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The R-SNARE Motif of Tomosyn Forms SNARE Core Complexes with Syntaxin 1 and SNAP-25 and Down-regulates Exocytosis*

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Tomosyn is a 130-kDa syntaxin-binding protein that contains a large N-terminal domain with WD40 repeats and a C-terminal domain homologous to R-SNAREs. Here we show that tomosyn forms genuine SNARE core complexes with the SNAREs syntaxin 1 and SNAP-25. *In vitro* studies with recombinant proteins revealed that complex formation proceeds from unstructured monomers to a stable four-helical bundle. The assembled complex displayed features typical for SNARE core complexes, including a profound hysteresis upon unfolding-refolding transitions. No stable complexes were formed between the SNARE motif of tomosyn and either syntaxin or SNAP-25 alone. Furthermore, both native tomosyn and its isolated C-terminal domain competed with synaptobrevin for binding to endogenous syntaxin and SNAP-25 on inside-out sheets of plasma membranes. Tomosyn-SNARE complexes were effectively disassembled by the ATPase *N*-ethylmaleimide-sensitive factor together with its cofactor α-SNAP. Moreover, the C-terminal domain of tomosyn was as effective as the cytoplasmic portion of synaptobrevin in inhibiting evoked exocytosis in a cell-free preparation derived from PC12 cells. Similarly, overexpression of tomosyn in PC12 cells resulted in a massive reduction of exocytosis, but the release parameters of individual exocytotic events remained unchanged. We conclude that tomosyn is a soluble SNARE that directly competes with synaptobrevin in the formation of SNARE complexes and thus may function in down-regulating exocytosis.

Neurotransmitter release is mediated by regulated exocytosis of synaptic vesicles. Exocytosis is carried out by membrane fusion machines involving evolutionary conserved proteins that are common to all fusion events of the secretory pathway in eukaryotic cells. Among the conserved proteins are the SNAREs,[1] small membrane proteins characterized by homologous stretches of 60–70 amino acids referred to as SNARE motifs (1). The SNAREs involved in neuronal exocytosis include the vesicle protein synaptobrevin (also termed VAMP), and the plasma membrane-associated proteins syntaxin 1 and SNAP-25 (acronym for synaptosome-associated protein of 25 kDa). These proteins undergo an assembly-disassembly cycle that involves the SNARE motifs and that is associated with large structural and energetic changes (2, 3). It is currently believed that assembly leads to a tight connection between the vesicle and the plasma membrane that initiates the opening of the fusion pore (for review see Refs. 4 and 5).

The components of the basic fusion apparatus are acted upon by arrays of control proteins that are responsible for the specific features of each fusion reaction. For the SNARE proteins involved in neuronal exocytosis many candidate regulator proteins have been identified, but their molecular mechanisms are only beginning to be understood (5). Considering the central role of SNARE assembly and disassembly for exocytosis, it is thus necessary to understand in detail which of such regulatory proteins interact with the SNARE conformational cycle and how exactly the cycle is influenced.

Tomosyn is a candidate regulatory protein that was first identified as a binding protein for syntaxin 1 (6). Tomosyn is a 130-kDa protein that lacks a membrane anchor and in which two domains are distinguished: a small C-terminal region with homology to the R-SNARE subfamily of SNAREs and a large N-terminal region that contains WD40 repeats and shares similarities with the *Drosophila* tumor suppressor lethal (2) giant larvae (l(2)gl) (7), its recently characterized mammalian homologue *Mgl*I (8), and the yeast proteins Sro7p and Sro77p (9). Three splicing variants of tomosyn are known, two of which are predominantly expressed in brain, whereas one is ubiquitously distributed (10).

Tomosyn has been reported to bind to the SNARE motif of syntaxin (11). In tissue extracts, tomosyn was found to be associated with syntaxin, SNAP-25, and synaptotagmin but not with synaptobrevin (6). Binding of synaptobrevin and tomosyn appear to be mutually exclusive leading to the suggestion that tomosyn may regulate synaptobrevin binding to the other SNAREs. Furthermore, it was suggested that tomosyn displaces Munc18 from syntaxin (6). Munc18 is a conserved protein essential for exocytosis that forms tight complexes with syntaxin 1 *in vitro*. Munc18 binding maintains syntaxin in an inactive state and inhibits formation of SNARE complexes (12, 13). Hence, it has been proposed that tomosyn may activate syntaxin, allowing it to interact with synaptobrevin in preparation for exocytosis (6). On the other hand, overexpression of tomosyn in PC12 cells resulted in a slight but significant inhibition of exocytosis (6, 13) suggesting that the role of tomosyn cannot be confined to an activator of membrane fusion.

Interestingly, the binding region of tomosyn could be assigned to the C-terminal region containing the R-SNARE motif

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[1] The abbreviations used are: SNAREs, soluble NSF attachment protein receptors; SNAP-25, soluble NSF attachment protein of 25 kDa; NPY, neuropeptide Y; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; CD, circular dichroism; PBS, phosphate-buffered saline; DTT, dithiothreitol; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-nexatriene *p*-toluenesulfonate; NSF, *N*-ethylmaleimide-sensitive factor.

raising the possibility that tomosyn may substitute as an R-SNARE in the formation of SNARE complexes (10, 11). SNARE complexes are represented by bundles of four α-helices in which each helix is contributed by a single SNARE motif (14, 15). All helices are aligned in parallel, with the transmembrane domains extending at one end. The α-helices are connected by layers of interacting amino acid side chains that are mostly hydrophobic and thus characteristic for coiled-coils. In the center, an unusual polar layer was observed consisting of three glutamines contributed by syntaxin and the two SNARE motifs of SNAP-25, and one arginine contributed by synaptobrevin (14). These residues are among the most highly conserved of the SNARE protein superfamily and led to the classification of SNAREs into Q- and R-SNAREs, respectively (16). If tomosyn substitutes as an R-SNARE for synaptobrevin in core complexes, it would be expected to interfere with synaptobrevin binding before fusion, thus acting as an inhibitor rather than an activator of SNARE function. However, it is still unknown whether tomosyn forms SNARE core complexes, whether it can interact with membrane-bound SNAREs, or whether it interferes with the SNARE association-dissociation cycle.

In the present study, we show that the C-terminal domain of tomosyn functions as a genuine R-SNARE motif. Tomosyn SNARE complexes exhibit features typical for SNARE core complexes, and they are sensitive to disassembly by the ATPase NSF. The R-SNARE motif of tomosyn directly competes with synaptobrevin during complex formation with the plasma membrane-resident Q-SNAREs syntaxin 1 and SNAP-25. Membrane binding is exclusively mediated by the SNARE motif of tomosyn that is also responsible for the inhibitory action of the protein on exocytosis. Neither binding nor NSF-mediated dissociation appears to be affected by the large N-terminal domain of tomosyn, leaving the function of this domain still unexplained.

