). Moreover, a study reported that neither Ca^{2+}-dependent nor -independent interactions occur at physiological ionic strengths (Tang et al., 2006). While many researchers have used bacterial-expressed and truncated recombinant fusion proteins with various affinity tags in binding studies, the results obtained with such recombinants are inconsistent and contradictory. Thus, despite the substantial research that has been conducted for the past 20 years, there is no general agreement of the exact properties of synaptotagmin-SNARE binding.

To better understand the synaptotagmin I-SNARE interaction, we herein examined their binding using solubilized natural proteins from the brain and full-length recombinant proteins

without any affinity tags that were expressed in mammalian cells. We found that synaptotagmin I and SNAREs bound each other in the absence of Ca^{2+} at physiological ionic strengths and that the Ca^{2+}-independent synaptotagmin I-SNARE complex binding was possibly mediated through a synaptotagmin I-syntaxin 1 interaction. Moreover, we demonstrated that synaptotagmin bound to syntaxin through electrostatic interactions between positive charges in the synaptotagmin C_2B domain and the partly negative charges in the N-terminal half of the syntaxin SNARE motif. These results provide biochemical and experimental evidence for synaptotagmin I-SNARE complex binding before Ca^{2+} influx into presynaptic nerve terminals.

Results

Ca^{2+}-independent synaptotagmin-SNARE complex binding in brain extracts

In order to examine the properties of synaptotagmin-SNARE complex binding, natural proteins from rat brain synaptosomes were solubilized with detergent, immunoprecipitated, and then analyzed by immunoblotting. When synaptotagmin was immunoprecipitated with an anti-synaptotagmin I antibody, all SNAREs were found to specifically coprecipitate in the presence of 0.15 M NaCl and 2 mM ethylene glycol tetraacetic acid (EGTA; Fig. 1A, 1st versus 3rd lanes). Under these conditions, 4.40 ± 0.23% of synaptotagmin was precipitated from the total brain extract by the antibody, while 0.68 ± 0.08% of syntaxin, 2.00 ± 0.14% of SNAP-25, and 0.75 ± 0.08% of synaptobrevin 2 were coprecipitated (mean ± SEM, n = 3). The specific coprecipitation of synaptotagmin and SNARE complexes was completely disrupted by adding NaCl to the brain extract at a final concentration of 1 M (Fig. 1A, 1st versus 4th lanes), which is consistent with the results reported by Rickman and Davletov (2003). When an anti-synaptobrevin 2 antibody was used for immunoprecipitation, a similar result was obtained with the efficiencies of precipitation as follows: 0.89 ± 0.16% for synaptotagmin, 2.70 ± 0.25% for syntaxin, 1.80 ± 0.15% for SNAP-25, and 9.01 ± 0.33% of synaptobrevin (Fig. 1A, 2nd and 5th lanes).

In order to test whether Ca^{2+} enhances synaptotagmin-SNARE binding, 1 mM $CaCl_2$ was added to the brain extracts, and immunoprecipitation was performed. Surprisingly, the addition of Ca^{2+} had no significant effects on the amount of SNAREs that coprecipitated with synaptotagmin: syntaxin was decreased by 11.7 ± 1.0%; SNAP-25 was decreased by 9.4 ± 2.9%;

and synaptobrevin 2 was increased by 6.4 ± 3.5% (mean ± SEM, n = 3; Fig. 1B, 1st versus 2nd lanes). Similar results were found for the amount of synaptotagmin that coprecipitated with the anti-synaptobrevin antibody (26.8 ± 8.0% increase; Fig. 1B, 3rd versus 4th lanes). Based on these results, we concluded that natural synaptotagmin and SNARE proteins from the brain that were solubilized bound to each other through electrostatic interactions in a Ca^{2+}-independent manner at physiological ionic strengths.

