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Regulation of the UNC-18–*Caenorhabditis elegans* Syntaxin Complex by UNC-13

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The *Caenorhabditis elegans unc-13*, *unc-18*, and *unc-64* genes are required for normal synaptic transmission. The UNC-18 protein binds to the *unc-64* gene product *C. elegans* syntaxin (*Ce* syntaxin). However, it is not clear how this protein complex is regulated. We show that UNC-13 transiently interacts with the UNC-18-*Ce* syntaxin complex, resulting in rapid displacement of UNC-18 from the complex. Genetic and biochemical evidence is presented that UNC-13 contributes to the modulation of the interaction between UNC-18 and *Ce* syntaxin.

Key words: UNC-18; Ce *syntaxin; UNC-13; SNARE complex; synaptic vesicle; exocytosis*

At chemical synapses, neurotransmitter release is accomplished by a series of interactive steps between synaptic vesicles and plasma membrane, including targeting, docking, fusion, and exocytosis (Kelly, 1993; Bennett and Scheller, 1994; Südhof, 1995; Martin, 1997). The regulatory targeting of synaptic vesicles to the plasma membrane requires a core complex of neuronal synaptic proteins, soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors, termed SNAREs (Söllner et al., 1993). These synaptic proteins include syntaxin, vesicle-associated membrane protein (VAMP) (also called synaptobrevin), and SNAP-25 (Calakos et al., 1994; Chaoman et al., 1994; Pevsner et al., 1994a; Hayashi et al., 1994).

In *Caenorhabditis elegans*, many potential presynaptic genes have been identified (Rand and Russell, 1984, 1985; Hosono et al., 1989; Hosono and Kamiya, 1991; Maruyama and Brenner, 1991; Nonet et al., 1993, 1998; Jorgensen et al., 1995; Miller et al., 1996). *unc-18*, one such candidate gene, is expressed in a neuron-specific manner, and its gene product (UNC-18) is present in presynaptic terminals (Hosono et al., 1992; Gengyo-Ando et al., 1993; Ogawa et al., 1998). The UNC-18 homolog n-Sec1 tightly associates with syntaxin (Hata et al., 1993; Pevsner et al., 1994b; Kee et al., 1995). n-Sec1 is not a component of either the 7S VAMP–SNAP-25 or 20S SNARE–SNAP–*N*-ethylmaleimide-sensitive factor protein complex, although syntaxin is present in both complexes (Pevsner et al., 1994b; Garcia et al., 1995). Binding of syntaxin to the component of the 7S complex is diminished in the presence of increasing concentrations of n-Sec1 (Pevsner et al., 1994a). From these results, it is hypothesized that n-Sec1 is associated with syntaxin before synaptic vesicle docking and may be a negative regulator of vesicle docking and/or release.

This hypothesis predicts that *C. elegans* null mutations of the *unc-18* gene would lead to increased neurotransmitter release. However, *unc-18* mutations have been shown to be associated with decreased transmitter release and with the accumulation of neurotransmitters at the presynaptic terminal (Hosono et al., 1987, 1989). These results are also consistent with observations in yeast (Novick et al., 1980). To examine these apparently conflicting observations, we analyzed *unc-18* mutants. We (Ogawa et al., 1998) and others (Saifee et al., 1998) found that *unc-64* encodes the mammalian syntaxin 1A homolog *C. elegans* (*Ce* syntaxin) and the product could bind to UNC-18. We further searched for factors interacting with UNC-18 and found the *unc-13* gene product. UNC-13 has a potential phorbol ester binding domain (C1) and two probable calcium phospholipid-binding domains (C2) (Ahmed et al., 1992; Brose et al., 1995; Kazanietz et al., 1995). These structural features suggest that UNC-13 contributes to the calcium- and diacylglycerol-dependent regulation of transmitter release. We therefore performed detailed genetic and biochemical analyses of the three genes and their products. We report here UNC-13 dissociates UNC-18 from the UNC-18–*Ce* syntaxin complex. We propose that the three genes have a critical role in synaptic vesicle docking and subsequent processes. During the preparation of our manuscript, a factor from rat cerebral cytosol was found (tomosyn) that dissociates Munc-18 from the syntaxin-1a–Munc-18 complex (Fujita et al., 1998). Tomosyn differs from the mammalian UNC-13 homolog Munc-13 in protein structure.

