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# Three Splicing Variants of Tomosyn and Identification of Their Syntaxin-Binding Region[1]

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**We have recently isolated a neural tissue-specific syntaxin-1-binding protein, named tomosyn, which is capable of dissociating Munc18/n-Sec1/rbSec1 from syntaxin-1 to form a 10S tomosyn complex, an intermediate complex converted to the 7S SNARE complex. We isolated here two splicing variants of tomosyn: one had 36 amino acids (aa) insertion and another had 17 aa deletion. We named original one m-tomosyn, big one b-tomosyn, and small one s-tomosyn. s-Tomosyn as well as m-tomosyn was mainly expressed in brain whereas b-tomosyn was ubiquitously expressed. All the isoforms bound to syntaxin-1, but not to syntaxin-2, -3, or -4, and had a region highly homologous to VAMP, another syntaxin-binding protein. This region was necessary but not sufficient for high-affinity binding of tomosyn to syntaxin-1.** 

We have recently isolated a novel syntaxin-binding protein, named tomosyn (1). Tomosyn is a neural tissue-specific protein with 1,116 aa. In neuron, it is highly concentrated in presynaptic nerve terminals. Syntaxin is a t-SNARE which is localized on the presynaptic plasma membrane and forms a complex with SNAP-25, another t-SNARE, and VAMP and synaptotagmin, v-SNAREs, in process of the docking and/or fusion of synaptic vesicles with the presynaptic plasma membrane during neurotransmitter release (for reviews, see Refs. 2-4). Syntaxin furthermore interacts with Munc18/n-Sec1/rbSec1 and this interaction inhibits the complex formation of syntaxin with other SNARE components (5). Syntaxin consists of at least six members (6). Tomosyn binds to syntaxin-1 with the highest affinity among many syntaxin-1-binding proteins, including SNAP-25, VAMP, and Munc18/n-Sec1/rbSec1, and is capable of dissociating Munc18/n-Sec1/rbSec1 from syntaxin-1 to form a 10S tomosyn complex, consisting of syntaxin-1, SNAP-25, and synaptotagmin, but not of VAMP (1). This complex is converted by replacement of tomosyn with VAMP to form a 7S SNARE complex, consisting of syntaxin-1, SNAP-25, VAMP, and synaptotagmin (1). The 7S complex is finally converted to a 20S complex by addition of $\alpha$-SNAP and NSF (7, 8) which may cause docking and/or fusion of synaptic vesicles with the presynaptic plasma membrane. Thus, tomosyn plays an important role in the neurotransmitter release processes.

Syntaxin-1 is neural tissue-specific, but other isoforms are ubiquitously expressed and implicated in vesicle trafficking (6, 9). We therefore attempted here to examine whether tomosyn has an isoform(s) which is expressed in non-neural tissues.

## MATERIALS AND METHODS

*Materials and chemicals.* GST-Syntaxin-1a was purified as described (10). DNA fragments encoding various fragments of m-tomosyn with the *Bam*HI and *Kpn*I sites were generated by PCR. Each DNA fragment was digested by *Bam*HI and inserted into the *Bam*HI site of pRSET plasmid. The $His_6$-tagged C-terminal fragment of m-tomosyn (1031-1116 aa) was purified from *Escherichia coli* overexpressing this protein (1).

*Plasmid construction of b- and s-tomosyns.* We prepared fragments including variant regions of b- and s-tomosyns by the PCR method using a rat kidney Quick-clone cDNA (Clontech) and the following primers: 5′-GGCCGGATCCGGTACCACAGAAACGTCAAGGTTACAGC-3′ and 5′-GGCCGGATCCGGTACCTTCAGCGTGATGACAAAGGC-3′. The fragments of b- and s-tomosyns were digested by *Sph*I and *Bgl*II and each resulting fragment was

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Abbreviations: aa, amino acids; SNARE, soluble NSF attachment protein receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; SNAP, soluble NSF attachment protein; NSF, N-ethylmaleimide-sensitive factor; GST, glutathione-*S*-transferase; RT, reverse transcriptase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; bp, base pairs.