EXPERIMENTAL PROCEDURES

Materials—The expression constructs of synaptobrevin 2 (1–96) S28C and of a chimera of human neuropeptide Y with EGFP (NPY-EGFP) were described previously (17, 18). NSF and α-SNAP were expressed and purified as described previously (19). Mouse monoclonal antibodies were used for the following proteins: syntaxin 1A/B (Cl 78.3), GDI (Cl 81.2), NSF (Cl 83.13, raised against recombinant NSF), SNAP-25 (Cl 71.1), synaptobrevin (Cl 69.1), α-SNAP (Cl 77.2), and c-Myc (9E10, purchased from ATCC). With the exception of Cl 83.13, which has not yet been published, all other antibodies were obtainable from Synaptic Systems (Göttingen, Germany). A rabbit polyclonal antibody raised against syntaxin 1A (R31) was used for the detection of syntaxin 1A (18). An antibody to tomosyn was raised in rabbits against a C-terminal fragment (tomosyn: residues 985–1051) that was expressed as a GST fusion protein in *Escherichia coli* using pGEX-KG (Amersham Biosciences). This antibody was purified on affinity chromatography of Sepharose 4B (Amersham Biosciences) coupled with GST-tomosyn-(985–1051).

Molecular Cloning—The cDNA corresponding to the open reading frame of rat *m*-tomosyn-(1–1116) was amplified by PCR using the *Pfu* Turbo DNA polymerase (Stratagene) from rat cerebellum cDNA libraries (Stratagene). The fragment was subcloned into pBluescript vector (Stratagene) and then sequenced to confirm the identity with the published sequence.

The sequences encoding for tomosyn-(1–1116), tomosyn-(1–1030), tomosyn (1031–1116) were amplified by PCR from pBlue-tomosyn-(1–1116). Unless indicated otherwise, the 5′-primers were designed to add an myc epitope upstream of the respective 5′-ends. The fragments were subcloned into the mammalian expression plasmids pCR3.1 (Invitrogen) or pBOB1.5 (pBOB1.5-EGFP was a gift from T. Hughes, Yale University, New Haven, CT) to express proteins fused with the N-terminal myc epitope for tomosyns, named, respectively, myc-tomosyn, myc-N-tom, and myc-C-tom. The sequences encoding tomosyn-(985–1051) and tomosyn-(1051–1116) were subcloned into the bacterial expression vector pGEX-KG to produce fusion proteins containing an N-terminal GST moiety.

Purification of Recombinant Proteins—The tomosyn fragment (residues 985–1051) and the tomosyn-SNARE-motif (residues 1051–1116) were expressed in *E. coli* as N-terminal GST-tagged proteins and purified by glutathione-Sepharose (Amersham Biosciences). The GST-tagged tomosyn-SNARE-motif-(1051–1116) was digested by thrombin to remove the tag and was further purified on a Mono-S column using an Äkta FPLC-system (Amersham Biosciences).

His6-tagged versions of the SNARE-motif (H3-domain) of syntaxin 1A-(180–262), SNAP-25 (1–206, all native cysteines replaced by serines), and the N- (1–83) and C-terminal (120–206) SNARE-motifs of SNAP-25 were purified by Ni^{2+}-agarose. After removal of the tag by thrombin, all proteins were further purified using a Mono-Q column on an Äkta system (Amersham Biosciences) essentially as described (20, 21). All proteins were 95% pure as determined by SDS-PAGE and Coomassie Blue staining.

For assembly of the tomosyn-containing SNARE core complex, equimolar amounts of the tomosyn-SNARE-motif-(1051–1116), syntaxin 1A-(180–262), and SNAP-25 were incubated overnight at 4 °C. The complex was purified by ion exchange and size exclusion chromatography (Mono-Q column) and HiLoad1660 Superdex 200 column (Amersham Biosciences).

Cell Culture, Fractionation, and Immunoprecipitation—For subcellular fractionation, PC12 cells, grown at ~70% confluence on two 150-mm dishes, were washed twice with ice-cold phosphate-buffered saline (PBS containing 150 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4). They were then homogenized by passage fifteen times through a 23-gauge needle in 2 ml of ice-cold homogenization buffer A (20 mM HEPES-KOH, pH 7.2, 0.25 M sucrose) containing a complete protease inhibitor mixture (Roche Diagnostics) and centrifuged at 2,500 rpm for 10 min at 4 °C in a microcentrifuge to remove nuclei and unbroken cells. The postnuclear supernatant was then centrifuged at 120,000 × *g* for 60 min at 4 °C to obtain a membrane pellet. For immunoprecipitations, this pellet was solubilized in 1% Triton X-100, 20 mM HEPES-KOH, pH 7.2, 2 mM EDTA, 100 mM KCl, 1 mM DTT containing the complete inhibitor mixture, incubated for 10 min at 4 °C, and then cleared by centrifugation at 13,000 rpm for 20 min at 4 °C in a microcentrifuge. Aliquots containing each 200 μg of protein were incubated for 30 min at 4 °C with affinity-purified antibodies against tomosyn (25 μg of protein), monoclonal antibodies against syntaxin 1 A/B (Cl 78.3), or monoclonal antibodies against the myc-epitope (100 μg of purified IgG in each case). Protein A-Sepharose or Protein G-Sepharose (Amersham Biosciences) was then added and allowed to incubate for 12 h at 4 °C with gentle rotation. The beads were sedimented in a microcentrifuge and washed four times in lysis buffer. All precipitates were treated with SDS-sample buffer and analyzed by SDS-PAGE and immunoblotting.

For measuring NSF-driven release of tomosyn, postnuclear supernatant of PC12 cells was treated with 4 mM *N*-ethylmaleimide for 30 min on ice to inactivate endogenous NSF and then quenched by adding 8 mM DTT before isolation of a membrane pellet. The pellets were resuspended in 20 mM HEPES-KOH, pH 7.2, 100 mM KCl, 1% (v/v) glycerol, 100 μM phenylmethylsulfonyl fluoride supplemented (where indicated) with 2 mM ATP, 4 mM $MgCl_2$, an ATP-regenerating system (20 mM creatine phosphate and 0.1 mg/ml creatine kinase), 0.6 μM NSF, and 6 μM α-SNAP with or without 10 mM EDTA, incubated for 30 min at 37 °C, and centrifuged again as above.

Competition Assay—The single cysteine at position 28 of synaptobrevin 2-(1–96)-S28C was labeled with a 10-fold molar excess of Alexa594 C5 maleimide (Molecular Probes Inc., Eugene, OR) according to the manufacture's instruction. The labeled protein was separated by gel filtration from the dye.

