**Mechanism of Calcium-independent Synaptotagmin Binding to Target SNAREs**

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Synaptic vesicle exocytosis requires three SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins: syntaxin and SNAP-25 on the plasma membrane (t-SNAREs) and synaptobrevin/VAMP on the synaptic vesicles (v-SNARE). Vesicular synaptotagmin 1 is essential for fast synchronous SNARE-mediated exocytosis and interacts with the SNAREs in brain material. To uncover the step at which synaptotagmin becomes linked to the three SNAREs, we purified all four proteins from brain membranes and analyzed their interactions. Our study reveals that, in the absence of calcium, native synaptotagmin 1 binds the t-SNARE heterodimer, formed from syntaxin and SNAP-25. This interaction is both stoichiometric and of high affinity. Synaptotagmin contains two divergent but conserved C2 domains that can act independently in calcium-triggered phospholipid binding. We now show that both C2 domains are strictly required for the calcium-independent interaction with the t-SNARE heterodimer, indicating that the double C2 domain structure of synaptotagmin may have evolved to acquire a function beyond calcium/phospholipid binding.

Syntxin, SNAP-25,¹ and synaptobrevin, also known as VAMP, are members of a large family of SNARE proteins that likely execute fusion in all intracellular compartments (1–3). Whereas most intracellular membrane fusion reactions are constitutive, neurotransmission relies on the coupling of neurotransmitter release to calcium influx into the nerve terminal (4). On calcium entry, a proportion of synaptic vesicles fuse with the presynaptic membrane and release neurotransmitter with a delay of less than 1 ms (5). The synchronization of neuronal exocytosis to calcium entry has been attributed to synaptotagmin, a major synaptic vesicle protein (6, 7). Synaptotagmin contains two calcium-binding C2 domains and is proposed to be a calcium sensor in neuronal exocytosis (8, 9).

Despite identification of the major players involved in neurotransmitter release, the molecular mechanisms responsible for the tight coupling between calcium influx and synaptic vesicle exocytosis are still under debate (10, 11). Previous biochemical studies implicated calcium-dependent binding of synaptotagmin to either syntaxin or SNAP-25 in vesicular exocytosis (12–14). However, the short delay between calcium influx and the fusion of synaptic vesicles suggests that synaptotagmin is linked to the fusion machinery prior to calcium influx into the nerve terminal. Indeed, synaptotagmin, in the absence of calcium, co-purifies with the SNARE complexes from brain material as demonstrated using anti-syntaxin immunoprecipitation and α-SNARE affinity chromatography (15, 16).

The calcium-independent molecular link between the calcium sensor and the SNARE fusion machinery is not well understood, and the previous studies using recombinant protein fragments yielded conflicting results (reviewed in Refs. 10 and 11). Therefore, we investigated this link using, for the first time, highly purified brain synaptotagmin and SNARE proteins as a starting point. We now demonstrate that brain-purified t-SNAREs, syntaxin and SNAP-25, form a stable heterodimer that can bind native synaptotagmin in the absence of calcium. Since this interaction can be reproduced using the recombinant cytoplasmic domain of synaptotagmin 1, we were able to address the requirement for C2 domains in this binding. Our results show that the calcium-independent binding of synaptotagmin to the t-SNARE heterodimer requires the double C2 domain structure.

**EXPERIMENTAL PROCEDURES**

**Isolation of SNARE Proteins and Synaptotagmin—**All procedures were carried out at 4 °C. 1 mg of anti-SNAP-25 SM1 SMI monoclonal antibody (Sternberger Monoclonals) was covalently coupled to 1 ml of CNBr-activated Sepharose-4B (Amersham Biosciences) according to the manufacturer’s instructions. 3.5 g of bovine cerebral cortex was homogenized in 50 ml of phosphate-buffered saline (PBS) containing 2 mM EDTA, and the membrane material was collected by centrifugation at 12,000 × g. Pelleted membranes were solubilized in PBS in the presence of 2% (v/v) Triton X-100 and Complete protease inhibitor mixture (Roche Molecular Biochemicals). The lysate was cleared by centrifugation and batch-incubated with the anti-SNAP-25-Sepharose for 2 h. Total protein in the loading material was estimated by BCA protein determination kit (Pierce) to be 250 mg. The beads were then washed in a column with 30 ml of 100 mM NaCl, 2 mM EDTA, 20 mM Hepes, pH 7.0, 0.1% Triton X-100 (buffer A) followed by 10 ml of buffer A adjusted to 0.6 mM NaCl followed by 10 ml of 0.25 M NaCl, 0.2 mM EGTA, 0.1% Triton X-100, 20 mM glycine HCl buffer, pH 2.5. Synaptotagmin in the 0.6 M NaCl eluate was further purified by a heparin affinity chromatography. Brain monomeric SNARE proteins were prepared as described previously (25). Plasmids encoding glutathione S-transferase (GST) fusion proteins of syntaxin 1A (amino acids 1–265), synaptobrevin 2 (amino acids 1–96), the wild-type cytoplasmic part C2AB of synaptotagmin 1 (amino acids 96–421), mutated C2AB G374D, C2A domain (amino acids 95–265), and C2B domain (amino acids 248–421) were described previously (17–19). Recombinant proteins, purified on glutathione-Sepharose beads, were released from GST, where necessary, by thrombin cleavage.

