Functional Properties of the unc-64 Gene Encoding a Caenorhabditis elegans Syntaxin*

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Phenotypes of Caenorhabditis elegans unc-18 and unc-64 gene mutations are similar. While unc-18 is known to be essential for normal synaptic transmission (Hosono, R., Hekimi, S., Kamiya, Y., Sassa, T., Murakami, S., Nishiwaki, S., Miwa, J., Taketo, A., and Kodaira, K.-I. (1992) J. Neurochem. 58, 1517–1525), the function of unc-64 remains unclear. Here we describe the cloning, and the molecular and genetic characterization of the unc-64 gene, especially in relation to unc-18. unc-64 encodes a protein (C. elegans syntaxin) showing sequence and structural similarities to mammalian syntaxin 1A. From unc-64, at least three types of poly(A)+ RNA are transcribed, which encode two types of syntaxin that differ in the deduced transmembrane domain. In gene expression, unc-64 closely resembles unc-18, that is, both are expressed in neural cells, especially in motor neurons, and neurons constituting head ganglia. C. elegans syntaxin binds to UNC-18 with high affinity. The unc-64 (e246) mutation producing a mild phenotype causes an Ala→Val conversion in the conserved COOH-terminal region in mammalian syntaxin 1A or Drosophila syntaxin-1A whose site is included in three types of transcripts. The binding of the mutant C. elegans syntaxin to UNC-18 is greatly reduced, indicating the mutation site contributes to the binding.

Synaptic transmission is regulated by neurochemical secretion from the presynaptic terminus. The process is accomplished by the docking of synaptic vesicles to the plasma membrane and their signal-dependent fusion. At the interaction, the proteins associated with synaptic vesicles and plasma membrane execute important roles (1). A number of membrane proteins functioning in the vesicles mediate transport from synaptic vesicles to the plasma membrane. Syntaxin is one such protein functioning in several steps of the secretory process (2–5). The mammalian syntaxin has been found in a 20 S complex thought to be responsible for the docking of synaptic vesicles at the presynaptic plasma membrane (6, 7). Synaptobrevin/vesicle-associated membrane protein-like proteins residing on transport vesicles are donated as vesicle-associated soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein-SNARE (v-SNAREs), and their target membrane proteins including syntaxin as target membrane-associated SNAP receptors (t-SNAREs) (8). Although the binding of synaptic vesicles to the plasma membrane is critical to the synaptic transmission, its regulation remains unknown. Recently syntaxin 1A has been shown to bind the C. elegans UNC-18 protein homolog, n-sec-1 or Munc-18 (9–11). Since the UNC-18 homolog does not copurify with stoichiometric amounts of other proteins in the 20 S fusion complex, it is suspected that it functions at a different step in this process (9).

We have been studying the C. elegans UNC-18 protein, which is thought to play an important role in mediating synaptic vesicle interaction with plasma membrane (12–15). UNC-18 is rich in charged and hydrophilic amino acids. The protein is expressed exclusively in neurons; a large amount is insoluble but a significant amount is in bound form. In unc-64 gene mutants, the release of neurotransmitters is defective, probably due to blocked presynaptic function (12, 13, 16).

We have sought genetic and biochemical evidence for interaction between UNC-18 and C. elegans syntaxin. To this end, we cloned the genome and cDNAs encoding C. elegans syntaxin. Genetic and biochemical analyses show that the unc-64 gene encodes C. elegans syntaxin and is functionally related to unc-18. In a preliminary study we showed that UNC-18 binds to GST-C. elegans syntaxin fusion protein (14). We report here the kinetics of the binding between UNC-18 and C. elegans syntaxin. During the study, a preliminary report that unc-64 encodes C. elegans syntaxin was published (17).

**EXPERIMENTAL PROCEDURES**

Strains—General methods for C. elegans culture and genetic manipulation were as described previously (18). The following mutations were used: LGIII, unc-64 (e246), dpy-18 (e364); LGX, unc-18 (e81, cn347, md1094, md1294), lon-2 (e678). Double mutants with unc-64 and unc-18 were generated from the progeny derived from unc-18/lon-2, unc-64/dpy-18. Homozygous unc-18/unc-18, unc-64/unc-64 genotype

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB008842, AB008843, and AB008844.

The C. elegans syntaxin family (SYN1, SYN2, SYN3, and SYN4) discussed in this paper has been submitted to the SwissProt Data Bank with accession number(s) Q20024, Q20574, Q20797, and Q91409.