MATERIALS AND METHODS

General handling. Culture, maintenance, and genetic manipulation were essentially as described previously (Brenner, 1974).

C. elegans *strains.* The wild-type Bristol strain N2 and the following mutations were used: LGI, *unc-13* (*n2823*, *e51*) and *dpy-5* (*e61*); LGIII, *unc-64* (*e246*) and *dpy-18* (*e364*); LGX, *unc-18* (*cn347*, *md118*, *md183*,

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Drs. Sassa and Harada contributed equally to this work.

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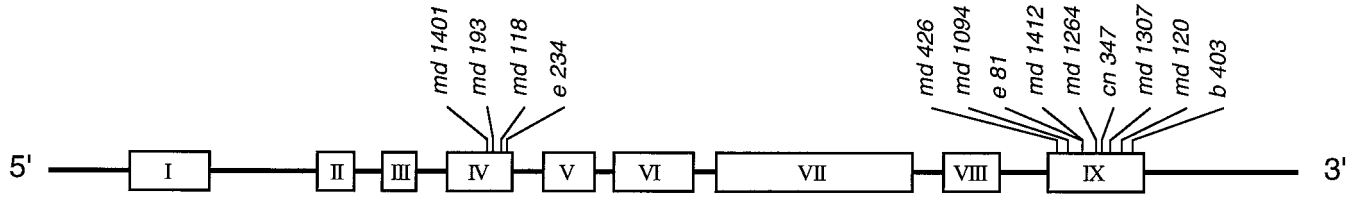
A



B

Allele	Mutations	
	Nucleotide	Amino acid
md 1401	A 397 G	I 133 V
md 193	Tc1 insertion	
md 118	Rearrangement	
e 234	C 421 T	Q 141 Stop
md 426	Tc1 insertion	
md 1094	Tc1 insertion	
e 81	C 1582 T	Q 530 Stop
md 1412	C 1582 T	Q 530 Stop
md 1264	Insertion	
cn 347	Tc1 insertion	
md 1307	Deletion	
md 120	Deletion	
b 403	G 1645 A	E 549 K

Figure 1. Sequences of *unc-18* mutant alleles. *A*, Map of sequenced mutations at the *unc-18* locus. *Boxes* indicate coding regions. *B*, The altered codons and positions of amino acid substitutions are shown.

md193, *b403*, *md1054*, *md1412*, *md1417*, *e234*, *md120*, *md1307*, *md1401*, *e81*, *md1264*, *md426*, *md1094*) and *lon-2* (*e678*).

Construction of unc double mutants. Double mutants of *unc-13*, *unc-18*, and *unc-64* were tested by making hermaphrodites that are homozygous in one *unc* mutation (u1) and heterozygous in another (u2). The u2 mutation is linked to a Dpy or a Lon morphological marker (a). Self-progeny from these hermaphrodites (u1/u1; + +/u2 a) were inspected to determine survival.

Analyses of mutant phenotypes and ACh assay. Locomotion, trichlorfon resistance, and assays of ACh level of mutant alleles were performed as described previously (Harada et al., 1994).

Sequence determination. To determine the mutation sites, total RNA from mutant alleles was isolated by the CsCl gradient method (Ogawa et al., 1998). Poly(A^+) RNAs were purified by Oligotex-dT30 (TaKaRa, Tokyo, Japan). The cDNAs amplified by reverse transcription-PCR were cloned into the pBluescript SK(+) vector and sequenced by the dye primer method or by the dideoxy chain termination method. The following oligonucleotides were used to amplify cDNAs and to determine their sequences: CE1845, GA**AAGCTT**ATGTCACTCAAACAAATCGTTGGGCA (+1 to +26); CE1846, TC*TCTAGA*TCATATGTCACGCGGTTTGTTC (+1755 to +1776); CE18SEQ2, AGCGTCGAGTTTTTGCTCAA (+608 to +627); CE18SEQ3, TGAGAGAAATGTTGAGCTCG (+579 to +598); CE18SEQ4, AACAGAATCAATCTGAGGCG (+1207 to +1226); and CE18SEQ5, GTTGATGGTGCCACTTTTGA (+1161 to +1180). Bold indicates a *Hin*dIII site, and italics indicates a *Xba*I site.

