STRUCTURAL AND FUNCTIONAL ANALYSIS OF TOMOSYN IDENTIFIES DOMAINS IMPORTANT IN EXOCYTOTIC REGULATION
Antionette L. Williams¶, Noa Bielopolski§, Daphna Meroz§§, Alice D. Lam¶¶, Daniel R. Passmore¶, Nir Ben-Tal§§, Stephen A. Ernst¶¶, Uri Ashery§, and Edward L. Stuenkel¶*

From the Departments of ¶Molecular and Integrative Physiology and ¶¶ Molecular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109, and the Departments of §Neurobiology and §§Biochemistry and Molecular Biology, George S Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Running head: Structural and Functional Analysis of Tomosyn

*Address correspondence to: Edward L. Stuenkel, Ph.D., 7807 Medical Science Building II, 1137 E. Catherine Street, Ann Arbor, Michigan 48109-5622. Fax: 734-936-8813; E-mail: esterm@umich.edu

Tomosyn is a 130kDa cytosolic R-SNARE protein that associates with Q-SNAREs and reduces exocytotic activity. Two paralogous genes, tomosyn-1 and -2, occur in mammals and via alternative splicing produce 7 different isoforms. Here, we map structural differences between the yeast homologue of m-tomosyn-1, Sro7, and tomosyn genes/isoforms to identify domains critical to tomosyn’s regulation of exocytotic activity that are outside the SNARE motif. Homology modeling of m-tomosyn-1 based on the known structure of yeast Sro7 revealed a highly conserved functional conformation, but with tomosyn containing 3 additional loop domains that emanate from a β-propeller core. Notably, deletion of loops 1 and 3 eliminate tomosyn inhibitory activity on secretion without altering its SNARE pairing with syntaxin1A. By comparison, deletion of loop 2, which contains the hypervariable splice region (HVR), did not reduce the ability of tomosyn to inhibit regulated secretion. However, exon variation within the HVR resulted in significant differences in protein accumulation of tomosyn-2 isoforms. Functional analysis of s-tomosyn-1, m-tomosyn-1, m-tomosyn-2 and xb-tomosyn-2 demonstrated that they exert similar inhibitory effects on elevated K⁺-induced secretion in PC12 cells, although m-tomosyn-2 was novel in strongly augmenting basal secretion. Finally, we report that m-tomosyn-1 is a target substrate for SUMO 2/3 conjugation and that mutation of this SUMO target site (K730) enhances m-tomosyn-1 inhibition of secretion without altering interaction with syntaxin1A. Together these results suggest that multiple domains outside the R-SNARE of tomosyn are critical to the efficacy of tomosyn’s inhibition of exocytotic secretion.

Synaptic vesicle fusion and subsequent release of neurotransmitter requires the formation of heterotrimeric SNARE complexes formed from plasma membrane proteins syntaxin1A and SNAP-25 (Q-SNAREs) with the synaptic vesicle membrane protein VAMP/Synaptobrevin (R-SNARE) (1-3). Present on opposing membranes, these SNAREs combine and engage in thermodynamically stable, coiled-coil interactions that bridge the two membranes and catalyze their fusion (4). The formation of SNARE complexes is spatially and temporally controlled by accessory components that lend additional specificity to SNARE pairing, arrest SNARE complex intermediates, and/or lower the energy required for fusion (4-6). Ultimately, it is the functional activity of these regulators on SNARE complex assembly that determines the dynamics of the exocytotic event.

Tomosyn is an important regulator of SNARE complex formation whose mechanism of action remains unclear. Initially identified in neurons (7-8), tomosyn, a soluble R-SNARE protein, was considered to be a negative effector of fusogenic SNARE complex assembly through interactions with syntaxin1A and SNAP-25 that preclude the binding of VAMP2, thereby resulting in “dead end”, nonfusogenic SNARE complexes (7-10). Cumulative biochemical evidence lends much support to this mechanism. Tomosyn overexpression inhibits exocytosis in C. elegans (11-14), in neurons (15-16) and a number of neuroendocrine secretory cell models.
Moreover, gene mutation studies in *C. elegans* along with a tomosyn knockout mouse model have demonstrated enhanced synaptic transmission in the absence of functional tomosyn (12-13,21). While there is clear evidence for a negative role for tomosyn in regulation of neurotransmitter release, additional tomosyn functions have also been reported. Recent studies have provided evidence for a second, permissive role for tomosyn regulation of neurotransmission. Tomosyn depletion by siRNA inhibits acetylcholine release from superior cervical ganglion neurons (22) and insulin secretion from insulin-secreting INS-1E cells (23). Subsequent to strong stimulation, tomosyn-overexpressing cells exhibited enhanced late-phase secretion in chromaffin cells (16,24) and asynchronous release in neurons (22) relative to controls. Moreover, in adipocytes, tomosyn was displaced by VAMP2 in vitro, and bound simultaneously to Munc18c and syntaxin 4 (19) in a complex that has been suggested to prime syntaxin on the plasma membrane for fusion (25).

In addition, while negative regulation by tomosyn on neurotransmitter release has been attributed to its C-terminal SNARE domain, this SNARE motif is absent from tomosyn homologues in yeast (Sro7p and Sro77p), the Drosophila tumor suppressor lethal giant larvae family, and the mammalian Mg/l family (26-30) whose functions are likewise attributed to interactions with cognate Q-SNAREs. Indeed, tomosyn’s R-SNARE motif occupies less than 10% of its sequence, yet it is the primary domain assigned a functional significance. Notably, one of the first reports on mammalian tomosyn demonstrated that both the N- and C-terminal regions were required for its inhibitory effect on secretion (8). More recently, an N-terminal deletion mutant of tomosyn was reported to still bind to syntaxin1A, yet it lacked the ability to inhibit secretion (24). Moreover, a tomosyn truncation mutant that lacked the R-SNARE domain and could not interact with syntaxin1A still demonstrated partial inhibition of secretion (24). Taken together, these studies indicated additional tomosyn regulatory domains.

Recently an autoregulatory function has been assigned to a tail domain of tomosyn in a manner likened to the autoregulation of Sro7 (31-32). Although the N-terminal β-propeller domains of tomosyn comprise a substantial proportion of the protein and are required for the full inhibitory effect of tomosyn on secretion (24), only a few specific protein interactions have been identified with this region. For example, synaptotagmin-1 binds in a Ca\(^{2+}\)-dependent manner directly to the N-terminal WD40 repeats to negatively regulate synaptotagmin-1 mediated neurotransmitter release (33). However, the specific structural motifs of tomosyn involved in this interaction have not been identified. The N-terminal portion of tomosyn has also been reported to enhance oligomerization of SNARE complexes facilitating inhibition of synaptic transmission (21).