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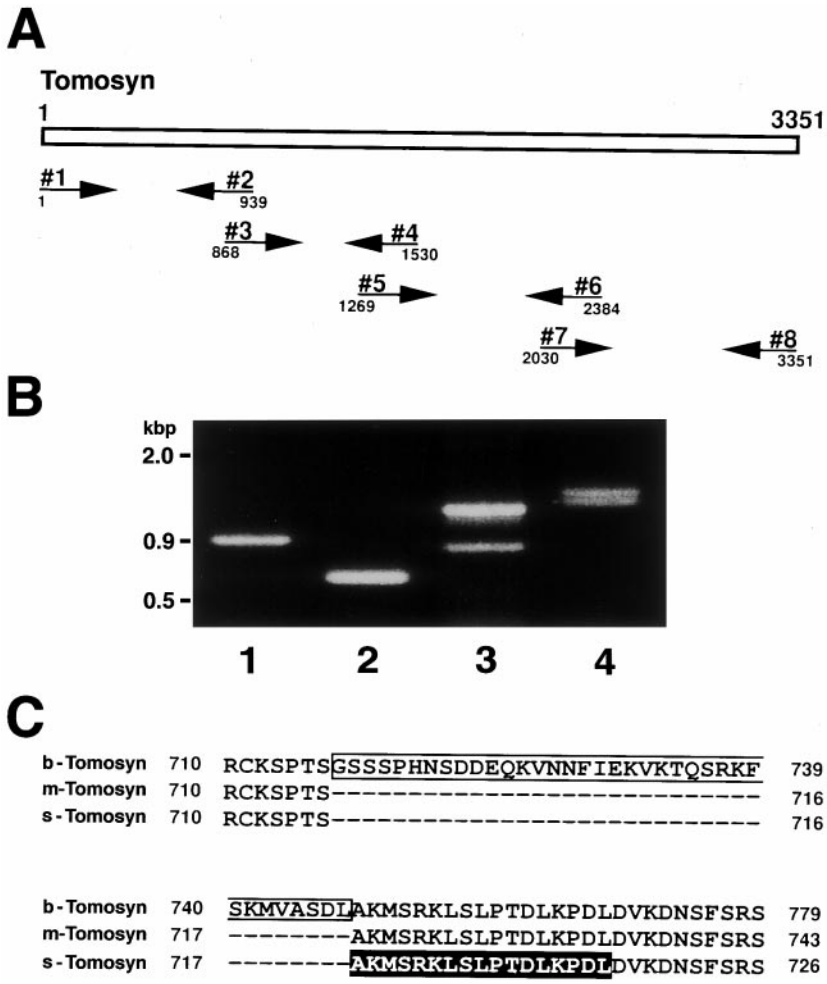


**FIG. 1.** Identification of splicing variants of tomosyn. PCR amplification was performed using a rat kidney Quick-clone cDNA and various sets of primers. *A*, schematic diagram of the primers. Primer #1, 5′-GGCCGGATCCGGTACCATGAGGAAATTCAACATCAGGAAGGTGC-3′; primer #2, 5′-GGCCGGATCCGGTACCTCACAAAATAATAAAAGGTTCCCCCG-3′; primer #3, 5′-GGCCGGATCCGGTACCCCCGAGCCGTGCAAGCCTAT-3′; primer #4, 5′-GGCCGGATCCGGTACCCCAGGAGATGATCTGAATGG-3′; primer #5, 5′-GGCCGGATCCGGTACCACAGAAACGTCAAGGTTACAGC-3′; primer #6, 5′-GGCCGGATCCGGTACCTTCAGCGTGATGACAAAGGC-3′; primer #7, 5′-GGCCGGATCCGGTACCTCCTTATCGGAGAGAACCGAGG-3′; and primer #8, 5′-GGCCGGATCCGGTACCTCAGAACTGGTACCACTTCTTATCTTTG-3′. *B*, electrophoresis of PCR products. Lane 1, primers #1 and #2; lane 2, primers #3 and #4; lane 3, primers #5 and #6; and lane 4, primers #7 and #8. *C*, aligned amino acid sequences of b-, m-, and s-tomosyns. The insertion and deletion are indicated by a box and black shading, respectively.

inserted into the *Sph*I and *Bgl*II sites of pGEX-4T2-m-tomosyn that was digested by *Sph*I and *Bgl*II. A DNA fragment encoding full-length b- or full-length s-tomosyn was prepared by digesting the plasmid by *Bam*HI. The fragment was inserted into the *Bam*HI site of pRSET plasmid. The plasmids were used as pRSET-b- and pRSET-s-tomosyns.

