

Tomosyn Interacts with the t-SNAREs Syntaxin4 and SNAP23 and Plays a Role in Insulin-stimulated GLUT4 Translocation*

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The Sec1p-like/Munc18 (SM) protein Munc18a binds to the neuronal t-SNARE Syntaxin1A and inhibits SNARE complex assembly. Tomosyn, a cytosolic Syntaxin1A-binding protein, is thought to regulate the interaction between Syntaxin1A and Munc18a, thus acting as a positive regulator of SNARE assembly. In the present study we have investigated the interaction between b-Tomosyn and the adipocyte SNARE complex involving Syntaxin4/SNAP23/VAMP-2 and the SM protein Munc18c, *in vitro*, and the potential involvement of Tomosyn in regulating the translocation of GLUT4 containing vesicles, *in vivo*. Tomosyn formed a high affinity ternary complex with Syntaxin4 and SNAP23 that was competitively inhibited by VAMP-2. Using a yeast two-hybrid assay we demonstrate that the VAMP-2-like domain in Tomosyn facilitates the interaction with Syntaxin4. Overexpression of Tomosyn in 3T3-L1 adipocytes inhibited the translocation of green fluorescent protein-GLUT4 to the plasma membrane. The SM protein Munc18c was shown to interact with the Syntaxin4 monomer, Syntaxin4 containing SNARE complexes, and the Syntaxin4/Tomosyn complex. These data suggest that Tomosyn and Munc18c operate at a similar stage of the Syntaxin4 SNARE assembly cycle, which likely primes Syntaxin4 for entry into the ternary SNARE complex.

Soluble N-ethylmaleimide-sensitive factor (NSF)¹ attachment protein (SNAP) receptors (SNAREs) play a critical role in vesicular transport by regulating membrane docking and fusion (1–4). Transport vesicles contain membrane proteins, known as v-SNAREs, that bind in a highly specific manner to cognate membrane proteins, t-SNAREs, present in the appro-

priate target membrane. Different sets of v-/t-SNAREs control discrete membrane transport steps. For example, synaptic vesicle exocytosis is facilitated by the t-SNAREs Syntaxin1A and SNAP25 that localize to the presynaptic plasma membrane and bind with high affinity to the v-SNARE, VAMP-2, present on synaptic vesicles (1). A defining feature of v- and t-SNAREs is the presence of a conserved α -helical domain in the juxtamembrane region of their cytosolic tail. During SNARE-mediated membrane fusion, four of these SNARE domains contribute to a parallel four-helical bundle arrangement (5, 6).

The assembly of functional SNARE complexes in eukaryotic cells occurs in several discrete stages, each of which represents a potential site of regulation. For example, there is the priming step where *cis*-SNARE complexes, found in both donor and target membranes, are disassembled by the ATPase NSF and α -SNAP (7, 8). Once primed, tethering machinery may guide the SNAREs in each membrane into close proximity, and a proof-reading machinery likely ensures fidelity of the aligned v-/t-SNARE pairs (9). Following this, formation of the high affinity *trans*-SNARE complex results in the committed step of docking (10). Many different molecules play a role in various stages of this SNARE assembly cycle. One family of proteins that regulates SNARE assembly is the Sec1p/Munc18 (SM) family. However, the role of SM proteins in SNARE assembly is complex and somewhat controversial (11).

The neuronal SM protein Munc18a binds with high affinity ($K_d = 80$ nm) to Syntaxin1A, reducing the affinity of Syntaxin1A for VAMP-2 (12). Consistent with the conclusion that Munc18a plays a negative role in SNARE complex formation is the observation that Munc18a does not bind to the Syntaxin1A/SNAP25/VAMP-2 ternary fusion complex (13–15). The Syntaxin1A monomer has been shown to adopt a closed conformation because of interactions between the amino-terminal Habc helical domain and the SNARE-forming H3 helix at the carboxyl terminus (16). In the ternary complex Syntaxin1A undertakes a more open conformation, thus facilitating interactions between the H3 domain and the SNARE motifs present in SNAP25 and VAMP-2 (16, 17). Because Munc18a binds to, and possibly stabilizes, Syntaxin1A in its closed conformation a major objective has been to identify factors that could disrupt the Munc18a/Syntaxin1A heterodimer, thus enhancing SNARE complex formation. Tomosyn was identified as a molecule with properties consistent with such a role. Tomosyn was found to interact specifically with Syntaxin1A and in so doing displace Munc18a (18).

Tomosyn contains a series of conserved amino-terminal WD-40 repeats and a carboxyl-terminal VAMP-2-like domain that is necessary for binding to Syntaxin1A and participation in a 10 S complex with SNAP25 and the synaptic membrane protein Synaptotagmin (18–20). Despite the fact that Tomosyn was shown to interact only with the neuronal t-SNARE Syntaxin1A its ubiquitous tissue distribution raised the possi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF516607 and AF516608.

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¹ The abbreviations used are: NSF, N-ethylmaleimide sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptors; VAMP, vesicle-associated membrane protein; SM proteins, Sec1/Munc18-like proteins; eGFP, enhanced green fluorescent protein; RT, reverse transcriptase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NEM, N-ethylmaleimide; PM, plasma membrane; LDM, low density microsomes.

bility that it may participate in vesicle transport steps controlled by other syntaxin isoforms (18, 20). Intriguingly, the v-SNARE VAMP-2, in addition to binding to Syntaxin1A, also binds to the ubiquitously expressed t-SNARE Syntaxin4 (14, 21). Further, Syntaxin4 has been shown to form an SDS-resistant SNARE complex with SNAP23 and VAMP-2 (22). This complex plays a role in a number of regulated exocytic processes including the insulin-regulated trafficking of the facilitative glucose transporter GLUT4 in fat and muscle cells (21–25). Furthermore, this SNARE complex resembles the synaptic ternary complex and is subject to regulation by the ubiquitously expressed SM protein Munc18c (21, 26, 27). We have investigated the role of b-Tomosyn in exocytosis of GLUT4 vesicles and in the assembly of Syntaxin4/SNAP23/VAMP-2 ternary complexes. The present study shows that b-Tomosyn binds with high affinity to Syntaxin4 via its VAMP-2 like domain and that overexpression of Tomosyn in 3T3-L1 adipocytes inhibits the insulin-dependent translocation of GLUT4-eGFP to the cell surface. In contrast to the situation in neurons, we found that the SM protein Munc18c associated with both the Syntaxin4/b-Tomosyn binary complex and with the Syntaxin4 SNARE complexes. Taken together, our data suggest a novel role for b-Tomosyn in SNARE complex formation and vesicle transport and support the hypothesis that Munc18c plays a fundamental role in the assembly of SNARE core complexes.

EXPERIMENTAL PROCEDURES

Cloning of 3T3-L1 Adipocyte Tomosyn—Total RNA was prepared from differentiated 3T3-L1 adipocytes by a single step guanidine isothiocyanate method (28). Reverse transcriptase (RT)-PCR was performed using a standard protocol with oligo(dT) primer (0.5 μ g) and total RNA (1 μ g). Subsequent PCR amplifications were performed using primers 1, 5'ccggatccaccatgaggaaattcaacatc3'; and 2, 5'ccgaattctcatgtgagaagtgac3'; according to a standardized PCR protocol. The PCR product of 1050 bp was cloned into a pGEMt easy vector kit (Promega, Madison, WI) and sequenced using an automated sequencer. The above PCR was carried out on two successive occasions, and the products were sequenced independently. Radiolabeled PCR product (rediprimeTM; Amersham Biosciences) was used to screen a 3T3-L1 adipocyte cDNA library constructed in λ ZAP, kindly provided by Dr. F. Fiedorek (University of North Carolina). Approximately 100,000 plaques were mutually screened, and one well isolated plaque was excised using Ex-AssistTM (interference-resistant helper phage with SOLRTM strain) according to the manufacturer's protocol (Stratagene, La Jolla, CA). The cDNA clone was fully sequenced on both strands, and the data were analyzed using SequencherTM 3.1.1s. 3T3-L1 adipocyte Tomosyn was 98% identical to the neuronal b-Tomosyn splice variant (GenBankTM accession number AF118889). Our sequencing data derived from the RT-PCR analysis of 3T3-L1 adipocyte Tomosyn differed from the cDNA-derived clone of Tomosyn at four amino acids, D238G, H247Y, A271T, and L301F. These changes do not appear to be random as the changes are either conserved between our cDNA clone and the published rat brain b-Tomosyn sequence (amino acids 238 and 247) or the RT-PCR product and the respective b-Tomosyn sequence (amino acids 271 and 301).