To date, structural and functional analysis of tomosyn has been relegated to one specific mammalian (rat) isoform, m-tomosyn-1. Yet, two tomosyn genes, tomosyn-1 and tomosyn-2, and 7 distinct isoforms have been identified in mice that arise from specific differential splicing within a domain termed the hypervariable region (HVR). Splicing of tomosyn-1 generates 3 distinct isoforms (s, m and b) (34), whereas splicing tomosyn-2 results in four isoforms (s, m, b, and xb) (35). All structural differences within the isoforms of each gene occur strictly within the HVR. Recent studies indicate the functional importance of the HVR. For example, expression of the HVR with the N-terminal portion of tomosyn was sufficient to elicit an inhibitory effect on vesicle priming (24). In addition, protein kinase A (PKA) phosphorylation within the HVR acts to negatively regulate tomosyn interaction with syntaxin1A and upregulate the readily releasable vesicle pool in superior cervical ganglion neurons (22). However, this phosphorylation site is not present in all tomosyn isoforms and, therefore, is not a general regulatory site of tomosyn function.

In this paper, we capitalize on differences between tomosyn-1 and tomosyn-2, and their isoforms to identify structural motifs of tomosyn that underlie its functional activity. Based on sequence analysis we find that rat m-tomosyn-1 shares a nearly identical structural conformation with the yeast homologue Sro7, but, importantly, contains an additional three loops...
that emanate free from the main β-propeller structure. Remarkably, deletion of loops 1 or 3, but not 2, resulted in a complete loss in negative regulation of tomosyn on secretion, although a strong interaction with syntaxin1A remained. We also identify a site within the HVR of m-tomosyn-1 that is subject to SUMOylation, and which regulates the extent of tomosyn inhibition on secretion. Taken together, these data identify multiple novel structural motifs of tomosyn that are critical to its negative regulation on secretory activity independent of SNARE-SNARE interactions.

**Experimental Procedures**

**Antibodies and chemicals**—The following antibodies were used: α-syntaxin1A clone HPC-1 (Sigma, St. Louis, MO); α-SNAP25 clone 71.1 (Synaptic Systems, Göttingen, Germany); α-tomosyn-1 clone 15 (BD Transduction Laboratories, San Jose, CA); α-V5 epitope (Invitrogen, Carlsbad, CA); α-Flag antibody (Sigma); α-SUMO1 and α-SUMO 2/3 (Abcam); and IRDye 800CW Goat α-Mouse IgG (H + L) and IRDye 680 Goat α-Rabbit IgG (H + L) (LiCoR Biosciences, Lincoln, NE). MG132, a cell-permeable, proteasome inhibitor was obtained from Sigma (St. Louis, Missouri).

**Expression constructs**—Tomosyn loop deletion mutants were initially created in plasmid pSFV. The segment N-terminal to each deletion region was PCR amplified while adding restriction sites, BamHI to the 5’ end and Ascl and BssHII to the 3’ end. DNA sequence corresponding to glycine residues was added upstream of the Ascl sites to replace the missing loop regions and prevent disruptions of the 3D structure of the protein. Then, the segment was ligated to the vector (pSFV-GFP) using the BamHI and BssHII restriction sites. The segment C-terminal to the deletion area was PCR amplified in a similar manner, adding the restriction enzyme sites Ascl and BssHII. This segment also included sequence corresponding to glycine residues to replace the deleted region. This plasmid construction was repeated for all loop deletion mutant constructs. As a final step, these mutated cDNA sequences were subcloned pEGFP for imaging in PC12 cells and pFlag for growth hormone secretion assay.

Plasmids containing full-length cDNA inserts for mouse (Mus musculus) m-tomosyn-1, in plasmid pcDNA3.1, and tomosyn-2 isoforms (s-, m-, b- and xb-) in plasmid pCR-ScriptSKII+ were generously provided by Dr. Alexander J.A. Groffen (Vrije Universiteit, Amsterdam, The Netherlands) and subcloned into vector pDNRCMV (Clontech). Monomeric mutants (A206K) of pECER-C1 (cureulain) and pEcYFP-C1 (citrine) vectors containing the LoxP sequence were used as recipient vectors for subcloning mouse m-tomosyn-1, and mouse (s-, m-, b- and xb-) tomosyn-2 to the C terminus of cureulain and citrine fusion tags using the Cre-recombinase-mediated Creator system (Clontech). Mouse s-tomosyn-1 was obtained by PCR-based deletion of the region corresponding to exon 22 of mouse m-tomosyn-1 in vector pDNRCMV using the QuickChange® Site-Directed Mutagenesis (SDM) kit (Stratagene). The resulting mutant was subsequently transferred by recombination to vectors pLoxP-cureulain and pLoxP-citrine.

SDM was also used to generate mutants of rat m-tomosyn-1. Mutants include a point mutation that introduces a premature stop codon at residue 1068 resulting in a tomosyn that lacks the C-terminal SNARE domain (ΔSNARE), and a Lys to Arg or Ala mutation at Lysine residue 730. The sequence fidelity of all constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core).

**Protein expression of GST, GST-syntaxin1A and biotinylated tomosyn**—BL21 DE3 cells were transformed with a pGex-KG vector encoding GST, or a soluble form of GST-syntaxin1A (residues 4-263). Cells were grown to an OD$_{600}$ of 0.4–0.6, induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and grown O/N with shaking at 23°C. Cells were harvested by centrifugation for 20 min at 6000 x $g$ (Beckman, Fullerton, CA, JA-14 rotor). The resulting pellet was resuspended in French Press Buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.05% Tween-20, 1 mM DTT) supplemented with a protease inhibitor mixture (Roche) at 20 ml/liter of culture, passed twice through the press (10,000 psi), and centrifuged at 20,000 x $g$ for 20 min at 4°C. The supernatant (S1) was
recovered and GST or GST-syntaxin1A was purified from the S1 using glutathione-Sepharose 4B beads (Amersham), according to the manufacturer’s protocol. A small sample of the bound beads was fractionated by SDS-PAGE alongside standard amounts of BSA followed by staining with Coomassie. The staining intensities of the Coomassie stained gels were captured and analyzed by Odyssey Infrared Imaging Systems (LiCor).

Mouse tomosyn-1 isoforms (s- and m-) and tomosyn-2 isoforms (m- and xb-) were PCR amplified and TOPO cloned into Gateway® entry vector pENTR/D-TOPO (Invitrogen) according to the manufacturer’s specifications. N-terminally tagged mammalian expression constructs were generated by performing LR recombination into the Gateway® destination vector pcDNA™3.2/capTEV™/V5-DEST. Mouse s- and b-tomosyn-2 mammalian expression constructs were generated by PCR-based deletion of the regions corresponding to exon 20 in mouse m-tomosyn-2 and xb-tomosyn-2 in Gateway® destination vector pcDNA™3.2/capTEV™/V5-DEST using SDM. Similar mutagenesis methods were used to generate additional tomosyn-2 deletion constructs by deleting the regions corresponding to exon 21 from s-tomosyn-2, m-tomosyn-2, and b-tomosyn-2 in mammalian expression vector pcDNA™3.2/capTEV™/V5-DEST to create null (ΔHVR), exon 20, and exon 22 tomosyn-2 mutants, respectively. The sequence fidelity of all constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core).

Cell culture and transfection—Rat adrenal pheochromocytoma (PC12) cells were plated and cultured in Kaighn’s media supplemented with 15% horse serum, 2.5% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO₂. Human embryonic kidney (HEK) 293A cells were cultured at 37 °C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% Glutamax (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. One hour before transfection, cells were placed into OptiMEM (Invitrogen) lacking antibiotics and serum. 4-6 h after the transfection, cells were returned to normal growth medium.