*RT-PCR analysis.* Total RNA was isolated from various rat tissues by using ISOGEN Reagent (Nippon Gene) and reverse-transcribed by using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech). PCR was performed by using a PE Applied Biosystems PCR kit with primers specific for each isoform. The PCR products were electrophoresed and visualized by staining with ethidium bromide.

*Blot overlay.* Various $^{35}$S-labeled fragments of m-tomosyn were generated using the TNT T7 quick coupled transcription/translation system (Promega Corporation). GST-Syntaxin-1a (5 pmol) was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was washed with phosphate buffered saline (PBS) three times at 4°C for 5 min and sequentially blocked with PBS containing 0.1% (w/v) Tween 20 and 5% (w/v) defatted powder milk at 4°C for 2 h. The membrane was then incubated with various $^{35}$S-labeled fragments of m-tomosyn (~1 pmol) in the presence or absence of various amounts of the $His_6$-tagged C-terminal fragment in PBS containing 0.1% (w/v) Tween 20 and 5% (w/v) defatted powder milk at 4°C for 12 h. After the incubation, the membrane was washed with PBS containing 0.1% (w/v) Tween 20 three times at 4°C for 5 min, followed by autoradiography using an image analyzer (Fujix BAS-2000).

*Other procedures.* SDS-PAGE was performed as described (11). Standard molecular biological techniques were used for construction of plasmids, DNA sequencing, and PCR (12). DNA sequences were determined using ALFred DNA sequencer (Amersham Pharmacia Biotech) and PCRs were performed using GeneAmp PCR System 2400 (PE Applied Biosystems).