Plasmid Construction—cDNAs encoding rat Syntaxins-1A, -2, -3, and -4 and VAMP-2 were generously provided by Dr. R. Scheller (Stanford University). Syntaxin-6- and -7-encoding cDNAs have been described (29). Dr. Robert Piper (University of Iowa) provided cDNA encoding full-length Syntaxin13. The SNAP23 pGEX-4T1 construct has been described elsewhere (22). Dr. Peter van der Sluijs (Utrecht University School of Medicine, Utrecht, The Netherlands) kindly provided full-length pRSETA-Rab4-encoding cDNA, which was subcloned into pGEX-2T as an *EcoRI* fragment. pcDNA3-Munc18c was constructed by subcloning a 2.5-kb *EcoRI* fragment from pMEXneo-Munc18c into the *EcoRI* site of pcDNA3 (Invitrogen) (21). The pBluescript KG-Munc18b and pRSETA-Munc18c constructs have been described (21, 30). pET20b-Syntaxin4 was kindly donated by Dr. Jenny Martin (Institute of Molecular Bioscience, Brisbane, Australia). Full-length Tomosyn cDNA was obtained by PCR using primers 3, 5'ccggatccaccatgaggaaattcaacatc3'; and 4, 5'ccggatccaccatgaggaaattcaacatc3' from the cDNAs described in the previous section and was subcloned into triple FLAG

epitope-tagged pMEX_{neo} using *Bam*HI and *Eco*RI. The FLAG epitope was constructed using the following primers: 5, 5'gaagatcgcgtcacaccatggactacaagaccatgacgggtgattataaagatcatgat3'; 6, 5'atcatgatctttataatcacgctcatggtcttttagtccatggtgtcagcagatccttc3'; 7, 5'gaagatcgcattacaagatcagcagatcagcagatccttc3'; 8, 5'ccggatccaccatgaggaaattcaacatc3'. Primers 5–8 were annealed and digested with *Eco*RV before being subcloned as a double stranded fragment into pMEX_{neo} using *Sal*I and *Bam*HI. Truncated Tomosyn cDNAs encoding bp 1–1050, 1–3225, 1050–3225, and 3199–3459 were obtained by PCR using primers 9, 5'ccgaattcaggaaattcaacatc3'; 10, 5'ccctcagtcagtcagagaagtcacac3'; 11, 5'ccctcagtcagtcagagaagtcacac3'; 12, 5'ccgaattcacactctgtgaaac3'; 13, 5'ccgaattcaggagatgctcctc3'; and 14, 5'ccctcagtcagagaagtcacac3'. The Tomosyn cDNAs were cloned into pBTM116 using *Eco*RI and *Sal*I. pBTM116-Munc18c and pAct-Syntaxin4 cDNAs were kind gifts from Dr. Y. Le Marchand-Brustel (Nice, France). eGFP-tagged GLUT4 (GLUT4-GFP-pcDNA3) was received from Dr. J. Pessin (University of Iowa). All cDNAs obtained by PCR were confirmed by sequencing.

Generation of Antibodies—Rabbit antiserum that specifically recognizes Munc18c was raised against a glutathione *S*-transferase (GST) fusion protein comprising a tandem repeat of the carboxyl-terminal 14 amino acids of mouse Munc18c (MLNKSQKDKVSFKDE). Following purification on glutathione-agarose beads (Sigma) the immunogen was dialyzed against phosphate-buffered saline (PBS) and used to immunize rabbits. Munc18c-specific antibodies were purified using a monomeric form of the peptide, immobilized on a Sulfolink gel (Pierce), and dialyzed against PBS. A polyclonal anti-Syntaxin4 antibody was used as described (21). Rabbit sera against the cytoplasmic domain of GST-VAMP-2 was raised according to a standard protocol, and the polyclonal anti-SNAP23 sera has been described (14, 22). Dr. Y. Takai (Osaka University Medical School, Osaka, Japan) kindly donated an anti-Tomosyn antibody (18). A monoclonal Caveolin1 antibody was donated by Dr. Rob Parton (Institute of Molecular Bioscience, Brisbane, Australia). The following antibodies were purchased: anti-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFP (Molecular Probes, Eugene, OR), and anti-LexA (Invitrogen).

Tomosyn Tissue Distribution—Mouse brain and 3T3-L1 adipocyte samples were prepared in HES buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA), supplemented with protease and phosphatase inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 25 μ g/ml phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM ammonium molybdate, and 10 mM sodium fluoride), using a glass homogenizer or by passing through a 22- and 27-gauge needle, respectively. The homogenates were subject to centrifugation at 100,000 $\times g_{av}$ for 1 h at 4 °C in a Beckman TLA-100.3 rotor. The supernatant from this spin was used as the cytosol fraction. Protein concentrations were determined using a Bio-Rad Bradford assay (Hercules, CA). Samples were analyzed by SDS-PAGE according to the method of Laemmli, transferred to polyvinylidene difluoride membrane (Millipore), and subject to immunoblot analysis (31). Signal was visualized using SuperSignal[®] West Dura Extended Duration Substrate (Pierce).

Protein Expression and Purification—Recombinant fusion proteins encoding GST or GST fused in-frame to the cytosolic domains of the Syntaxin isoforms-1A, -2, -3, -4, -6, -7, -13, -VAMP-2, -SNAP23, or full-length -Rab4 were produced in *Escherichia coli* BL21 (DE3) and purified using glutathione-Sepharose 4B beads (Amersham Biosciences) according to standard procedures. Histidine (His₆)-tagged Munc18c and Syntaxin4 were made in Sf9 and BL21 (DE3) cells, respectively, and purified using TALONTM Cobalt metal affinity resin according to the supplier's protocol (Clontech, Palo Alto, CA). The recombinant proteins were dialyzed against PBS, except His₆-Munc18c, which was dialyzed against PBS containing 2 mM 2-mercaptoethanol, and concentrated using Aquacide (Calbiochem). The protein concentration and purity of all recombinant fusion proteins were determined by Bio-Rad Bradford protein assays and SDS-PAGE followed by staining with Coomassie Blue. GST-VAMP-2 was cleaved with thrombin (25 units/ml) (Amersham Biosciences) in 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 0.1%, 2-mercaptoethanol. Phenylmethylsulfonyl fluoride (25 μ g/ml) was added to the reaction, and the thrombin-cleaved protein was dialyzed against PBS.

Recombinant Fusion Proteins—Recombinant GST fusion proteins (5–15 μ g) were attached to glutathione-Sepharose beads (15–25 μ l) in a total volume of 300–350 μ l of PBS for 1 h at 20 °C and recovered by brief centrifugation. His₆-tagged fusion proteins were incubated with TALONTM resin (25–50 μ l) in the same way. Binary (Syntaxin4/SNAP23; Syntaxin4/VAMP-2; SNAP23/VAMP-2; Syntaxin4/Munc18c) or ternary (Syntaxin4/SNAP23/VAMP-2) complexes were formed by mixing approximately equimolar amounts of recombinant proteins and incubated

either for 2 h or overnight at 4 °C in PBS or PBS containing 0.1–0.2% Triton X-100 and 0.1% bovine serum albumin (BSA). The complexes were further incubated with resin as described above.

In Vitro Binding Assay—Recombinant GST or His₆-tagged proteins, attached to glutathione-Sepharose or metal affinity resin, were incubated with 0.8–1.0 ml of purified 3T3-L1 adipocyte cytosol (1–3 mg/ml) or mouse brain cytosol, containing 120 mM NaCl, 0.1% BSA, and 1% Triton X-100, for 2 h at 4 °C. Alternatively, recombinant SNARE proteins were incubated with 20 μl of [³⁵S]methionine-labeled Munc18-b or -c (TNT® Coupled Reticulocyte Lysate System; Promega) in 0.5 ml of binding buffer (20 mM HEPES, pH 7.5, 120 mM NaCl, 0.2% Triton X-100, and 0.1% BSA) as described above. After washing the samples three to four times with PBS or binding buffer (lacking BSA), the proteins attached to the affinity beads were solubilized in 20 μl of 2 × SDS-sample buffer containing 100 mM dithiothreitol and heated for 5 min at 100 °C. Data were analyzed by SDS-PAGE followed by Autoradiography (Eastman Kodak Co. BioMax MR film; Rochester, NY) or immunoblotted using specific antibodies and stained with 0.5% Ponceau S in 0.5% trichloroacetic acid or Coomassie Blue stain.

Yeast Two-hybrid Methods—A standard lithium acetate/single-stranded carrier DNA/polyethylene glycol method for transformation into yeast strain L40 was used, and expression of proteins fused to LexA was checked by SDS-PAGE and immunoblotted. Transcriptional activation of LacZ was determined using the X-Gal filter lift assay and a quantitative liquid assay, using the substrate nitrophenyl-β-D-galactopyranoside as described (32, 33). For the filter lift assay color development within 0–3 h was scored (+++) and development within 3–6 h was scored (++) ; no color after 16 h was scored negative.