Real-time quantitative PCR—Quantitative analysis of mRNA levels was performed in duplicate by real-time monitoring of SYBR Green I fluorescence on a Mastercycler® ep realplex² real-time PCR system (Eppendorf). Prior to cDNA synthesis, RNA was extracted from cells transfected with a mammalian expression plasmid coding for biotinylated tomosyn-1 (s- or m-), tomosyn-2 (s-, m-, b-, or xb-) or their mutants (ΔHVR, s-tomosyn-2 [20], or s-tomosyn-2 [22]) using the RNasy mini kit (Qiagen). Following treatment with RNase-free DNase I (Qiagen) to degrade trace amounts of genomic DNA, 3 μl total RNA was used for Oligo(dT)₁₅-primed cDNA synthesis by AMV reverse transcriptase (Promega) for 15 min at 42 °C. Each qPCR reaction was composed of 2 μl template cDNA, 70 nM primers, 12.5 μl 2X FastStart SYBR Green qPCR Master mix (Roche), and PC-grade water in a total reaction volume of 25 μL. The thermal cycler program included an initial polymerase activation step (10 min at 95 °C), followed by 40 cycles of denaturation (15 s at 95 °C), annealing (30 s at 50 °C) and polymerization (20 s at 68 °C). Finally, a dissociation curve was determined for each sample (15 s at 95 °C and 15 s at 50 °C, followed by a slow ramp to 95 °C under continuous monitoring of fluorescence intensity). In all cases, the dissociation curves excluded a significant contribution of relatively short by-products to the measured fluorescence intensities. All measurements were performed in duplicate. To allow for normalization with respect to the amount of template cDNA in different samples, we also measured the mRNA levels for the small subunit (SSU) 18S rRNA housekeeping gene measured in duplicate. For each reaction, the cycle threshold value was calculated as the fractional cycle number at which the fluorescence intensity exceeds a threshold value of 10 standard deviations above background fluorescence. Relative gene expression was calculated using the comparative CT, or 2⁻ΔΔCT, method (36-37).

Homologous proteins for structural modeling—Homologues were collected in order to build a
multiple sequence alignment, from which the pair-wise alignment between target and template would be extracted. The homologues taken here for each family; Sro7, LGl and tomosyn, are the same as in (26).

**Multiple sequence alignment**— A multiple sequence alignment (MSA) of all the homologues was created for each protein family (Sro7, tomosyn and LGl) separately, using the alignment software MUSCLE (38). Each MSA was edited according to secondary structures as indicated in (26) and cross-referenced with secondary structure prediction by PSIPRED (39). This was followed by a profile-to-profile alignment between the tomosyn and LGl profiles, with editing of the MSA (see above) and finally run a profile-to-profile alignment between the tomosyn-LGL profile and the Sro7 profile. This last MSA was modified, again, according to secondary structure elements. Ultimately the pair-wise alignment of interest, between the m-tomosyn-1 and Sro7 of the *S. cerevisiae*, was extracted and used for structural modeling.

**Modeling**—The model structure of the tomosyn protein was built with the NEST modeling software (40). The modeling was implemented with optimization refinement in insertion and deletion regions (usually loop regions).

**Model validation**—The compatibility of the model structure with general properties of proteins was examined by calculating Ramachandran plots. Another indicator of structural quality is the correlation between the degree of evolutionary conservation of amino acid positions and their location: buried residues are usually highly conserved while peripheral residues are variable. The evolutionary conservation was calculated using MSA of homologues (Sro7, LGl and tomosyn) and the ConSurf server (41).

**Human growth hormone (hGH) secretion assay**—PC12 cells were plated onto poly-D-Lysine coated 24-well plates and co-transfected with a plasmid coding for human growth hormone (hGH), in addition to full-length tomosyn-1 (s, or m isoform), tomosyn-2 (s, m, b, or xb isoform), or an empty vector control. Each of the mouse tomosyn genes were expressed as N-terminal biotinylated V5-epitope tagged fusions in pcDNA3.2/capTEV/V5-DEST vectors (Invitrogen). The total concentration of transfected DNA was held equal across all treatments. Secretion assays were performed 48 h following transfection. To measure secretion, cells were rinsed for 5 min in a physiological saline solution, PSS (145 mM NaCl, 5.6 mM KCl, 15 mM NaHEPES, 0.5 mM MgCl₂, 2.2 mM CaCl₂, pH 7.3 containing 5.6 mM glucose, 2 mg/ml sodium ascorbate and 2 mg/ml fatty acid-free bovine serum albumin). Cells were subsequently incubated in either an additional application of saline or stimulated to secrete by treatment with saline supplemented with 100 mM K⁺ (equimolar replacement for Na⁺), each for 10 min. The saline solution containing the secreted hGH was collected, and cells were lysed (2% Triton X-100) to determine total hGH content. Secreted and total hGH was measured from sample aliquots using an hGH enzyme-linked immunosorbent assay kit (Roche). Each experiment was performed with duplicate replicates for each treatment with experiments repeated a minimum of 3 times.

**Syntaxin1A Q-SNARE-tomosyn V-SNARE binding analysis**—GST or soluble GST-syntaxin1A was prepared as described above. GST or GST-syntaxin1A bound to glutathione-Sepharose 4B beads was then incubated for 16 h at 4 °C with lysates prepared from HEK293A cells expressing mouse tomosyn isoforms that were N-terminal biotinylated, V5 or of rat m-tomosyn-1 variants that were Flag-tagged. The lysis buffer contained 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 200μM EDTA, 1.5 mM MgCl₂, 0.1% NP40, and protease inhibitors (Roche). The beads were then collected by centrifugation and washed three times with lysis buffer. Bound proteins were analyzed by SDS-PAGE followed by immunoblot analysis with α-V5 antibody (Invitrogen).

**Analysis of tomosyn SUMOylation**—N-terminal fused V5 epitope tagged constructs of m-Tomosyn-1, s-Tomosyn-1 or an empty vector control were transfected into HEK293 cells. At 48 hours post-transfection the cells were lysed and Tomosyn was immunoprecipitated using Dynabeads® Protein G (Invitrogen) beads prebound with α-V5 antibody. After extensive washing, immunoprecipitates were resuspended in 2X SDS reducing sample buffer and fractionated by SDS-PAGE followed by
immunoblot analysis with α-SUMO1 or α-SUMO2/3 antibodies.

Fluorescence Microscopy of Cerulean and Citrine Fusion Proteins in Live Cells—PC12 cells were cultured for 48 h post transfection on poly-D-lysine coated glass coverslips (No. 1, Fisher) attached to the bottom of 35mm culture dishes. Prior to fluorescence imaging, culture media bathing the cells was removed and replaced with PSS. Confocal imaging of the subcellular localization of expressed fluorophore-tagged proteins was performed on an Olympus FluoView 500 Laser Scanning Confocal Microscope (Olympus, Melville, NY), using an LD405 laser, 60X objective (1.2 numerical aperture) and a pinhole aperture of 260μm.

