Role of SNAP23 in Insulin-induced Translocation of GLUT4 in 3T3-L1 Adipocytes

MEDIATION OF COMPLEX FORMATION BETWEEN SYNTAXIN4 AND VAMP2

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Masatoshi Kawanishi, Yoshikazu Tamori‡, Hideki Okazawa, Satoshi Araki, Hiroaki Shinoda, and Masato Kasuga

From the Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Both syntaxin4 and VAMP2 are implicated in insulin regulation of glucose transporter-4 (GLUT4) trafficking in adipocytes as target (t) soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) and vesicle (v)-SNARE proteins, respectively, which mediate fusion of GLUT4-containing vesicles with the plasma membrane. Synaptosome-associated 23-kDa protein (SNAP23) is a widely expressed isoform of SNAP25, the principal t-SNARE of neuronal cells, and colocalizes with syntaxin4 in the plasma membrane of 3T3-L1 adipocytes. In the present study, two SNAP23 mutants, SNAP23-ΔC8 (amino acids 1 to 202) and SNAP23-ΔC49 (amino acids 1 to 161), were generated to determine whether SNAP23 is required for insulin-induced translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes. Wild-type SNAP23 (SNAP23-WT) promoted the interaction between syntaxin4 and VAMP2 both in vitro and in vivo. Although SNAP23-ΔC49 bound to neither syntaxin4 nor VAMP2, the SNAP23-ΔC8 mutant bound to syntaxin4 but not to VAMP2. In addition, although SNAP23-ΔC8 bound to syntaxin4, it did not mediate the interaction between syntaxin4 and VAMP2. Moreover, overexpression of SNAP23-ΔC8 in 3T3-L1 adipocytes by adenovirus-mediated gene transfer inhibited insulin-induced translocation of GLUT4 but not that of GLUT1. In contrast, overexpression of neither SNAP23-WT nor SNAP23-ΔC49 in 3T3-L1 adipocytes affected the translocation of GLUT4 or GLUT1. Together, these results demonstrate that SNAP23 contributes to insulin-dependent trafficking of GLUT4 to the plasma membrane in 3T3-L1 adipocytes by mediating the interaction between t-SNARE (syntaxin4) and v-SNARE (VAMP2).

A primary function of insulin is to stimulate the transport of glucose into target tissues, prominent among which are skeletal muscle, cardiac muscle, and adipose tissue. Insulin achieves this effect by inducing the translocation of GLUT4 glucose transporters from an intracellular vesicular compartment to the plasma membrane. Under basal conditions, GLUT4 cycles slowly between this intracellular compartment and the plasma membrane (1, 2). However, activation of insulin receptors triggers a large increase in the rate of exocytosis of GLUT4-containing vesicles and a smaller decrease in the rate of GLUT4 internalization by endocytosis (3–5), with the former action likely contributing most to the insulin-induced increase in the amount of GLUT4 in the plasma membrane (6).

Intracellular membrane fusion is mediated by evolutionarily conserved membrane proteins known as soluble N-ethylmaleimide-sensitive factor (NSF)1 attachment protein receptors (SNAREs) (7, 8). SNARE proteins that contribute to neuronal exocytosis include the synaptic vesicle protein synaptobrevin (also referred to as VAMP) and the plasma membrane proteins synaptosome-associated 25-kDa protein and syntaxin1A. These proteins readily assemble into a stable ternary complex; however, disassembly of this complex can be reversibly induced by the ATPase NSF in conjunction with soluble cofactors termed SNAPs (soluble NSF-attachment proteins) (9). The formation of the SNARE complex is thought to be a critical step in the pathway leading to membrane fusion.

The insulin-stimulated trafficking of GLUT4 vesicles in adipocytes shares several features with the regulated pathway of synaptic vesicle exocytosis. Thus, members of the synaptobrevin (VAMP) family of proteins were shown to localize to GLUT4 vesicles in rat adipocytes (10). These proteins were identified as VAMP2 and cellubrevin in 3T3-L1 adipocytes (11) and were subsequently shown to be essential for insulin-stimulated GLUT4 translocation in these cells (12, 13). Furthermore, syntaxin isoform 4 was shown to contribute to the translocation of GLUT4 in 3T3-L1 adipocytes (14). In addition, SNAP23, a widely expressed isoform of SNAP25 (15), was shown to be present in the plasma membrane of 3T3-L1 adipocytes (16) and to be colocalized with syntaxin4 in these cells (17). Thus, SNAP23 in adipocytes, like SNAP25 in neurons, may function in the exocytosis of intracellular vesicles.