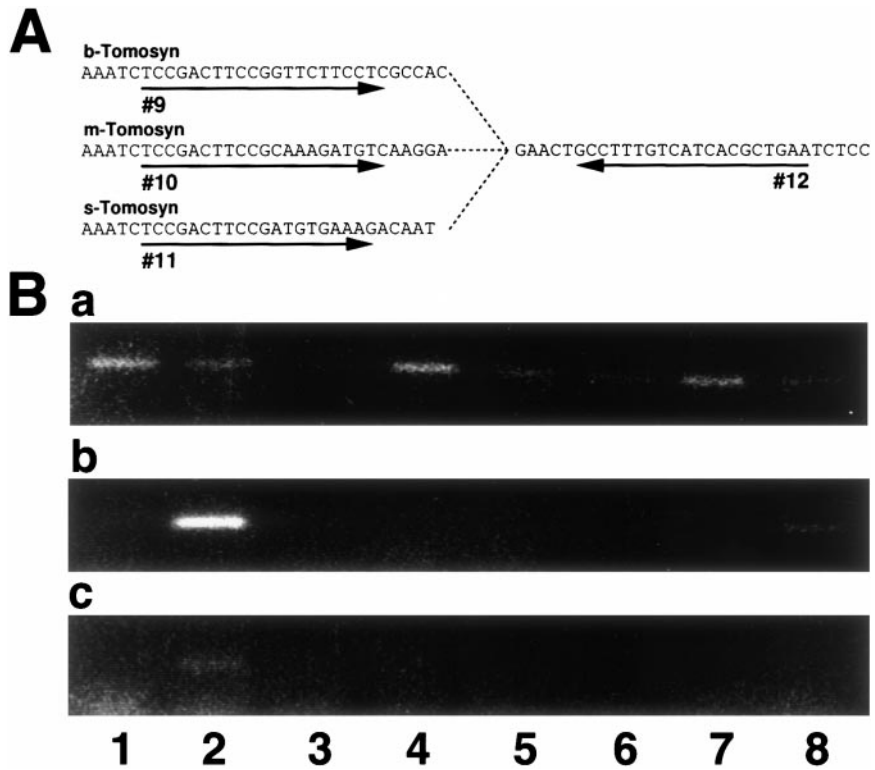


**FIG. 2.** RT-PCR analyses of the b-, m-, and s-tomosyn mRNAs of various rat tissues. RT-PCR analysis was performed using a set of primers specific for each tomosyn isoform. *A,* schematic diagram of the primers. Primer #9, 5′-GGCCGGATCCGGTACCTCCGACTTCCG-GTTCTTCCTC-3′; primer #10, 5′-GGCCGGATCCGGTACCTCCGACTTCCGCAAAGATGTC-3′; primer #11, 5′-GGCCGGATCCGGTACCTC-CGACTTCCGATGTGAAAG-3′; and primer #12, 5′-GGCCGGATCCGGTACCTTCAGCGTGATGACAAAGGC-3′. Primers #9 and #12, primers #10 and #12, and primers #11 and #12 were used as sets of primers specific for b-, m-, and s-tomosyns, respectively. *B,* electrophoresis of PCR products. *a,* b-tomosyn; *b,* m-tomosyn; and *c,* s-tomosyn. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; and lane 8, testis.

## RESULTS

*Identification of splicing variants of tomosyn.* We screened a rat kidney cDNA library using a nick-translated probe encoding full-length tomosyn. Two positive clones were isolated and the nucleotide sequence of each clone was determined. Of the clones isolated, one clone encoded the C-terminal region of tomosyn with 17 aa deletion and the other clone encoded the C-terminal region of tomosyn with 36 aa insertion. These results suggest that tomosyn has at least two splicing variants.

To confirm that these isolated clones are splicing variants of tomosyn, PCR amplification was performed using a rat kidney Quick-clone cDNA and primers shown in Fig. 1A. When PCR amplification was performed by use of primers #1 and #2 or primers #3 and #4, only one amplified fragment was detected (Fig. 1B). When PCR amplification was performed by use of primers #5 and #6, three amplified fragments were mainly detected. When nucleotide sequences of the PCR products were determined, a fragment of tomosyn with 36 aa insertion and one with 17 aa deletion were detected in addition to an original fragment of tomosyn. The nucleotide sequences of the insertion and deletion were identical to those of the clones isolated in the rat kidney cDNA library. When PCR amplification was performed by use of primers #7 and #8, two amplified fragments were mainly detected. When nucleotide sequences of the PCR products were determined, a fragment of tomosyn with the same insertion and one with the same deletion were detected in addition to an original fragment of tomosyn.

These results indicate that tomosyn has at least three isoforms. We named original one m-tomosyn (GenBank accession number U92072), big one b-tomosyn (GenBank accession number AF118889), and small one s-tomosyn (GenBank accession number AF118890). The reduced amino acid sequences of the variable regions are shown in Fig. 1C.

*Tissue distribution of tomosyn isoforms.* Tissue distribution of tomosyn isoforms was examined by RT-PCR using primers specific for each isoform shown in Fig. 2A. When primers specific for b-tomosyn were used, a fragment with about 350 bp was detected in heart, brain, lung, liver, skeletal muscle, kidney, and testis (Fig. 2Ba). When primers specific for m-tomosyn

