BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 257–262 (1997)
ARTICLE NO. RC976560

Inhibition of the Binding of SNAP-23 to Syntaxin 4 by Munc18c

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Received March 21, 1997

SNARE proteins have been implicated in the insulin-induced translocation of vesicles containing the GLUT4 glucose transporter to the plasma membrane of adipocytes. The role of the target SNARE SNAP-25 or its homologs in this process was investigated by screening a mouse adipocyte cDNA library with rat SNAP-25 and human SNAP-23 cDNA probes. Both positive clones isolated encoded a protein with 87% sequence identity to human SNAP-23, and which was therefore designated mouse SNAP-23. Immunoblot and immunofluorescence analyses revealed that SNAP-23 is located predominantly in the plasma membrane of 3T3-L1 adipocytes incubated in the absence or presence of insulin. Of syntaxins 1 to 5, SNAP-23 bound with the highest affinity to syntaxins 1 and 4 in the yeast two-hybrid system. Expression of SNAP-23, syntaxin 4, and the syntaxin-binding protein Munc18c in COS cells revealed that Munc18c reduced the amount of SNAP-23 bound to syntaxin 4 in a concentration-dependent manner. These results suggest that the binding of SNAP-23 to syntaxin 4 is inhibited by Munc18c in adipocytes.

Insulin stimulates glucose uptake in muscle and adipose tissue by promoting the translocation of vesicles containing the GLUT4 glucose transporter to the plasma membrane. Although the exact mechanism of insulin-induced GLUT4 translocation is not known, SNARE proteins have been implicated in the docking and fusion of GLUT4-containing vesicles with the plasma membrane. In neurons, the specific targeting of synaptic vesicles is thought to be achieved through the selective interaction of SNARE proteins in the vesicle membrane (v-SNAREs, such as synaptobrevin/VAMP) with the cognate SNARE proteins in the target membrane (t-SNAREs, such as syntaxin and SNAP-25) [1]. Several isoforms of VAMP and syntaxin have been identified, some of which are expressed outside the nervous system. Thus, VAMP-2 and cellubrevin [2-4] as well as syntaxin isoform 2, 3, 4, and 5 [5] have been detected in adipocytes and skeletal muscle. Three isoforms (Munc18a, -b, and -c) of the syntaxin-binding protein Munc18/n-Sec1/rbSec1 have also been identified in 3T3-L1 adipocytes [6]. However, although several studies have indicated the importance of SNARE proteins in the translocation of GLUT4-containing vesicles [4, 5, 7], the t-SNARE SNAP-25 does not appear to be abundant in adipocytes [5, 8, 9]. A cDNA that encodes a homolog of SNAP-25, designated SNAP-23, was recently cloned from human lymphocytes [10]. Thus, adipocytes may also express such homologs that act in a manner similar to SNAP-25. To investigate whether SNAP-25 or homologs such as SNAP-23 contribute to GLUT4 translocation in adipocytes, we have searched for the corresponding cDNAs in a mouse adipocyte library. We cloned mouse SNAP-23 cDNA from this library and examined the potential role of the encoded protein as a t-SNARE in adipocytes.

MATERIALS AND METHODS

Antibodies and cDNA clones. Polyclonal antibodies to SNAP-23, syntaxin 4, and Munc18c were generated by immunizing rabbits with a glutathione S-transferase (GST) fusion protein of mouse SNAP-23, a GST fusion protein containing the cytoplasmic region of rat syntaxin 4 (residues 1 to 273), or a synthetic peptide corresponding to the COOH-terminal portion of mouse Munc18c (KMLNKSKDKVSFKDE), respectively. Rabbit polyclonal antibodies specific for the COOH-terminal portion of GLUT4 were kindly provided by S. W. Cushman (NIH, Bethesda, MD), and syntaxin cDNAs by R. H. Scheller (Stanford University, Stanford, CA).