Ca^{2+}-independent recombinant synaptotagmin-SNARE binding

In order to determine which protein in the SNARE complex binds to synaptotagmin in the absence of Ca^{2+}, syntaxin 1, SNAP-25, or synaptobrevin 2 was coexpressed with synaptotagmin I in HEK293 cells by the transfection of plasmids that encoded full-length proteins without any tags. The expression levels of the recombinant proteins in the cotransfected cells were examined by immunoblotting. All 4 proteins were expressed at the expected molecular weights, and cotransfection of each SNARE had no effect on synaptotagmin expression levels (Fig. 2A). After solubilizing the transfected cells, synaptotagmin was immunoprecipitated from the cell lysate. Immunoblotting analysis of the precipitates revealed that syntaxin, and not SNAP-25 and synaptobrevin, specifically coprecipitated with synaptotagmin (Fig. 2B). When SNAP-25 was coexpressed with synaptotagmin, a faint band that migrated to a position similar to that of SNAP-25 was detected. This band was believed to be nonspecific because it was also found in immunoprecipitates from the cell lysates that did not coexpress SNAP-25 with a control antibody. Ca^{2+}-independent synaptotagmin-syntaxin binding was disturbed almost completely (97%) in the presence of 1 M NaCl in the cell lysate (Fig. 2C), indicating that the binding was an electrostatic

interaction that was mediated by the cytoplasmic regions and not by nonspecific hydrophobic interactions between the transmembrane regions in detergent micelles. Thus, these results suggest that synaptotagmin I binds electrostatically to SNARE complexes through an interaction with syntaxin 1, and not with SNAP-25 or synaptobrevin 2, in the absence of Ca^{2+}.

Syntaxin-binding sites on synaptotagmin

In order to determine the region of synaptotagmin that is involved in Ca^{2+}-independent binding to syntaxin, we performed point mutagenesis. Each synaptotagmin C_2 domain has a curved β-sandwich structure that is composed of 2 4-stranded β sheets with loops, and the surface of the concave β sheet was assumed to be an effector-binding region of this protein (Fig. 3A) (Fernandez et al., 2001; Sutton et al., 1995). At the edge of the concave sheet, multiple highly conserved basic amino acid residues are adjacently located in the fourth β (β4) strand (C_2A, 189–200; C_2B, 321–332). We then examined the involvement of these basic residues of synaptotagmin C_2 domains in the binding to syntaxin.

All 3 lysine (Lys, K) residues in the C_2A (Lys^{191}, Lys^{192}, Lys^{196}) and C_2B (Lys^{326}, Lys^{327}, Lys^{331}) domains were replaced with glutamine (Gln, Q), and these mutants were designated C_2A 3KQ and C_2B 3KQ, respectively (Fig. 3A). Such mutations neutralize the positive charge of each Lys residue without greatly affecting the overall size of the side chain. Cells with wild-type (WT) and mutant synaptotagmin I were cotransfected with syntaxin 1 into HEK293 cells, and the effects of the charge-neutralizing mutations on their binding were examined. Immunoblotting analysis showed no significant differences in the expression levels of the proteins in the 3 types of transfected cells, indicating that the WT and mutant synaptotagmins were expressed and stably

existed in the transfected cells and that they had no significant effects on syntaxin expression levels (Fig. 3B).

When synaptotagmin was immunoprecipitated from the cell lysate, the C_2A 3KQ mutant coprecipitated syntaxin at levels similar to those of the WT synaptotagmin, indicating that mutation of the Lys residues in the C_2A domain did not impair the ability of synaptotagmin to bind syntaxin in the absence of Ca^{2+} (Fig. 3C and D). In contrast, mutation of the Lys residues in the C_2B domain inhibited coprecipitation with syntaxin by 80% (Fig. 3C and D). Although the Ca^{2+}-binding sites of synaptotagmin were intact, Ca^{2+} addition to the cell lysates did not alter the syntaxin binding in all 3 types of synaptotagmin (Fig. 3C and D), which was similar to the results with the brain extract demonstrated in Fig. 1B. These results indicated that the Lys residues in the β4 strand of the synaptotagmin I C_2B domain were critical for its Ca^{2+}-independent binding to syntaxin 1.

Synaptotagmin-binding sites on syntaxin

As described above, the positively charged Lys residues in the C_2B domain constitute the syntaxin 1-binding sites on synaptotagmin I, suggesting that the synaptotagmin I-binding sites on syntaxin 1 could be formed by negatively charged acidic residues. Previous studies have shown that many syntaxin-binding proteins interact with the third C-terminal of the syntaxin H3 domain that is absolutely required for neurotransmitter release (Wu et al., 1999). The H3 domain contains 14 negatively charged amino acid residues [glutamate (Glu, E) and aspartate (Asp, D)] that are highly conserved across species (Fig. 4A). We thus attempted to determine the synaptotagmin-binding sites by focusing on these residues.