Cell Culture—3T3-L1 adipocytes were cultured as described (34). Experiments were performed using adipocytes between 7 and 17 days post-differentiation. Before experimental use the cells were starved in Dulbecco's modified Eagle's medium lacking fetal calf serum overnight at 37 °C with 5% CO₂ (v/v) and either used in a basal condition or stimulated with insulin (4 μg/ml) for 15 min. *N*-Ethylmaleimide (NEM) treatment was carried out using 1 mM NEM in Me₂SO for 15 min at 37 °C before subcellular fractionation. Electroporation was performed essentially as described by Pessin and co-workers (26), using 100 μg of cesium chloride-purified GLUT4-eGFP-pcDNA3 and 400 μg of pMEX_{neo}, pMEX_{neo}-Munc18c, or pMEX_{neo}-FLAG-Tomosyn. The electroporated cells were seeded on 1% gelatin-coated coverslips or 10-cm tissue culture dishes and either fixed in 2% paraformaldehyde or subjected to subcellular fractionation (35). Fixed cells were quenched using 50 mM ammonium chloride and blocked/permeabilized in BB (2% BSA and 0.1% saponin in PBS) before GLUT4-eGFP was visualized using a primary GFP antibody (1:300) and Alexa 488-conjugated secondary antibody (1:150) in BB. Immunofluorescence images were obtained using a Zeiss Axioskop 40 fluorescence microscope (×63 objective) and analyzed using Zeiss AxioVision software.

Subcellular Fractionation of 3T3-L1 Adipocytes—Subcellular fractionation was carried out according to an established protocol to generate membrane fractions that are enriched in markers of the plasma membrane (PM), endoplasmic reticulum and endosomes (high density microsomes, HDM), Golgi membranes, recycling endosomes, and the majority of the intracellular GLUT4 responsive compartment (low density microsomes, LDM), mitochondria/nuclei, and cytosol (35, 36).

Statistical Analysis—Statistical analyses were performed using Excel software. Statistical significance was established using a Student's *t* test.

RESULTS

Tomosyn has been shown to disrupt the neuronal Munc18a/Syntaxin1A complex by binding to Syntaxin1A and has thus been proposed to be a positive modulator of SNARE complex formation, overcoming the negative regulatory role of Munc18a (18). We have demonstrated previously (22) that VAMP-2 forms a high affinity SNARE complex with Syntaxin4 and SNAP23. This complex regulates a variety of exocytic transport events including the translocation of GLUT4 to the plasma membrane of adipocytes and the translocation of water channels to the cell surface of kidney cells (23, 37–39). Tomosyn binds to Syntaxin1A via a helical domain that is homologous to the VAMP-2 SNARE motif, and a recently described molecule, Amisyn, contains a Tomosyn-like SNARE motif and has further been shown to interact with both Syntaxin1A and Syntaxin4 from rat brain (18, 20, 40). Therefore, we set out to

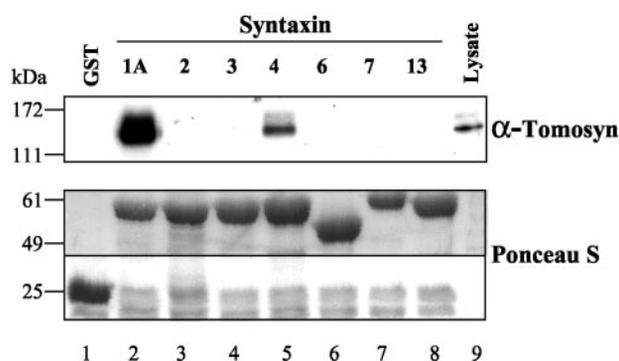


FIG. 1. b-Tomosyn binds to Syntaxin1A and Syntaxin4. Recombinant GST or GST-Syntaxin isoforms-1A, -2, -3, -4, -6, -7, and -13 (15 μg) were used in an *in vitro* pull-down assay. The fusion proteins were pre-adsorbed to glutathione-Sepharose and incubated with purified 3T3-L1 adipocyte cytosol, as a source of b-Tomosyn, for 2 h at 4 °C before extensive washing. Interacting proteins were separated by SDS-PAGE (10%) and immunoblotted with an anti-Tomosyn antibody. The right lane shows a small amount (~0.5%) of the total cytosol lysate used for the binding assay. Recombinant proteins were visualized with 0.5% Ponceau S in 0.5% trichloroacetic acid.

investigate whether Tomosyn binds to Syntaxin4 and whether it plays a similar role in destabilizing the Syntaxin4/Munc18c complex to that observed in neurons.

Tomosyn Is a Ubiquitously Expressed Protein—Tomosyn was originally identified as a 120–130-kDa Syntaxin1A-binding protein that is highly expressed in neuronal tissue. An immunoreactive species was also demonstrated to be present in other tissues (18). Using a Tomosyn-specific antibody we verified the ubiquitous expression of this protein in tissue including heart, spleen, lung, skeletal muscle, liver, and kidney (data not shown). We also showed that Tomosyn is expressed in 3T3-L1 adipocytes, a cell line that is commonly used to study the insulin-dependent trafficking of GLUT4 (18, 41). Takai and co-workers (20) identified three Tomosyn splice variants referred to as s-, m-, and b-Tomosyn (20). To determine which isoform is expressed in adipocytes we cloned Tomosyn from a 3T3-L1 adipocyte cDNA library (see “Experimental Procedures”) and verified that it corresponds to the b-Tomosyn isoform found in rat brain. This is consistent with RT-PCR data from Takai and co-workers (20) showing that b-Tomosyn is ubiquitously expressed.

Tomosyn Specifically to the VAMP-2 t-SNAREs Syntaxin1A and Syntaxin4—In light of the observation that Tomosyn binds to Syntaxin1A via its VAMP-2-like domain and that VAMP-2 binds to Syntaxins-1A and -4, we set out to investigate the Syntaxin binding spectra of b-Tomosyn (12, 14, 18, 20, 21). We assessed the ability of GST fusion proteins containing the entire cytosolic domain of Syntaxin-1A, -2, -3, -4, -6, -7, or -13 to bind b-Tomosyn from 3T3-L1 adipocyte cytosol *in vitro* (Fig. 1). It was necessary to use cytosol as a source of b-Tomosyn, because, as reported previously (18), we found that recombinant Tomosyn was insoluble when produced as a bacterial fusion protein. Immunoblot analysis of subcellular 3T3-L1 adipocyte fractions, obtained through differential centrifugation, demonstrated that b-Tomosyn is predominantly cytosolic, and therefore adipocyte cytosol was used as a source of Tomosyn in all our *in vitro* binding experiments (see Fig. 6) (35). In agreement with Fujita *et al.* (18), b-Tomosyn bound avidly to Syntaxin1A (Fig. 1, lane 2). In addition, Tomosyn also bound to Syntaxin4 (lane 5). Similar results were obtained using rat brain cytosol as a source of Tomosyn (data not shown). There was no detectable binding of Tomosyn to GST alone or GST-Syntaxins-2, -3, -6, -7, and -13. These studies were performed using similar concentrations of each fusion protein as indicated by the Ponceau S stain. There was seven

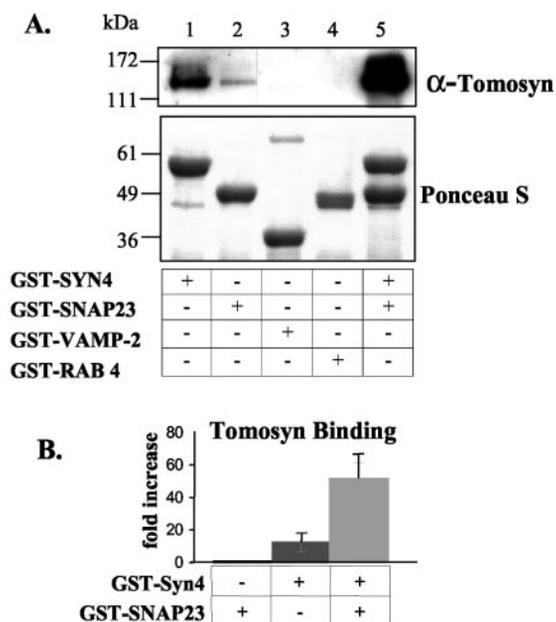


FIG. 2. b-Tomosyn forms a ternary complex with the t-SNAREs Syntaxin4 and SNAP23. *A.*, GST-tagged Syntaxin4, -SNAP23, -VAMP-2, -Rab4, and -Syntaxin4/-SNAP23 (binary complexes) (15 μ g) were mixed with glutathione-Sepharose and incubated with purified adipocyte cytosol as described in the legend to Fig. 1. Samples were analyzed by SDS-PAGE and immunoblotted using the Tomosyn antibody. Recombinant fusion proteins were detected with 0.5% Ponceau S stain. *B.*, autoradiograms from three to four independent experiments were scanned using a densitometer (Molecular Dynamics) and analyzed using IP Lab GelH software (Signal Analytics). Data represent mean \pm S.D.

times more b-Tomosyn bound to Syntaxin1A than to Syntaxin4, indicating that it has a stronger avidity for the neuronal isoform. This parallels the binding affinity of the v-SNARE VAMP-2, which has also been reported to bind with greater affinity to Syntaxin1A than to Syntaxin4 (12, 21).