Preparation of recombinant proteins. pGEX vectors (Pharmacia Biotech, Uppsala, Sweden) for the bacterial expression of glutathione *S*-transferase (GST) fusion proteins containing the cytoplasmic domain of *Ce* syntaxin (amino acids 1–267) and the N-terminal domain of UNC-13 (UNC-13N) (amino acids 1–266) were constructed using PCR procedures. Expression of GST fusion proteins and subsequent purification using glutathione–Sepharose 4B beads were performed as described previously (Ogawa et al., 1996). Soluble recombinant syntaxin or UNC-13N was purified from the GST fusion protein by cleavage with Factor Xa or thrombin, respectively, and these digestions were stopped by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). UNC-18 was prepared using a baculovirus expression system as described previously (Ogawa et al., 1996). Protein concentrations were estimated by Coomassie blue staining of protein bands after SDS-PAGE with bovine serum albumin as a standard.

In vitro *binding assay.* UNC-18 and GST fusion protein (either *Ce* syntaxin or UNC-13N) were incubated in 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM PMSF, 10 μg/ml leupeptin, and 0.1% NP-40 for 1 hr at 4°C, and then glutathione–Sepharose 4B beads were added. After a 1 hr incubation, the beads were washed three times with 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, and 0.5 mM PMSF and then washed once with 50 mM Tris-HCl, pH 8.0. Proteins were eluted with 10 mM reduced glutathione and then analyzed by Western blotting using antibodies to UNC-18 after SDS-PAGE. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL system; Amersham, Arlington Heights, IL). The binding ability was quantitated by using NIH Image software. Proteins were also visualized by autoradiography of ^{125}I-labeled sheep anti-rabbit secondary antisera (Amersham) and by phosphorimaging.

Table 1. Phenotypes of *unc-18* mutant alleles

Allele	Trichlorfon resistance[a] (μM)	Locomotion[b] forward(sec)	Locomotion[b] backward(sec)	ACh levels (nmol/mg protein)
+/+	20	3.3 ± 0.5	3.3 ± 0.7	0.45 ± 0.09
md1401	20	7.6 ± 1.6	6.8 ± 1.3	0.67 ± 0.02
md193	300	>60	>60	4.61 ± 0.02
md118	300	>60	>60	2.62 ± 0.34
e234	>300	>60	>60	4.53 ± 0.07
md426	100	8.4 ± 2.8	9.5 ± 2.3	4.74 ± 0.66
md1094	50	8.0 ± 1.2	11.0 ± 2.4	1.27 ± 0.25
e81	300	>60	>60	1.34 ± 0.07
md1412	>300	>60	>60	4.54 ± 0.21
md1264	20	4.9 ± 0.9	5.2 ± 0.9	1.80 ± 0.16
cn347	200	>60	>60	3.39 ± 0.59
md1307	300	>60	>60	6.22 ± 0.15
md120	250	53.0 ± 3.2	37.3 ± 4.3	3.09 ± 0.07
b403	300	>60	>60	5.82 ± 0.75

[a] Three larvae were grown on nematode growth medium-agar plates containing eight different concentrations of trichlorfon (10, 20, 50, 100, 150, 200, 250, and 300 μM). Shown are the highest concentrations of trichlorfon at which the animal in triplicate experiments was able to produce F_2 progeny within 10 d.

[b] The mean time for entire animals to cross a line was recorded when either the head or the tail regions were touched with toothpicks. Locomotion of 10 animals was measured.

Table 2. Epistatic relationship between *unc-13*, *unc-18*, and *unc-64* gene mutant alleles

Allele	Trichlorfon resistance[a] (μM)	Locomotion[b] Forward (sec)	Locomotion[b] Backward (sec)	ACh levels (nmol/mg protein)
+/+	20	3.3 ± 0.5	3.3 ± 0.7	0.45 ± 0.09
unc-13(e51)	50	>60	>60	1.48 ± 0.07
unc-13(n2823)	50	5.2 ± 1.1	6.5 ± 2.4	0.69 ± 0.03
unc-18(cn347)	200	>60	>60	3.39 ± 0.59
unc-18(md1094)	100	8.0 ± 1.2	10.9 ± 2.4	1.27 ± 0.25
unc-64(e246)	200	11.7 ± 5.0	14.1 ± 4.0	2.61 ± 0.14
unc-13(e51); unc-18(cn347)	lethal			
unc-18(cn347); unc-64(e246)	lethal			
uunc-13(e51); unc-64(e246)	lethal			
unc-13(n2823); unc-18(md1094)	150	13.1 ± 4.2	13.1 ± 2.7	6.47 ± 1.02
unc-18(md1094); unc-64(e246)	150	>60	>60	4.93 ± 0.46
unc-13(n2823); unc-64(e246)	>300	>60	>60	6.69 ± 0.24

[a, b] See legend of Table 1.