Sensitized emission FRET was detected using an Olympus IX71 microscope equipped with a 60X 1.49 NA oil immersion objective. Two lasers, a 45 mW, 442 nm diode-pumped solid state laser (Toptica Photonics, Grafelfing, Germany) and a 225 mW Argon-ion laser (National Laser Company, Salt Lake City, UT) were combined into an accousto-optic tunable filter whose output was coupled via a unimodal fiber optic into the microscopes back port. The microscope’s filter cube contains a triple band laser clean-up filter (z442/514/594x) and laser polychroic mirror (z442/514/594rpc). Fluorescence emission from cerulean and citrine were separated with a Quad-View beam splitter (Mag Biosystems, Pleasanton, CA) containing two dichroic mirrors and emission filters for Cerulean (HQ480/40m) and citrine (HQ540/30m), as well as mCherry (HQ630/50m) and infrared (D700/10m). All filters were from Chroma Technology (Rockingham, Vt). Images were acquired with a Photometrics QuantEM 512SC EM-CCD camera (Roper Scientific, Tuscon AZ). A final pixel size of 67 nm (object coordinates) was achieved by insertion of a 1.6x slide magnifier and a 2.5x beam expander in the emission light path. Raw FRET image sets (donor excitation, donor emission; donor excitation, acceptor emission; acceptor excitation, acceptor emission) were aligned, background subtracted and analyzed offline using MATLAB (The MathWorks, Natick, MA) scripts. Sensitized emission FRET analysis used the FRET stoichiometry method (42). Calibration parameters specific to the optical system and fluorophores that are required for FRET analysis were determined as previously described (43). The apparent FRET efficiency of the acceptor in complex with the donor (EA), apparent FRET efficiency of the donor in complex with the acceptor (ED), and molar ratio of total acceptor/donor (Ratio) were determined by pixel-by-pixel analysis of the three required images.

Data analysis and statistics—Statistical analysis was performed using IGOR PRO (Wavemetrics Inc.) and Excel (Microsoft Office 2007) software. Population data were expressed as means ± S.E., and statistical significance was determined using Student's unpaired t tests or for multiple comparisons using ANOVA with Dunnett's post hoc test or a Mann-Whitney U test for non-parametric data. Significant differences are defined in the corresponding figure legends and indicated by asterisks.

RESULTS

Homology modeling of tomosyn. The crystal structure of a close homologue of tomosyn, S. cerevisiae Sro7 (gi: 6325289) has been determined at 2.4Å resolution (pdb id: 2OAJ) and reveals a structure (residues 61-962) comprised of two, seven-bladed WD40 β-propellers, which lead to a sequence ‘tail’ domain bound to the β-propeller loops (Figure 1A) (26). This structure was used as a template for modeling of the rat m-tomosyn-1 (gi: 3790389) protein. The sequence identity between target and template was low (~19%), but the core domains of m-tomosyn-1 (Figure 1B) were generally well modeled based on the Sro7 template (Figure 1A). This structure was used as a template for modeling of the rat m-tomosyn-1 (gi: 3790389) protein. The sequence identity between target and template was low (~19%), but the core domains of m-tomosyn-1 (Figure 1B) were generally well modeled based on the Sro7 template (Figure 1A). This is reflected in the compatibility of the evolutionary conservation profile of the protein with the structure (Figure 1D, right panel). As anticipated, the structural core is highly conserved while the periphery is variable; same as in the Sro7 template (Figure 1A). This is reflected in the compatibility of the evolutionary conservation profile of the protein with the structure (Figure 1D, right panel). As anticipated, the structural core is highly conserved while the periphery is variable; same as in the Sro7 template (Figure 1A). However, the primary tomosyn sequence is significantly longer and exhibits three loops that deviate from the Sro7 template. These loops result from insertions in m-tomosyn-1 compared to Sro7, creating regions in the model with no
The loops emanate from the main β-propeller backbone as shown in Figure 1B. These loops are conserved among the tomosyn genes and isoforms, and encompass amino acid residues of rat b-tomosyn-1 and xb-tomosyn-2 as shown in Figure 1C. Loop two, is the most extensive, occurring in an exposed region of the protein. A separate homologous template for this region was not found. The individual loops are likely to be functionally important, as phosphorylation of a serine residue within loop 2 of rat m-tomosyn-1 has been reported to regulate tomosyn interaction with syntaxin1A and the extent of tomosyn mediated secretory inhibition (22).

To validate the accuracy of the tomosyn model, a Ramachandran plot was created using the Swiss pdb-viewer (44). The plot revealed discrepancies that rendered the model inadequate. However, going over the residues in the disallowed regions, it was clear that they were all part of the three template-less loops that were arbitrarily modeled. Consequently, a second Ramachandran plot was created without the three undefined loops resulting in a highly significant correlation. Residues that remained located in the disallowed regions were restricted to sequence defined above as the loop regions.

Effect of tomosyn loop regions on inhibition of secretion in PC12 cells. To test for functional effects of each loop region, deletion mutants of rat m-tomosyn-1 were constructed and transfected individually into PC12 cells along with a reporter of the regulated secretory pathway, human growth hormone (hGH) (45). As shown in Figure 2A, wild type m-tomosyn-1 inhibited elevated K⁺-induced hGH secretion by approximately 36% (27.7 ± 1.8% of total hGH, control; 17.8 ± 2.8%, m-tomosyn-1; mean ± SE, n=4) relative to control cells transfected with empty vector. Importantly, deletion of loop 1 (Δ-loop 1) or loop 3 (Δ-loop 3) resulted in complete elimination of inhibition by tomosyn of K⁺-induced hGH secretion. By comparison, deletion of loop 2 (Δ-loop 2) had no significant effect on tomosyn’s ability to inhibit secretion. Notably, these differences did not result from reduced expression of the Δ-loop mutants relative to wild type m-tomosyn-1 (Figure 2A inset). In addition, there were no significant effects of m-tomosyn-1 or the Δ-loop mutants on basal secretion.

Tomosyn inhibition of secretion is believed to depend primarily on its high affinity interaction with syntaxin1A and SNAP25 via its C-terminal VAMP-like R-SNARE domain. Therefore, we next determined if the lack of secretory inhibition by the Δ-loop 1 and Δ-loop 3 tomosyn mutants resulted from reduced syntaxin1A binding relative to wild-type m-tomosyn-1. Experiments used GST-syntaxin1A immobilized on glutathione sepharose beads to pull-down each Δ-loop mutant from HEK293 cell lysate that was then compared to pull-down by wild type tomosyn. The recombinant tomosyn constructs were expressed in HEK293A cells to facilitate maturation and folding of the protein in a eukaryotic environment. The HEK293 cell line does not demonstrate endogenous expression of neuronal SNARE proteins (46) and, therefore, provides a clean background upon which to test interactions with the various tomosyn deletions. Immunoblots of the pull down reactions show that each of the Δ-loop mutants bound GST-syntaxin1A equivalent to that of wild type m-tomosyn-1 (Figure 2B). N-terminal epitope Flag tags on each tomosyn construct were used for detection to avoid potential differences in reactivity of the tomosyn deletion mutants to the tomosyn antibody. Membrane targeting of tomosyn occurs via direct interaction with syntaxin1A at the plasma membrane (20,24). Therefore, as an additional test for interaction between the tomosyn mutants and syntaxin1A, we compared membrane targeting of each Δ-loop tomosyn mutant relative to wild type tomosyn when coexpressed with mCherry-syntaxin1A in PC12 cells. Figure 2C shows that each tomosyn mutant targeted to the membrane, indicating the capacity of these mutants to interact with syntaxin1A in vivo. Thus, loop domains one and three are required for the inhibitory activity of m-tomosyn-1 on regulated secretion, independent of tomosyn’s ability to bind syntaxin1A.