Tomosyn Binding to the Binary Syntaxin4/SNAP23 Complex—We went on to determine whether b-Tomosyn could form a complex with the t-SNAREs Syntaxin4 and SNAP23 similar to VAMP-2. Fig. 2 demonstrates that there was a weak but specific interaction between b-Tomosyn and SNAP23 alone (*lane 2*). These data are consistent with the reports by Scheller and co-workers (40, 42), who demonstrated an interaction between the carboxyl terminus of Tomosyn and SNAP23 in a yeast two-hybrid study and a weak interaction between Amisyn and SNAP25. There was no detectable binding of b-Tomosyn to either recombinant VAMP-2 or Rab4. Strikingly, the amount of Tomosyn that was bound to the Syntaxin4/SNAP23 SNARE complex (*lane 5*) was substantially greater than with either monomeric Syntaxin4 (*lane 1*) or SNAP23 (*lane 2*) alone. Quantitation of data from three to four separate experiments revealed that the amount of Tomosyn bound to either Syntaxin4 or to the Syntaxin4/SNAP23 dimer was 12.5-fold and 52-fold greater, respectively, than that bound to SNAP23 alone (Fig. 2*B*). This indicates that these proteins form a high affinity ternary complex similar to that reported previously for Syntaxin4/SNAP23/VAMP-2 (22, 23).

The VAMP-2-like Domain of Tomosyn Is Sufficient for the Syntaxin4 Interaction—To confirm that b-Tomosyn binds to Syntaxin4 via its VAMP-2-like domain we performed competition binding studies using recombinant VAMP-2. Fig. 3*A* shows the binding of b-Tomosyn to the Syntaxin4/SNAP23 dimer in the presence of increasing concentrations of VAMP-2. We observed quantitative inhibition of b-Tomosyn binding to the Syntaxin4/SNAP23 heterodimer when VAMP-2 was present in

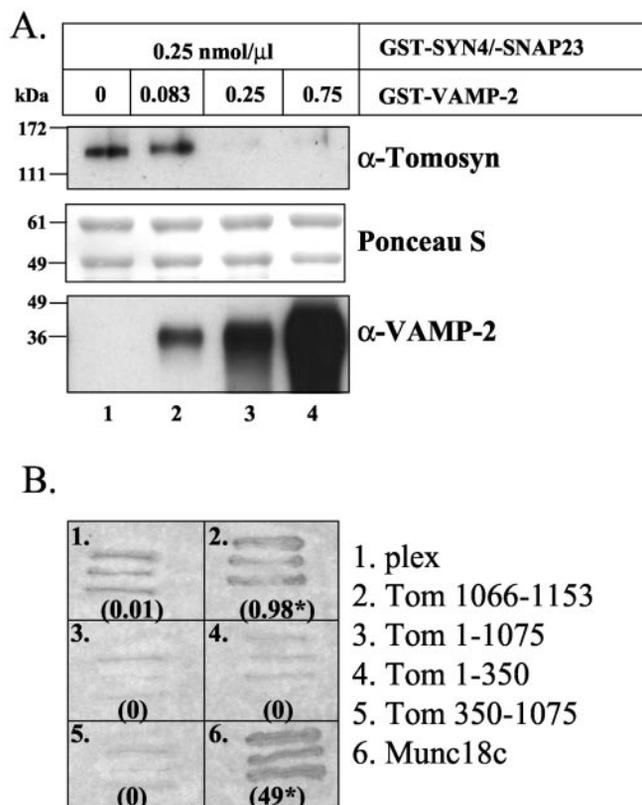


FIG. 3. b-Tomosyn binds to Syntaxin4 via its VAMP2-like domain. *A.*, preformed GST-Syntaxin4/-SNAP23 complexes were incubated with increasing amounts of GST-VAMP-2 (0, 0.083, 0.25, or 0.75 nmol/ μ l). The recombinant protein complexes were isolated using glutathione-Sepharose and assayed for their ability to bind b-Tomosyn from adipocyte cytosol. *B.*, baits, pLex empty vector (1), Tomosyn amino acids 1075–1153 (2), Tomosyn 1–1075 (3), Tomosyn 1–350 (4), Tomosyn 350–1075 (5), and full-length Munc18c (6), were co-expressed with Syntaxin4 (prey) in yeast strain L40. Transcriptional activation of LacZ was assessed using an X-Gal filter lift assay. The X-Gal filter lift assay shown is representative of both 3- and 16-h incubations in the presence of substrate. A quantitative liquid assay was carried out using nitrophenyl- β -D-galactopyranoside as substrate, and the results are shown in parentheses in milli β -Gal units below each relevant track. Interactions that were significantly different from the negative control are represented as *, $p < 0.01$.

approximately equimolar concentrations (0.25 nmol/ μ l) (*lane 3*). The Ponceau S stain indicates that each lane contains a constant amount of GST-Syntaxin4 and GST-SNAP23. A recent study has shown that mammalian lethal giant larvae, a homolog of *Drosophila* tumor suppressor protein lethal (2) giant larvae and of Tomosyn, interacts with Syntaxin4 in the basolateral membrane of Madin-Darby canine kidney cells (43). This was surprising, as both the family of lethal (2) giant larvae proteins and the yeast homolog of Tomosyn, Sro7/77, do not contain a SNARE motif. However, there is significant sequence conservation in the amino-terminal part among lethal giant larvae proteins, Sro7/77 and Tomosyn, including a series of WD-40 repeats (19, 44). We therefore investigated whether this conserved amino-terminal part of Tomosyn could interact with Syntaxin4 independently of the VAMP-2 like domain. Four different Tomosyn truncations were constructed, comprising amino acids 1–350, 350–1075, 1–1075, and 1066–1153, and used as baits in a yeast two-hybrid study where Syntaxin4 was prey (Fig. 3*B*). None of the Tomosyn baits showed intrinsic transcriptional activation in the absence of prey and *vice versa* for the prey alone (data not shown). Consistent with our *in vitro* binding studies, we observed a strong interaction between the VAMP-2-like domain of Tomosyn, amino acids 1066–1153, and

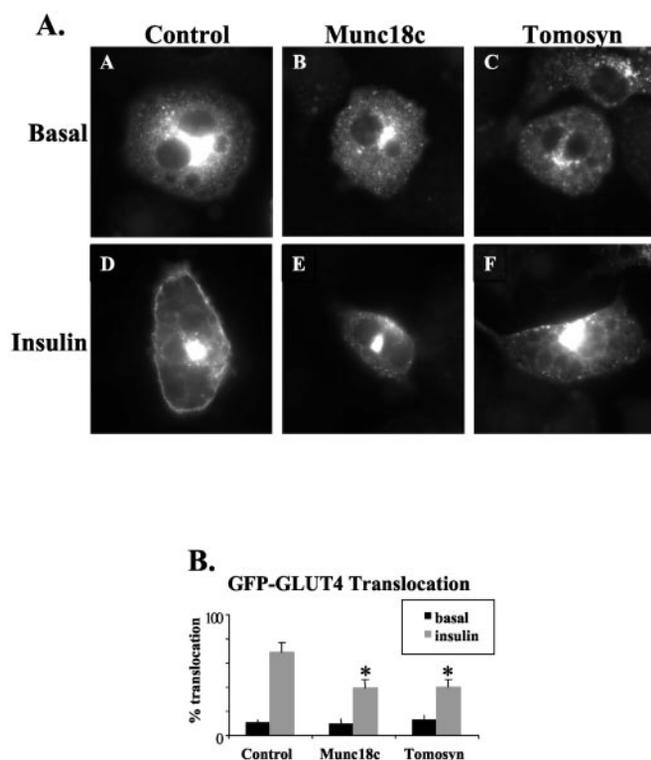


FIG. 4. Overexpression of Tomosyn and Munc18c inhibits insulin-stimulated GFP-GLUT4 movement to the PM. *A*, differentiated 3T3-L1 adipocytes were co-electroporated with 100 μ g of GFP-GLUT4-pcDNA3 and 400 μ g of either pMEX_{neo}, pMEX_{neo}-Munc18c, or pMEX_{neo}-FLAG-Tomosyn and replated onto gelatin-coated coverslips. At 36 h after replating the cells were incubated in the absence (*A*, *B*, and *C*) or presence of insulin (10^{-7} M) for 15 min (*D*, *E*, and *F*) before fixation in 2% paraformaldehyde. Shown are representative immunofluorescence images. *B*, quantitation of surface staining expressed as percent cells demonstrating plasma membrane staining. An average of 200 cells/coverslip, from randomly picked fields, was scored per condition. Shown are mean data \pm S.D. of four separate coverslips per condition from two separate experiments. *, $p < 0.01$ compared with control cells incubated with insulin.