We have now investigated the possible role of SNAP23 as a functional plasma membrane t-SNARE in insulin-stimulated GLUT4 translocation. Our data demonstrate that SNAP23 is required for insulin-induced GLUT4 translocation to the plasma membrane and that it mediates the formation of a complex between syntaxin4 and VAMP2.

EXPERIMENTAL PROCEDURES

Antibodies and cDNAs—Rabbit polyclonal antibodies to VAMP2 were kindly provided by N. Takahashi (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan). Rabbit polyclonal antibodies specific for

1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble NSF-attachment protein receptor; v- and t-SNARE, vesicle and target SNARE, respectively; SNAP23, synaptosome-associated 23-kDa protein; WT, wild type; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; MOI, multiplicity of infection; PFU, plaque-forming unit.
the COOH-terminal portion of GLUT1 (18) and rabbit polyclonal antibodies generated in response to a gluthathione S-transferase (GST) fusion protein containing the cytoplasmic portion (residues 1 to 273) of syntaxin4 (16) were prepared as described previously. A mouse monoclonal antibody (1F9) to GLUT4 and a rabbit polyclonal antibody to the COOH terminus of GLUT4 were kindly provided by J.-P. Verheijen (University of Queensland, Australia) and S. W. Cushman (NIH, Bethesda, MD), respectively. Mouse monoclonal antibody 12CA5 to the hemagglutinin (HA) epitope and rabbit polyclonal antibodies to HA were obtained from Roche Molecular Biochemicals and Zymed Laboratories Inc., respectively. Mouse monoclonal antibody 9E10 to c-MYC was obtained from Santa Cruz Biotechnology. Mouse full-length SNAP23 cDNA was obtained as described previously (16). Rabbit full-length syntaxin4 cDNA and rat full-length VAMP2 cDNA were kindly provided by R. H. Scheller (Stanford University, CA) and M. Takahashi (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan), respectively.

Cell Culture—3T3-L1 fibroblasts were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Adipogenesis was induced by treatment of the cells with insulin, dexamethasone, and isobutylmethylxanthine as described previously (19), and the cells were subjected to experiments after 8 to 13 days. COS cells and 293 cells were also maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Analysis of Syntaxin4, SNAP23, and VAMP2 in COS Cells—COS cells were transiently transfected with the expression vector pcDL-SRα (20), encoding the cytoplasmic portion of syntaxin4, HA epitope-tagged SNAP23, or the MYC epitope-tagged cytoplasmic portion of VAMP2 with the use of Lipofectin (Life Technologies, Inc.). Two days after transfection, cells were solubilized with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and subjected to immunoprecipitation with monoclonal antibodies to the HA or MYC tags. The immunoprecipitates were then subjected to immunoblot analysis with antibodies to syntaxin4, to HA, or to MYC.

Assay of Binding of SNAP23 to Syntaxin4 or to VAMP2—With the use of Lipofectin, COS cells were transiently transfected with pcDL-SRα encoding HA epitope-tagged wild-type (WT) or the ΔC8 or ΔC49 mutants of SNAP23 (see “Results”) or with the same vector encoding the cytoplasmic portion of syntaxin4. The cells were subsequently solubilized with lysis buffer, and the resulting extracts were incubated with constant agitation at 4 °C for 1 h with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) that had been conjugated with GST fusion proteins containing the cytoplasmic portions of either syntaxin4 or VAMP2. The beads were washed with ice-cold lysis buffer, after which proteins bound to the beads were eluted with Laemmli sample buffer and subjected to immunoblot analysis with rabbit antibodies to either HA (for SNAP23) or syntaxin4.