In order to neutralize the negative charges of these amino acid residues, Glu and Asp were replaced with Gln and asparagine (Asn, N), respectively, in combination with an adjacent residue, and a series of mutations was generated (designated as E194/196Q, E201/206Q, E211Q/D214N, D218N, E224/228Q, D231N/E234Q, E238Q/D242N, and D250N). All mutant syntaxins were expressed at levels similar to those of WT syntaxin in HEK293 cells cotransfected with synaptotagmin (Fig. 4B). The cell lysate containing synaptotagmin and the WT or mutant syntaxin was immunoprecipitated with an anti-synaptotagmin antibody and subjected to immunoblotting. Three double-mutant syntaxins with mutations in the N-terminal half of the H3 domain (E194/196Q, E201/206Q, and E211Q/D214N) decreased the Ca^{2+}-independent syntaxin-synaptotagmin binding by ~50% compared to WT syntaxin (Figs. 4C and D). In contrast, syntaxins with mutations in the C-terminal half of the H3 domain (D218N, E224/228Q, D231N/E234Q, E238Q/D242N, and D250N) were coprecipitated with synaptotagmin at similar or slightly higher levels than WT syntaxin, but these differences were not statistically significant. In order to further probe the role of the acidic residues in the N-terminal half of the syntaxin H3 domain, mutations were simultaneously introduced into 5 Glu residues (E194/196/201/206/211Q). The quintuple-mutant syntaxin (5EQ) was expressed in transfected cells at levels similar to those of WT syntaxin (Fig. 4E), and synaptotagmin binding was inhibited to 35% compared to the WT (Figs. 4D and E). These results indicate that the N-terminal half of the syntaxin H3 domain contains redundant synaptotagmin-binding sites that are formed by multiple negatively charged amino acid residues.

Discussion

In Ca^{2+}-dependent neurotransmitter release, synaptotagmin I may play a role as a Ca^{2+} sensor in order to synchronize SNARE-mediated synaptic vesicle exocytosis. Therefore, elucidating the exact properties of the synaptotagmin-SNARE complex binding is a critical problem. Here, we examined the interaction between solubilized native synaptotagmin and SNAREs from the rat brain. We found that synaptotagmin bound to SNARE complexes at physiological ionic strengths in the absence of Ca^{2+}. Moreover, using recombinant proteins that were overexpressed in cultured mammalian cells, we demonstrated that synaptotagmin directly bound to syntaxin, but not to SNAP-25 or synaptobrevin 2, in a Ca^{2+}-independent manner. Multiple negatively charged amino acid residues in the syntaxin H3 domain were at least partially involved in the binding to the synaptotagmin C_2B domain. These results indicate the possibility that synaptotagmin I may act on the SNARE complex through the binding to syntaxin 1 in presynaptic nerve terminals at resting Ca^{2+} levels.

Although many studies have shown an interaction between synaptotagmin and syntaxin, its physiological significance has been uncertain. Our results reveal for the first time that the Ca^{2+}-independent binding of synaptotagmin to syntaxin 1 required the adjacent Lys residues localized on the β4 strand of the synaptotagmin I C_2B domain, and these residues are important for the actions of synaptotagmin during neurotransmitter release. Our findings are consistent with the previous findings of a NMR analysis that demonstrated that the C_2B β4 strand participates in Ca^{2+}-independent synaptotagmin-SNARE complex binding (Dai et al., 2007). Moreover, mutations in those Lys residues abolish synaptotagmin-t-SNARE heterodimer binding (Rickman et al., 2004). Thus, these findings suggest that synaptotagmin I apparently binds to SNARE

complexes through syntaxin 1. Microinjection of a synthetic peptide that is homologous to the β4 strand completely blocks neurotransmitter release (Bommert et al., 1993). In rescue experiments, synaptotagmin I with mutations in its Lys residues in the C_2B β4 strand lost the ability to rescue transmitter release from synaptotagmin-deficient neurons (Borden et al., 2005; Li et al., 2006; Mackler and Reist, 2001). In *Drosophila*, these residues were required for Ca^{2+}-independent docking/priming of synaptic vesicles at the neuromuscular junction (Loewen et al., 2006). Given these findings, we conclude that synaptotagmin I-syntaxin 1 binding at resting Ca^{2+} levels is essential for synaptotagmin I's function in neurotransmitter release.