Syntaxin4 (Fig. 3*B*), whereas no significant interaction was observed for the amino terminus, the middle domain, or full-length Tomosyn lacking the SNARE motif (*panels 3 to 5*). Full-length Munc18c and pLex (a potent autoactivator) were used as positive controls for LacZ transcriptional activation (*panels 6 and 1*, respectively). The data obtained from the liquid-assay are shown in parentheses below each *panel* (Fig. 3*B*). Collectively, these data preclude a role for the conserved amino-terminal domain of Tomosyn in binding to Syntaxin4, a role assigned to the VAMP-2-like SNARE motif of Tomosyn.

Expression of Tomosyn in 3T3-L1 Adipocytes Inhibits GLUT4-eGFP Translocation—Dissociation of the Munc18c/Syntaxin4 heterodimer may represent a key regulatory event in insulin-dependent translocation of GLUT4. Because Tomosyn has been implicated in controlling this event in neurons, and our studies indicate that Tomosyn is involved in *t-v*-SNARE complex formation, *in vitro*, we examined the role of Tomosyn in insulin-stimulated GLUT4 trafficking using a 3T3-L1 adipocyte model. FLAG-epitope tagged Tomosyn or empty vector were co-expressed with GLUT4-eGFP in adipocytes by electroporation. The electroporated cells were either used in the basal state or stimulated with insulin for 15 min, fixed in 2% paraformaldehyde, and scored for plasma membrane staining using a fluorescence microscopy assay as described previously (Fig. 4*A*) (26). To validate the GLUT4-eGFP assay, Munc18c, which is known to inhibit GLUT4 translocation, was co-expressed with GLUT4-eGFP and scored in an

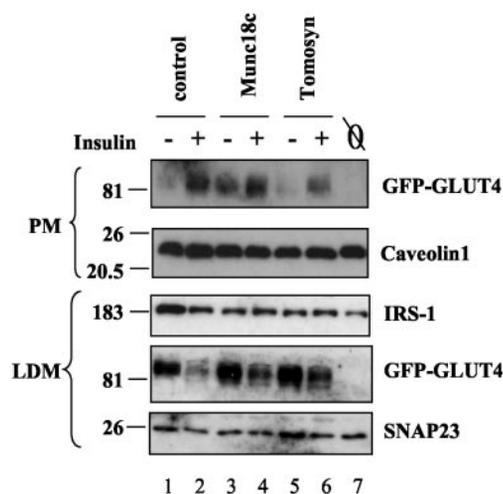


FIG. 5. Expression of exogenous Tomosyn and Munc18c inhibit the translocation of GFP-GLUT4 vesicles to the PM. Electroporation of adipocytes was carried out as described in the legend for Fig. 4. The cells were allowed to recover on gelatin-coated tissue culture dishes, incubated in the absence or presence of insulin, and subjected to subcellular fractionation to obtain membranes enriched for PM and LDM markers. Equivalent amounts of protein were loaded and analyzed by SDS-PAGE (10%) and immunoblotted using antibodies against GFP, Caveolin1, IRS-1, and SNAP23. Lane 7 shows cells not expressing GFP-GLUT4. The relative migration of molecular weight standards is shown at the left.

identical manner to the Tomosyn-expressing cells (*panels b and e*) (26, 45). Insulin caused a 7-fold increase in the total number of cells displaying positive GLUT4-eGFP surface labeling (Fig. 4). Co-expression of either Munc18c or Tomosyn caused an approximate 50% decrease in the number of cells displaying GLUT4 surface labeling following insulin treatment. The expression of GLUT4-eGFP was similar in each of the treatment groups, suggesting that Tomosyn or Munc18c overexpression had no significant effect on the expression of the reporter. To further substantiate the effects of Tomosyn overexpression on GLUT4 translocation, we next performed subcellular fractionation on electroporated adipocytes (Fig. 5) (35). The subcellular distribution of GLUT4-eGFP was similar to that described previously for endogenous GLUT4. In the absence of insulin, GLUT4-eGFP was sequestered in an intracellular compartment, highly enriched in the LDM fraction, and translocated to the cell surface from the LDM with insulin (Fig. 5, *lanes 1 and 2*) (46–49). Consistent with our immunofluorescence data, overexpression of Tomosyn caused a 40% block in the movement of GLUT4-eGFP containing vesicles to the plasma membrane (0.64 ± 0.06 , $p < 0.05$). Intriguingly, overexpression of Munc18c caused an increase in the amount of GLUT4-eGFP found at the PM under basal conditions. However, the incremental increase at the plasma membrane in response to insulin was less in cells overexpressing Munc18c compared with control cells. This was readily apparent from the reduced insulin-stimulated decrement in GLUT4-eGFP in the LDM fraction from both the Munc18c and Tomosyn overexpressing cells. Taken together, these data implicate Tomosyn in the regulation of GLUT4 trafficking, similar to Munc18c.

Tomosyn and Munc18c Dissociate from Membranes in the Presence of NEM—The *t*-SNARE Syntaxin4 is an integral membrane protein, highly enriched in the PM, whereas both Tomosyn and Munc18c encode soluble proteins, lacking transmembrane domains (21, 50). To ascertain the subcellular distribution of Tomosyn, we employed subcellular fractionation as described in Fig. 5. Consistent with previous studies Syntaxin4 was highly enriched in the PM fraction (Fig. 6*A*) (21, 26). The majority of Tomosyn was soluble and found in the cytosol

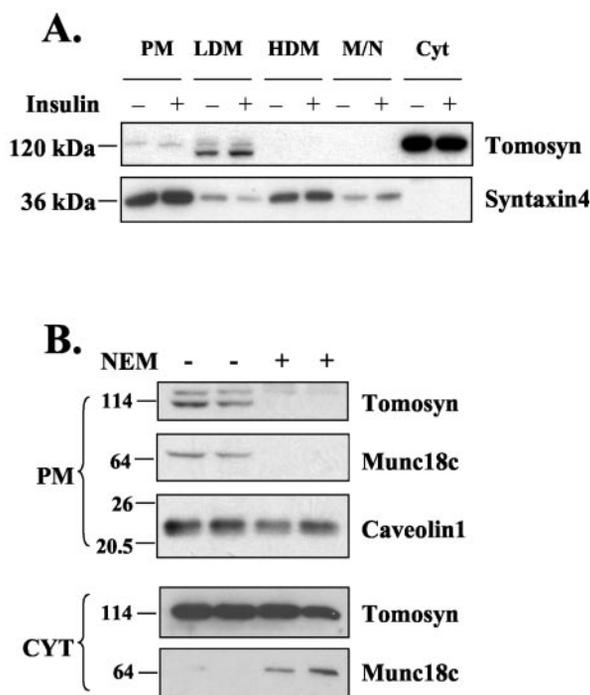


FIG. 6. Intracellular localization of Tomosyn and Syntaxin4 in 3T3-L1 adipocytes. *A*, 3T3-L1 adipocytes were subjected to subcellular fractionation to obtain PM, LDM, high density microsomes (*HDM*), mitochondria/nuclei (*M/N*), and cytosol (*Cyt*) fractions as previously described (35, 36). Equivalent amounts of protein for each fraction was analyzed by SDS-PAGE (10%) and immunoblotted with antibodies specific for Tomosyn and Syntaxin4. *B*, 3T3-L1 adipocytes were incubated in the absence or presence of 1 mM NEM. The cells were subjected to subcellular fractionation, and equivalent amounts of protein were immunoblotted with antibodies specific for Tomosyn, Munc18c, and Caveolin1. The relative migration of molecular weight standards is shown at the left.

fraction (~98%), but interestingly, a significant amount was also present in the PM and LDM fractions (Fig. 6A). Similar to Syntaxin4, the subcellular distribution of Tomosyn did not change with insulin (Fig. 6). We have attempted to determine whether the interaction of Tomosyn with the membrane is facilitated via Syntaxin4 binding using immunoprecipitation. However, these studies have failed to identify such an interaction possibly because of the affinity of the interaction or maybe because of the presence of endogenous factors that dissociate the complex. To overcome such problems we have attempted to inhibit the enzyme NSF by incubating cells with NEM (51–53) reasoning that this enzyme may catalyze the disassembly of Tomosyn complexes. Although we observed an increase in Syntaxin4 ternary complex formation with NEM (data not shown) surprisingly under these conditions we found that Tomosyn dissociated from membranes into the cytosol (Fig. 6B). Strikingly, Munc18c was also released from the membrane in response to NEM in a similar manner to Tomosyn.