RESULTS

Sequences and phenotypes of the *unc-18* mutants

Mutation sites of the *unc-18* alleles other than *cn347* had not been determined (Hosono et al., 1992). We therefore analyzed all available *unc-18* alleles by DNA sequencing and compared their phenotypes. Interestingly, mutations were all clustered in exons IV and IX (Fig. 1*A*). Three mutations, *md193*, *md426*, and *md1094*, were found to result from Tcl insertions as observed in *cn347*. Four mutations, *md118*, *md1264*, *md1307*, and *md120*, were small DNA rearrangements, including deletions and insertions. The remaining five mutations were single base changes; three mutations, *e234*, *e81*, and *md1412*, had stop codons, and two, *md1401* and *b403*, had amino acid substitutions.

All *unc-18* mutations had uncoordinated behaviors, abnormal ACh accumulation, resistance to AChE inhibitors, and a significantly slower growth rate than wild type. However, the severity of the behavioral impairment of mutants varied; that is, some of these mutants were quite uncoordinated (Table 1). Putative null alleles (stop codons or frameshifts) showed the strongest phenotype; that is, the animals were severely paralyzed, strongly resistant to trichlorfon, and accumulated ACh. We were especially interested in two missense mutations described in greater detail below; *md1401* leads to mild behavioral defects, whereas *b403* leads to a severe mutant phenotype.

Genetic interactions between *unc-13*, *unc-18*, and *unc-64* mutations

Genetic, molecular, and biochemical analyses suggest that *unc-13*, *unc-18*, and *unc-64* gene products are all involved in neurotransmitter release (Miller et al., 1996). We therefore constructed double mutants to examine possible genetic interactions among these three genes. For these constructions, we used a mild (*md1094*) and a severe (*cn347*) allele of *unc-18*, a mild (*n2823*) and a severe (*e51*) allele of *unc-13*, and the relatively mild (*e246*) allele of *unc-64* (Table 2). The double mutants of *unc-13* and

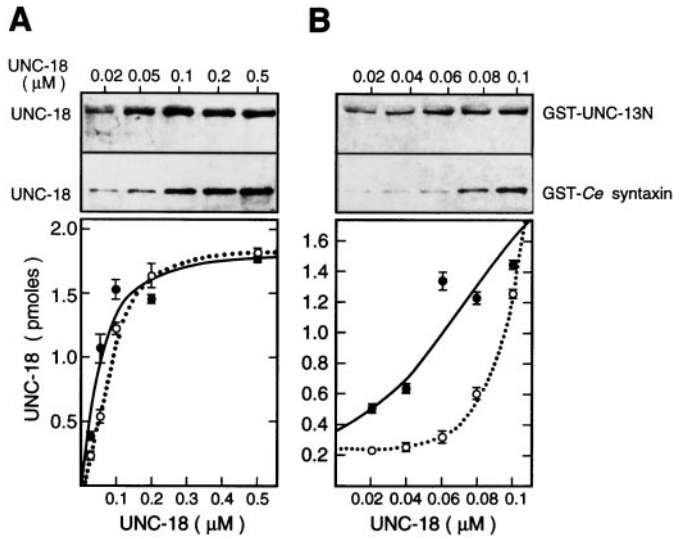


Figure 2. Binding of UNC-18 to UNC-13 and to *Ce* syntaxin. One micromolar GST alone or the GST fusion proteins consisting of the UNC-13N (*top column*, *GST-UNC13N*) or the cytoplasmic domain of *Ce* syntaxin (*bottom column*, *GST-Ce syntaxin*) was incubated with concentrations of UNC-18 ranging from 0.02 to 0.5 μM (*A*) and from 0.02 to 0.1 μM (*B*). Amounts of bound UNC-18 were determined by SDS-PAGE and immunoblotting. The ECL signal intensities were quantitated using NIH Image software, and the total value of each band was converted to picomoles on standard curves for UNC-18. The results are the mean of three experiments. *Top*, Immunoblot results from a single experiment. *Ce* syntaxin, ○; UNC-13N, ●.