Syntaxin 1 was originally identified as a synaptotagmin I-binding protein from rat brain extracts in the absence of Ca^{2+} (Bennett et al., 1992; Yoshida et al., 1992). A subsequent study showed that synaptotagmin can be coimmunoprecipitated with syntaxin and the other components of SNARE complexes from bovine brain extracts in the presence of a chelating agent (Söllner et al., 1993). Thus, our results confirmed these previous findings and showed that tagless full-length recombinant synaptotagmin and syntaxin expressed in mammalian cells possessed their intrinsic binding properties. Many studies have examined the synaptotagmin-SNARE interaction, and most of them have demonstrated that the binding was highly Ca^{2+} dependent (Bai et al., 2004b; Dai et al., 2007; Fernández-Chacón et al., 2001; Hui et al., 2009; Wang et al., 2003; Zhang et al., 2002). One of the reasons for the Ca^{2+}-dependent binding observed in previous studies could be the use of soluble cytoplasmic fragments that were fused with various tags that were designed so that the protein could be rapidly and simply purified by affinity chromatography. Further studies will be needed in order to clarify these discrepancies.

A previous study reported that synaptotagmin I did not bind to syntaxin 1 alone, even when binding experiments were performed using proteins purified from brain (Rickman et al., 2004).

Whereas the synaptotagmin-syntaxin complex was observed in the cotransfected cell lysates (Fig. 2), we did not observe their binding when the proteins were expressed individually in HEK293 cells and then mixed together after solubilizing (data not shown). These findings suggest that at least 1 of either of the proteins might need to be integrated into the cell membrane in order to bind Ca^{2+} independently. The conformation of syntaxin in solution can be different from that in the plasma membrane because botulinum type C neurotoxin can cleave syntaxin 1 only if the protein is incorporated into a lipid bilayer (Schiavo et al., 1995).

Although synaptotagmin-syntaxin binding was not increased by Ca^{2+} under our experimental conditions, many studies have reported Ca^{2+}-dependent enhancement of the binding. The Ca^{2+}-dependent binding to syntaxin requires the synaptotagmin C_2A domain (Kee and Scheller, 1996; Li et al., 1995), and mutating the Asp residues in the C_2A Ca^{2+}-binding sites completely abolishes the binding (Davis et al., 1999; Shao et al., 1997). However, synaptotagmin with the same mutation still possesses the ability to trigger neurotransmitter release (Robinson et al., 2002). Therefore, we suggest that the physiological significance of the Ca^{2+}-enhanced synaptotagmin I-syntaxin 1 interaction might be for reasons other than Ca^{2+}-dependent synaptic vesicle exocytosis.

We did not detect direct synaptotagmin I-SNAP-25 binding in the absence of Ca^{2+}, which is in agreement with a previous study (Rickman and Davletov, 2003). However, some studies have reported a Ca^{2+}-independent interaction between them (Gerona et al., 2000; Schiavo et al., 1997). A possible interpretation for this discrepancy is that their lower binding affinity is due to the fact that chemical cross-linking is needed in order to efficiently detect the synaptotagmin-SNAP-25 complex (Zhang et al., 2002). Similarly, NMR analysis indicated the possibility of their lower

binding specificity (Dai et al., 2007). Further studies are required to clarify these differences in synaptotagmin-SNAP-25 binding.