Cell culture and subcellular fractionation. 3T3-L1 adipocytes were cultured in 100-mm dishes containing Dulbecco's modified Ea-

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The nucleotide sequence data of mouse SNAP-23 reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following Accession Number AB000822.

0006-291X/97 $25.00

gle's medium (DMEM) supplemented with 10% fetal bovine serum. Differentiated adipocytes were subjected to subcellular fractionation for the isolation of intracellular membrane (ICM) and plasma membrane (PM) fractions as described previously [4, 11, 12]. COS cells were also maintained in DMEM supplemented with 10% fetal bovine serum.

Screening of a mouse adipocyte cDNA library. The full-length human SNAP-23 and rat SNAP-25B cDNAs were obtained by reverse transcription and the polymerase chain reaction with specific oligonucleotide primers [SNAP-23, 5′-CATGGATAATCTGTCATCAGAAG-3′ (sense) and 5′-TTAGCTGTCAATGAGTTTCTTTGC-3′ (antisense); SNAP-25, 5′-CATGGCCGAGGACGCAGACATG-3′ (sense) and 5′-TTAACCACTTCCCAGCATCTTTG-3′ (antisense)] and total RNA from either human lymphocytes or rat brain, respectively. Duplicate filters of a mouse adipocyte cDNA library ($\sim 3 \times 10^5$ plaques) (Clontech) were screened by incubating first for 4 h at 42° C in a solution containing 20% (v/v) formamide, 5× SSPE (saline, sodium phosphate, EDTA), 5× Denhardt's reagent, 0.1% SDS, and boiled salmon sperm DNA (100 μg/ml), and then for 12 h at 42° C with ^{32}P-labeled, full-length human SNAP-23 or rat SNAP-25 cDNAs as probes. Phage DNA from the positive plaques was isolated and, after digestion with EcoRI, subcloned into a pUC18 plasmid vector (TaKaRa). All DNA sequences were determined on both strands by the dideoxynucleotide sequencing method.

Immunoblot analysis. Subcellular fractions of 3T3-L1 adipocytes and extracts of transfected COS cells were subjected to SDS-polyacrylamide gel electrophoresis with the Laemmli discontinuous buffer system [13], and immunoblot analysis was performed as described previously [14, 15], with minor modifications. Primary antibodies were used at the following dilutions: affinity-purified antibodies to SNAP-23 (1:300), antibodies to the COOH-terminal of GLUT4 (1:500), affinity-purified antibodies to Munc18c (1:1000), and affinity-purified antibodies to syntaxin 4 (1:300). Immune complexes were detected with horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin G and the ECL detection system (Amersham).

Immunolocalization of SNAP-23 in 3T3-L1 adipocytes. 3T3-L1 adipocytes grown in Lab-Tek chamber slides (Nunc) were fixed with 2% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 20° C, washed, and permeabilized by freezing and thawing twice. The cells were then incubated for 30 min at 20° C with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS), and then for 1 h at 20° C with antibodies to SNAP-23 (1:50 dilution in TBS containing 1% BSA). After washing for 15 min with three changes of PBS, the cells were incubated for 60 min at 20° C with Texas red-conjugated donkey antibodies to rabbit immunoglobulin G (1:100 dilution in TBS containing 1% BSA) (Amersham). The cells were again washed for 15 min with three changes of PBS, mounted in PBS containing 50% (v/v) glycerol, and finally examined with an Olympus BX-50-FLA microscope at 400× magnification.

Yeast two-hybrid system. Protein-protein interactions were analyzed with the MATCHMAKER Two-Hybrid System kit (Clontech). Briefly, full-length rat SNAP-25, human SNAP-23, and mouse SNAP-23 cDNAs were cloned into the bait and prey yeast expression vectors pGBT9 and pGAD424. cDNAs encoding the cytoplasmic regions of syntaxin 1A (residues 1 to 265), syntaxin 2 (residues 1 to 266), syntaxin 3 (residues 1 to 263), syntaxin 4 (residues 1 to 273), syntaxin 5 (residues 1 to 284) [16] were cloned into the same vectors. Yeast strain YGH1 (kindly provided by D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was transfected with bait and prey vectors by the lithium acetate method [17]. Transformants were plated on selection medium lacking Trp and Leu. After growth at 30° C, they were inoculated into SD medium lacking Trp and Leu, and then placed in a shaking incubator at 30° C until the optical density at 600 nm reached 0.5 to 1.0. The activity of β-galactosidase was then assayed in cell extracts.

