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-Tomo- syn and the adipocyte SNAP23 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 appropriate 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 juxtamem-

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; PDS, phosphatase-buffered saline; BSA, bovine serum albumin; NEM, N-ethylmaleimide; PM, plasma membrane; LDM, low density microsomes.

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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 facultative 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 a ubiquitous and ubiquitously 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 μg) and total RNA (1 μg). Subsequent PCR amplifications were performed using primers 1, 5′-cggatcctcgaattcagcaacctgaagttgattataaagttcag3′; 2, 5′-cggatcctcgaattcatccttgtaatcgatatcttc-3′; and 4, 5′-cggatcctcgaattcatccttgtaatcgatatcttc-3′. Primers 5–8 were annealed and digested with EcoRI before being subcloned as a double stranded fragment into pMEXneo, using SureII and BclII. Truncated Tomosyn cDNAs encoding amino acids 1–525, 1050–2255, and 3195–3459 were obtained by PCR using primers 9, 5′-cggatcctcgaattcatccttgtaatcgatatcttc-3′; 10, 5′-cggatcctcgaattcatccttgtaatcgatatcttc-3′. Tomosyn cDNAs were cloned into pBTM116 using EcoRI and SalI. pET20b-Syntaxin4 was kindly donated by Dr. Jenny Martin (Institute of Molecular Biology, Brisbane, Australia). 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 carboxy-terminal 14 amino acids of mouse Munc18c (MLNKSKDKVSFKDE). 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 monoclonal antibody to a GST-pET20b-Syntaxin4 cDNA fusion 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. D. Sabatini (Columbia University, New York). All cDNAs obtained by PCR were confirmed by sequencing.

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 Syntaxin4 were expressed in E. coli BL21 (DE3) cells, 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 TALON™ Cobalt metal affinity resin (Clontech, Palo Alto, CA). The recombinant proteins were dialyzed against PBS, except His6-Munc18c, which was dialyzed against PBS containing 2% glycerol, and concentrated using Aquacide (Calbiochem). The protein concentration and purity of all recombinant fusion proteins were determined by Bio-Rad Bradford assay protocols 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 μg/ml) was added to the reaction, and the thrombin-cleaved protein was dialyzed against PBS.

Racemontine Proteins—Recombinant GST fusion proteins (5–15 μg) were attached to glutathione-Sepharose beads (15–25 μl) in a total volume of 200–350 μl of PBS for 1 h at 20 °C and recovered by brief washes with GST elution buffer (25 μg/ml) in the same way. Binary (Syntaxin4/SNAP23; Syntaxin4/VAMP-2; Syntaxin4/Munc18c) or ternary (Syntaxin4/SNAP23/VAMP-2) complexes were formed by mixing approximately equimolar amounts of recombinant proteins and incubated 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 (Her- cules, 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).

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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 His-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 Munc18c-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 Method**—A standard lithium acetate/single-stranded carrier DNA/polystyrene 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 visualization of LacZ was determined using the X-Gal filter lift assay and a X-Gal filter lift assay color development (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.

**In Vitro Binding Assay**—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 either for 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.

**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 over night 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 MeSO 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 recombinant clathrin-positive GLUT4-GFP-pcDNA3 and 400 μg of pMEX_m, pMEX_m-Munc18c, or pMEX_m-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-GFP 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.

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**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, Amisy, 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 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 Binds 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 Syntaxin1A, -2, -3, -4, -6, -7, and -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
times more β-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 β-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 β-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 Amin and SNAP25. There was no detectable binding of β-Tomosyn to either recombinant VAMP-2 or Rab4. Strikingly, the amount of Tomosyn that was bound to the Syntaxin4/SNAP23 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. 2B). 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 β-Tomosyn binds to Syntaxin4 via its VAMP-2-like domain we performed competition binding studies using recombinant VAMP-2. Fig. 3A shows the binding of β-Tomosyn to the Syntaxin4/SNAP23 dimer in the presence of increasing concentrations of VAMP-2. We observed quantitative inhibition of β-Tomosyn binding to the Syntaxin4/SNAP23 heterodimer when VAMP-2 was present in approximately equimolar concentrations (0.25 nmol/µl) (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 larva, 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 larva 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 larva proteins and Sro7/77 and Tomosyn, including a series of WD-40 repeats (19, 44). We therefore investigated whether this conserved amino-terminal domain 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. 3B). 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 competition binding studies, we observed a strong interaction between the VAMP-2-like domain of Tomosyn, amino acids 1066–1153, and...
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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 μg of GFP-GLUT4-pcDNA3 and 400 μg of either pMEX3c-Munc18c or pMEX3c-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, were scored per condition. Shown are mean data ± S.D. of four separate experiments. *, p < 0.01 compared with control cells incubated with insulin.

Syntaxin4 (Fig. 3B), 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. 3B). 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 electrooporated 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. 4A) (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 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. 6A) (21, 26). The majority of Tomosyn was soluble and found in the cytosol...
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 Syntaxin4 (Fig. 7A), and no direct interaction between b-Tomosyn and His<sub>a</sub>-Munc18c was detected. Subsequently, we used preformed GST-Syntaxin4/His<sub>a</sub>-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.

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porins, platelet \(\alpha\)-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 syntaxin binding (60). Based on the crystal structure of the Munc18a/Syntaxin1A dimer, domain 1 in Munc18a contacted regions in both the H_{13} and H_{13} 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 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 we 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 Tomosyn and Munc18c from the plasma membrane into the cytosol. Because NEM is known to have a profound effect on both Tomosyn and Munc18c from the plasma membrane into the cytosol. Because NEM is known to have a profound effect on 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.

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