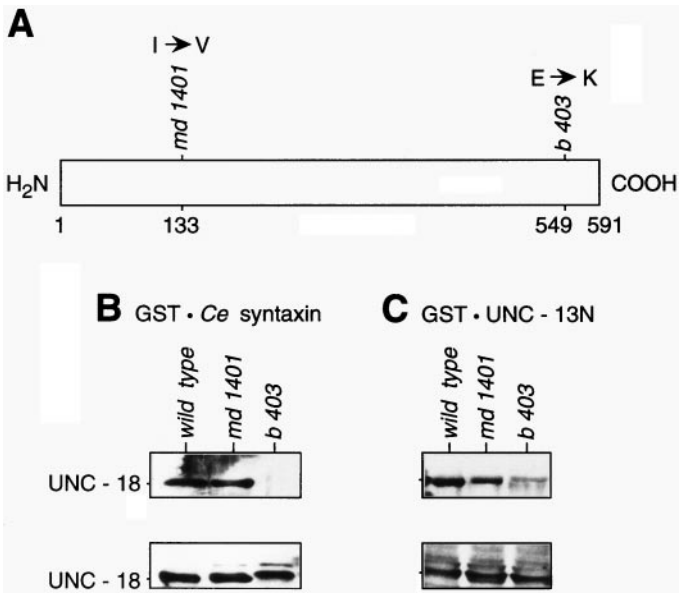


Figure 3. Binding of *md1401* and *b403* mutant UNC-18 proteins to GST-*Ce* syntaxin (*B*) and GST-UNC-13N (*C*). *A*, The protein structure of UNC-18. The mutation sites of *md1401* at 133 and *b403* at 549 are indicated by *arrows*. *B*, *C*, Amounts of UNC-18 in bound forms (*top*) and used for the incubations (*bottom*) were determined by Western blotting.

unc-18 had phenotypes suggesting approximate additivity. Thus, the strain containing the two mild mutations was somewhat more severe than either of the single mutants, but not markedly so, and the strain containing the two severe mutations was lethal. In contrast, although the *unc-64* (*e246*) allele is relatively mild, it greatly enhanced the phenotype of mutation with which it was

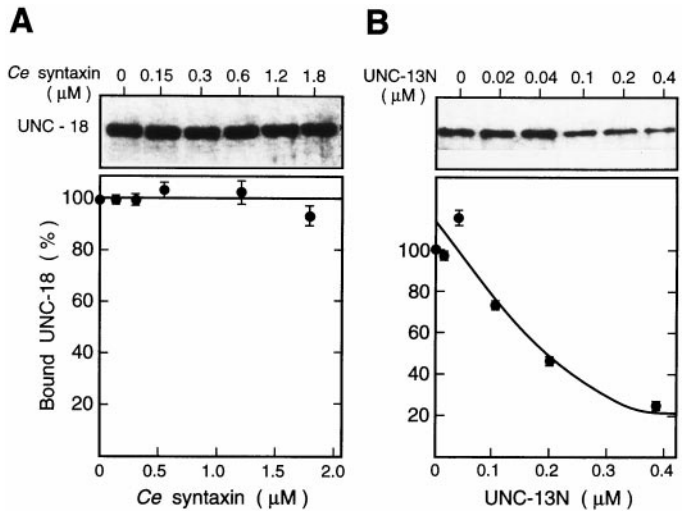


Figure 4. Interaction of UNC-13N, UNC-18, and *Ce* syntaxin. *A*, Binding of UNC-18 to GST-UNC-13N in the presence of *Ce* syntaxin. UNC-18 (0.2 μM) and GST-UNC-13N (1.2 μM) were incubated with the indicated concentrations of *Ce* syntaxin. *B*, UNC-18 (0.2 μM) and GST-*Ce* syntaxin (1.0 μM) were incubated with the indicated amount of UNC-13N. The value of each band of UNC-18 bound to immobilized GST-UNC-13N (*A*) or GST-*Ce* syntaxin (*B*) was expressed relative to that bound without *Ce* syntaxin (*A*) or UNC-13N (*B*). *Top*, Immunoblot results from a single experiment.