Rat brain cytosol (1 mg) was incubated for 30 min at 4 °C with anti-tomosyn antibody or control IgG (100 μg each) in KGlu buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA) and added to 30 μl of Protein A-Sepharose (50% slurry) to deplete endogenous tomosyn in the cytosol. After incubation for 1 h at 4 °C with gentle rotation, supernatant fraction was collected by centrifugation (immunodepleted cytosol fraction).

PC12 cells were grown on poly-L-lysine-coated coverslips and disrupted as previously described (23) using a 100-ms ultrasound treatment in ice-cold sonication buffer (KGlu buffer containing 10 mM EGTA, 2 mM ATP, 4 mM $MgCl_2$, and 0.5 mM DTT). The membrane sheets were then incubated in KGlu buffer containing 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 30 mg/ml bovine serum albumin, and 5 μM Alexa 594-labeled synaptobrevin 2-(1–96)-S28C together with competitors (synaptobrevin 2-(1–96)-S28C, tomosyn-(1051–1116), lysozyme, or rat brain cytosol (1 mg/ml each)) for 30 min at 37 °C. Membranes were washed twice in KGlu buffer quickly and fixed for 30 min at room temperature in 4%

paraformaldehyde in PBS. They were then washed twice in PBS for 20 min each. Imaging of the membrane sheets and quantification of fluorescence was performed as previously described (24).

In Vitro Exocytosis Assay—For the *in vitro* exocytosis assay, PC12 cells expressing the secretory granule marker neuropeptide Y-EGFP (18, 22) were used. At 40–50 h after transfection, membrane sheets were generated as described above and preincubated for 10 min at room temperature in KGlu buffer with 80 μM of the inhibitors as indicated (synaptobrevin 2-(1–96)-S28C, tomosyn-(1051–1116), or lysozyme). After preincubation, membrane sheets with at least 15 brightly fluorescent granules were stimulated using KGlu buffer containing 0.5 mg/ml rat brain cytosol, 0.5 μM $[Ca^{2+}]_{free}$ buffered with 10 mM EGTA (see Ref. 25) and 80 μM of the corresponding inhibitors. For controls, membrane sheets were incubated with KGlu buffer containing rat brain cytosol and 2 mM EGTA. The membrane sheets were imaged every min for 30 min. The focal position of the objective was controlled throughout all experiments using a low voltage piezo translator drive and a linear variable transformer displacement sensor/controller (Physik Instrumente, Waldbronn, Germany). Finally, the membrane sheets were visualized using TMA-DPH (Molecular Probes). Imaging and image analysis were performed as previously described (25).

Carbon Fiber Amperometry—Carbon fiber amperometry was used essentially as described previously (26). Amperometric signals from PC12 cells were recorded with an EPC-7 amplifier (Heka Electronics) with a holding potential of the carbon fiber set to +800 mV. The tip of the fiber was brought into direct contact with the plasma membrane to minimize temporal distortion of the release signal and diffusional loss of the released transmitter amount. Experiments were performed in Ringer's solution containing 130 mM NaCl, 4 mM KCl, 10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 44 mM glucose. Using a multichannel perfusion pipette (27) a depolarizing stimulus was applied by rapid superfusion with 80 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 44 mM glucose, 10 mM Hepes, pH 7.4. Stimulation periods were bracketed by superfusion with Ringer's solution. Signals were filtered with 3 and 10 kHz, digitized at 25 kHz, and stored on a personal computer. For data analysis, the AutesW software (NPI Electronics, Tamm, Germany) was used as described in a previous study (26). Signals were again digitally filtered at 3 kHz (overall bandwidth, 2.1 kHz). Additionally, a routine for analysis of foot events preceding the main amperometric spike was included (see Table I for details).

PC12 cells (28) (passage numbers 27–33) were cultured as described previously (29). For transfection, cells were electroporated (Gene Pulser, Bio-Rad, settings: resistance 50 ohm, capacitor 50 microfarads, voltage 1.1 kV, cuvette 4-mm gap) in a solution containing 85 mM KCl, 10 mM NaCl, 10 mM KH_2PO_4, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM EGTA, 0.15 mM $CaCl_2$, 50 mM glucose, 25 mM Hepes-KOH, pH 7.6. Control cells were sham-transfected with pCDNA3 (containing no insert, Invitrogen), whereas tomosyn-overexpressing cells were transfected with pBOB1.5-tomosyn encoding for the entire open reading frame of *m*-tomosyn-(1–1116) with a myc-tag fused to the N-terminal end of the protein. Overexpressing cells were identified by co-transfection with pBOB1.5-EGFP coding for EGFP as marker protein. A molar ratio of 5 to 1 for pBOB1.5-tomosyn *versus* pBOB1.5-EGFP was used to guarantee a high co-transfection efficiency (>85%) as judged from immunosignals of transfected cells processed in parallel. For amperometric recordings, only cells that exhibited strong EGFP signals were used. Cells were plated on collagen-coated culture dishes (Falcon 3001) at densities between 1×10^6 and 1.5×10^6 cells/dish and were used for recordings 3–4 days after transfection.

Other Methods—SDS-PAGE and immunoblotting was carried out using standard procedures. Electroblotting was performed on Immobilon-P membranes (Millipore, Bedford, MA). As secondary antibodies, we used horseradish peroxidase-conjugated anti-rat IgG or anti-mouse IgG, and the blots were developed by enhanced chemiluminescence using SuperSignal West (Pierce) according to the manufacture's instructions. Multiangle laser light scattering measurements and CD spectroscopy were performed as described (3, 21).

RESULTS

The C-terminal Domain of Tomosyn Forms SNARE Core Complexes—SNARE core complexes display several highly unusual properties, including progression from unstructured monomers to helical bundles upon assembly, extraordinary heat stability, and profound hystereses upon unfolding-refolding cycles (3). To examine whether the C-terminal domain of tomosyn can function as a genuine R-SNARE in the formation of core complexes, purified recombinant SNARE motifs of tomosyn, syntaxin 1, and SNAP-25 were combined, and the features of the resulting complexes were studied.

First, we used circular dichroism (CD) spectroscopy to examine whether binding of tomosyn to syntaxin 1 and SNAP-25 involves a transition from unstructured monomers into a helical bundle. As shown in Fig. 1*A*, a large increase in α-helical content was observable when the SNARE motif of tomosyn was mixed with SNAP-25 and syntaxin. No secondary structure was detectable in the CD spectrum of the isolated SNARE motifs (Fig. 1*A*). Furthermore, no structural change was observed when the SNARE motif of tomosyn was combined with SNAP-25 or with its isolated SNARE motifs, and only a minor increase was observed upon mixing with the H3 domain of syntaxin 1 (Fig. 1*B*). For further characterization, tomosyn-containing SNARE complexes were purified by ion exchange chromatography. When analyzed by size-exclusion chromatography and multiangle laser light scattering, the purified complex migrated largely as a single peak with a molecular mass of ~45,000 Da (data not shown), as expected for a complex with a 1:1:1:1 stoichiometry.