Figure 5 illustrates our model of the role of Ca^{2+}-independent synaptotagmin-SNARE complex binding in neurotransmitter release. After vesicle docking to the presynaptic membrane, SNARE assembly may be initiated at the N-terminal end and then proceed toward the C-terminal transmembrane regions in a process called zippering (Melia et al., 2002; Sørensen et al., 2006). In this model, we propose that synaptotagmin I binds the partially zipped SNARE complexes through syntaxin. Synaptotagmin I has 2 roles in neurotransmitter release: triggering synchronous release and suppressing asynchronous release (Nishiki and Augustine, 2004b; Shin et al., 2003; Yoshihara and Littleton, 2002). We previously showed that Ca^{2+} binding to the C_2B domain is essential for synchronizing transmitter release but not for suppressing asynchronous release (Nishiki and Augustine, 2004a). Synaptotagmin I inhibits SNARE-mediated liposome fusion in the absence of Ca^{2+} (Chicka et al., 2008). More recently, the poly-Lys motif has also been shown to be critical for synaptotagmin II, which is closely related to synaptotagmin I, in suppressing asynchronous release (Kochubey and Schneggenburger, 2011). Given these findings, we postulate that synaptotagmin I suppresses SNARE-mediated membrane fusion by binding to syntaxin 1 before Ca^{2+} influx.

When an action potential arrives, Ca^{2+} influxes into the presynaptic nerve terminals, binds to synaptotagmin, and triggers neurotransmitter release. Although we observed no effects of Ca^{2+} on synaptotagmin-SNARE binding under our experimental conditions, interestingly, Ca^{2+} has been shown to elicit the dissociation of synaptotagmin from the SNARE complexes in intact plasma membranes (Leveque et al., 2000). If this is the case, Ca^{2+} may release SNARE complexes from clamping by synaptotagmin in the presence of phospholipids and allow the complex to

completely assemble. Moreover, the Ca^{2+}-dependent penetration of synaptotagmin into lipid bilayers may trigger SNARE-mediated membrane fusion (Bai et al., 2004a; Herrick et al., 2006; Hui et al., 2009; Martens et al., 2007). A key question, which remains to be addressed, is how Ca^{2+}-bound synaptotagmin triggers membrane fusion.

Experimental methods

Antibodies

Monoclonal antibodies against synaptotagmin I (1D12), syntaxin 1 (9A7), and SNAP-25 (BR05) and polyclonal antibodies against synaptobrevin 2 were prepared as described previously (Leveque et al., 1992; Nishiki et al., 1994; Oho et al., 1995; Shimazaki et al., 1996; Takahashi et al., 1991; Yoshida et al., 1992). A monoclonal antibody against botulinum type B neurotoxin (B-102; Kozaki et al., 1998) was used as a control IgG.

Solubilization of rat brain synaptosomes

Synaptosomes were prepared from rat brains by a modification of the procedures of Whittaker (Nishiki et al., 1994; Whittaker, 1959). The animal maintenance and experimental procedures were approved by the Okayama University Animal Experimentation Committee. Crude synaptosomal fractions were suspended in 20 mM Hepes-NaOH buffer (pH 7.4) containing 0.15 M NaCl (HBS) and then mixed with an equal volume of the same buffer containing 1% (w/v) Triton X-100 and Complete protease inhibitor cocktail (EDTA-free, Roche Diagnostics Corporation, Indianapolis, IN, USA). After 30 min on ice, the insoluble materials were removed by centrifugation at 100,000 × *g* for 1 h. The supernatant is hereafter referred to as the brain extract. Protein concentrations were determined to be 2 mg/mL by the bicinchoninic acid method using bovine serum albumin as a reference protein (Smith et al., 1985).

Expression constructs and site-directed mutagenesis

A plasmid encoding full-length synaptotagmin I was described previously (Nishiki and Augustine, 2004a). Plasmids encoding syntaxin 1A, SNAP-25B, and synaptobrevin 2 were kind gifts from Dr. M. Takahashi (Kitasato University School of Medicine, Sagamihara, Japan). cDNAs were amplified by PCR using the following primers: syntaxin 1, 5′-ATGAAGGACCGAACCCAGGA-3′ (sense) and 5′-CTATCCAAAGATGCCCCCGAT-3′ (antisense); SNAP-25, 5′-ATGGCCGAGGACGCAGACAT-3′ (sense) and 5′-TTAACCACTTCCCAGCATCTTTGTT-3′ (antisense); and synaptobrevin 2, 5′-ATGTCGGCTACCGCTGCCA-3′ (sense) and 5′-TTAAGTGCTGAAGTAAACGATGATGATG-3′ (antisense). The amplified products were ligated into a pCR-Script vector (Agilent Technologies, Inc., Santa Clara, CA, USA) and then subcloned into pIRES2-DsRed2 (Clonthech Laboratories, Inc., Mountain View, CA, USA) with *Eco*R I and *Sac* II restriction enzyme sites. Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) and the following mutagenic primers: C_2A 3KQ, 5′-CAGCAATTTGAGACTCAAGTCCACCGGAAAACC-3′ (sense); C_2B 3KQ, 5′-GCAACAGAGGACGATTCAGAAGAACACACTCAA-3′ (sense); E194/196Q, 5′-GCCCTCAGTCAGATCCAGACCAGGCACAG-3′ (sense); E201/206Q, 5′-GTCAGATCATCAAGTTGCAGAACAGCATCCGGG-3′ (sense); E211Q/D214N, 5′-CACGATATGTTCATGAACATGGCCATGCTGGTGG-3′ (sense); D218N, 5′-CACGATATGTTCATGAACATGGCCATGCTGGTGG-3′ (sense); E224/228Q; 5′-GCAGAGCCAGGGGCAGATGATTGACAG-3′ (sense); D231N/E234Q, 5′-GGAGATGATTAACAGGATCCAGTACAATGTGGAACACG-3′ (sense); E238Q/D242N,