Subcellular Fractionation of 3T3-L1 Adipocytes and Immunoprecipitation—Subcellular fractionation of 3T3-L1 adipocytes was performed as described previously (21) with minor modifications. Cells were scraped from the dishes and resuspended in TES buffer (300 mM Tris-HCl (pH 7.4), 1 mM EDTA, 225 mM sucrose), and the homogenate was centrifuged at 16,000 × g. The resulting pellet was layered on top of a 1.12 M sucrose cushion and centrifuged at 101,000 × g. The plasma membrane fraction was collected from the interface of the two solutions and suspended in lysis buffer, after which overexpressed SNAP23 proteins were immunoprecipitated with the monoclonal antibody to the HA epitope tag. The immunoprecipitates were then subjected to immunoblot analysis with rabbit antibodies to syntaxin4, to the HA tag, or to VAMP2.

Construction of and Infection with Adeno virus Vectors Encoding SNAP23 Proteins—Recombinant adenovirus vectors were generated by cloning cDNAs into pSH-Cavt (22), which contains the CAg promoter (23), and cotransfection into 293 cells with DNA-terminal protein complex, as described previously (24). Protein-encoding viruses were screened by immunoblot analysis and cloned by limiting dilution. Adenovirus vectors were propagated by a standard procedure and then purified and titrated as described (25). Ten to 12 days after induction of differentiation, 3T3-L1 adipocytes were infected with adenovirus vectors for 2 h. The cells were subjected to experiments 48 h after infection.

Assay of 2-Deoxy-d-Glucose Transport—3T3-L1 cells were deprived of serum by incubation for 2 h in 12-well plates containing Dulbecco's modified Eagle's medium. The cells were then incubated with 100 nM insulin for 20 min in 450 µl of KRH buffer (25 mM Hepes-NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.3 mM K2HPO4). Glucose transport was initiated by the addition of 50 µl of KRH buffer containing 0.5 mM 2-deoxy-d-[1,2-3H]glucose (0.25 μCi) to each well, and after 5 min, transport was terminated by washing the cells three times with ice-cold KRH buffer. The cells were solubilized with 0.5% SDS, and the incorporated radioactivity was measured by liquid scintillation counting.

Con cofocal Immunofluorescence Microscopy—3T3-L1 adipocytes grown in Lab-Tek chamber slides (Nunc) were fixed and permeabilized with methanol at −20 °C. The cells were incubated for 30 min at 20 °C with 5% (v/v) bovine serum albumin in Tris-buffered saline, and then for 1 h at 20 °C with rabbit antibodies to HA (1:1000 dilution in Tris-buffered saline containing 1% bovine serum albumin). After washing for 15 min with three changes of phosphate-buffered saline (PBS), the cells were incubated for 60 min at 20 °C with tetramethylrhodamine isothiocyanate-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech). The cells were again fixed for 15 min with three changes of PBS, mounted in FluoroGuard Antifade Reagent (Bio-Rad), and examined with a confocal fluorescence microscope (Bio-Rad).

Plasma Membrane Lawn Assay—3T3-L1 or GLUT4 to the plasma membrane was measured by the plasma membrane lawn assay as described previously (26). In brief, 3T3-L1 cells cultured on coverslips were washed in PBS and treated with poly-L-lysine (0.5 mg/ml) in PBS. They were then incubated in a hypotonic solution (30 mM Hepes-NaOH (pH 7.5), 70 mM KCl, 5 mM MgCl2, 3 mM EGTA), disrupted by placement under an ultrasonic microprobe in the same solution containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and fixed in 2% paraformaldehyde. The fixed cells were incubated first with rabbit polyclonal antibodies to GLUT1 and mouse monoclonal antibodies to GLUT4 and then, after washing three times with PBS, with tetramethylrhodamine isothiocyanate-conjugated antibodies to rabbit immunoglobulin G and fluorescein isothiocyanate-conjugated antibodies to mouse immunoglobulin G. The cells were washed with PBS, mounted in FluoroGuard Antifade reagent, and examined with a confocal fluorescence microscope.