In vivo assays of syntaxin 4 binding to SNAP-23 and Munc18c. The full-length mouse Munc18c cDNA was obtained by the polymerase chain reaction with specific oligonucleotide primers [5′-GATGGCGCCGCCGGTATCG-3′ (sense) and 5′-GTTACTCATCCTTAAAGGAAAC-3′ (antisense)] and a mouse adipocyte cDNA library (Clontech). COS cells were transiently transfected, with the use of lipofectin reagent (GIBCO), with cDNAs encoding full-length mouse SNAP-23 and the cytoplasmic region of syntaxin 4 that had been cloned into the expression vector pcDL-SRα [18], together with various amounts of full-length Munc18c cDNA cloned into the same vector. Forty-eight hours after transfection, the cells were lysed in a solution containing 20 mM Hepes-KOH (pH 7.5), 140 mM potassium acetate, 1 mM $MgCl_2$, 1 mM EGTA, and 1% Triton X-100, and the lysates were subjected to immunoprecipitation with antibodies to syntaxin 4. After washing, the immunoprecipitates and the total cell lysates were boiled in SDS sample buffer and subjected to immunoblot analysis with antibodies to SNAP-23, to syntaxin 4, or to Munc18c.

RESULTS

Cloning of mouse SNAP-23 cDNA. In an attempt to isolate cDNAs encoding SNAP-25 and its homologs from mouse adipocytes, we screened $\sim 3 \times 10^5$ plaques from a mouse adipocyte cDNA library at low stringency with randomly labeled cDNAs for rat SNAP-25 and human SNAP-23. Two positive clones were isolated, both of which were shown by sequencing to encode the same protein. The initiator methionine was identified in the cDNA sequence by comparison with the cDNA

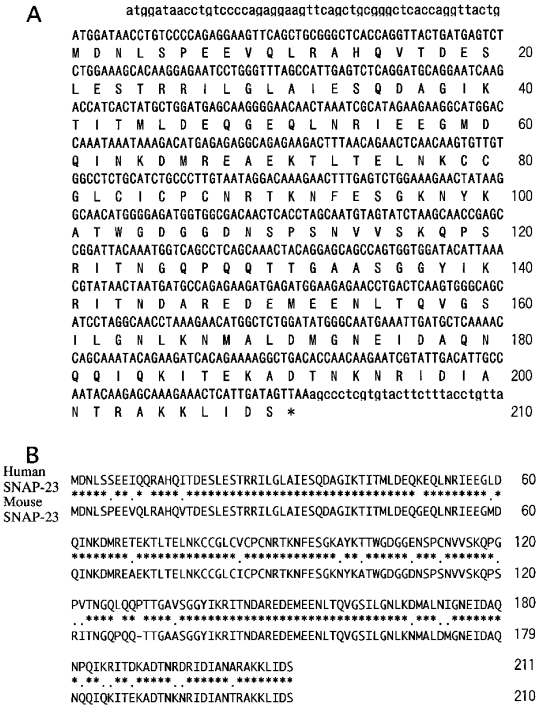

FIG. 1. (A) Nucleotide and predicted amino acid sequences of mouse SNAP-23. The amino acid sequence is shown in single-letter code below the nucleotide sequence, with residue numbers on the right. Asterisk indicates termination codon. (B) Alignment of the amino acid sequences of mouse and human SNAP-23. Asterisks and dots indicate identical and similar amino acids, respectively. Alignment was optimized with the GENETYX-MAC (version 7.3) program.