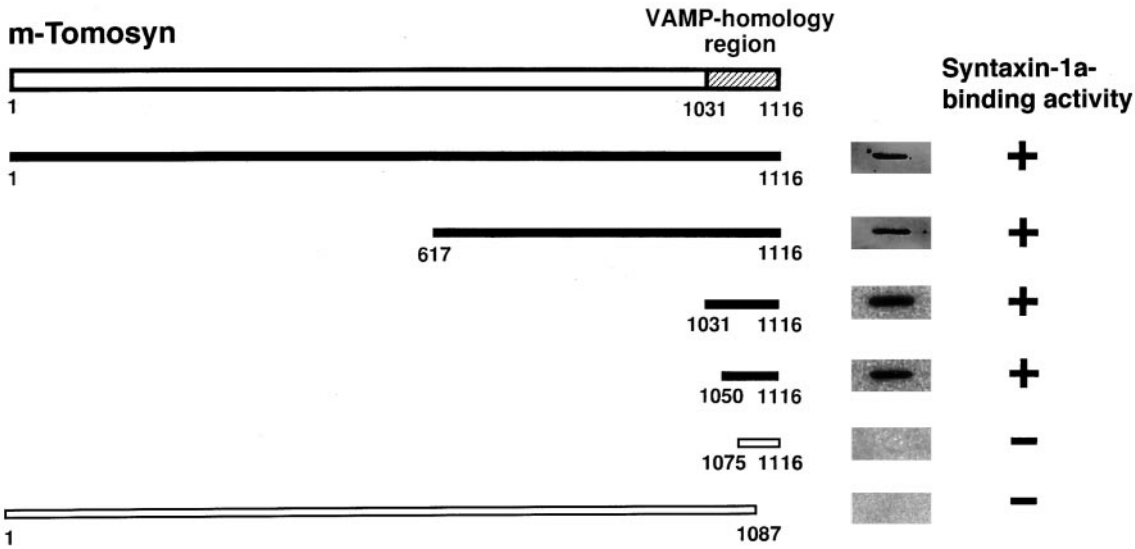


**FIG. 3.** Syntaxin-1-binding region of m-tomosyn. GST-Syntaxin-1a was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with various $^{35}$S-labeled fragments of m-tomosyn. The $^{35}$S-labeled fragment bound to GST-syntaxin-1a was detected.

were used, a fragment with about 230 bp was mainly detected in brain and to a much lesser extent in testis (Fig. 2Bb), consisting with our earlier observation (1). When primers specific for s-tomosyn were used, a fragment with about 200 bp was mainly detected in brain (Fig. 2Bc). The length of each fragment was consistent with that estimated from the respective nucleotide sequences. These results indicate that b-tomosyn was ubiquitously expressed whereas s-tomosyn as well as m-tomosyn was mainly expressed in brain.

*Syntaxin-1-binding region of tomosyn.* We determined the syntaxin-1-binding region of m-tomosyn using blot overlay. The $^{35}$S-labeled fragment (1050-1116 aa), but not the $^{35}$S-labeled fragment (1075-1116 aa) or the $^{35}$S-labeled fragment (1-1087 aa), bound to GST-syntaxin-1a (Fig. 3). The aa sequences of the syntaxin-1a-binding region (1050-1116 aa) were identical among three tomosyn isoforms. We have previously shown that m-tomosyn binds to syntaxin-1, but not to syntaixn-2, -3, or -4 (1). Consistently, full-length b- and s-tomosyns as well as full-length m-tomosyn bound to syntaxin-1a, but not to syntaxin-2, -3, or -4 (data not shown). The $^{35}$S-labeled fragment (1050-1116 aa) did not bind to syntaxin-2, -3, or -4, either (data not shown).

To confirm that the C-terminal region of m-tomoysn binds to syntaxin-1, we prepared recombinant $His_6$-tagged C-terminal fragment (1031-1116 aa) of tomosyn and examined the inhibitory effect of this fragment on the binding of $^{35}$S-labeled full-length m-tomosyn to GST-syntaxin-1a. The C-terminal fragment showed an inhibitory effect in a dose-dependent manner, but the amount of the C-terminal fragment necessary for the inhibition of the binding of $^{35}$S-labeled full-length m-tomosyn to GST-syntaxin-1a was more than 100-fold of that of $^{35}$S-labeled full-length m-tomosyn (Fig. 4).

These results indicate that the C-terminal region (1050-1116 aa) of the three tomosyn isoforms is necessary but not sufficient for high-affinity binding of tomosyn to syntaxin-1.