Effect of the HVR tomosyn loop 2 on tomosyn expression. All structural differences between the isoforms of each tomosyn gene occur strictly within the HVR that is contained within the loop 2 region. Differential splicing of tomosyn-1
generates 3 distinct isoforms (s, m and b), whereas splicing of tomosyn-2 results in four isoforms (s, m, b, and xb) (Figure 3A). As noted above, deletion of loop 2 did not reduce the level of inhibition exerted on secretion by rat m-tomosyn-1. On the other hand, allosteric regulation within loop 2 via PKA-mediated phosphorylation (S724) has been reported to reduce tomosyn’s inhibition of secretion (22). This suggests that variation in loop 2 between the tomosyn isoforms may be a critical component of tomosyn regulation. Indeed, the PKA phosphorylation site is absent in the s- and m-tomosyn-2 isoforms, as well as s-tomosyn-1. To compare the physiological effects of the different loop 2 region splice variants, mouse tomosyn-1 (except b-tomosyn-1) and tomosyn-2 isoforms were transfected into HEK293 cells. Surprisingly, although m- and s-tomosyn-1 were well expressed, only the m-tomosyn-2 isoform and to a lesser extent, xb-tomosyn-2, demonstrated protein overexpression detectable on Western blots (Figure 3B). These expression differences resulted from differences in the HVR, as each tomosyn isoform was subcloned similarly into the pcDNA™3.2 vector and DNA sequencing confirmed that structural differences were limited to the HVR for each tomosyn gene. Moreover, RT–PCR of RNA extracted from HEK293A cells transfected with each tomosyn isoform demonstrated presence of each construct of appropriate size in all cases (data not shown). To determine if differences in protein expression resulted from differences in levels of mRNA expression we employed real-time qPCR to quantify the mRNA of each splice variant in transfected cells relative to that of m-tomosyn-1. As a control for differences in transfection efficiency, an additional set of primers were included in the qPCR reactions that corresponded to the neomycin resistance gene found in the pcDNA3.2 plasmids. No significant differences were found in the levels of mRNA between the endogenous tomosyn isoforms relative to that of m-tomosyn-1 (Figure 3C).

To identify specific exons within the HVR that may be responsible for the lowered protein levels of most of the tomosyn-2 variants relative to m-tomosyn-2, we generated additional mutants where sequence corresponding to exon 21 in the s-, m- and b-tomosyn-2 isoforms was deleted to create tomosyn-2 cDNA with no HVR (null), exon 20 only or exon 22 only, respectively (Figure 3A). Each construct was then transfected into HEK293 cells and protein and mRNA expression examined. As shown in the western blot of Figure 3B, none of these mutants generated appreciable tomosyn protein relative to that exhibited by m-tomosyn-2. Importantly, mRNA levels quantified by real time qPCR demonstrated no significant difference in mRNA expression between an exon null and exon 20 only m-tomosyn-2 construct with that of m-tomosyn-2 (Figure 3C). RT-PCR confirmed mRNA products expressed were of appropriate size (data not shown). The combination of exons for tomosyn-2 that leads to the highest protein levels is the combination of exon 20 and 21 (m-tomosyn-2), with the addition of exon 22 (xb-tomosyn-2) demonstrating limited expression. By comparison, each tomosyn-1 HVR splice variant, as well as the null mutant, expressed mRNA and protein similar to that of m-tomosyn-1, indicating effects of HVR splicing on protein levels were specific to tomosyn-2. Considered together, the differences in protein levels between tomosyn genes/isoforms indicate that exon 19 in tomosyn-1 may stabilize the protein and that the combination of exons 20 and 21 in tomosyn-2 act in a partial manner to reduce degradation/turnover of tomosyn-2 protein.

To evaluate if ubiquitin proteasome-mediated tomosyn-2 degradation may be responsible for limited accumulation of tomosyn-2 protein, the effect of a proteasome inhibitor, MG132, was studied. Figure 3D illustrates that the levels of m-tomosyn-1, m-tomosyn-2 and to a lesser extent xb-tomosyn-2, but not s-tomosyn-2, were substantially increased following MG132 (100 µM) treatment in transfected PC12 cells.

Functional differences between tomosyn-1 and tomosyn-2 on secretion from PC12 cells. To date, functional evaluation of tomosyn has relied exclusively on the m-tomosyn-1 isoform. To extend analysis to the tomosyn-2 gene we compared the effects of overexpression of mouse m-tomosyn-1, s-tomosyn-1, m-tomosyn-2 and xb-tomosyn-2 on secretion using PC12...
cells cotransfected with hGH. Notably, each of the tomosyn genes/isoforms inhibited elevated K\(^+\)-induced hGH secretion to a similar extent (Figure 4A). By comparison, a significant increase in basal secretion occurred with m-tomosyn-2 overexpression when expressed relative to the empty plasmid control (Figure 4A). Real time qPCR and western blots performed on samples of the cells used to assess secretory responsiveness demonstrated only slightly lower expression of m-tomosyn-2 relative to that of the tomosyn-1 isoforms (Figure 4C). Differences in resting [Ca\(^{2+}\)] cannot account for the m-tomosyn-2 enhancement of basal secretion, as no significant difference in resting free [Ca\(^{2+}\)] was found between the empty vector transfected controls and PC12 cells overexpressing m-tomosyn-2 or m-tomosyn-1 (Figure 4D). Together, these data demonstrate a lack of functional distinction between the tomosyn genes/isoforms relative to inhibition of evoked secretion, but pinpoint a novel function for m-tomosyn-2 as a positive regulator of basal secretion.

To determine if m-tomosyn-2 differed in its interaction with syntaxin1A relative to other tomosyn isoforms we next compared the subcellular distribution patterns of citrine-tomosyn isoforms when expressed individually, and when coexpressed with cerulean-syntaxin1A in PC12 cells. Alone, citrine-tomosyn isoforms showed a diffuse cytosolic distribution (Figure 5A) consistent with the previously described localization of rat m-tomosyn-1 (20). By comparison, coexpression with cerulean-syntaxin1A dramatically altered the citrine-tomosyn distribution resulting in strong targeting of each tomosyn isoform to the plasma membrane region. To confirm that the redistribution reflects a direct interaction between the citrine-tomosyn isoforms and cerulean-syntaxin1A we performed sensitized emission FRET imaging. The FRET results demonstrate that each of the tomosyn isoforms directly interact with syntaxin1A in vivo (Figure 5B). Indeed, m-tomosyn-1 and s-tomosyn-1 show equivalent apparent FRET efficiency with syntaxin1A (ED, 10.92% ± 0.783, m-tomosyn-1; 10.94% ± 0.817 s-tomosyn-1), while FRET efficiency for m-tomosyn-2 and xb-tomosyn-2 were found to be significantly greater. Taken together, these data indicate that facilitation of basal secretion by m-tomosyn-2 overexpression relative to other tomosyn isoforms does not result from a reduced interaction with syntaxin1A.