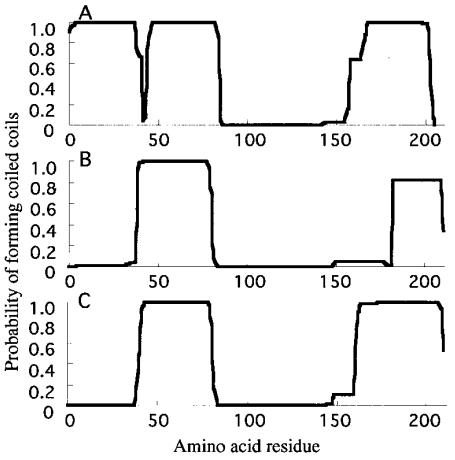

FIG. 2. Probability of the formation of coiled-coil structures for rat SNAP-25B (A), human SNAP-23 (B), and mouse SNAP-23 (C). The amino acid sequence of each protein was compared by COILS program to a database of known parallel two-stranded coiled coils and a similarity score was obtained. By comparing this score to the distribution of scores in globular and coiled-coil proteins, the program then calculates the probability that the sequence will adopt a coiled-coil conformation [22, 23].

sequences of human SNAP-23 and rat SNAP-25, and its position was confirmed by the presence of upstream stop codons. The new cDNA sequence encodes a predicted protein of 210 amino acids (Fig. 1A) that shows 98.6% similarity and 86.7% identity to human SNAP-23 (Fig. 1B). We therefore concluded that the adipocyte cDNA encodes mouse SNAP-23. The cysteine residues clustered around residue 90 of SNAP-25 [19], which are though to contain a palmitoylation site through which the protein is anchored to the plasma membrane

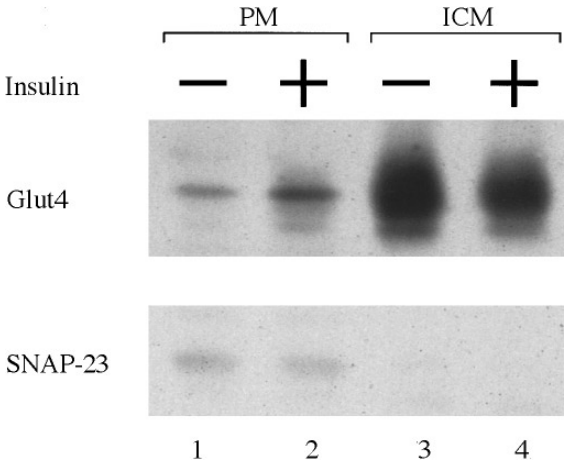

FIG. 3. Subcellular distribution of SNAP-23 in 3T3-L1 adipocytes. Cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.1 μM insulin at 37°C for 30 min, after which PM (lanes 1 and 2) and ICM (lanes 3 and 4) fractions were prepared and subjected (50 μg of protein per lane) to immunoblot analysis with antibodies to SNAP-23 or to GLUT4.