Binding of Tomosyn to the Munc18c/Syntaxin4 Complex—Neuronal Tomosyn has been proposed to regulate exocytosis of small synaptic vesicles by competing with Munc18a for Syntaxin1A binding (18). In support of this hypothesis, Tomosyn and Munc18a binding to Syntaxin1A were shown to be mutually exclusive and precede ternary complex formation (18). To ascertain whether Tomosyn similarly regulates Syntaxin4/SNAP23/VAMP-2 ternary complex formation, we set out to investigate the interactions between b-Tomosyn, Syntaxin4, and Munc18c (Fig. 7). *In vitro* binding assays were performed using purified adipocyte cytosol, as a source of b-Tomosyn, and recombinant fusion proteins. Consistent with our previous data (see Figs. 1 and 2) b-Tomosyn bound avidly to recombinant

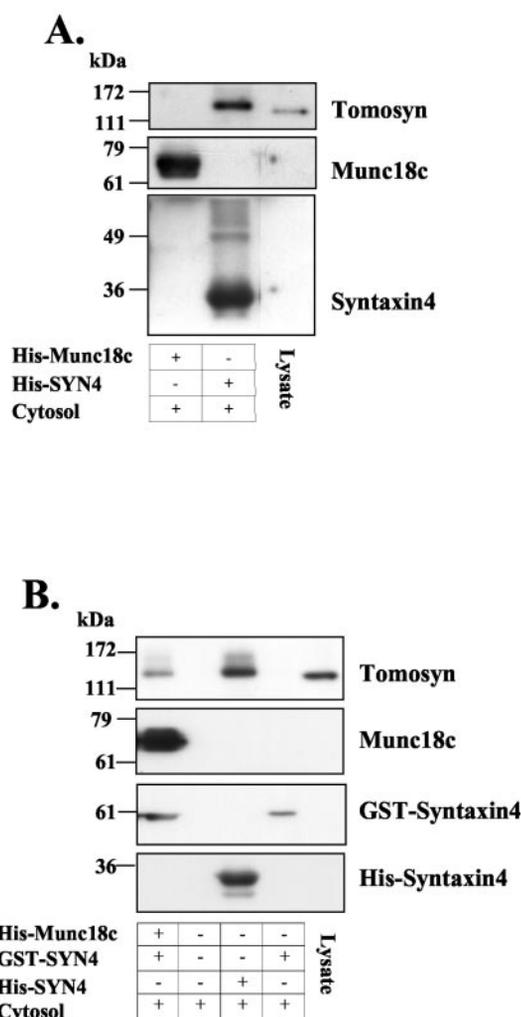


FIG. 7. Munc18c and b-Tomosyn binding to Syntaxin4 is not mutually exclusive. *A*, purified His₆-tagged Munc18c (14 μ g) or His₆-Syntaxin4 (12 μ g) were incubated with purified 3T3-L1 adipocyte cytosol for 2 h at 4 °C, recovered using TALONTM metal affinity resin and washed extensively before analysis. *B*, His₆-Munc18c (14 μ g) and GST-tagged Syntaxin4 (12 μ g) were either mixed or assayed individually for their ability to bind b-Tomosyn as described above. Samples were analyzed by SDS-PAGE (10%) and immunoblotted with antibodies against Tomosyn, Munc18c, and Syntaxin4. *Right-hand lanes* show an aliquot of cytosol (7.5 μ g). Recombinant fusion proteins were visualized using either Ponceau S or Coomassie Blue.

His₆-Syntaxin4 (Fig. 7A), and no direct interaction between b-Tomosyn and His₆-Munc18c was detected. Subsequently, we used preformed GST-Syntaxin4/His₆-Munc18c complexes for binding of Tomosyn from adipocyte cytosol (Fig. 7B). Importantly, under these conditions Tomosyn could only be bound to the Munc18c/Syntaxin4 dimer, because Tomosyn does not bind to Munc18c alone. Interestingly, we observed a significant interaction between b-Tomosyn and the Munc18c/Syntaxin4 dimer (Fig. 7B). This was not because of an interaction between b-Tomosyn and Syntaxin4 alone, as there was no detectable binding of b-Tomosyn when the same binding reaction was performed in the absence of recombinant Munc18c (*lane 4*). These data suggest that b-Tomosyn can bind both to the Syntaxin4 monomer and to the Syntaxin4/Munc18c complex. On occasions we observed a different mobility of b-Tomosyn present in the cytosol lysate compared with immunoreactive Tomosyn in the SNARE complex samples, seen in Fig. 7. This aberrant mobility of b-Tomosyn in the lysate was likely because of the high protein and detergent concentrations in this fraction.

Munc18c Binds to the Syntaxin4 SNARE Complex—The ob-

servation that Tomosyn and Munc18c can bind to Syntaxin4 simultaneously was surprising in view of previous findings using Munc18a and the neuronal SNAREs (18). Moreover, because b-Tomosyn binds to Syntaxin4 in a manner that parallels VAMP-2 binding, we reasoned that Munc18c might interact with other Syntaxin4 containing complexes, including the ternary fusion competent SNARE complex. To determine whether Munc18c binds to SNARE complexes, it was necessary to purify these complexes without the presence of monomeric Syntaxin4. This was achieved using recombinant fusion proteins comprising His₆-Syntaxin4, GST-SNAP23, GST-VAMP-2, or thrombin-cleaved VAMP-2, in various combinations to produce binary and ternary complexes, which were subsequently isolated using glutathione-Sepharose (Fig. 8). Recombinant Munc18-b and -c were produced as untagged, *in vitro* translated, [³⁵S]methionine-labeled entities for detection by autoradiography. Interestingly, we observed significant binding of Munc18c to the SNARE complex comprised of His₆-Syntaxin4/GST-SNAP23/GST-VAMP-2 and VAMP-2 (Fig. 8A, lanes 1 and 2). In addition, the His₆-Syntaxin4/GST-VAMP-2 dimer and to a lesser extent the His₆-Syntaxin4/GST-SNAP23 binary complex also interacted specifically with Munc18c in our assay (lanes 3 and 5). We failed to detect any significant interaction between Munc18c and the GST-SNAP23/VAMP-2 heterodimer (lane 4) or to GST-SNAP23 alone (lane 6). The amount of nonspecific binding of radiolabeled Munc18c or His₆-Syntaxin4 to the glutathione-Sepharose was negligible, as seen by the appropriate controls (lanes 10 and 11). In addition, no binding of the ubiquitously expressed SM protein Munc18b to the recombinant SNARE complexes was detected, showing that nonspecific binding to coiled-coil proteins in our binding assay was minimal (Fig. 8B). These data suggest that Munc18c is capable of interacting with Syntaxin4 while present in complexes with its SNARE partners SNAP23 and VAMP-2, including the ternary fusion competent complex.

DISCUSSION

We have further characterized the properties of Tomosyn, originally identified as a Syntaxin1A-binding protein in neural tissue, and report several novel observations extending earlier studies (18). First, we show that in addition to binding to Syntaxin1A, b-Tomosyn also interacts with the ubiquitously expressed t-SNAREs Syntaxin4 and SNAP23. This binding was specific, as we failed to observe an interaction between b-Tomosyn and other Syntaxin isoforms. Further, this interaction was mediated via the carboxyl-terminal VAMP2-like domain in Tomosyn, indicating that the amino-terminal part encodes an alternate function. Second, we have obtained functional data showing that overexpression of full-length Tomosyn in adipocytes inhibits insulin-dependent translocation of GLUT4. Finally, we show that Munc18c interacts both with the Tomosyn/Syntaxin4 complex, as well as the Syntaxin4 containing SNARE complex. Collectively these data implicate a novel role for Tomosyn in the insulin-regulated trafficking of GLUT4 and suggest that it may act in concert with the SM protein Munc18c to prime the t-SNARE for subsequent docking.

Tomosyn was originally described as a Syntaxin-binding protein with a function confined to regulated exocytosis of synaptic vesicles, utilizing Syntaxin1A as a t-SNARE (18). However, our studies demonstrate that ubiquitously expressed b-Tomosyn binds with high affinity to the ubiquitously expressed t-SNAREs Syntaxin4 and SNAP23 (see Figs. 1–3), suggesting that b-Tomosyn plays a more widespread role in regulating exocytosis in all cells. The t-SNAREs Syntaxin4 and SNAP23 have been implicated in a variety of cell surface transport events, including the insulin-regulated translocation of GLUT4 containing vesicles, vasopressin-regulated trafficking of aqua-