**Tomosyn HVR is a substrate for SUMOylation.** SUMO modification of proteins can alter their localization, activity, stability, or transcriptional regulation as a result of alteration of inter- or intramolecular interactions (47-48). SUMO acceptor sites map to a consensus sequence Ψ-K-x-D/E, where Ψ is a hydrophobic residue. SUMO modification results in an isopeptide bond between the C-terminal glycine residue of SUMO and the lysine side chain. Examination of the rat m-tomosyn-1 sequence using SUMOplot™ Prediction (Abgent, San Diego, CA) revealed two highly predicted SUMOylation sites, K730 and K298 (Figure 6A). The K730 site (LKPD) resides in exon 22 of m-tomosyn-1. As a result of alternative splicing of exons within the HVR, the K730, sumoylation site is absent in s-tomosyn-1, while all tomosyn-1 isoforms carry a K298 site. Initial experiments examined if m-tomosyn-1 and s-tomosyn-1 were substrate targets for SUMOylation. For these experiments, N-terminal fused V5 epitope tagged constructs of m-tomosyn-1 and s-tomosyn-1 and empty vector were transfected into HEK293 cells. Following a 48 hour expression period the cells were lysed, and overexpressed tomosyn immunoprecipitated using α-V5 antibody. Immunoprecipitates were then fractionated by SDS-PAGE, and the resulting immunoblots probed with α-SUMO antibody recognizing SUMO2/3 isoforms. Both m-tomosyn-1 and s-tomosyn-1 demonstrated SUMO 2/3 labeling, with specificity confirmed by a lack of signal for the empty vector control (Figure 6B). Importantly, comparison of anti-SUMO immunoreactivity demonstrated no significant difference between s- and m-tomosyn-1 samples when corrected for sample input, suggesting no altered SUMOylation at an alternative site than K730, or that multiple SUMOylation sites are present. To further evaluate whether K730 is sumoylated in m-tomosyn-1, we repeated the experiment, but compared the level of SUMO immunoreactivity between wild type and an m-tomosyn-1 mutant K730R. The data demonstrate a selective loss of
SUMOylation in the mutant (Figure 6C), thereby demonstrating K730 as a substrate site for m-tomosyn-1 SUMOylation.

To evaluate the functional consequence of SUMOylation at K730 on regulated secretion we compared the effects of overexpressed wild-type m-tomosyn-1 to SUMOylation site mutants of m-tomosyn-1. As shown in Figure 6D, mutation of K730 to either alanine or arginine significantly enhanced tomosyn’s inhibitory effect (normalized hGH secretion: tomosyn (wt) 0.76 ± 0.07; tomosyn K730A 0.52 ± 0.04; tomosyn K730R 0.50 ± 0.1). The enhanced inhibition of secretion by the K730R mutant was not attributable to enhanced expression compared to wild-type tomosyn, as demonstrated in Western blots of HEK cell lysates overexpressing V5-tagged tomosyn constructs (Figure 6D inset). Moreover, the similarity between the K730R and K730A mutants suggests that the increased inhibitory function was unlikely to be related to mutation of the positive charge of the lysine residue.

Typically, only a small fraction of a given protein is SUMOylated and this modification has been shown to result in altered protein localization and binding partners. We hypothesized that if tomosyn’s inhibitory function was related to its ability to form SNARE complexes, then mutation of the K730 residue should be associated with an increase in FRET with syntaxin1A compared to wild-type tomosyn. However, we found that FRET between tomosyn K730R and syntaxin1A was no different from that with wild-type tomosyn, when measured across a range of molar expression ratios (Figure 6E).

**DISCUSSION**

Homology modeling of m-tomosyn-1 onto its yeast counterpart, Sro7, was used in the present study to identify novel structural motifs in tomosyn that may be important to its activity on the regulated secretory pathway. Our findings reveal an evolutionarily conserved N-terminal β-propeller structure in which tomosyn differs by three unique regions of loops. Remarkably, deletion of loop1 and loop3, but not loop 2, of m-tomosyn-1 resulted in the complete elimination of its ability to inhibit secretion. By comparison, loop 2 was found important in determining the extent of tomosyn protein accumulation, although this effect was restricted to tomosyn-2 isoforms. On the other hand, the HVR of loop 2 in those tomosyn-1 isoforms containing exon 22 (b- and m-tomosyn-1) was demonstrated to be a target substrate for SUMOylation. Finally, while functional analysis of tomosyn-1 and tomosyn-2 activity had previously been restricted to the m-tomosyn-1 isoform, our data indicate that tomosyn-1 and tomosyn-2 isoforms exert similar inhibitory activity on exocytic secretion.

Tomosyn is largely believed to inhibit secretion via direct competition with VAMP for reactive Q-SNARE proteins (49). This interpretation is consistent with studies demonstrating that tomosyn overexpression substantially reduces exocytosis in neurosecretory cells (8,10,15-20) and neurons (21-22) by inhibiting vesicle priming reactions that generate fusogenic SNARE core complexes. Moreover, mutations of the ortholog to vertebrate tomosyn in C. elegans, Tom-1, demonstrated that Tom-1 normally negatively regulates vesicle priming at neuromuscular synapses in vivo (11-14,50). These functional studies are complemented by an extensive analysis of tomosyn interactions attained through in vitro binding studies and co-immunoprecipitation, which demonstrate that tomosyn’s R-SNARE domain is required for high affinity interaction with syntaxin1A (10,21,34), and that it also mediates interaction with SNAP25, SNAP23 and syntaxin4 (19). The affinity of the interaction is sufficient to compete with and dissociate Munc18-1 from a conformational closed form of syntaxin1A (8,20). Although tomosyn exhibits heterodimer interaction with these Q-SNAREs, it also readily forms into stable heterotrimeric SNARE core complexes in vitro (10,19,21). Structurally, these tomosyn-containing SNARE core complexes are very similar to fusion competent syntaxin1A-SNAP25-VAMP SNARE complexes, although tomosyn complexes failed to exhibit tight binding of complexin (9).