We then tested whether the tomosyn-SNARE complex is heat-stable, and if so, whether heat denaturation and re-naturation would display hysteresis characteristic for SNARE core complexes (3). Fig. 1*C* shows that the tomosyn SNARE complex has an unfolding transition temperature of over 80 °C whereas re-folding requires temperatures below 60 °C. Together, these properties are indistinguishable from SNARE complexes containing synaptobrevin (3) documenting that the R-SNARE motif of tomosyn is fully functional in substituting for synaptobrevin as R-SNARE.

The N-terminal Domain of Tomosyn Does Not Interfere with the Activity of the R-SNARE Motif—Tomosyn possesses a large N-terminal domain of unknown function that could block or down-regulate the activity of the SNARE motif, at least in intact preparations. We therefore investigated whether tomosyn is complexed with syntaxin and SNAP-25 in intact cells, and if so, if the presence of the N-terminal domain has any influence on this interaction.

Tomosyn was immunoprecipitated from detergent extracts of PC12 cells and tested for association of SNARE proteins (Fig. 2*A*). Both syntaxin 1 and SNAP-25 co-precipitated with tomosyn, showing that native tomosyn interacts with these SNAREs in intact cells. Furthermore, a minor amount of synaptobrevin was found in the immunoprecipitate, which may be due to interactions involving the transmembrane domains rather than the SNARE motifs (30). Conversely, tomosyn co-precipitated with syntaxin when a monoclonal antibody specific for the N-terminal domain of syntaxin was used (Fig. 2*B*).

To estimate the contribution of the N-terminal domain of tomosyn to the formation of SNARE complexes, we transfected PC12 cells with expression vectors coding for myc-tagged versions of full-length tomosyn, the N-terminal domain (residues 1–1030), and the C -terminal region carrying the R-SNARE motif (residues 1031–1116). Immunoprecipitations were then performed from detergent extracts of PC12 cells as above using an anti-myc antibody. As shown in Fig. 2*C*, both syntaxin 1 and SNAP-25 co-precipitated with the tomosyn variants containing the R-SNARE motif. In contrast, these SNAREs were not detectable in immunoprecipitates obtained from cells transfected with the N-terminal domain of tomosyn. In this case, synaptobrevin was not detectable in any of the immunoprecipitates.

Next we investigated whether the R-SNARE motif of tomosyn substitutes for synaptobrevin when SNARE complexes are formed in intact membranes. To address this issue, we took advantage of our recent finding that the recombinant R-SNARE motif of

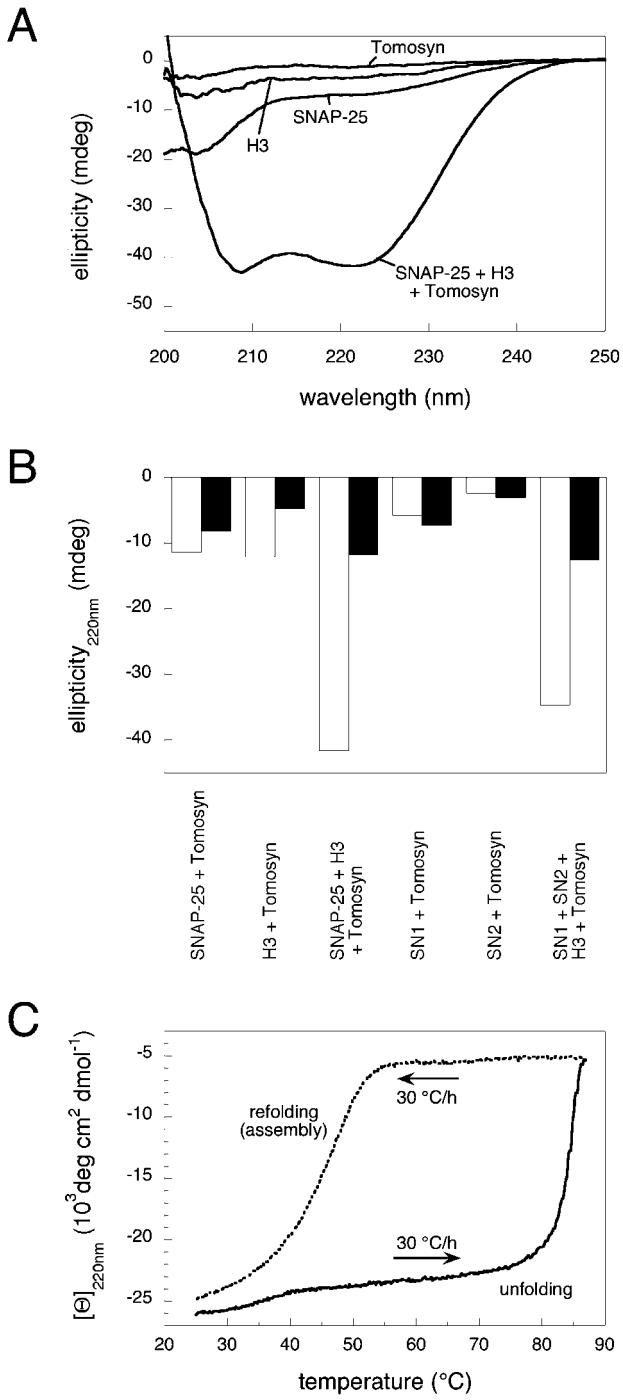

FIG. 1. **The SNARE motif of tomosyn forms SNARE core complexes with syntaxin 1 and SNAP-25.** *A*, CD spectra of purified SNAP-25, of the H3-domain of syntaxin 1 (residues 180–262), of the C-terminal region of tomosyn (residues 1051–1161), and of a mixture of all three proteins. The spectra of the monomers show no evidence for secondary structure. Note the decrease in ellipticity of the mixture, with minima at 208 and 222 nm, which are typical for α-helices. *B*, significant increases in α-helicity are only observed when tomosyn is combined with both SNARE motifs of SNAP-25 and that of syntaxin. The figure shows a comparison of the ellipticities (220 nm) of the theoretically non-interacting spectra (*black bars*) and the measured spectra (*open bars*). *H3*, H3 domain of syntaxin; *SN1*, N-terminal SNARE motif of SNAP-25 (residues 1–83); *SN2*, C-terminal SNARE motif of SNAP-25 (residues 120–206). *C*, tomosyn-SNARE complexes show profound hysteresis upon thermal unfolding-refolding cycles. Folding was determined by monitoring helical content with CD spectroscopy at 220 nm. Complexes were formed with tomosyn-(1051–1161), syntaxin-(180–262), and full-length SNAP-25, purified by anion exchange and size-exclusion chromatography and subjected to heating and cooling at the rates indicated (see Ref. 3, for further details).