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The abbreviation used are: NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein; v-SNARE, vesicle-associated soluble NSF attachment protein-SNARE receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; t-SNAREs, target membrane-associated SNAP receptors; ACh, acetylcholine; GST, glutathione S-transferase; YAC, yeast artificial chromosome; RT, reverse transcriptase; PCR, polymerase chain reaction; LG, linkage group; bp, base pair(s); kb, kilobase pair(s).

1 S. Harada and R. Hosono, unpublished results.
was determined by crossing with +/unc-64 and +/+ males.

Isolation and Analysis of cDNA Clones—cDNAs encoding C. elegans syntaxins were screened from a C. elegans cZAP cDNA library (a gift from R. Barstead and R. Waterston) using the RT-PCR product and the cm04d2 C. elegans cDNA (provided by C. Martin and R. Waterston) as probes. Longer clones (10 in all) were characterized by restriction analysis and sequenced after subcloning into M13 vectors using the BLAST and/or FASTA programs.

Germine Transformation and unc-64 Rescue—Germine transformation was performed using standard techniques (19). Cosmid clones located within the unc-64 gene and the genomic clones were microinjected into the mitotic germine of hermaphrodites, according to the method developed by Mello et al. (19). For rescue experiments, the relevant DNA was injected at concentration of 5–100 μg/ml pRF4, a plasmid containing the rol-6 (sa1006) allele, was cojected as a marker to identify transgenic animals. Since unc-64 mutants are abnormal in locomotion, a line was considered to be rescued if the transgenic homozygous unc-64 animals could generate a normal rolling phenotype.

GFP Constructs—Expression constructs were made using the 6.0-kbp HindIII fragment including the putative unc-64 5'-regulatory region to direct expression of the GFP expression plasmid pPD 95.75.3 This comprised nucleotides of genomic DNA immediately upstream of the initiator methionine codon in exon 1 plus 3.5 kb of additional down stream genomic sequence including exon 1 through exon 5.

GST-Syntaxin Fusion Proteins—Vectors encoding GST fusion proteins with the C. elegans syntaxin were constructed using PCR products and the pGEX vector essentially as described previously (14).

Molecular Biology—Standard molecular biology protocols (20) were followed except where noted. Total RNA was extracted by the guanidinium thiocyanate/cesium chloride centrifugation method, and poly(A)+ RNA was isolated with Oligotex-dT30 (Takara). Northern blots were performed using approximately 5 μg of poly(A)+ RNA per lane and transferred to Hybond N+ (Amersham, Buckinghamshire, UK) for hybridization. The Northern blot was probed with the full-length C. elegans syntaxin A cDNA, which had been labeled according to the manufacturer’s directions (Strategenes). DNA sequence analysis was performed by an Applied Biosystems 377 sequencer. Sequencing reaction was performed according to dye primer protocol (Applied Biosystems Inc.).

Identification of unc-64 Mutation—The unc-64 (e246) cDNA was prepared by RT-PCR using four different primers: STX-1 (5'-GA-CAGTTCCACAACTAGGAAC-3', -58 to -39), STX-2 (5'-ATTAGGC-CAGTGATGTTAGT-3', 842–823), STX-3 (5'-ACAGAAGTATAC-GAG-3', 412–430), and STX-4 (5'-TTTCCCTCAGCCATTCT-3', 505–486). These primers cover the entire C. elegans syntaxin A cDNA. After 30 cycles of amplification using LA Taq polymerase (Takara), the products were cloned into the pCR TMII cloning vector (Invitrogen).

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In Situ Hybridization—The antisense riboprobe was labeled with digoxigenin-UTP. The mean length of the in vitro transcribed RNA was reduced to 100–200 bp by alkaline hydrolysis. In situ hybridization was performed as described by Mitani et al. (21), with slight modifications. The collected worms were fixed in 3.7% formaldehyde. After washing three times with methanol for 5 min, specimens were treated twice for 5 min each time with 100 mtrriethanolamine (pH 8.0) and then with
Hybridization was done in hybridization buffer containing 50% formamide, 5\times\text{SSC}, 100\,\text{mg/ml} salmon sperm DNA, 100\,\text{mg/ml} yeast RNA, 0.1% SDS, and 1\,\text{mg/ml} RNA probe at 60 °C overnight. For detecting the hybridized signal, specimens were incubated with anti-digoxigenin antibody tagged on alkaline phosphatase. Visualization of bound alkaline phosphatase was performed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride as substrates.