In spite of extensive biochemical evidence demonstrating tomosyn’s capacity to form tomosyn-SNARE core complexes, it remains uncertain if this association alone is necessary
and sufficient to limit availability of reactive Q-SNAREs in vivo for interaction with VAMP and inhibit vesicle priming. Indeed, in the present study deletion of loops 1 or 3 (44 and 22 residues in size, respectively) in the N-terminal region of tomosyn ablate tomosyn’s inhibitory action on exocytosis. We believe that the loop deletion is unlikely to have induced loss of the integrity of the large β-propeller structure, as Sro7 which lacks these loops is a β-propeller structure. In addition, these tomosyn mutants retained membrane targeting and syntaxin1A interaction, suggesting that the mutants were still capable of forming tomosyn-containing heterotrimeric SNARE complexes. Overall, these results suggest that tomosyn R-SNARE/plasma membrane Q-SNARE interactions do not, per se, limit Q-SNARE availability for vesicle priming, but instead dictate targeting of tomosyn to sites where Q-SNAREs are actively transitioning into SNARE core complexes. These data complement previous reports demonstrating that tomosyn’s R-SNARE domain is essential in live cells for targeting of tomosyn to the plasma membrane via directinteraction with syntaxin1A (20) and for forming a SNARE complex containing SNAP25 (10). Of specific importance, loop 1 and 3 deletion data demonstrate that domains of tomosyn outside the R-SNARE mediate control over formation of VAMP-containing SNARE complexes in vesicle priming and consequent exocytotic activity.

In support of an inhibitory function to regions N-terminal to tomosyn’s R-SNARE are reports demonstrating that tomosyn continued to exert secretory inhibition in transfected PC12 (18,20) and chromaffin cells (24) when key R-SNARE residues required for efficient binding of syntaxin were mutated (18) or when the R-SNARE domain was deleted (20,24). In addition, the tomosyn gene family includes Drosophila tumor suppressor lethal giant larvae with homologues in yeast (Sro7p, Sro77p) (29-30) and mammals (Lgl) (28,51) which all lack a defined R-SNARE motif, yet retain the ability to interact with Q-SNARE proteins and perhaps regulate vesicle targeting and SNARE complex assembly.

Recently, three separate, but not mutually exclusive mechanisms have been proposed by which the N-terminal region of tomosyn may control calcium-regulated exocytotic activity. These include, 1) an N-terminal mediated oligomerization of SNARE core complexes that reduces the probability of a vesicle SNARE to form reactive monomeric SNARE complexes at presumptive sites of exocytosis (21); 2) a structural rearrangement of the 60 residue tail motif found immediately proximal to the SNARE domain that regulates the efficacy and stability of tomosyn’s R-SNARE interaction with Q-SNAREs (31-32); and, 3) direct binding of the calcium sensor synaptotagmin-1 to tomosyn’s N-terminal region, which results in negative regulation of synaptotagmin-1 function (33).

It is notable that loops 1 and 3 of tomosyn, identified here as essential for tomosyn’s inhibition of secretion, are located on the bottom surface of the N-terminal β-propeller. By comparison, the bottom surface of the β-propeller region of Sro7 forms the binding interface upon which the Sro7 tail interacts, with many of the residues conserved in tomosyn. The additional sequence forming the loop regions in tomosyn raises the possibility that these loops may exert regulation on conformational rearrangements of tomosyn’s tail domain. Indeed, although the low affinity Q-SNARE binding site within the N-terminal region of tomosyn has not yet been mapped, allosterically regulated conformational shifts in the tail region of Sro7, which lacks an R-SNARE motif, enables direct binding of the Q-SNARE Sec9 (26). An orthologous tail region in tomosyn may, therefore, regulate low affinity Q-SNARE interactions. Alternatively, loops 1 and 3 may present scaffolding platforms for effectors that allosterically regulate Q-SNARE binding and assembly of SNARE complex oligomers, or that stabilize synaptotagmin-1 interaction.

The level of tomosyn interaction with Q-SNAREs has been reported to be regulated by protein kinase A mediated phosphorylation of serine 724 (S724) of m-tomosyn-1 within the HVR (22), and of Rho/ROCK phosphorylation of serine 14 of syntaxin1A (20,52). In this study we have extended potential for allosteric regulation of tomosyn-1 by identifying a SUMOylation site (K730) within the loop 2 HVR region of b- and m-tomosyn-1. This site
was sumoylated by SUMO 2/3 proteins, and SUMO modification was eliminated in a (K730R) site directed mutant of m-tomosyn-1. In addition, functional analysis of tomosyn carrying mutations of the SUMOylation site resulted in an increase in tomosyn inhibition of secretion without alteration in the extent of tomosyn interaction with Q-SNAREs. The signaling pathway utilized to regulate SUMOylation at this site as well as the mechanism by which SUMOylation reduces the inhibitory activity of tomosyn remains to be elucidated. However, SUMOylation frequently allows novel interactions of the modified target protein (53). Furthermore, our data imply that the HVR within loop 2 is a regulatory unit for adjusting the extent of accumulation of tomosyn-2, but not tomosyn-1 protein. This regulation may involve susceptibility of specific tomosyn 2 isoforms to rapid turnover via proteosomal degradation.

In summary, our findings are important as they identify and characterize new structural motifs of tomosyn N-terminal to the SNARE region that substantially impact tomosyn’s ability to inhibit secretion independent of alterations in the strength of tomosyn’s interaction with syntaxin1A. Furthermore, the results establish that regulation of secretion by tomosyn involves cooperative action between the newly identified N-terminal domains of tomosyn that regulate assembly of tomosyn-containing SNARE complexes and tomosyn’s R-SNARE that targets tomosyn to the plasma membrane.

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FOOTNOTES

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The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive attachment receptors; SNAP25, synaptosome-associated protein 25 kDa; VAMP, vesicle associated membrane protein; HVR, hypervariable region; hGH, human growth hormone; PKA, protein kinase A; FRET, Förster resonance energy transfer

FIGURE LEGENDS

Fig. 1. Homology modeling of m-tomosyn-1 sequence to Sro 7. A. Model representing the crystal structure of Sro7 including N-terminal (purple) and C-terminal (green) sequence. B. Homology model of m-tomosyn-1 based on Sro7 demonstrating preservation of the β-propellers and addition of 3 undefined loops (L1, L2,L3). C. Schematic of the two longest isoforms of mouse tomosyn genes 1 and 2. Regions corresponding to the left- (grey) and right-sided (blue) β-propeller structures, conserved tail domain (green), R-SNARE (black) and residues comprising the three predicted loops (L1, L2, L3) are shown. D. Evolutionary conservation profiles of Sro7 (left) and tomosyn (right). The residues are colored according to their conservation grades, with turquoise-through-maroon indicating variable-through-conserved. The most highly conserved amino acids are presented using space-filled atoms.