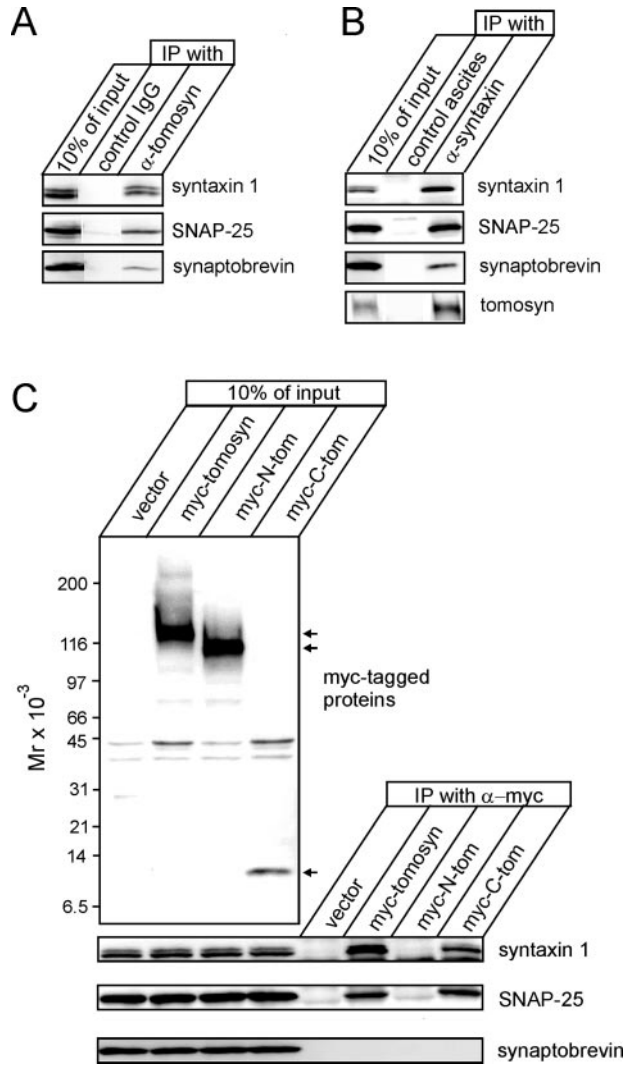

FIG. 2. **Tomosyn forms SNARE complexes with syntaxin and SNAP-25 in PC12 cells.** *A* and *B*, co-immunoprecipitation of tomosyn, syntaxin, and SNAP-25 from detergent extracts of PC12 cell membranes using either affinity-purified anti-tomosyn antibodies (*A*) or an anti-syntaxin monoclonal antibody (*B*). *C*, co-immunoprecipitation of myc-tagged tomosyn variants expressed in PC12 cells with SNARE proteins. PC12 cells were transfected with expression plasmids carrying N-terminally fused myc-tags that encode full-length tomosyn (*myc-tomosyn*), the N-terminal domain of tomosyn (residues 1–1030, *myc-N-tom*), and the C-terminal domain of tomosyn containing the R-SNARE motif (residues 1031–1116, *myc-C-tom*). Two days after transfection, immunoprecipitations were performed from membrane extracts using anti-myc antibodies. All immunoprecipitates were analyzed by SDS-PAGE and immunoblotting.

synaptobrevin forms core complexes with endogenous membrane-resident SNAP-25 and syntaxin 1 when added to freshly prepared inverted sheets of plasma membranes (24). PC12 cells were grown on coverslips and subjected to brief sonication to disrupt the cells, resulting in the generation of inside-out membrane sheets. The membrane sheets were incubated for 30 min at 37 °C with recombinant synaptobrevin (lacking the transmembrane domain) that was labeled with the fluorescent dye Alexa 594. As shown in Fig. 3, synaptobrevin bound to syntaxin clusters in these membrane sheets resulting, as shown previously (24), in the formation of genuine cis-core complexes. As expected for a specific and saturable interaction, binding of labeled synaptobrevin was progressively reduced when increasing amounts of unlabeled protein were added (Fig. 3*G*). When synaptobrevin was replaced with the SNARE motif of tomosyn in these experiments, competition with the binding of labeled synaptobrevin was of comparable efficacy. These data show