**Re-assembly of SNARE and Synaptotagmin Complexes and Their Quantification—**SNARE proteins or synaptotagmin (~2 μg each) attached to appropriate Sepharose beads (~15 μl bed volume) were incubated for 30 min at room temperature in 200 μl buffer A with 1–2 μM protein to be tested for binding. Beads were washed three times by low speed centrifugation with 1 ml of buffer A, and bound protein was eluted in sample buffer followed by SDS-PAGE in 12% Ready gels (Bio-Rad) and Coomassie staining. For determination of the stoichiometry of binding, proteins bound to the beads were eluted into sample....
Calcium-independent Interaction of Synaptotagmin with SNAREs

RESULTS

We used a preparative anti-SNAP-25 immunoaffinity chromatography to test an interaction of synaptotagmin with the SNAREs in the absence of calcium in native material, namely bovine brain membranes. The brain membrane extract was incubated with an anti-SNAP-25 antibody covalently attached to Sepharose beads in the presence of 2 mM EDTA, a calcium-chelating agent. Following a two-step elution, bound proteins were analyzed by SDS-PAGE and Coomassie staining (Fig. 1, left panel). For identification of protein bands both Western immunoblotting and mass spectrometry were used (Fig. 1 and data not shown). The 0.6 M NaCl eluate contained synaptotagmin 1, whereas the pH 2.5 eluate contained the three SNAREs and complexin. To determine the specificity of protein isolation on the anti-SNAP-25 column, we performed a chromatography of the brain extract using control beads with covalently attached bovine serum albumin. Myelin basic protein, but not synaptotagmin nor complexin, bound to the control beads (Fig. 1, right panel). Thus, synaptotagmin bound the t-SNARE heterodimer, but none of

and complexin are the major proteins that specifically co-purify with the SNARE complexes, in the absence of divalent cations.

Several scenarios can account for the co-purification of synaptotagmin with the SNAREs on the anti-SNAP-25 column. Synaptotagmin may interact with monomeric SNAP-25 as suggested previously (20). Alternatively, it may interact with SNAP-25 complexes containing SNAP-25. Thus, we investigated whether SNAP-25 can form stable complexes with syntaxin and synaptobrevin. Fig. 2A shows that SNAP-25, prebound to anti-SNAP-25-Sepharose beads, was able to bind syntaxin and could only bind synaptobrevin when syntaxin was present. Therefore, co-purification of synaptotagmin 1 during anti-SNAP-25 chromatography may be due to its interaction with either SNAP-25 alone and/or the t-SNARE heterodimer and/or the full ternary SNARE complex. To distinguish between these possibilities, we prepared anti-SNAP-25-Sepharose beads carrying either SNAP-25 alone, the t-SNARE heterodimer, or the full SNARE complex and incubated them with brain-purified synaptotagmin 1. After washing, bound protein was analyzed by SDS-PAGE and Coomassie staining (Fig. 2B). The native synaptotagmin, in the absence of calcium, was able to interact with the t-SNARE heterodimer and the ternary SNARE complex, but not with SNAP-25 alone. We conclude that, in the case of brain-purified proteins, the interaction of synaptotagmin with the native or re-assembled SNARE complexes does not require calcium.

Since in the SNARE assembly pathway the stable t-SNARE heterodimer exists in brain (21) and precedes the ternary SNARE complex (22), we analyzed in more detail the calcium-independent interaction of synaptotagmin with syntaxin and SNAP-25. First, we tested whether the recombinant cytoplasmic part of synaptotagmin 1 (C2AB, amino acids 96–421) has the ability to bind monomeric SNAREs and/or the t-SNARE heterodimer. In the absence of calcium, the cytoplasmic part of synaptotagmin bound the t-SNARE heterodimer, but none of...
the monomeric SNAREs (Fig. 3A). To determine the binding efficiency we analyzed the stoichiometry of interaction using fluorescent Sypro Orange staining, which labels proteins quantitatively (23). The two t-SNAREs were incubated with the immobilized cytoplasmic domain of synaptotagmin and, after washing, the bound protein was analyzed by SDS-PAGE followed by Sypro Orange staining. Quantification of bound dye in relation to the molecular masses of the bound proteins yielded a molar ratio of 1:1.28:1.2 for synaptotagmin, syntaxin, and SNAP-25, respectively (Fig. 3B). We conclude that the binding of GST-C2AB to the t-SNAREs is approximately equimolar. We next investigated the dependence for binding of SNAP-25 to synaptotagmin as a function of syntaxin concentration. Syntaxin at concentrations ranging from 27 nM to 1.7 μM was added to GST-C2AB beads in the presence of a constant concentration of SNAP-25. Syntaxin promoted binding of SNAP-25 to synaptotagmin at concentrations above 100 nM with the half-maximal binding measured at 290 nM (Fig. 3C).