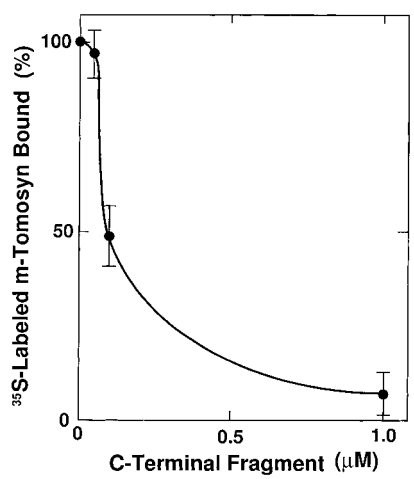


**FIG. 4.** Inhibition of the binding of m-tomosyn to syntaxin-1 by the C-terminal fragment of m-tomosyn. GST-Syntaxin-1a was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with the $^{35}$S-labeled m-tomosyn in the presence of various amounts of the $His_6$-tagged C-terminal fragment of m-tomosyn. The $^{35}$S-labeled m-tomosyn bound to GST-syntaxin-1a was detected. The amount of the $^{35}$S-labeled m-tomosyn bound to syntaxin-1a was expressed as a percentage of that in the absence of the $His_6$-tagged C-terminal fragment of m-tomosyn. The values are mean ± S.E. of three independent experiments.

## DISCUSSION

We have shown here that tomosyn has at least two splicing variants, named b- and s-tomosyns. Although s-tomosyn as well as m-tomosyn (the originally isolated type) is mainly expressed in brain, b-tomosyn is expressed in most tissues examined. We have previously shown that neural tissue-specific m-tomosyn specifically binds to neural tissue-specific syntaxin-1. Both b- and s-tomosyns specifically bind to syntaxin-1. The results, that b-tomosyn is expressed in many tissues and that syntaxin-1 is specifically expressed in neural tissue, have raised a possibility that b-tomosyn interacts with a protein other than syntaxin. Tomosyn is homologous to the *l(2)gl* gene product (L(2)gl), known as a tumor suppressor (13, 14). L(2)gl is involved in establishing cell polarity and plays a role in a signaling pathway regulating cell growth and differentiation. Moreover, L(2)gl is associated with non-muscle myosin II heavy chain (15) and part of a cytoskeletal network (16). b-Tomosyn may play a role similar to that of L(2)gl in non-neural tissues.

It has recently been shown that a fragment of tomosyn is obtained by the yeast two-hybrid screening with syntaxin-4 as a bait from a murine mast cell line MC-9 cDNA library (17). A computer analysis has revealed that this fragment of tomosyn has a region highly homologous to VAMP, another syntaxin-binding protein. The region of tomosyn (1036-1116 aa) shares 22% identity with VAMP1. On the basis of these observations, it has been predicted that tomosyn binds to syntaxin-1 through this VAMP-like region (17). We have confirmed here this region by blot overlay assay and shown that tomosyn indeed binds to syntaxin-1 through this VAMP-like region and that the fragment containing this region indeed inhibits the binding of full-length tomosyn to syntaxin-1. However, a large amount of this fragment is required for the inhibition of the binding of full-length m-tomosyn to syntaxin-1. These results suggest that the VAMP-like region is necessary but not sufficient for high-affinity binding of full-length tomosyn to syntaxin-1. The exact reason for marked difference of the affinities between the VAMP-like region alone and full-length tomosyn is not known, but other regions of tomosyn may make the ternary structure of the VAMP-like region more sensitive to syntaxin-1. This interpretation is consistent with our earlier observation that deletion of the N-terminal or C-terminal region of tomosyn results in loss of syntaxin-1-binding activity presumably due to marked decrease of the affinity of tomosyn to syntaxin-1 (1). The VAMP-like region is found in all the three tomosyn isoforms which specifically bind to syntaxin-1. This result suggests that the inserted region of b-tomosyn or the deleted region of s-tomosyn is not involved in the high-affinity binding of m-tomosyn to syntaxin-1.

We have previously shown that tomosyn in the tomosyn complex is not replaced by VAMP to form the 7S complex in a cell-free system even when VAMP is added in a large amount (1). This result is consistent with the present and previous results that the affinity of tomosyn to syntaxin-1 is much higher than that of VAMP to syntaxin-1 and that tomosyn has a VAMP-like region which may bind to syntaxin-1 in a manner competitive with VAMP. To replace tomosyn with VAMP in the tomosyn complex to form the 7S complex, there should be a mechanism which lowers the affinity of tomosyn to syntaxin-1. Clarification of this mechanism is important for our understanding of the formation of the SNARE complex.

## ACKNOWLEDGMENTS

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