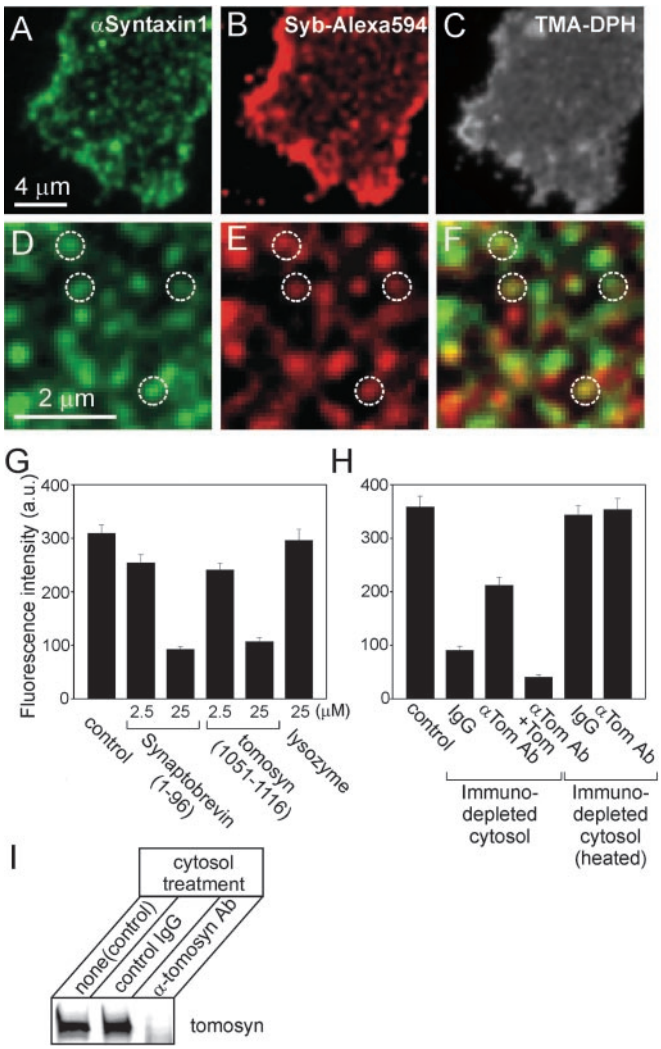

FIG. 3. **Tomosyn competes with Alexa594-labeled synaptobrevin 2 (1–96, S28C) in binding to inverted lawns of plasma membranes derived from PC12 cells.** *A–F*, Alexa594-labeled synaptobrevin 2-(1–96) binds to endogenous syntaxin 1 clusters that were immunolabeled after the binding reaction. *A*, immunostaining for syntaxin 1A. *B*, bound Alexa-labeled synaptobrevin. *C*, the position and the integrity of the membrane sheets were documented with TMA-DPH, a lipophilic membrane dye. *D* and *E*, magnified views from *A* and *B*; see *F* for overlay. *G*, competition of binding of Alexa594-labeled synaptobrevin with unlabeled recombinant proteins. All sheets were washed and fixed after the binding reaction, and the fluorescence intensity of the membrane sheets was measured (see "Experimental Procedures"). Values are presented as means ± S.E. (n = 30 membrane sheets). *H*, competition of synaptobrevin binding using tomosyn-containing cytosol that was either immunodepleted with control IgG or with anti-tomosyn antibodies. Treatment with anti-tomosyn antibodies led to near quantitative removal of tomosyn (immunoblot analysis shown in *I*). As control, all cytosol fractions were heat-inactivated prior to the experiment.

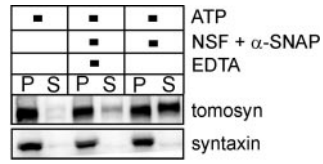

FIG. 4. **Tomosyn is released from membranes by NSF and α-SNAP.** Immunoblots of PC12 cell membranes and the corresponding supernatants that were incubated with NSF and α-SNAP. Incubation was carried out for 30 min at 37 °C with either an ATP-regenerating system (*ATP*) or an ATP-regenerating system supplemented with EDTA to inactivate ATP-cleavage due to chelation of Mg^{2+}. Where indicated, purified recombinant NSF and α-SNAP (each at 6 μM final concentration) were added. The membrane fractions were pretreated with 4 mM *N*-ethylmaleimide on ice for 30 min prior to membrane isolation to inactivate endogenous NSF (see "Experimental Procedures"). After the incubation, membranes were sedimented by high speed centrifugation, and equal proportions of the pellet (*P*) and supernatant (*S*) fractions were analyzed by SDS-PAGE and immunoblotting.

that the proteins bind to the same site with similar affinities.

We next investigated whether full-length tomosyn is also capable of competing with synaptobrevin binding in this assay. Unfortunately, we have so far been unable to prepare recombinant full-length tomosyn for binding studies. We therefore took an indirect approach and used whole brain cytosol as a source for endogenous tomosyn. To account for effects by factors other than tomosyn, part of the cytosol was depleted of endogenous tomosyn by immunoprecipitation resulting in near-quantitative removal of the endogenous protein (Fig. 3*I*) with no detectable changes in the overall protein pattern (not shown). Addition of cytosol to the binding reaction that was pretreated with a control IgG resulted in efficient competition of synaptobrevin binding (Fig. 3*H*). Equal concentrations of immunodepleted cytosol were significantly less efficient, although some inhibitory activity remained. Addition of the recombinant SNARE motif of tomosyn to the reaction was as effective in competing with synaptobrevin as in the absence of cytosol. Thus, native tomosyn forms SNARE complexes with syntaxin and SNAP-25, and there appears to be no interference by cytosolic factors with the R-SNARE activity of the protein.

The data described so far suggests that the large N-terminal domain does not interfere with the ability of the C-terminal SNARE motif to enter SNARE complexes. However, it remains to be established whether the same applies to the "reverse" reaction, *i.e.* whether SNARE complexes containing native tomosyn can be disassembled by the ATPase NSF.

Membrane fractions, prepared from PC12 cells, were pretreated with *N*-ethylmaleimide to block endogenous NSF and then incubated with ATP either in the absence or the presence of recombinant NSF and α-SNAP. NSF treatment resulted in a release of tomosyn from the membrane that was dependent on the presence of Mg-ATP (Fig. 4). Similarly, tomosyn could be removed from the plasma membrane of digitonin-permeabilized PC12 cells upon incubation with ATP-NSF (data not shown). We conclude that neuronal SNARE complexes containing tomosyn are effectively disassembled by NSF.

The SNARE Motif of Tomosyn Is Responsible for Downregulating Exocytosis in PC12 Cells—So far we have established that the R-SNARE motif of tomosyn forms SNARE complexes with syntaxin and SNAP-25 that can be disassembled by NSF and that are similar to core complexes containing synaptobrevin. It has previously been shown that overexpression of tomosyn causes partial inhibition of exocytosis in PC12 cells (6), and it has been proposed that the tomosyn may mediate its inhibition by functionally substituting an R-SNARE motif.

To investigate whether the R-SNARE motif of tomosyn is indeed responsible for inhibiting exocytosis in PC12 cells, we took advantage of a recently developed cell-free system for exocytosis that is based on the preparation of membrane sheets similar to those described above. We have shown previously that such membrane sheets contain bound secretory granules that retain their ability to undergo Ca^{2+}-dependent exocytosis for some time after cell disruption (23). PC12 cells whose secretory granules were fluorescently labeled by expression of neuropeptide Y-EGFP (18) were grown on coverslips and sonicated to produce plasma membrane sheets containing labeled secretory granules (Fig. 5). Exocytosis was monitored by changes in the fluorescence intensity as described (23, 25). After a preincubation period of 10 min in the presence of cytosol and ATP, calcium was added, resulting in a calcium-dependent exocytic activity of ~21% of all granules during the subsequent 30

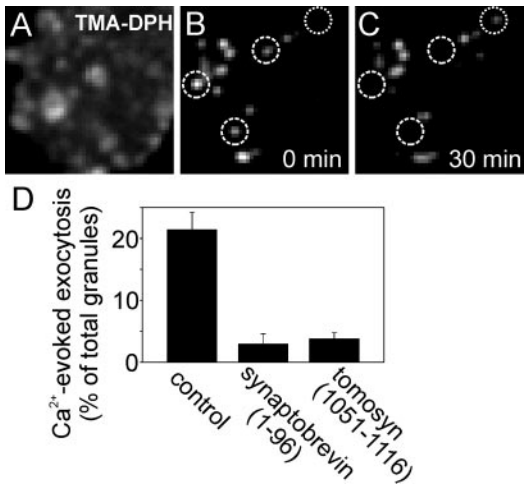

FIG. 5. **The C-terminal region of tomosyn (residues 1051–1116) inhibits Ca^{2+}-dependent exocytosis in a semi-intact system derived from PC12 cells.** PC12 cells were transfected with NPY-EGFP as a granule content marker, followed by cell disruption, leading to the generation of inverted lawns of plasma membranes (see *A* for visualization of phospholipid membranes with TMA-DPH) containing bound secretory granules (*B* and *C*). For the triggering and monitoring of exocytosis, ATP, cytosol, and 500 nM free calcium were added, and membrane sheets were imaged every minute for 30 min (see *B* and *C* for the first and last image of a sequence). Exocytic activity of individual granules resulted in abrupt changes in fluorescence intensity in the sequence leading either to dimming (due to loss of GFP, compare *dashed circles* in *B* and *C*) or brightening (due to pH-dequenching of GFP, compare *dotted circles* in *B* and *C*) of individual granules (25). The percentage of active granules (*D*) was related to all GFP-labeled granules present at the beginning (for details see Ref. 25). For each condition, the background exocytic activity in the absence of calcium was measured separately and subtracted. For each experiment 6–14 (mean = 10) membrane sheets were analyzed. Values are presented as means ± S.E. The concentration of synaptobrevin and tomosyn fragments was 80 μM.