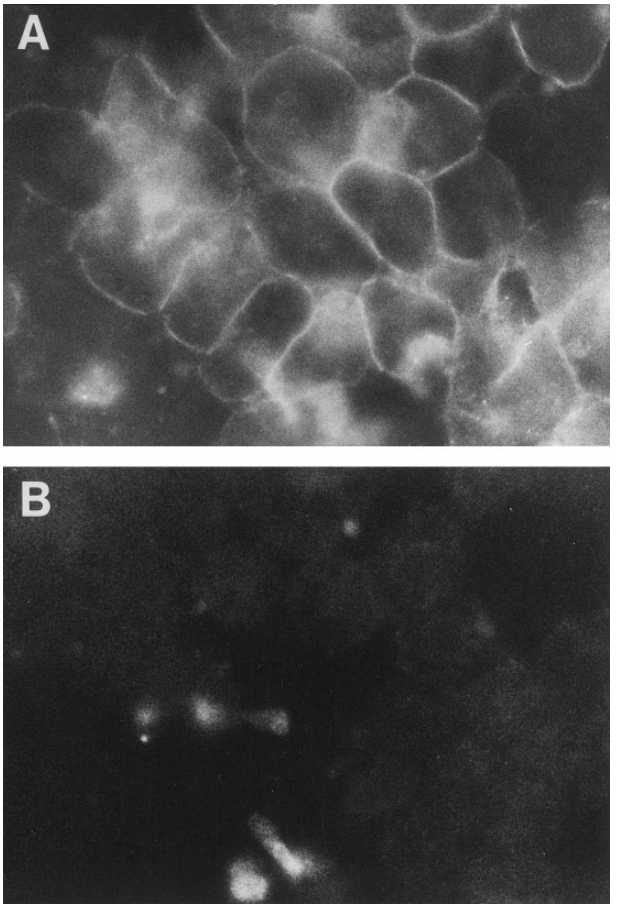

FIG. 4. Immunofluorescence microscopic analysis of SNAP-23 in 3T3-L1 adipocytes. Fixed and permeabilized 3T3-L1 adipocytes were incubated with antibodies to SNAP-23 in the absence (A) or presence (B) of recombinant mouse SNAP-23 protein (0.1 mg/ml).

[20], are conserved in mouse SNAP-23. The putative phosphorylation site at Ser^{187} of SNAP-25 [21] appears to be converted to Thr^{192} in mouse SNAP-23. Structural predictions performed with the COILS program through the internet (http://ulrec3.unil.ch/software/COILS_form.html) [22, 23] revealed that mouse and human SNAP-23 contain two putative coiled-coil domains, whereas SNAP-25 contains three (Fig. 2).

Localization of SNAP-23. Subcellular fractionation of 3T3-L1 adipocytes and immunoblot analysis revealed that most SNAP-23 was present in the PM fraction under basal conditions and that insulin stimulation of cells did not affect the localization of SNAP-23; in contrast, insulin induced the translocation of GLUT4 from the ICM fraction to the PM fraction (Fig. 3).

Immunofluorescence labeling confirmed that SNAP-23 was expressed predominantly in the plasma membrane of 3T3-L1 adipocytes (Fig. 4A). Incubation of cells with recombinant SNAP-23 protein in addition to anti-SNAP-23 antibodies prevented staining of the plasma membrane (Fig. 4B). The location of SNAP-23 in adipo-

TABLE 1

Interactions of SNAP-23 and SNAP-25 with Syntaxins in the Yeast Two-Hybrid System

	β-Galactosidase activity (arbitrary units)							
	pGBT9 (bait vector)							
pGAD424 (prey vector)	Syntaxin 1	Syntaxin 2	Syntaxin 3	Syntaxin 4	Syntaxin 5	Rat SNAP-25	Human SNAP-23	Mouse SNAP-23
Syntaxin 1	25.6 ± 1.1					6330 ± 550	4720 ± 370	2870 ± 260
Syntaxin 2		3.46 ± 2.11				4680 ± 300	2250 ± 50	1000 ± 20
Syntaxin 3			1.51 ± 0.30			1490 ± 130	372 ± 7	56.9 ± 1.7
Syntaxin 4				40.4 ± 1.2		7240 ± 330	4480 ± 90	2490 ± 70
Syntaxin 5					6.51 ± 0.82	38.6 ± 0.4	2.17 ± 0.63	1.77 ± 0.55
Rat SNAP-25	8590 ± 1390	4820 ± 1200	3430 ± 490	6980 ± 1000	15.5 ± 0.7	183 ± 4		
Human SNAP-23	393 ± 7	6.07 ± 1.11	23.4 ± 0.9	266 ± 6	9.31 ± 0.98		20.6 ± 0.8	
Mouse SNAP-23	81.7 ± 3.5	14.3 ± 0.2	38.9 ± 0.9	23.4 ± 1.3	9.69 ± 0.46			2.31 ± 0.25

Yeast clones transformed with the pGAD424 and pGBT9 vectors containing the indicated combinations of cDNAs were selected on supplemented plates lacking Trp and Leu and then grown in the presence of selection medium in liquid culture. β-Galactosidase activity in cell lysates was measured in triplicate. Data are means ± SD.

cytes was thus similar to that of SNAP-25 in neural cells.