5′-GTGCAACACGCTGTGAACTACGTGGAGAGGG-3′ (sense); and D250N, 5′-GAGGGCCGTGTCTAACACCAAGAAGGCC-3′ (sense). All antisense primers were designed to be complementary to corresponding sense primers. The nucleotide sequences of all constructs were verified by DNA sequencing.

Expression of recombinant proteins in HEK293 cells

HEK293FT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), penicillin-streptomycin (100 units/mL and 100 μg/mL, respectively), and 0.5 μg/mL geneticin in a humidified 5% CO_2 atmosphere at 37°C. Only cells from passages ≤25 were used.

HEK293 cells were plated on a 10-cm plastic culture dish and transfected the next day using Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's instructions. In brief, DNA plasmids (4 μg) and Lipofectamine 2000 reagents (7.2 μL) were diluted separately with 400 μL of DMEM, mixed, and then added to each dish containing the cells. After incubation at 37°C for 4–5 h in a CO_2 incubator, the medium was replaced with a fresh growth medium. Three days after transfection, cells were rinsed twice with 4 mL of HBS and solubilized with 1 mL of HBS containing 0.5% (w/v) Triton X-100 and protease inhibitors (lysis buffer) at 4°C for 20 min with gentle shaking. The insoluble materials were removed by centrifugation at 104,000 × *g* for 1 h, and the supernatant was referred to as the cell lysate.

Immunoprecipitation

All procedures were performed at 4°C. The brain extracts (1 mg in 500 μL) or cell lysates (400 μL) were incubated with an antibody (50 μL) for 1 h in the presence of 1 mM $CaCl_2$ or 2 mM EGTA, which was followed by a further 1-h incubation with Protein G Sepharose beads (10 μL, GE Healthcare, Piscataway, NJ, USA). The beads were washed twice by low speed centrifugation with 750 μL of lysis buffer, and precipitated proteins were analyzed by SDS-PAGE and immunoblotting (Nishiki et al., 1994). Immunoreactive bands were visualized by enhanced chemiluminescence using ECL plus (GE Healthcare) and Bio-Rad Versadoc (Model 5000, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified using a computer software (Quantity One, Bio-Rad Laboratories, Inc.). All measurements were performed within the linear range of the assay, as determined from a standard curve. In Figures 2–4, the amount of coprecipitated syntaxin was corrected by the corresponding synaptotagmin signal and then normalized to the data for 0.15 M NaCl (Fig. 2), WT synaptotagmin (Fig. 3), or WT syntaxin (Fig. 4).

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Figure captions

Fig. 1. Ca^{2+}-independent synaptotagmin-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex binding in the brain extract. (A) Proteins were immunoprecipitated (IP) from the brain extracts (1 mg of protein) containing 2 mM ethylene glycol tetraacetic acid (EGTA) using an anti-synaptotagmin (Syt1), anti-synaptobrevin 2 (Syb2), or control (Cont.) antibody in the presence of 0.15 or 1.0 M NaCl (see Experimental methods). Twenty percent of the precipitates and the brain extract (1 μg) were analyzed by immunoblotting for Syt1, syntaxin 1 (Stx1), 25-kDa synaptosomal-associated protein (SNAP25), and Syb2. (B) Effects of Ca^{2+} on synaptotagmin-SNARE complex binding. The brain extracts containing 0.15 M NaCl were immunoprecipitated in the presence of 2 mM EGTA (−) or 1 mM Ca^{2+} (+) and analyzed as described in A. The differences in the signal intensity of the immunoreactivity between A and B were due to different exposure times. The data shown are representative of 3 independent experiments.