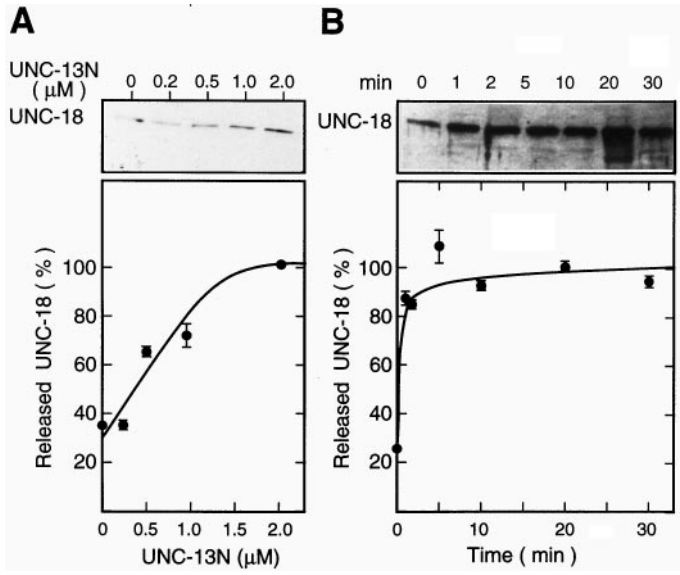


Figure 5. Dissociation of UNC-18 from the UNC-18–*Ce* syntaxin complex by UNC-13N. *A*, UNC-18–*Ce* syntaxin complex immobilized to GST beads was prepared using UNC-18 (0.2 μM) and GST-*Ce* syntaxin (1.0 μM) as described in Materials and Methods. The GST beads were incubated with the indicated concentrations of UNC-13N at 30°C for 10 min. *B*, Time course of the displacement of UNC-18 by UNC-13N. The UNC-18–GST-*Ce* syntaxin complex was incubated with UNC-13N (2.0 μM) at 30°C and then centrifuged at the indicated times. The concentration of the released UNC-18 in the supernatant was expressed relative to the concentration of the released UNC-18 when the complex was incubated with 10 mM reduced glutathione.

combined, i.e., the *unc-64* (*e246*) mutation in combination with either the mild *unc-13* or the mild *unc-18* mutations led to a severely paralyzed phenotype and, when combined with either the severe *unc-13* allele or the severe *unc-18* allele, led to a synthetic lethality.

In addition, the extent of the elevation of ACh levels in double mutants is not comparable with the extent of the loss of coordination. Although the ACh level is only slightly elevated in *unc-13* (*n2823*) animals, in the double mutants with *unc-18* (*md1094*) and *unc-64* (*e246*), it was quite high.

UNC-18 binds to an UNC-13 fragment in addition to *Ce* syntaxin

We have shown previously that UNC-18 binds to the *unc-64* gene product *Ce* syntaxin (Ogawa et al., 1996, 1998). We tested interaction between UNC-13, UNC-18, and *Ce* syntaxin. In a preliminary work, proteins were visualized both by ECL and ^{125}I-labeled secondary antisera (T. Sassa, unpublished results). Here, we present the former results because significant difference between the methods was not observed. A half-maximal binding (ED_{50}) of UNC-18 to *Ce* syntaxin occurred at 0.08 μM. Furthermore, we found that UNC-13N also bound to UNC-18 (Fig. 2), with an ED_{50} of 0.04 μM. Parallel incubations with GST alone demonstrated that the binding was dependent on the fusion protein (data not shown).

The binding ability of mutant UNC-18 proteins derived from *b403* and *md1401* was also tested. The *md1401* UNC-18 protein bound normally to both UNC-13N and *Ce* syntaxin, whereas UNC-18 from the *b403* mutant was defective in binding to *Ce* syntaxin and had greatly reduced ability to bind to UNC-13N (Fig. 3).

An UNC-13 fragment can regulate the UNC-18–*Ce* syntaxin complex

Ce syntaxin did not interfere with the formation of the UNC-13N–UNC-18 complex (Fig. 4*A*). However, the amount of UNC-18–*Ce* syntaxin complex decreased when incubated with UNC-13N (Fig. 4*B*). These results suggest that UNC-13N either functions as an inhibitor of the complex formation or causes the displacement of UNC-18 from the complex.