Association of SNAP-23 with syntaxin isoforms. To investigate whether SNAP-23 binds to syntaxins, we used the yeast two-hybrid system [24]. We cloned SNAP-25 or SNAP-23 cDNAs into the bait vector (pGBT9) and syntaxin cDNAs into the prey vector (pGAD424), and the affinity of interactions between SNAP-25 or SNAP-23 and syntaxins was assessed on the basis of β-galactosidase activity in yeast cell extracts. These experiments revealed that human and mouse SNAP-23 associated most strongly with syntaxins 1 and 4, moderately with syntaxin 2, weakly with syntaxin 3, and hardly at all with syntaxin 5 (Table 1). SNAP-25 showed a similar rank order of preference for the syntaxin isoforms, but the affinities of the interactions were higher than those for the SNAP-23-syntaxin complexes. When syntaxin cDNAs were cloned into pGBT9 and SNAP-25 or SNAP-23 cDNAs into pGAD424, only SNAP-25 retained its pattern of binding to syntaxins, SNAP-23 associated very weakly with the syntaxin isoforms (Table 1).

Effect of Munc18c on the binding of SNAP-23 to syntaxin 4 in vivo. The effect of the syntaxin-binding protein Munc18c on the association of syntaxin 4 and SNAP-23 in intact cells was investigated by expressing syntaxin 4, SNAP-23, and various amounts of Munc18c in COS cells. Cell lysates were then subjected to immunoprecipitation with antibodies to syntaxin 4. The total cell lysates and the immunoprecipitates were analyzed by immunoblotting with antibodies to each of the three proteins. Both SNAP-23 and Munc18c were coprecipitated with syntaxin 4, showing that syntaxin 4 actually binds to these two proteins in vivo (Fig. 5). In addition, as the amount of transfected Munc18c cDNA increased, the amount of SNAP-23 that was coprecipitated with syntaxin 4 decreased.

DISCUSSION

Recent studies of synaptic vesicle exocytosis have revealed the important roles of SNARE proteins in this process. However, the wide tissue distribution of SNARE proteins suggests that they also function in vesicle transport in nonneural cells. Indeed, these proteins have been implicated in the translocation of GLUT4 vesicles to the plasma membrane in adipocytes. Thus, both the translocation of GLUT4 and glucose transport induced by insulin were reduced in permeabilized 3T3-L1 adipocytes in which both VAMP-2 and

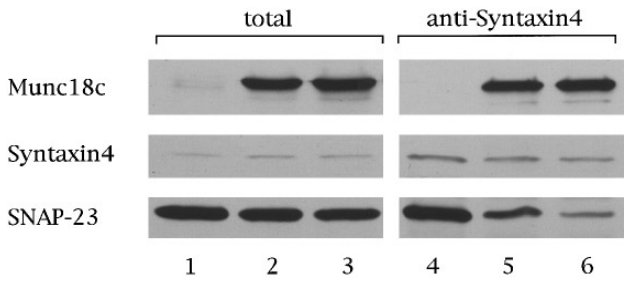

FIG. 5. Inhibition of SNAP-23 binding to syntaxin 4 by Munc18c. COS cells were cotransfected with expression vectors containing full-length mouse SNAP-23 cDNA (5 μg) and a cDNA encoding the cytoplasmic region of syntaxin 4 (3 μg), as well as 0 (lanes 1 and 4), 5 (lanes 2 and 5), or 10 μg (lanes 3 and 6) of the same vector containing full-length Munc18c cDNA. After 48 h, cells were lysed and subjected to immunoprecipitation with antibodies to syntaxin 4. The total cell lysates (lanes 1 to 3) and the immunoprecipitates (lanes 4 to 6) were analyzed by immunoblotting with antibodies to Munc18c, syntaxin 4, or SNAP-23.