Fig. 2. Effect of tomosyn loop regions on secretion from PC12 cells. A. Comparison of basal (grey bars) or elevated K⁺-induced (100mM, 10 min; black bars) secretion of hGH from PC12 cells expressing full-length m-tomosyn 1 (wt) or m-tomosyn-1 deletion constructs (Δloop 1, Δloop 2, or Δloop 3) from which the indicated loop domain regions were deleted. hGH secreted was calculated as a percentage of the total cell content using duplicate samples and then normalized to the control condition. Bar graphs depict the mean values ±SEM. (n=4 replicate experiments; ** p<0.05 compared to control values). Tomosyn protein (wt and loop deletion mutants) was expressed at nearly equivalent levels as shown by Western blot of corresponding PC12 cell lysates probed with α-flag antibody (inset). Each tomosyn construct was epitope flag-tagged on its N-terminus. B. Representative immunoblot showing in vitro GST-syntxin1A pulldown of indicated Flag-tagged tomosyn proteins. Recombinant GST-syntxin1A or GST (control) was immobilized on glutathione sepharose beads and incubated with HEK293A cell lysates expressing tomosyn wt or indicated tomosyn loop deletion construct. Tomosyn immunoreactivity in each fraction was determined using a α-Flag antibody. C. Confocal fluorescence images of EGFP-tomosyn (wt and
Fig. 3. Effect of loop 2/HVR region on tomosyn protein expression.  A. Schematic of the HVR of tomosyn genes/isoforms and recombinant mutant deletion constructs.  B. Representative immunoblots resulting from lysate from HEK293 cells transfected with the tomosyn gene/isoform or mutant construct indicated.  Tomosyn was detected by probing Western blots with antibodies against N-terminal V5 (tomosyn) or Flag (tomosyn Δloop2) epitopes, or β-actin (loading control).  C. Averaged qPCR results comparing relative RNA transcript expression levels in transfected HEK293 cells for corresponding conditions shown in B.  Relative tomosyn (top, black bars; normalized to m-tomosyn-1 expression) and control gene (neomycin; bottom, grey bars) transcript levels were calculated using the comparative CT method.  D. Effects of MG132 treatment on tomosyn protein expression levels.  Immunoblot analysis was performed on lysates from transfected HEK293A cells.  Cells were treated ±MG132 (100 μM) for 6h prior to lysis.  Region of tomosyn immunoreactivity is indicated (►).  Actin is shown as a loading control (α-β actin).  A degradation product detected in the s-tomosyn-2 protein sample is indicated by an asterisk (*).  All experiments shown were performed in triplicate.

Fig. 4. Effect of overexpression of tomosyn gene/isoforms on regulated secretion.  PC12 cells expressing tomosyn gene/isoforms indicated were incubated in physiological saline (A), or stimulated with 100 mM K+ containing saline (B) for 10 min at 30°C.  Collection of aliquots of saline and cell lysate for each condition were used to determine the percent of total hGH content secreted.  (n≥4 replicate experiments with duplicate sample analysis; ** p<0.05, ***p<0.005 compared to control)  C. Relative tomosyn gene expression determined by qPCR analysis of transfected PC12 cells.  Inset shows representative immunoblot confirming V5 epitope tagged tomosyn genes/isoform protein expression corresponding to each transfection condition.  D. Comparison of averaged measurements of resting intracellular calcium for m-tomosyn-1 and m-tomosyn-2 transfected PC12 cells (control, citrine alone expressing).  Relative [calcium] expressed as F340/F380 ratio based on imaging of Fura-2 (Molecular Probes).

Fig. 5.  Syntaxin1A-dependent plasma membrane targeting of tomosyn gene/isoforms.  A. Confocal fluorescence images of indicated tomosyn isoforms, expressed alone or in combination with syntaxin1A in live PC12 cells.  Monomeric mutants of citrine and cerulean were fused to the N-terminus of tomosyn and syntaxin1A proteins, respectively.  B. Averaged apparent FRET efficiency between citrine-tomosyn and cerulean-syntaxin1A proteins measured by sensitized-emission FRET in live transfected PC12 cells.

Fig. 6. SUMOylation of m-tomosyn-1within HVR of loop 2.  A. Table of predicted SUMOylation sites in rat m-tomosyn-1 based on sequence analysis.  Sites were predicted and scored from high to low probability using SUMOplot™ Analysis Program (Abgent).  B. Immunoblots probed with α-SUMO 2/3 (left) antibody to detect SUMOylation of V5 epitope-tagged s- or m-tomosyn-1 immunoprecipitated and detected using α-V5 antibody (right).  HEK293 cells were transfected with the tomosyn constructs, or empty vector control, 48 hours prior to cell lysis and immunoprecipitation.  C. SUMOylation is strongly reduced in a K730R point mutation of m-tomosyn-1 expressed in HEK293 cells.  D. Effect of mutation of tomosyn SUMOylation site (K730) on tomosyn inhibition of hGH secretion from transfected PC12 cells (mean ± SE, n=3).  Inset, Immunoblot probed with α-V5 and α-transferrin receptor antibodies to demonstrate equivalent expression upon transfection of HEK293 cells of wild type and a K730R mutant of tomosyn..  E. Averaged apparent FRET efficiency between citrine-tomosyn (wild type or K730R) and cerulean-syntaxin1A proteins measured by sensitized-emission FRET over a range of acceptor:donor fluoroprotein expression levels in live transfected PC12 cells.
Figure 1

A. 

B. 

C. 

D. 

b-Tomosyn-1

L1 (537-578) L3 (933-955)

L2 (675-789)

xb-Tomosyn-2

L1 (548-587) L3 (966-988)

L2 (683-825)
Figure 2

A. 

B. 

C. 

Figure 2
Figure 3

A. Tomosyn-1

B. Tomosyn-2

C. qPCR Tomosyn

D. Tomo-1 ΔLOOP mutants
Figure 4

A. Relative hGH secretion

B. Basal Relative hGH secretion

C. Relative mRNA

D. Intensity Ratio (F340/F380)
Figure 5

A. coexpression

| Tomosyn-1 | Tomosyn-1 | Syn1A | Merge |
|-----------|-----------|-------|-------|
| Tomosyn-1 | Tomosyn-1 | Syn1A | Merge |
| Tomosyn-2 | Tomosyn-2 | Syn1A | Merge |
| Tomosyn-2 | Tomosyn-2 | Syn1A | Merge |

B. Apparent FRET Efficiency (ED)

| tomosyn-1  | m | s | m | xb |
|------------|---|---|---|----|

+ syntaxin1A
Figure 6

A. 

| No. | Pos.  | Group | Score |
|-----|-------|-------|-------|
| 1   | K730  | SLPTD LKPD LDVKD | 0.91  |
| 2   | K298  | PCKPI LKVE FKTTR | 0.91  |
| 3   | K285  | PHGKQ LKD GKPPEP | 0.73  |
| 4   | K288  | QKLDK GKKP EPCKP | 0.57  |
| 5   | K852  | HNVPE EKDE KEKLK | 0.50  |
| 6   | K230  | WDLKS KKDAD YRYTY | 0.48  |

B. 

WB: SUMO-2/3 Tomosyn

DNA: M S M S Tomosyn isoform

C. 

IP:V5

WB: SUMO-2/3 Tomosyn

DNA: M K730R M K730R Tomosyn isoform

D. 

Relative hGH Secretion

ctrl wt K730A K730R blank V5-Tomo Trf-R

E. 

Apparent FRET Efficiency (ED)

Tomo WT Tomo K730R Syntaxin1A + Tomosyn

Molar Ratio ([Accepter] : [Donor])
Structural and functional analysis of tomosyn identifies domains important in exocytotic regulation
Antionette L. Williams, Noa Bielopolski, Daphna Meroz, Alice D. Lam, Daniel R. Passmore, Nir Ben-Tal, Stephen A. Ernst, Uri Ashery and Edward L. Stuenkel

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