Fig. 2. Recombinant synaptotagmin-SNARE binding in the absence of Ca^{2+}. (A) Coexpression of synaptotagmin (Syt1) with syntaxin (Stx1), SNAP25, or synaptobrevin 2 (Syb2) in HEK293 cells. (B) Immunoprecipitation of synaptotagmin from the cell lysate coexpressing one of the SNAREs in the presence of 0.15 M NaCl and 2 mM EGTA using an anti-synaptotagmin antibody (Syt) or control IgG (Cont.). Precipitates were analyzed by immunoblotting as described in Fig. 1A. Nonspecific immunoreactive bands are indicated by an asterisk (*). (C) Coimmunoprecipitation of synaptotagmin and syntaxin in the presence of 0.15 or 1 M NaCl. Results that are representative of the 3 independent experiments are shown on the left. The immunoreactive

bands of coprecipitated syntaxin were measured, corrected by the corresponding synaptotagmin signal, and normalized to the value obtained with 0.15 M NaCl. The averaged data is shown in the bar graph on the right. Values are mean ± SEM from 3 experiments. ***p = 0.0002, by t-test.

Fig. 3. Effects of mutations in the poly-Lysine (Lys) regions in the C_2 domains on Ca^{2+}-independent synaptotagmin-syntaxin binding. (A) Structures of the C_2A (left, Sutton et al., 1995) and the C_2B (right, Fernandez et al., 2001) domains in the presence of Ca^{2+} (spheres) that were generated using the ICM software (MolSoft L.L.C., San Diego, CA, USA). The locations of the Lys residues that were mutated in this study are shown in black with their corresponding residue numbers. (B) Overexpression of synaptotagmin and syntaxin in HEK293 cells. Syntaxin (Stx1) was cotransfected with either wild-type (WT) or mutant synaptotagmin (C_2A 3KQ or C_2B 3KQ) in HEK293 cells. (C) The immunoprecipitation of synaptotagmin with syntaxin from the cell lysate in the presence of EGTA or Ca^{2+}. Precipitates were analyzed by immunoblotting. The data shown are representative of 3 independent experiments. (D) Syntaxin signals were corrected by the corresponding synaptotagmin signal and averaged. Values are mean ± SEM from 3 experiments. ***p < 0.001, by one-way ANOVA with Tukey's test.

Fig. 4. Effects of mutations in the H3 domain of syntaxin on Ca^{2+}-independent binding to synaptotagmin. (A) Alignment of the H3 domain sequences of syntaxin 1 from multiple organisms. The conserved negatively charged residues (E and D) are highlighted. (B) Overexpression of synaptotagmin (Syt1) and WT or mutant syntaxin (Stx1) that were cotransfected in HEK293 cells. (C) Coimmunoprecipitation of synaptotagmin with the WT or mutant syntaxin (E194/196Q–D250N). The cell lysate was immunoprecipitated with an

anti-synaptotagmin antibody and analyzed by immunoblotting for synaptotagmin (Syt1) and syntaxin (Stx1). (D) The immunoreactive bands of coprecipitated syntaxin were measured, corrected by the corresponding synaptotagmin signal, and normalized to the WT syntaxin. Values are mean ± SEM from 3–4 experiments. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, by one-way ANOVA with Dunnett's test compared to the WT syntaxin. (E) Coimmunoprecipitation of synaptotagmin with WT or quintuple mutant (5EQ) syntaxin.

Fig. 5. A model of synaptotagmin-syntaxin binding in neurotransmitter release. Symbols represent synaptotagmin I (syt), syntaxin 1 (stx), SNAP-25 (sn25), and synaptobrevin 2 (syb); SV indicates a synaptic vesicle, and PM indicates the presynaptic plasma membrane; Ca^{2+} ions are shown as small, dark gray spheres.