To know whether UNC-13N displaces UNC-18, we assayed the amount of UNC-18 released in the soluble fraction (Fig. 5*A*). The amount of UNC-18 in the soluble fraction increased depending on the concentration of added UNC-13N. We then assayed the velocity of the release of UNC-18 from the UNC-18–*Ce* syntaxin complex. The release is very rapid, with most UNC-18 released within 1 min (Fig. 5*B*). The amount of UNC-18 released from the complex by UNC-13N (2 μM) is the same as that released by adding 10 mM reduced glutathione (data not shown).

DISCUSSION

Some synaptic genes, such as *cha-1*, *snb-1*, *unc-17*, and *unc-64*, are lethal if they are completely defective (Rand, 1989; Alfonso et al., 1993; Nonet et al., 1998; Saifee et al., 1998). In contrast, null mutations of *snt-1* are viable but uncoordinated (Nonet et al., 1993). The *unc-18* gene appears to fall into the latter category because null mutations, although causing severe paralysis, were viable.

To explore the function of the *unc-18* gene, we first analyzed the different mutations and their mutant phenotypes. Among them, we found two missense mutations, *md1401* and *b403*. These mutation sites are located at the N and C terminal of UNC-18, respectively. However, although both of these mutations occur in predicted α-helices, they have very different amino acid substitutions, and they lead to quite different phenotypes. The N-terminal mutation *md1401* is mildly defective, whereas the C-terminal mutation *b403* shows a severely defective phenotype. Based on the computational three-dimensional structure, N. Hayashi (Fujita Health University, Aichi, Japan) predicted that the conformation is lost by the E-to-K substitution in the *b403* mutation (personal communication). I-to-V substitution in the *md1401* mutation may not bring out great conformational alteration, and the ability of the mutant UNC-18 to bind *Ce* syntaxin and UNC-13 appears intact.

We have shown that UNC-18 has the ability to bind to UNC-13N, in addition to *Ce* syntaxin. A mammalian UNC-18 homolog, Munc-l8, can bind the Doc2 and Mint proteins, in addition to syntaxin (Okamoto and Südhof, 1997; Verhage et al., 1997). Therefore, UNC-18 also may bind to multiple proteins. The function of such interactions is not clear at present. It is noteworthy that UNC-18, once bound to *Ce* syntaxin, can lose its syntaxin-binding ability by interaction with UNC-13N. This step may be important for the late stage of synaptic transmission, including fusion and/or exocytosis. We suggest that UNC-18 induces a conformational change in *Ce* syntaxin, which then acquires the ability to bind synaptic vesicles after the UNC-13N-dependent release of UNC-18 from the complex. The mammalian homolog Munc-13–1 has been shown to bind both syntaxin and Doc2 (Betz et al., 1997; Orita et al., 1997). Assays with full-length UNC-13 and UNC-18 were not done, however, because we have not yet established a method for preparation of the recombinant UNC-13 corresponding to the C-terminal region. Betz et al. (1997) performed coprecipitation and yeast two-hybrid experiments with Munc-13–1 and Munc-18–1. However, they did not observe the interaction.

We have found that UNC-13N, UNC-18, and *Ce* syntaxin form complexes with each other, although stable complex formation between UNC-13N and *Ce* syntaxin was not observed. We were unable to detect a ternary complex consisting of the three proteins. Instead, we observed that the UNC-18–*Ce* syntaxin complex is dissociated by UNC-13N. Recently, Betz et al. (1998) found that UNC-13 has binding ability to phorbor ester and diacylglycerol. Therefore, it is likely that UNC-13 activated by either Ca^{2+} or diacylglycerol dissociates UNC-18 from the UNC-18–*Ce* syntaxin complex.

In summary, we have demonstrated directly that the UNC-18–*Ce* syntaxin complex is dissociated by UNC-13N without forming a stable ternary complex. However, our findings do not exclude additional roles for the three proteins in synaptic transmission, given that the three proteins interact with numerous synaptic factors. For example, the syntaxin-1a–Munc-18 complex is dissociated by tomosyn (Fujita et al., 1998).

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