Statistical Analysis—All qualitative data are representative of at least three independent experiments. Quantitative data are presented as means ± S.E. and were compared with Student's t test. A P value of <0.05 was considered statistically significant.

RESULTS

Interactions among VAMP2, Syntaxin4, and SNAP23—To investigate the potential role of SNAP23 in mediating the formation of a ternary SNARE complex with syntaxin4 and VAMP2, both of which are thought to play important roles in GLUT4 translocation in 3T3-L1 adipocytes, we examined whether SNAP23 binds to syntaxin4 and to VAMP2. COS cells were transiently transfected with various combinations of pcDL-SRα expression vectors encoding SNAP23 tagged at its NH2 terminus with the HA epitope (YPYDVPDYA), the cytoplasmic portion of syntaxin4 (residues 1 to 273), or the cytoplasmic portion of VAMP2 (residues 1 to 93) tagged at its COOH terminus with the MYC epitope (AEEQKLISEEDLLK). Extracts of the transfected cells were subsequently subjected to immunoprecipitation with mouse monoclonal antibodies to HA or to MYC, and immunoprecipitated proteins were subjected to immunoblot analysis with rabbit antibodies to mouse monoclonal antibodies to HA or to MYC, or mouse immunoglobulin G and fluorescein isothiocyanate-conjugated antibodies to mouse immunoglobulin G. The cells were washed with PBS, mounted in FluoroGuard Antifade reagent, and examined with a confocal fluorescence microscope.
COS cells were cotransfected with the in-syntaxin4, and SNAP23 in COS cells. C8 interacted with syntaxin4 (Fig. 3). To investigate the DBVAMP2 (Fig. 3), whereas both SNAP23-WT and SNAP23-antibodies to HA. Only SNAP23-WT bound to immobilized portions of VAMP2 or syntaxin4, and proteins that bound to immobilized GST fusion proteins containing the cytoplasmic mutants. Cell extracts were subsequently incubated with bead-expression vectors encoding SNAP23-WT and each of the two in vitro as to the light chain of immunoglobulin G syntaxin4, SNAP23, and VAMP2 as well as to the light chain of immunoglobulin G are indicated.

The immunoprecipitates were then subjected to immunoblot analysis with rabbit antibodies to syntaxin4 or to HA or with mouse antibodies to MYC. The positions of bands corresponding to recombinant syntaxin4, SNAP23, and VAMP2 as well as to the light chain of immunoglobulin G were indicated.

Interactions of COOH-terminal Truncation Mutants of SNAP23 with Syntaxin4 and VAMP2—SNAP25 possesses three putative coiled-coil regions, which are thought to be important in its association with VAMP2 and syntaxin1, whereas SNAP23 contains only two such regions (16). The NH2-terminal portion of SNAP25, which contains two of the three coiled-coil structures, is required for binding to syntaxin1, whereas the entire SNAP25 protein is required for interaction with VAMP2 (29, 30). To investigate the structural requirements for the interaction of SNAP23 with syntaxin4 and VAMP2, we generated two COOH-terminally truncated mutants of SNAP23 and examined their association with these two proteins. The SNAP23-ΔC8 and SNAP23-Δ49 mutants lack the 8 and 49 COOH-terminal residues of the wild-type protein (SNAP23-WT). SNAP23-ΔC8 corresponds to the fragment of SNAP25 generated by botulinum neurotoxin A, and SNAP23-ΔC49 lacks the COOH-terminal coiled-coil region (Fig. 2). Both mutants, like the wild-type protein, were tagged with the HA epitope at their NH2 termini.