min (Fig. 5*D*). When recombinant synaptobrevin-(1–96) was added, exocytosis was largely inhibited. A very similar inhibition was observed when the C-terminal part of tomosyn was added.

In the final set of experiments, we addressed whether tomosyn overexpression has an impact on the exocytotic fusion event itself. It has previously been reported that tomosyn overexpression in PC12 cells leads to a slight reduction in the release of co-transfected human growth hormone (6). To gain more insight into the underlying mechanism, we analyzed the effect of tomosyn overexpression on the frequency and release parameters of dopamine release from single secretory vesicles. PC12 cells were co-transfected with full-length tomosyn and EGFP to identify transfected cells, and exocytosis was monitored by carbon fiber amperometry. Upon depolarizing stimulation with high K^+-containing solution control cells respond with an initial phase of high exocytotic activity (2.1 ± 0.8 events/s), which slowly declined to proceed at a more constant rate (1.1 ± 0.4 events/s) as long as the stimulus was applied (Fig. 6). Tomosyn-expressing cells showed no biphasic response but exocytosed vesicles with an average rate of about 0.25 ± 0.1 events/s. The lack of an initial phase with higher exocytotic activity suggests that the number of rapidly releasing granules is strongly diminished upon overexpression of tomosyn. In contrast, no significant changes were observed in the analysis of the single spike parameters, including the quantal size, amplitude, rise time, and half-width of the event and in the parameters of the amperometric foot signal that represents the opening kinetics of the exocytotic fusion pore (Table I). Taken together, the data suggest that tomosyn governs the readiness of the secretory organelles for exocytosis rather than the molecular mechanism of membrane merger.

DISCUSSION

In the present study we have shown that the C-terminal portion of tomosyn acts as a fully functional R-SNARE motif with similar properties to the R-SNARE motif of synaptobrevin. It mediates membrane binding by forming SNARE complexes with syntaxin 1 and SNAP-25 that are sensitive to disassembly by NSF. Furthermore, it mimics the inhibitory action of the SNARE motif of synaptobrevin on exocytosis (31, 32). In contrast to synaptobrevin, tomosyn does not contain a membrane anchor domain but has a large N-terminal domain that does not appear to interfere with the SNARE activity of the C terminus.

As outlined in the introduction, tomosyn is related to the *Drosophila* tumor suppressor lethal (2) giant larvae (l(2)gl) (7), its mammalian homologue *Mgl*I (8) and the yeast proteins Sro7p and Sro77p (9). The homology is confined to the N-terminal domain containing WD40 repeats, protein binding domains with a conserved structure of about 40 residues, each containing a central Trp-Asp motif. WD40 domains are involved in specific protein-protein interactions, best characterized in G protein-mediated signal transduction (33). In contrast to tomosyn, none of the hitherto known relatives possess a SNARE motif (based on unpublished sequence comparisons, see also "Discussion" in Ref. 8). Intriguingly, however, several of the other family members have not only been demonstrated to play a role in membrane traffic of polarized cells but also to directly interact with intracellular SNAREs. In yeast, cells deficient in SRO7 and SRO77 exhibit a defect in vesicle transport from the Golgi apparatus to the plasma membrane, and the protein Sro7p binds to the yeast SNAP-25 homologue Sec9p (9). Furthermore, *Mgl*I appears to play a role in polarized membrane trafficking as it becomes associated with the lateral membrane when MDCK cells establish polarity, and membrane binding of *Mgl*I seems to be mediated by binding to syntaxin 4 (8). These findings contrast with our observation that the N-terminal domain of tomosyn does not interact with membranes. Furthermore, Yokoyama *et al.* (10) were unable to detect syntaxin binding with this domain using a blot overlay assay, corroborating the findings reported here. It is tempting to speculate that the acquisition of the C-terminal SNARE motif is due to a gene fusion event during evolution and that SNARE binding of the N-terminal domain subsequently degenerated. However, we cannot exclude that the N-terminal domain of tomosyn (particularly the WD40 repeats) interacts with other intracellular proteins, either as an independent entity or as a regulator of the C-terminal SNARE motif. In the latter case, the SNARE motif would be constitutively "on" in the intact molecule, because the presence of the N-terminal domain did not have any measurable effect on the SNARE-activity of the C-terminal domain.

Our data show that the SNARE motif of tomosyn does indeed function as an R-SNARE in SNARE assembly and disassembly reactions. The complex formed with syntaxin and SNAP-25 is remarkably similar to the ternary SNARE complex formed with synaptobrevin, strongly suggesting that it is represented by a four-helix bundle characteristic for all SNAREs. The similarities include 1:1:1:1 stoichiometry of the participating SNARE motifs, unusual heat resistance, strong hysteresis during folding-unfolding transitions, and major conformational changes during assembly that proceeds from unstructured monomers to a highly helical complex (Ref. 3, see Ref. 5 for review). Furthermore, the recombinant SNARE motif of tomosyn binds to membrane-bound syntaxin 1 and SNAP-25 in a manner indistinguishable from the cytoplasmic part of synaptobrevin. Synaptobrevin was previously shown to form cis-core complexes under these conditions (24).