Two versions of the synaptotagmin cytoplasmic domain were cloned, one with a mutation of glycine to aspartic acid at residue 374 in the second C2 domain (C2B) (19, 24). This mutation has been shown to adversely effect folding of the C2B β-strand barrel structure (25). Fig. 4A shows that the t-SNARE heterodimer bound the wild-type cytoplasmic part, but not its mutated version, suggesting that either both C2 domains or the C2B domain alone are required for this interaction. We therefore analyzed whether, in the absence of calcium, individual C2 domains from the wild-type cytoplasmic part can be the t-SNARE heterodimer. Neither of the individual C2 domains was able to bind the t-SNARE heterodimer, but when linked together they exhibited efficient binding (Fig. 4C).

**DISCUSSION**

Synaptotagmin, the proposed calcium sensor, is essential for coupling the calcium signal to the synaptic vesicle exocytosis (6, 7). In this study we addressed whether such coupling could be due to a physical link between the calcium sensor and the SNARE fusion machinery. We demonstrated by an anti-
SNAP-25 immunoaffinity approach that synaptotagmin, in the absence of calcium, can specifically co-purify with the SNAREs from bovine brain detergent extract (Fig. 1). This is in agreement with the previously observed calcium-independent association of synaptotagmin with the SNARE complexes by anti-synaptin immunoprecipitation from rat brain (15) and by α-SNAP affinity chromatography from bovine brain (16). Moreover, synaptin itself was originally identified as a protein that co-purifies in the absence of calcium with synaptobrevin by anti-synaptotagmin immunoisolation (26). It is notable that further studies, using recombinant synaptin, questioned an efficient calcium-independent link between synaptotagmin and syntaxin (12, 13), suggesting that either synaptin may require other proteins for this interaction or the bacterially expressed SNARE fragments are deficient in some of their properties (11).

To address the molecular basis for the calcium-independent association between the SNAREs and synaptotagmin, we used for the first time brain-purified SNARE proteins and native synaptotagmin in parallel with its truncated versions. Three SNARE proteins, when mixed, formed the ternary complex that can bind synaptotagmin (Fig. 2). We further analyzed the possible calcium-independent association of synaptotagmin with the SNAREs in the stages preceding the ternary SNARE complex. Syntaxin and SNAP-25 can form a stable intermediate on the pathway from the three monomeric SNAREs to their ternary complex, consistent with a central role for the t-SNARE heterodimer in SNAP-25 complex formation (Fig. 24) (21, 22). Remarkably, the t-SNARE heterodimer, but not monomeric SNAREs, was able to bind synaptotagmin with high affinity and stoichiometrically in the absence of calcium (Fig. 3). Association of syntaxin with SNAP-25 at the release sites in the presynaptic membrane, therefore, can be an important step for further engagement of both vesicular synaptotagmin and synaptobrevin. As synaptobrevin is potently inhibited on synaptic vesicle membrane (27), synaptotagmin acts in the absence of calcium with monomeric SNAP-25 to position the vesicles at the release sites (20). However, this hypothesis is inconsistent with the observation that SNAP-25 is found throughout the plasma membrane (21, 29). In addition, further studies could only detect binding of synaptotagmin to SNAP-25 by Western immunoblotting (14, 30), suggesting a closely aligned double C2 domain structure (33, 34).

A quantitative electron microscopy study of synapse morphology in the absence of synaptotagmin revealed that synaptic vesicles are no longer maintained in close proximity to the presynaptic membrane (28). One attractive mechanism for this phenomenon was put forward, whereby synaptotagmin interacts in the absence of calcium with monomeric SNAP-25 to position the vesicles at the release sites (20). However, this hypothesis is inconsistent with the observation that SNAP-25 is found throughout the plasma membrane (21, 29). In addition, further studies could only detect binding of synaptotagmin to SNAP-25 by Western immunoblotting (14, 30), suggesting a closely aligned double C2 domain structure (33, 34).

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