COS cells were transiently transfected separately with expression vectors encoding SNAP23-WT and each of the two mutants. Cell extracts were subsequently incubated with bead-immobilized GST fusion proteins containing the cytoplasmic portions of VAMP2 or syntaxin4, and proteins that bound to the beads were subjected to immunoblot analysis with rabbit antibodies to HA. Only SNAP23-WT bound to immobilized VAMP2 (Fig. 3B), whereas both SNAP23-WT and SNAP23-ΔC8 interacted with syntaxin4 (Fig. 3C). To investigate the ability of the SNAP23 mutants to mediate the formation of a ternary complex with syntaxin4 and VAMP2, we cotransfected COS cells with an expression vector encoding the cytoplasmic domain of syntaxin4 separately with each of the vectors encoding the three SNAP23 proteins. Cell extracts were then again incubated with immobilized VAMP2, and bound proteins were subjected to immunoblot analysis with antibodies to HA or to syntaxin4. When syntaxin4 was expressed in the cells alone, it was not able to bind to VAMP2 (Fig. 3E). However, when expressed together with SNAP23-WT, syntaxin4 bound to the immobilized VAMP2, indicating that SNAP23-WT mediated the association between syntaxin4 and VAMP2. Neither SNAP23-ΔC8 nor SNAP23-ΔC49 was able to mediate the interaction between syntaxin4 and VAMP2, suggesting that the complex of syntaxin4 and SNAP23-ΔC8 could not associate with immobilized VAMP2.

Removal of the nine COOH-terminal residues of SNAP25, which yields a fragment corresponding to that generated by botulinum neurotoxin A, reduced the extent of the interaction between SNAP25 and VAMP2 but not of that between SNAP25 and syntaxin1 (29). Botulinum neurotoxin A does not cleave SNAP23 at the position corresponding to that targeted in SNAP25 (31), but SNAP23-ΔC8 corresponds to the fragment of SNAP25 generated by neurotoxin cleavage. Moreover, the nine residues cleaved from SNAP25 show sequence similarity to the eight residues removed from SNAP23 to form SNAP23-ΔC8, and both truncated proteins exhibit a reduced affinity for VAMP2 but not for syntaxin.

Effects of Overexpression of Wild-type and Mutant SNAP23 Proteins on Glucose Transport—To assess the functional role of SNAP23 in the translocation of GLUT4 in 3T3-L1 adipocytes, we first assessed the effects of SNAP23-WT, SNAP23-ΔC8, and SNAP23-ΔC49 on glucose transport activity. Each of the three HA-tagged SNAP23 proteins was overexpressed in 3T3-L1 adipocytes with the use of adenovirus-mediated gene transfer. Immunoblot analysis with rabbit antibodies to HA revealed that the three proteins were expressed to similar extents in a multiplicity of infection (MOI)-dependent manner (Fig. 4A). The distribution of overexpressed SNAP23 proteins in the 3T3-L1 adipocytes was examined by immunofluorescence labeling and confocal microscopy. The wild-type and mutant SNAP-23 proteins were all localized exclusively in the plasma membrane (Fig. 4B), which is the predominant site of localization of the native protein in 3T3-L1 adipocytes (16). Overexpression of neither SNAP23-WT nor SNAP23-ΔC49 had a significant effect on insulin-induced glucose transport activity (Fig. 4C). However, overexpression of SNAP23-ΔC8 inhibited insulin-induced glucose transport in an MOI-dependent manner, with 59.3% inhibition apparent at an MOI of 60 plaque-forming units (PFU)/cell.

Effects of Wild-type and Mutant SNAP23 Proteins on GLUT1 and GLUT4 Translocation in 3T3-L1 Adipocytes—In addition to GLUT4, 3T3-L1 adipocytes express the glucose transporter GLUT1. Insulin also induces the translocation of GLUT1 to the plasma membrane but to a lesser extent than it does that of GLUT4 (32). To determine which transporter isoform was responsible for the reduced stimulation of glucose transport in 3T3-L1 adipocytes overexpressing SNAP23-ΔC8, we performed a plasma membrane assay (26) with polyclonal antibodies generated in response to a synthetic COOH-terminal peptide of GLUT1 and with a monoclonal antibody to GLUT4. Insulin induced a marked increase in immunoreactivity corresponding to both GLUT1 and GLUT4 in the plasma membrane of non-
infected 3T3-L1 adipocytes (Fig. 5). Overexpression of SNAP23-ΔC8, but not that of SNAP23-WT or SNAP23-ΔC49, by adenovirus-mediated gene transfer inhibited the insulin-induced increase in GLUT4 immunoreactivity in the plasma membrane. None of the three recombinant SNAP23 proteins had a marked effect on the insulin-stimulated redistribution of GLUT1 protein.