cellubrevin had been cleaved with botulinum neurotoxin [4]. Moreover, GST fusion proteins encoding soluble forms of VAMP-2 or syntaxin 4 inhibited the insulin-induced translocation of GLUT4 in permeabilized 3T3-L1 adipocytes [7]. However, despite its important role in the docking and fusion of synaptic vesicles, SNAP-25 has rarely been detected in nonneural tissues, with the exception of pancreatic islets of Langerhans [25, 26], adrenal chromaffin cells [27], and anterior pituitary cells [28]. Thus, homologous proteins may substitute for SNAP-25 in other tissues. In our attempt to clarify potential role of SNAP-25 or its homologs in GLUT4 translocation in adipocytes, we searched for corresponding cDNAs in a mouse adipocyte library. Both positive clones isolated were shown to encode mouse SNAP-23. Thus, SNAP-23 mRNA would appear to be more abundant in mouse adipocytes than SNAP-25 mRNA. Jagadish *et al.* [8] recently detected SNAP-25 mRNA and protein in adipocytes by sensitive methods (reverse transcription-polymerase chain reaction plus Southern hybridization, and immunoblot analysis of purified plasma membranes from large-scale subcellular fractionation). Timmers *et al.* [5] and Wong *et al.* [9] did not detect SNAP-25 in adipocytes either. Thus, adipocytes apparently contain only small amounts of SNAP-25, which leads us to propose that SNAP-23, rather than SNAP-25, functions as the predominant t-SNARE in adipocytes.

Syntaxin proteins contain heptad repeats characteristic of α-helices that form coiled coils [29]. Chapman *et al.* [30] proposed that the binding of syntaxin to SNAP-25 may be mediated by the α-helical domains of each protein, that form coiled-coil structures. The COILS program identified two coiled-coil regions in SNAP-23 and three in SNAP-25. This difference may underlie our observation that the association of syntaxins with SNAP-25 was stronger than that with SNAP-23.

Of syntaxins 1 to 5, syntaxins 1 and 4 bound to SNAP-23 with the highest affinity in the yeast two-hybrid system. However, syntaxin 1 is expressed predominantly in neural cells [16], the major isoforms in adipocytes being syntaxins 2, 3, 4, and 5 [5]. Therefore, in adipocytes, SNAP-23 likely binds preferentially to syntaxin 4. Indeed, we observed that SNAP-23 and syntaxin 4 coimmunoprecipitate from lysates of 3T3-L1 adipocytes (data not shown).

Munc18 was isolated as a syntaxin-binding protein and turned out to be a mammalian homolog of *Caenorhabditis elegans unc-18* [31]. Mammalian homologs of yeast Sec1 were also identified by the polymerase chain reaction and named n-Sec1 and rbSec1 [32, 33]. All three mammalian proteins (Munc18, n-Sec1, and rbSec1) are identical. Munc18 inhibits the association of syntaxin 1 with SNAP-25 [34]. Given that the isoforms Munc18a and Munc18b bind to syntaxins 1, 2, and 3 but not to syntaxin 4 [35], and that Munc18c is expressed in adipocytes [6], we examined the effect of the latter isoform on the association of SNAP-23 and syntaxin 4. Our observation that the amount of SNAP-23 associated with syntaxin 4 decreased as the amount of Munc18c increased suggests that Munc18c may regulate the formation of the SNAP-23-syntaxin 4 SNARE complex in adipocytes.

ACKNOWLEDGMENTS

We thank S. W. Cushman for the antibodies to GLUT4, R. H. Scheller for syntaxin cDNAs, and D. Beach for yeast strain YGH1.

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