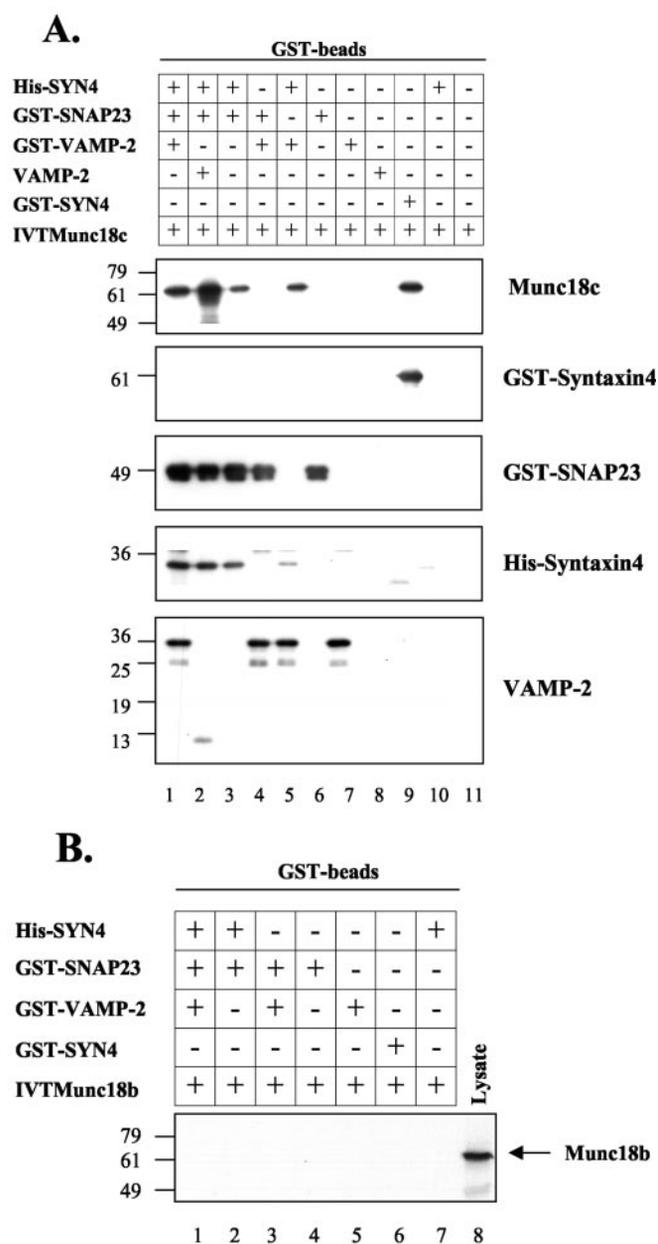


FIG. 8. Munc18c binds to the ternary SNARE complex. A and B, His₆-Syntaxin4, GST-SNAP23, GST-VAMP-2, or thrombin-cleaved VAMP-2 containing complexes (~10 μg each) were preincubated with PBS containing 0.2% Triton X-100 and 0.1% BSA at 4 °C overnight and then incubated with *in vitro* translated [³⁵S]methionine-labeled Munc18c (A) or Munc18b (B), respectively. Recombinant protein complexes were recovered by addition of glutathione-Sepharose and washed extensively before Munc18 binding was determined by SDS-PAGE (10 and 15%) followed by Coomassie Blue staining or Western blotting of the samples from A were carried out to confirm loading. The relative migration of molecular weight standards is shown at the left.

porins, platelet α-granule secretion, and IgE receptor-induced degranulation (22–25, 38, 39, 54, 55). Hence, it is conceivable that b-Tomosyn plays a role in each of these regulatory processes.

Structural studies have revealed that Syntaxin1A may exist in either a closed or an open conformation and that Munc18a selectively binds to the closed conformation, thus acting as a clamp on vesicle transport (16, 17). Tomosyn was described as a molecule with potential to displace Munc18a from Syntaxin1A, thus stimulating vesicle transport (18). Based on our study it is clear that the Syntaxin4 SNARE complex is

regulated in a different manner to its neuronal counterpart. Munc18c binds to both the Syntaxin4 monomer and the Syntaxin4 SNARE complex containing VAMP-2 and/or SNAP23 (Fig. 8). Notably, two alternate SM proteins in yeast, Sly1p and Vps45p, have also been shown to interact both with their cognate Syntaxins and SNARE complexes (56–59). Hence, these data suggest that SM proteins, including Munc18c, can bind both the open and closed form of Syntaxin, or alternatively that the non-neuronal Syntaxins do not adopt a closed conformation. These data do not support a model where SM proteins act as negative regulators of SNARE assembly. Consequently, it was perhaps not surprising that the interaction we observed between Tomosyn and Syntaxin4 did not conform to that described previously in neurons. Our data indicate that the interaction between Tomosyn and Syntaxin4 is not prevented by the presence of Munc18c, as both molecules could bind simultaneously (Fig. 7). These data are consistent with the observation that Tomosyn binds to Syntaxin4 via its VAMP-2-like domain and that both VAMP-2 and Tomosyn bind to Syntaxin4 in a manner that does not preclude an interaction with Munc18c. A previous study showed that the first 139 amino acids of Munc18c (domain 1) are sufficient for syntaxin4 binding (60). Based on the crystal structure of the Munc18a/Syntaxin1A dimer, domain 1 in Munc18a contacted regions in both the H_{abc} and H₃ domains of Syntaxin1A (17). Thus, it will be informative to determine the contact sites between Syntaxin4 and Munc18c, particularly because recent studies using yeast SM proteins have revealed distinct modes of interaction (61, 62).

Our new data place the point of action of Tomosyn close to that of Munc18c in the SNARE assembly cycle. First, both proteins bind to Syntaxin4 and can interact with Syntaxin4 binary complexes. Second, when overexpressed in adipocytes both Tomosyn and Munc18c inhibited insulin-stimulated GLUT4 translocation to a similar extent (see Figs. 4 and 5). Moreover, overexpression of Munc18c also appeared to enhance the level of GLUT4-eGFP in the plasma membrane under basal conditions (Fig. 5). This may reflect a role for Munc18c in promoting SNARE assembly, a concept that has been reported previously (63) for other SNARE complexes. As to why Munc18c overexpression should enhance cell surface levels of GLUT4-eGFP under basal conditions but block insulin action remains to be determined. Perhaps the inhibitory effect on insulin action is mediated via an interaction between Munc18c and some part of the insulin-regulated vesicle transport machinery. It is noteworthy that we² and others (64) have observed insulin regulation of the exocyst complex in adipocytes, and so it will be of interest to determine whether this effect is inhibited by over expression of Munc18c. Moreover, it will also be important to establish the mechanism for the inhibitory effect of Tomosyn overexpression on GLUT4 trafficking, because this may involve its interaction with the t-SNAREs Syntaxin4 and SNAP23 or some other regulatory machinery. Third, incubation of cells with NEM stimulated the release of both Tomosyn and Munc18c from the plasma membrane into the cytosol. Because NEM is known to have a profound effect on SNARE assembly through NSF, these data suggest that both Tomosyn and Munc18c may cycle on and off membranes at a similar stage of the vesicle transport cycle. We have shown recently (65) that the SM protein Vps45p cycles on and off membranes during the SNARE cycle in a manner that is regulated by phosphorylation. Hence, it will be interesting to examine the potential role of insulin-induced phosphorylation concerning the interaction of Munc18c with the plasma membrane.

Intriguingly, the domain structure of Tomosyn resembles that of the Golgi tethering protein p115. Tomosyn and p115 are of similar size and importantly contain a coiled-coil domain at their carboxyl termini that resembles the SNARE motif (66, 67). p115 is thought to act as a tethering molecule by first linking Giantin on COP1-coated transport vesicles with the Golgi protein GM130 and subsequently promoting SNARE complex assembly on the target membrane (67, 68). This role is consistent with the observation that expression of full-length Tomosyn was required to inhibit exocytosis, *in vivo*, and might indicate a separate but necessary role for the conserved amino-terminal domain of Tomosyn (18). It will be of interest to determine whether Tomosyn binds to GLUT4 containing vesicles and if so, to determine the molecular basis for this interaction. However, it is also conceivable that Tomosyn acts as a substitute coiled-coil, forming a soluble four-helix bundle with the PM t-SNAREs. Such t-SNARE/Tomosyn or Amisyn bundles could represent activated and fusogenic SNARE complexes (40). The t-SNARE Tlg2p has been shown previously (40, 69) to require activation by its cognate v-SNARE, Snc2p, or a peptide encompassing the coiled-coil region of Snc2p, suggesting a potential role for Tomosyn/Amisyn-like proteins in t-SNARE activation. In addition, t-SNARE/Tomosyn and Amisyn complexes have been co-immunoprecipitated from solubilized membranes, although these complexes are not abundant or of a transient nature in intact cells, as only a fraction of the t-SNAREs co-sedimented (18, 40). These studies raise a number of important questions for future investigation. What is the role of the Tomosyn amino terminus in vesicle transport? Is there also a role for the related proteins Amisyn and mammalian lethal giant larvae in insulin-regulated GLUT4 trafficking in adipocytes? These proteins have been described as additional Syntaxin4-binding proteins, yet they do not possess an apparent VAMP2-like domain like that found in Tomosyn (18, 20, 40, 43). So the mechanism for their interaction with the t-SNARE remains to be determined.