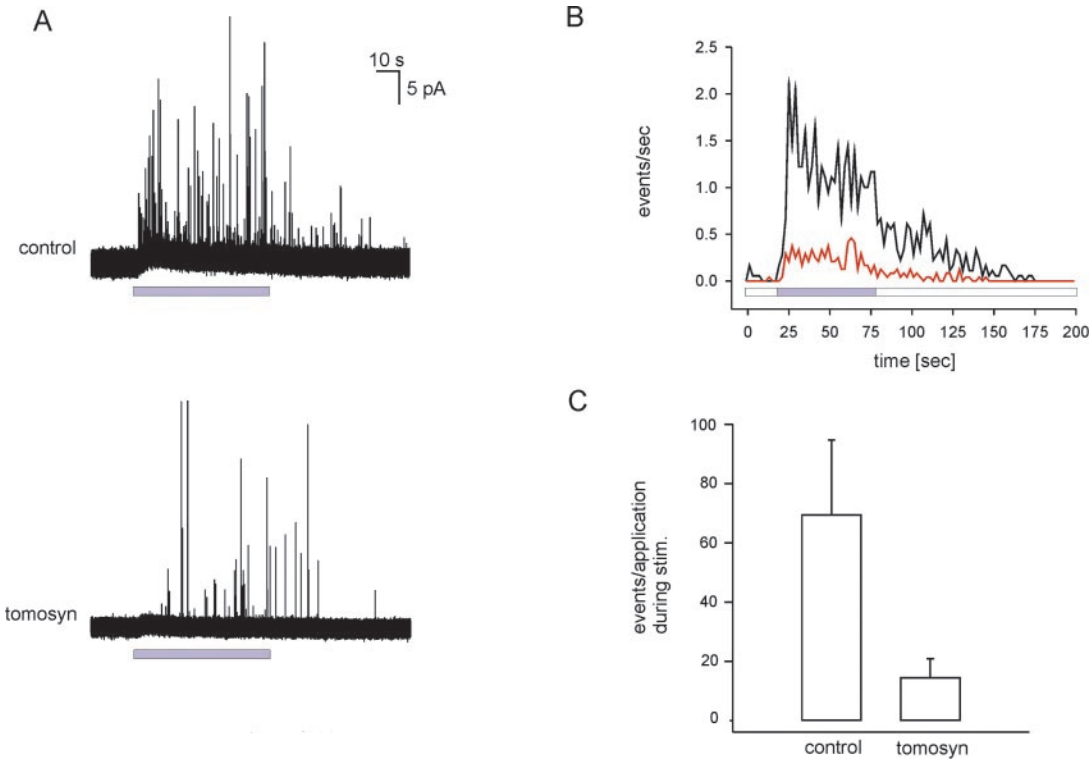

FIG. 6. **Overexpression of tomosyn profoundly inhibits exocytosis from PC12 cells.** *A*, representative amperometric recordings from control cells (expressing EGFP) and cells overexpressing tomosyn full-length protein. *Bar*, stimulation of exocytosis with high K^+-containing Ringer's solution. *B*, average exocytotic activity of control ($n = 9$) and tomosyn-transfected cells ($n = 12$). Note the lack of an initial phase with higher exocytotic activity in tomosyn-expressing cells (compare with *A*). *Bar*, stimulation with K^+-containing Ringer's solution. Only amperometric events with peak amplitudes > 3 pA were included. *C*, overexpression of tomosyn leads to a strong reduction in the number of spikes evoked during stimulation (control, 69 ± 25 events; tomosyn-transfected, 14 ± 6 events).

TABLE I

Properties of individual amperometric signals in control cells and cells overexpressing tomosyn full-length protein

Amperometric events were characterized with respect to charge (*fC*) reflecting the area under spike, maximum amplitude (pA), 50–90% rise time, and halfwidth (representing the duration of the spike at the 50% value of its maximum amplitude). Only events with an amplitude larger than 3 pA were included. Foot signals were analyzed with respect to charge (fC), current amplitude (pA) and duration (microseconds). The start of a foot signal was defined as the time point when the current amplitude exceeds two times the average baseline noise standard deviation; its end is indicated by the inflection point in the current signal marking the transition between the slow and the fast increase of the current amplitude during the foot and spike phase, respectively. Values are given as mean ± S.E.

(>3.0 pA)	Control (9 cells, 859 events)	Tomosyn (12 cells, 212 events)
Event parameters		
Charge (fC)[a]	34.9 ± 1.2	27.5 ± 1.8
Amplitude [pA]	11.9 ± 1.2	9.7 ± 0.8
50–90% rise time	668 ± 34	576 ± 63
Halfwidth (μs)	2723 ± 89	2400 ± 147
Foot parameters	$n = 153$	$n = 50$
Charge (fC)	6.1 ± 0.6	6.1 ± 0.9
Amplitude (pA)	4.4 ± 0.2	3.8 ± 0.4
Duration (μs)	2562 ± 204	2936 ± 401

[a] fC, femtoCoulomb.

The fact that tomosyn functions as a SNARE in the formation of SNARE core complexes is not easily reconcilable with the idea that the tomosyn-SNARE complex is an intermediate in an activation sequence during which it removes Munc18 from syntaxin and "hands" it over to its cognate SNARE partner synaptobrevin (see *e.g.* Ref. 10). Rather, tomosyn-containing SNARE complexes require the chaperone ATPase NSF for disassembly that acts upon all partially and fully assembled SNARE complexes. Insofar, tomosyn acts as a competitive inhibitor of synaptobrevin by occupying its binding site. Thus it is possible that tomosyn fine-tunes the availability of the plasma membrane SNAREs SNAP-25 and syntaxin for entering fusion-competent trans-complexes. Such a scenario is in agreement with the fact that an increase of intracellular tomosyn concentrations down-regulates exocytosis.

We have recently established that the plasma membrane pools of syntaxin and SNAP-25 are essentially uncomplexed and active under steady-state conditions (24). This indicates that NSF maintains all SNAREs in a predominantly disassembled state. Thus, the membrane pool of tomosyn is probably relatively small, although we cannot exclude that disassembly of tomosyn-SNARE complexes is slower than that of genuine SNARE complexes, or down-regulated by its N-terminal domain. It is conceivable that tomosyn becomes locked at the membrane as soon as NSF activity is reduced, which may result in an overestimate of the membrane-bound pool in some of our experiments. That overexpression of tomosyn reduces the rate of evoked exocytosis does not necessarily imply that a reduction of tomosyn would lead to an increase in exocytosis. In any case we can exclude that tomosyn affects the exocytotic fusion event itself, because no changes were observed in the kinetics of single vesicle exocytosis.

It remains to be established to what extent the function of tomosyn is confined to regulated exocytosis. Three splicing variants of tomosyn, all containing the R-SNARE motif, were described, one of which is ubiquitously expressed (10). The SNARE motif of tomosyn was identified in yeast two-hybrid screens searching for binding partners of syntaxin 4 and SNAP-23, although binding to syntaxin 4 was not observed in pull-down assays. Both syntaxin 4 and SNAP-23 are thought to be involved in fusion events at the plasma membrane suggest-

ing that tomosyns may be specific regulators of exocytosis in many different cell types.

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Membrane Transport, Structure, Function, and Biogenesis:

The R-SNARE Motif of Tomosyn Forms SNARE Core Complexes with Syntaxin 1 and SNAP-25 and Down-regulates Exocytosis

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