**RESULTS**

**Phenotypes of the unc-64 Mutation and Genetic Interactions with unc-18**—Genetic and biochemical analyses revealed that the unc-64 and unc-18 genes are functionally related (16, 22). That is, both gene mutations result in uncoordinated locomotion, abnormal accumulation of acetylcholine, resistance to acetylcholinesterase inhibitors, and developmental retardation (18, 22, 24). The phenotype is recessive and is probably produced by a defect in neurotransmitter release. To elucidate further the relationship between the two genes, we constructed double mutant strains carrying unc-64 and unc-18 mutations (Table I). A double mutation of unc-18 allele, e81 or cn347 and unc-64 (e246) was lethal. Double mutants showing mild phenotype were more severely defective in locomotion and development than the single mutants. ACh levels were elevated by 100 mM triethanolamine containing 0.5% acetic anhydride for 5 min. Hybridization was done in hybridization buffer containing 50% formamide, 5\times\text{SSC}, 100\,\text{mg/ml} salmon sperm DNA, 100\,\text{mg/ml} yeast RNA, 0.1% SDS, and 1\,\text{mg/ml} RNA probe at 60 °C overnight. For detecting the hybridized signal, specimens were incubated with anti-digoxigenin antibody tagged on alkaline phosphatase. Visualization of bound alkaline phosphatase was performed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride as substrates.

**Cloning of the unc-64 Gene**—All members of the syntaxin family contain a highly conserved sequence located in the cytoplasmic domain. C. elegans syntaxin homologs were amplified by RT-PCR using degenerated primers derived from two short regions in the carboxyl-terminal portion of mouse syntaxin 1A. A C. elegans cDNA library was screened with the 0.5-kb PCR product as probe, and 58 cDNA clones were obtained. The cDNAs were mapped at the region close to unc-64 on the right of chromosome III (Fig. 1A). We tested cosmids B0502 and C26A4 in germline transformation experiments with unc-64 gene allele e246, and an 8.0-kb XbaI genomic DNA fragment was also injected. Wild-type locomotion was restored for the unc-64 allele following the injection of the two cosmids and the genomic clone, indicating that unc-64 encodes C. elegans syntaxin.

Most cDNA clones were sequenced and classified into three groups (A, B, and C C. elegans syntaxin cDNAs), all of which were transcribed by trans-splicing SL1. The A, B, and C cDNAs contained a highly conserved sequence located in the cytoplasmic domain. C. elegans syntaxin cDNAs all encode the potential transmembrane domain at the 3’ regions.
sequences differ in only 92, 109, and 214 bp, respectively, at the 3' termini. The three clones contain a long open reading frame encoding 291 amino acids (Fig. 2). Clone B differs from A in 24 amino acid residues, a potential integral domain. Clone C differs from B only at the 3' noncoding region.

We sequenced 14 kb of genomic DNA including sequences of C. elegans syntaxin cDNAs. By comparing the genomic and cDNA sequences, we determined that the unc-64 gene is composed of eight exons. The eighth exon in the unc-64 B and C cDNAs results from an alternative splicing within intron 8 for the A cDNA (Fig. 1B).

Molecular Identification of unc-64 Mutation—We analyzed the unc-64 mRNA from e246 animals in the hope of identifying the defect. The mutant RNA is unchanged in content and in size. We generated cDNA from e246 by RT-PCR and sequenced it by linear amplification PCR. The e246 mutation occurs in the coding region of the mRNA (Figs. 2A and 3C), in which conserved alanine at position 248 is converted to valine.

Protein Structure of C. elegans Syntaxin—We compared the sequence of the predicted syntaxin with human and Drosophila syntaxins (Fig. 3A). C. elegans syntaxin shares extensive overall sequence homology with other syntaxins: 63% identical to human, and 66% identical to Drosophila syntaxin (25). C. elegans syntaxin is hydrophilic at the N terminus (Fig. 3B). The predicted secondary structure is an amphipathic α-helical wheel with a hydrophobic face (Fig. 3C). That is, basic amino acids are aligned along one face of the helix and hydrophobic amino acids along the other. Amphipathic helices are

