**Brief Report**

**nSec1 Binds a Closed Conformation of Syntaxin1A**

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**Abstract.** The Sec1 family of proteins is proposed to function in vesicle trafficking by forming complexes with target membrane SNAREs (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein [SNAP] receptors) of the syntaxin family. Here, we demonstrate, by using in vitro binding assays, non-denaturing gel electrophoresis, and specific neurotoxin treatment, that the interaction of syntaxin1A with the core SNARE components, SNAP-25 (synaptosome-associated protein of 25 kD) and VAMP2 (vesicle-associated membrane protein 2), precludes the interaction with nSec1 (also called Munc18 and rbSec1). Conversely, association of nSec1 and syntaxin1A prevents assembly of the ternary SNARE complex. Furthermore, using chemical cross-linking of rat brain membranes, we identified nSec1 complexes containing syntaxin1A, but not SNAP-25 or VAMP2. These results support the hypothesis that Sec1 proteins function as syntaxin chaperons during vesicle docking, priming, and membrane fusion.

**Key words:** membrane fusion • synaptic vesicles • exocytosis • SNARE • synapse

**Introduction**

Two of the most intensively studied membrane transport processes are the fusion of Golgi apparatus-derived vesicles with the plasma membrane in yeast and the secretion of neurotransmitter from the mammalian presynaptic nerve terminal (Bennett and Scheller, 1994; Jahn and Südhof, 1994; Rothman, 1994). These membrane fusion events are mediated by a set of structurally and likely functionally related proteins called SNAREs (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein [SNAP] receptors; Bennett, 1995). These proteins include vesicle-associated membrane proteins (VAMP) 1 and 2 (synaptobrevin), a synaptosome-associated protein of 25 kD (SNAP-25), and syntaxin1A and B in mammals; and their yeast orthologues Snc1 and 2, Sec9, and Sso1 and 2, respectively (Bennett and Scheller, 1994; Brennwald et al., 1994). The pairing of SNAREs across membranes to form four-stranded helical bundles is a late event in the fusion process, perhaps driving the actual mixing of the lipid bilayers (Poirier et al., 1998; Sutton et al., 1998; Weber et al., 1998; Chen et al., 1999).

In addition to SNAREs, a critical component of the fusion process is the Sec1p protein, known in mammals as munc18, nSec1, or rbSec1 (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994a). A model for Sec1/syntaxin function was suggested from an early study demonstrating that binding of the NH₂-ter-
exclusive interactions of nSec1/syntaxin1A and SNAREs

Previously, it was shown that nSec1 interacts with syntaxin1A with high affinity and that this complex prevents syntaxin from binding to the target SNARE, SNAP-25, in vitro (Pevsner et al., 1994a). However, it was never deter-
mined whether the binary association of syntaxin and SNAP-25 could prevent the nSec1/syntaxin interaction. To test this possibility, we incubated purified syntaxin1A, SNAP-25, or binary SNARE complex (syntaxin1A/SNAP-25) with GST-nSec1-bound glutathione-agarose beads. nSec1-bound agarose beads retained syntaxin, but not SNAP-25 (Fig. 1, lanes 10 and 11), consistent with previous results. The nSec1/syntaxin1A interaction, however, was prevented by syntaxin1A/SNAP-25 binary association (Fig. 1, lane 12). As a control, GST beads did not retain any SNAREs (Fig. 1, lanes 5–8). Furthermore, we tested the ability of nSec1 to prevent assembly of the complete ternary SNARE complex. The glutathione-agarose beads conjugated with the nSec1/syntaxin1A complex were incubated with either SNAP-25, VAMP2, or both. The results showed that the high-affinity nSec1/syntaxin1A interaction completely prevented binding among the SNAREs (Fig. 1, lanes 14 and 15) and assembly of the ternary SNARE complex (Fig. 1, lanes 16 and 17). These results indicate that association of syntaxin with SNAREs in either binary or ternary complexes and the association of syntaxin with nSec1 are mutually exclusive.