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REFERENCES

- Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* **362**, 318–324
- Rothman, J. E. (1994) *Adv. Second Messenger Phosphoprotein Res.* **29**, 81–96
- Sudhof, T. C. (1995) *Nature* **375**, 645–653
- Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) *Cell* **92**, 759–772
- Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y. K., and Bennett, M. K. (1998) *Nat. Struct. Biol.* **5**, 765–769
- Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) *Nature* **395**, 347–353
- Ungermann, C., Nichols, B. J., Pelham, H. R., and Wickner, W. (1998) *J. Cell Biol.* **140**, 61–69
- Mayer, A., and Wickner, W. (1997) *J. Cell Biol.* **136**, 307–317
- Zerial, M., and McBride, H. (2001) *Nat. Rev. Mol. Cell Biol.* **2**, 107–117
- Ungermann, C., Sato, K., and Wickner, W. (1998) *Nature* **396**, 543–548
- Jahn, R. (2000) *Neuron* **27**, 201–204
- Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994) *Neuron* **13**, 353–361
- Yang, B., Steegmaier, M., Gonzalez, L. C., Jr., and Scheller, R. H. (2000) *J. Cell Biol.* **148**, 247–252
- Calakos, N., Bennett, M. K., Peterson, K. E., and Scheller, R. H. (1994) *Science* **263**, 1146–1149
- Pevsner, J., Hsu, S. C., and Scheller, R. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1445–1449
- Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T. C., and Rizo, J. (1999) *EMBO J.* **18**, 4372–4382
- Misura, K. M., Scheller, R. H., and Weis, W. I. (2000) *Nature* **404**, 355–362
- Fujita, Y., Shirataki, H., Sakisaka, T., Asakura, T., Ohya, T., Kotani, H., Yokoyama, S., Nishioka, H., Matsuura, Y., Mizoguchi, A., Scheller, R. H., and Takai, Y. (1998) *Neuron* **20**, 905–915

² J. Lopez, M. Girotti and D. E. James, unpublished data.

19. Lehman, K., Rossi, G., Adamo, J. E., and Brennwald, P. (1999) *J. Cell Biol.* **146**, 125–140
20. Yokoyama, S., Shirataki, H., Sakisaka, T., and Takai, Y. (1999) *Biochem. Biophys. Res. Commun.* **256**, 218–222
21. Tellam, J. T., Macaulay, S. L., McIntosh, S., Hewish, D. R., Ward, C. W., and James, D. E. (1997) *J. Biol. Chem.* **272**, 6179–6186
22. Rea, S., Martin, L. B., McIntosh, S., Macaulay, S. L., Ramsdale, T., Baldini, G., and James, D. E. (1998) *J. Biol. Chem.* **273**, 18784–18792
23. Kawanishi, M., Tamori, Y., Okazawa, H., Araki, S., Shinoda, H., and Kasuga, M. (2000) *J. Biol. Chem.* **275**, 8240–8247
24. Cheatham, B., Volchuk, A., Kahn, C. R., Wang, L., Rhodes, C. J., and Klip, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15169–15173
25. Olson, A. L., Knight, J. B., and Pessin, J. E. (1997) *Mol. Cell Biol.* **17**, 2425–2435
26. Thurmond, D. C., Ceresa, B. P., Okada, S., Elmendorf, J. S., Coker, K., and Pessin, J. E. (1998) *J. Biol. Chem.* **273**, 33876–33883
27. Tamori, Y., Kawanishi, M., Niki, T., Shinoda, H., Araki, S., Okazawa, H., and Kasuga, M. (1998) *J. Biol. Chem.* **273**, 19740–19746
28. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
29. Mullock, B. M., Smith, C. W., Ihrke, G., Bright, N. A., Lindsay, M., Parkinson, E. J., Brooks, D. A., Parton, R. G., James, D. E., Luzio, J. P., and Piper, R. C. (2000) *Mol. Biol. Cell* **11**, 3137–3153
30. Tellam, J. T., McIntosh, S., and James, D. E. (1995) *J. Biol. Chem.* **270**, 5857–5863
31. Laemmli, U. K. (1970) *Nature* **227**, 680–685
32. Montano, M. M. (2001) *Methods Mol. Biol.* **177**, 99–106
33. Byrd, A., and St-Arnaud, R. (2001) *Methods Mol. Biol.* **177**, 107–119
34. Frost, S. C., and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 2646–2652
35. Piper, R. C., Hess, L. J., and James, D. E. (1991) *Am. J. Physiol.* **260**, C570–C580
36. Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B., and Cushman, S. W. (1983) *Biochim. Biophys. Acta* **763**, 393–407
37. Nielsen, S., Marples, D., Birn, H., Mohtashami, M., Dalby, N. O., Trimble, M., and Knepper, M. (1995) *J. Clin. Invest.* **96**, 1834–1844
38. Mandon, B., Chou, C. L., Nielsen, S., and Knepper, M. A. (1996) *J. Clin. Invest.* **98**, 906–913
39. Inoue, T., Nielsen, S., Mandon, B., Terris, J., Kishore, B. K., and Knepper, M. A. (1998) *Am. J. Physiol.* **275**, F752–F760
40. Scales, S. J., Hesser, B. A., Masuda, E. S., and Scheller, R. H. (2002) *J. Biol. Chem.* **277**, 28271–28279
41. Bryant, N. J., Govers, R., and James, D. E. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 267–277
42. Masuda, E. S., Huang, B. C., Fisher, J. M., Luo, Y., and Scheller, R. H. (1998) *Neuron* **21**, 479–480
43. Musch, A., Cohen, D., Yeaman, C., Nelson, W. J., Rodriguez-Boulan, E., and Brennwald, P. J. (2002) *Mol. Biol. Cell* **13**, 158–168
44. Kagami, M., Toh-e, A., and Matsui, Y. (1998) *Genetics* **149**, 1717–1727
45. Thurmond, D. C., Kanzaki, M., Khan, A. H., and Pessin, J. E. (2000) *Mol. Cell Biol.* **20**, 379–388
46. Holman, G. D., and Cushman, S. W. (1994) *Bioessays* **16**, 753–759
47. Kandror, K. V., and Pilch, P. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8017–8021
48. Ross, S. A., Scott, H. M., Morris, N. J., Leung, W. Y., Mao, F., Lienhard, G. E., and Keller, S. R. (1996) *J. Biol. Chem.* **271**, 3328–3332
49. Martin, L. B., Shewan, A., Millar, C. A., Gould, G. W., and James, D. E. (1998) *J. Biol. Chem.* **273**, 1444–1452
50. Bennett, M. K., Garcia-Ararras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D., and Scheller, R. H. (1993) *Cell* **74**, 863–873
51. Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T., and Rothman, J. E. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7852–7856
52. Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E., and Sollner, T. (1994) *Cell* **78**, 937–948
53. Fleming, K. G., Hohl, T. M., Yu, R. C., Muller, S. A., Wolpensinger, B., Engel, A., Engelhardt, H., Brunger, A. T., Sollner, T. H., and Hanson, P. I. (1998) *J. Biol. Chem.* **273**, 15675–15681
54. Flaumenhaft, R., Croce, K., Chen, E., Furie, B., and Furie, B. C. (1999) *J. Biol. Chem.* **274**, 2492–2501
55. Pombo, I., Martin-Verdeaux, S., Iannascoli, B., Le Mao, J., Deriano, L., Rivera, J., and Blank, U. (2001) *J. Biol. Chem.* **276**, 42893–42900
56. Grabowski, R., and Gallwitz, D. (1997) *FEBS Lett.* **411**, 169–172
57. Peng, R., and Gallwitz, D. (2002) *J. Cell Biol.* **6**, 6
58. Nichols, B. J., Holthuis, J. C., and Pelham, H. R. (1998) *Eur. J. Cell Biol.* **77**, 263–268
59. Coe, J. G., Lim, A. C., Xu, J., and Hong, W. (1999) *Mol. Biol. Cell* **10**, 2407–2423
60. Grusovin, J., Stoichevska, V., Gough, K. H., Nunan, K., Ward, C. W., and Macaulay, S. L. (2000) *Biochem. J.* **350**, 741–746
61. Dulubova, I., Yamaguchi, T., Wang, Y., Sudhof, T. C., and Rizo, J. (2001) *Nat. Struct. Biol.* **8**, 258–264
62. Dulubova, I., Yamaguchi, T., Gao, Y., Min, S. W., Huryeva, I., Sudhof, T. C., and Rizo, J. (2002) *EMBO J.* **21**, 3620–3631
63. Bryant, N. J., and James, D. E. (2001) *EMBO J.* **20**, 3380–3388
64. Inoue, M., Chang, L., Hwang, J., Chiang, S. H., and Saltiel, A. R. (2003) *Nature* **422**, 629–633
65. Bryant, N. J., and James, D. E. (2003) *J. Cell Biol.* **161**, 691–696
66. Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3046–3051
67. Shorter, J., Beard, M. B., Seemann, J., Dirac-Svejstrup, A. B., and Warren, G. (2002) *J. Cell Biol.* **157**, 45–62
68. Sonnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B., and Warren, G. (1998) *J. Cell Biol.* **140**, 1013–1021
69. Paumet, F., Brugger, B., Parlati, F., McNew, J. A., Sollner, T. H., and Rothman, J. E. (2001) *J. Cell Biol.* **155**, 961–968

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