**Fig. 3.** Protein structure of C. elegans syntaxin. A, similarity of the predicted amino acid sequences for human syntaxin 1A, Drosophila syntaxin-1A, and C. elegans syntaxin A. Alignment of sequences is shown with gaps introduced to optimize matches with GENETYX software. The residues marked by stars are identical. B, hydrophobicity of C. elegans syntaxins. (-----) C. elegans syntaxin A, (-----) C. elegans syntaxin B and C. C, helical-wheel representations of the residues Glu33–Ala50 (I), Asp78–Leu95 (II), and Met204–Ala221 (III). Hydrophobic residues are represented by circles. The mutation site of e246 at the Ala248 → Val is indicated by an arrow above the schematic representation of the coding region.
known to be frequently involved in both intra- and intermolecular protein-protein interactions (26, 27). A common motif among mammalian vesicle-associated membrane protein, SNAP-25, and syntaxin proteins is present in the first helical wheel. The amino acid sequence of the potential helical wheel domain in C. elegans syntaxin is very similar to that in other syntaxins; 15 of 18 amino acids are identical. In addition, positive amino acids are clustered in the C-terminal portion. In structure, C. elegans syntaxin A differs from C. elegans syntaxin B and C in 24 C-terminal amino acids, the potential transmembrane domain.

The unc-64 Gene Transcripts—To examine the expression of the unc-64 gene transcript, Northern blot analyses were performed. As shown in Fig. 4A, at least four bands were detected using the C. elegans syntaxin A cDNA as a probe. The developmental profile of the expression pattern of the major transcripts, 1.0- and 3.0-kb RNAs, are similar (Fig. 4B). That is, two of the transcripts are most abundant at the embryonic stage and decrease with the progress of development. The 1.0-kb transcript is identical to the C. elegans syntaxin cDNAs in size. To identify the source of the 3.0-kb RNA, Northern blotting using various DNA fragments from the unc-64 genome as probe was carried out. However, we failed to find the region of the genome corresponding to the large transcript.

Expression of the unc-64 Gene—To determine where and when the unc-64 gene is expressed during C. elegans development, we used in situ hybridization and transformation with the unc-64::GFP reporter gene. The gene is specifically expressed in neurons, and the transcript is exclusively localized also in neurons (Fig. 5). The pattern of unc-64 gene expression and transcriptional localization is very similar to that of the unc-18 gene. However, the unc-18 transcript presents abundantly in the hermaphrodite specific neurons, whereas the unc-64 transcript is not detectable in this type of neuron. Further, the unc-64 gene is expressed in almost all neurons constituting the head ganglion, while the unc-18 gene is expressed in only a limited number of neurons, including AVE, SIBD, and SIBV interneurons.4

Interaction between C. elegans Syntaxin and UNC-18—We already showed that in the cytoplasm C. elegans syntaxin binds to UNC-18 (14). To examine further the interaction of the two proteins, we assessed the binding of purified recombinant C. elegans syntaxin to UNC-18 (Fig. 6). Both the N-terminal and C-terminal halves of C. elegans syntaxin were devoid of the binding ability. Only the intact cytoplasmic domain has the binding ability (Fig. 6, A and B), indicating that a full-length of C. elegans syntaxin is necessary for the binding. We compared the binding of the wild-type and mutant C. elegans syntaxin to UNC-18 (Fig. 7). Half-maximal binding of the wild-type C. elegans syntaxin to UNC-18 occurred at 50 nM. The binding ability is approximately identical to the ability between n-sec-1 and syntaxin 1A (∼80 nM) (10). However, the binding affinity between the mutant C. elegans syntaxin and UNC-18 was greatly reduced with half-maximal binding at 300 nM.

**DISCUSSION**

*unc-64 Encodes the Syntaxin 1A Homolog—* During the transport of synaptic vesicles, docking/fusion is regulated by synaptic proteins. A previous genetic and biochemical study suggested that the *unc-64* gene is required for the synaptic transmission (16). Mutation in *unc-64* results in resistance to inhibitors of acetylcholinesterase and in an abnormal accumulation of ACh, probably due to a defect in synaptic transmission (13). Here we demonstrate that the gene encodes a mammalian

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4 T. Ishihara, personal communication.
syntaxin 1A homolog.

By Northern blotting, we detected at least four positive bands (3.0- and 1.0-kb major bands, and 3.3- and 1.6-kb minor bands) using the *C. elegans* syntaxin A cDNA as probe. Yeast artificial chromosome grids covering almost all the *C. elegans* genome were constructed by Coulson et al. (28). By hybridization to the YAC grid filter, we detected three YAC clones, Y47C12, Y54C1, and Y21D2, other than Y43F4 covering *unc-64*. Y47C12 hybridized strongly and was located to the right of *lev-10* on chromosome I (LG1). Y54C1 and Y21D2 hybridized weakly and were located to the left side of *unc-64* (LGIII), and between *gpa-3* and *gpa-1* (LGV), respectively. These results show that *C. elegans* syntaxins constitute a family as to mammalian syntaxins. We are currently analyzing *C. elegans* genes encoding syntaxins other than *C. elegans* syntaxin. The *C. elegans* genome sequence project identifies several genes encoding syntaxins: SYN1, SYN2, SYN3, and SYN4. The predicted peptide sequences of these genes are not strikingly similar to *C. elegans* syntaxin: SYN1 (27.2% identity), SYN2 (24.2%), SYN3 (22.4%), and SYN4 (25.6%). The SYN1 coding peptide is more homologous to *C. elegans* syntaxin in amino acid sequence and size (299 amino acids) than the other three SYN genes. The SYN1 gene is located close to the region covered by Y21D2 on LGV, suggesting that the gene may encode a member of the *C. elegans* syntaxin family.