Syntaxin Bound to nSec1 Does Not Form SNARE Complexes, and nSec1 Does Not Associate with the Preassembled SNARE Complex

To test the possibility that SNARE complexes act as receptors for nSec1 (Carr et al., 1999), either purified syntaxin or nSec1/syntaxin1A complex was incubated with SNAP-25 and VAMP2 in solution. Free syntaxin, along with the other SNAREs, formed a SDS-resistant complex, as previously reported (Fig. 2 A). Similarly, the nSec1-bound syntaxin was capable of forming an SDS-resistant SNARE complex (Fig. 2 A). This nSec1/syntaxin1A-derived SNARE complex required all SNARE protein components for SDS resistance (data not shown) and dissociated into individual monomers after boiling (Fig. 2 A), indicating that the complex is identical to the ternary SNARE complex. To test whether nSec1 remains associated with the SDS-resistant SNARE complex, we separated the protein complexes on a nondenaturing gel. SNAP-25, nSec1/syntaxin1A, and syntaxin1A ran as distinct bands on the native gel, whereas nSec1 and the positively charged VAMP2 did not enter the gel (Fig. 2 B, lanes 1–5). Surprisingly, in contrast to the results obtained by SDS gel electrophoresis, the native gel showed that the mixture of nSec1/syntaxin1A, SNAP-25, and VAMP2, instead of running as a unique complex, ran as individual components (Fig. 2 B, lane 6). In the absence of nSec1, the mixture of SNAREs ran as a unique band that represents the assembled complex (Fig. 2 B, lane 7), indicating that this assay is capable of detecting assembled SNARE complex. Therefore, we conclude that the ability of the nSec1/syntaxin1A complex to form SNARE complexes in the presence of SDS is due to the SDS-induced dissociation of the nSec1/syntaxin1A complex, which allows the released syntaxin to bind the other SNAREs. Furthermore, we tested for the possible association between nSec1 and the preassembled SNARE complex using a bead binding assay. As shown in Fig. 3, nSec1 beads failed to retain any of the ternary SNARE complex.

nSec1 Prevents Syntaxin from Forming a Neurotoxin-Resistant SNARE Complex

A assembly of binary and ternary SNARE complexes results in protection from neurotoxin digestion, whereas free SNAP-25 and VAMP2 are specifically cleaved by botulinum toxin E or D, respectively (Hayashi et al., 1994). To
further characterize whether both free syntaxin1A and prebound nSec1/syntaxin1A complex were able to form stable, neurotoxin-protected SNARE complexes in solution, we incubated either free syntaxin (Fig. 4, lanes 1, 2, 5, and 6) or prebound nSec1/syntaxin (Fig. 4, lanes 3, 4, 7, and 8) with SNAP-25 and VAMP2, followed by treatment with either botulinum toxin E or D. Without toxin treatment (Fig. 4, lanes 1–4), both free syntaxin and nSec1/syntaxin1A were capable of forming thermally sensitive SNARE complexes as assayed by SDS-PAGE. A's shown in Fig. 2, the nSec1/syntaxin complex is dissociated by SDS, followed by SNARE complex formation. Upon boiling in the presence of SDS, the complexes dissociated into SNARE monomers, as demonstrated by the increase in the density of both the SNAP-25 and VAMP2 protein bands. With either botulinum toxin E or D treatment, the complexes formed by syntaxin1A, SNAP-25, and VAMP2 were greatly protected from digestion, as intact SNAP-25 and VAMP2 bands were observed after boiling (Fig. 4, lanes 5 and 6). In contrast, treatment of nSec1/syntaxin1A, SNAP-25, and VAMP2 with botulinum toxin E or D resulted in digestion of SNAP-25 and VAMP2 bands were observed after boiling (Fig. 4, lanes 7 and 8), indicating that neither SNAP-25 nor VAMP2 were protected as part of an assembled SNARE complex. These results further indicate that prebound nSec1/syntaxin1A, SNAP-25, and VAMP2 are unable to assemble into a complex in solution without addition of SDS.

**In Vivo nSec1 Is Present in a Complex Containing Syntaxin1A, but Not SNAP-25 or VAMP2**

Next, to investigate the in vivo interactions between nSec1 and the SNARE proteins, we cross-linked detergent-solu-
bated with or without DSS (1 mM) for 2 h on ice. Lysates (20 μg/ lane) were analyzed by Western blot with the antisynaptobrevin antibody (anti-VAMP2), or anti-VAMP2 antibodies as indicated.

Figure 4. A association between nSec1 and syntaxin prevents toxin-resistant SNARE complex formation. Either syntaxin1A (SYN1; lanes 1, 2, 5, and 6) or nSec1/syntaxin1A complex (SYN1/nSec1; lanes 3, 4, 7, and 8) was combined with full-length SNAP-25 (SN25) and VAMP2. The mixtures were incubated for 1 h at 4°C. Then, either botulinum toxin E to digest SNAP-25 (BotT E; upper panel of lanes 5–8) or botulinum toxin D to digest VAMP2 (BotT D; lower panel of lanes 5–8) was added and incubated 30 min at 25°C. After incubation, sample buffer (final concentration of 2% SDS) was added, and half of the mixture was boiled, whereas the other half was kept at room temperature. Proteins were then separated on a 16% SDS-polyacrylamide gel and visualized by Coomassie blue staining.

Figure 5. Chemical cross-linking of rat brain membrane reveals complexes containing syntaxin1A and nSec1, but not VAMP2 or SNAP-25. Rat brain membrane detergent extracts were incubated with or without DSS (1 mM) for 2 h on ice. Lysates (20 μg/lane) were analyzed by Western blot with the antisynaptobrevin antibody (anti-VAMP2), or anti-VAMP2 antibodies as indicated. A asterisk-labeled complexes are described in Results. There are also two major unidentified cross-linked bands with molecular weights ranging from 45–60 kD recognized by anti-calsequestrin antibody. There is one major 56-kD cross-linked band recognized by anti-VAMP2 antibody corresponding to synaptophysin/VAMP2 complex as previously demonstrated (Calakos and Scheller, 1994). Molecular mass markers are indicated on the left in kD.

Discussion

Using recombinant proteins purified to homogeneity, we have investigated the interactions between nSec1 and the SNAREs. Several observations, obtained from in vitro binding assays, nondenaturing gel electrophoresis, and specific neurotoxin treatment, suggest that the interaction of syntaxin1 with the core SNARE components, SNAP-25 and VAMP2, precludes the interaction of syntaxin1 with nSec1. Inversely, association of nSec1 and syntaxin1 prevents assembly of the binary and ternary SNARE complex. Furthermore, chemical cross-linking of rat brain membrane extracts with the noncleavable cross-linked DSS. The extracts were analyzed by Western blotting with antibodies against either nSec1 or the different SNARES, as indicated in Fig. 5. Cross-linking generated two higher molecular mass complexes (apparent molecular weights: 105 and 140 kD), both of which were immunoreactive with antisyntaxin1A and anti-nSec1. Significantly, however, these two bands were not recognized by either anti-SNAP-25 or anti-VAMP2. Furthermore, chemically cross-linking purified rat brain membrane before solubilization with detergent gave the same results (data not shown). Therefore, in the mammalian case, we see no evidence for the existence of assembled SNARE complexes bound to nSec1.
When the vesicle arrives at the acceptor membrane, a series of events, beginning with target recognition and culminating with membrane fusion, ensues. Our data support a model in which the Sec1/syntaxin complex is at least one component of the target membrane receptor for the vesicle. Consistent with earlier hypothesis, we favor the idea that the Sec1/syntaxin complex is recognized by the vesicle, perhaps through a Rab/Rab effector interaction, and that conformational changes then occur that allow syntaxin to unfold to an open state. The molecular events leading to these proposed recognition events and conformational changes are not known and represent one of the most important issues in the membrane trafficking field. The open state of syntaxin is suggested to then interact with other SNAREs, leading to the formation of core complexes and membrane fusion. The question of whether Sec1 is totally dissociated from the SNARE complex or may remain bound deserves further investigation.

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References


Brennwald, P., B. Kearns, K. Champion, S. K eranen, V. Bankaitis, and P. No vick. 1994. Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell. 79:245–258.