To identify the source of the 3-kb RNA detected by the *C. elegans* syntaxin cDNA probe, we carried out Northern blotting with *unc-64* genomic DNA containing the upstream 5'-noncoding regions, introns, and the downstream 3'-noncoding regions as probe. Northern blotting with the 3'-noncoding region as a probe detected only the 0.7-kb band (data not shown). We therefore extensively screened the *C. elegans* cDNA library and got the cDNA clones with 3-kb long inserts. However, we were unable to find the corresponding genomic sequence. Therefore, it is unlikely that the 3-kb RNA is transcribed by the polycistronic transcription of the *unc-64* gene. We have not yet determined the nature of the 0.7-kb transcript.

**unc-64 Gene Expression**—The pattern of *unc-64* expression suggests the gene is directly involved in sensory transduction. *unc-64::GFP* gene fusions are expressed at high levels in the nerve ring and ventral nerve cord. It is not yet clear whether the three transcripts are differently expressed, either spatially or temporally. *In situ* hybridization and *unc-64::GFP* transgenic reporters revealed that *unc-64* is specifically expressed in neurons. However, the two methods gave different weights to each expression pattern that is, the *unc-64::GFP* fusions were expressed around the nerve ring and ventral nerve cord, whereas *in situ* hybridization detected more *unc-64* gene transcripts in motor neurons than in interneurons. These differences might reflect that *in situ* hybridization is quantitative, whereas GFP expression is qualitative.

**Interaction between unc-18 and unc-64**—We provide direct evidence that *unc-18* and *unc-64* genetically interact. First, the double mutation results in a more severe defect than do single mutations. Second, both gene products have high binding affinity. Third, the binding ability was reduced by the *unc-64* mutation. *C. elegans* syntaxin binds to UNC-18 with high affinity (Fig. 7). The cytoplasmic domain of *C. elegans* syntaxin

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**Fig. 6. Interaction of *C. elegans* syntaxin and UNC-18.** *A*, the structure of *C. elegans* syntaxin and UNC-18. *B*, *in vitro* binding of each part of *C. elegans* syntaxin (STX) and UNC-18. Under incubation with GST-STX 6 (lane 7), bound UNC-18 (67 kDa) was eluted with 10 mM glutathione. Lane 1, size markers; lane 2, GST-STX 2; lane 3, GST-STX 1; lane 4, GST-STX 3; lane 5, GST-STX 4; lane 6, GST-STX 5; lane 7, GST-STX 6.
has clustered, reactive hydrophobic amino acids consisting of helical wheels. To elucidate which domain contributes to the binding with UNC-18, we carried out a binding assay. The experiments assessing the ability of the binding domain (Fig. 6, A and B) suggest that the intact cytoplasmic domain of *C. elegans* syntaxin is essential for binding. Mutation at the C-terminus of *C. elegans* syntaxin reduces the binding ability to UNC-18. However, it is unlikely that only the C-terminal region contributes to the binding. While the interaction could be mediated directly, the simplest model is that *C. elegans* syntaxin interacts with UNC-18 as receptor and ligand, respectively. Vertebrate syntaxin binds to synaptobrevin, a component of synaptic vesicles. UNC-18 has a regulatory function in synaptic vesicle-mediated directly, the simplest model is that syntaxin functions following the docking of synaptic vesicles to the plasma membrane through the transmitter release. The UNC-18 (e246) mutant may be defective in two directly independent steps of the synaptic vesicle pathway which additively affect the transmitter release. Although the protein is yet to be found interacting in the region of the e246 mutation site, it is noticeable that charged amino acids (five Lys and two Arg) are clustered near the mutation site. A cluster of eight basic residues in K-Ras may electrostatically interact with the acidic phospholipids on the leaflet of the plasma membrane (24, 29). If this is the case in *C. elegans* syntaxin, it is likely that conversion of 248 alanine to valine affects the interaction between UNC-18 and *C. elegans* syntaxin.

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