Moreover, we confirmed by immunoblot analysis with the specific antibodies to GLUT1 and GLUT4 that overexpression of SNAP23-ΔC8 but not SNAP23-WT or SNAP23-ΔC49 inhibited the insulin-induced translocation of GLUT4 to plasma membrane, although none of the three types of SNAP23 proteins had remarkable effects on the insulin-induced translocation of GLUT1 (Fig. 6).

We also investigated the effects of the SNAP23 proteins on the translocation of GLUT4 induced by endothelin-1, which increases glucose transport and GLUT4 translocation to the plasma membrane through a pathway different from that mediated by the insulin receptor and insulin receptor substrate-1 (33). Exposure of uninfected 3T3-L1 adipocytes to 10 nM endothelin-1 for 30 min resulted in the translocation of GLUT4 to the plasma membrane (Fig. 7), albeit to a lesser extent that observed with insulin. Overexpression of SNAP23-ΔC8, but not that of SNAP23-WT or SNAP23-ΔC49, also inhibited endothelin-1-induced GLUT4 translocation to the plasma membrane. These results suggest that SNAP23-ΔC8-induced inhibition of insulin-stimulated GLUT4 translocation does not result from inhibition of the insulin receptor or insulin receptor substrate-1 but rather from inhibition of the translocation process per se.

Interaction between Overexpressed SNAP23 Proteins and En...
Finally, we examined the interaction between overexpressed SNAP23 proteins and endogenous syntaxin4 or VAMP2 in 3T3-L1 adipocytes. SNAP23-WT, SNAP23-ΔC8, or SNAP23-ΔC49 were immunoprecipitated with the monoclonal antibody to HA from detergent extracts of the plasma membrane fraction of adenovirus-infected cells (Fig. 8). Both endogenous syntaxin4 and VAMP2 were coprecipitated with overexpressed SNAP23-WT. Furthermore, endogenous syntaxin4 was coprecipitated with SNAP23-ΔC8, although little endogenous VAMP2 coprecipitated with this mutant. Neither endogenous syntaxin4 nor VAMP2 were coprecipitated to a substantial extent with SNAP23-ΔC49. These results are consistent with those of the in vitro binding assay (Fig. 3), showing that SNAP23-WT binds to both syntaxin4 and VAMP2, that SNAP23-ΔC8 binds to syntaxin4, and that SNAP23-ΔC49 binds to neither syntaxin4 nor VAMP2.

DISCUSSION

We have shown that the SNAP23 mutant SNAP23-ΔC8, which binds to syntaxin4 but not to VAMP2, inhibits the translocation of GLUT4, but not that of GLUT1, to the plasma membrane in 3T3-L1 adipocytes. This inhibition of GLUT4 translocation is likely attributable to prevention of the formation of a ternary SNARE complex among SNAP23, syntaxin4, and VAMP2 at the plasma membrane of these cells. Thus, SNAP-23 appears to function as a t-SNARE in the translocation of GLUT4 in 3T3-L1 adipocytes.

SNAP23 was identified as a widely expressed homolog of SNAP25 (15). Although SNAP25 is essential for exocytic
membrane fusion in neurons, this t-SNARE does not appear to be expressed in most nonneural tissues, with the exception of pancreatic islets of Langerhans (34, 35), adrenal chromaffin cells (36), and anterior pituitary cells (37). Although Jagadish et al. (38) detected SNAP25 mRNA and protein in fat cells and 3T3-L1 adipocytes by sensitive methods, Timmer et al. (39) and Wong et al. (40) did not detect SNAP25 in adipocytes. In contrast, SNAP-23 was shown not only to be expressed in 3T3-L1 adipocytes (16) but also to be colocalized in these cells with syntaxin4 (17) and VAMP2 (41), both of which are implicated as important mediators of the translocation of GLUT4 (12–14). These observations thus suggest that SNAP23 plays an important role as a plasma membrane t-SNARE, functioning together with syntaxin4 and VAMP2 in the translocation of GLUT4 in 3T3-L1 adipocytes. Furthermore, the observation that botulinum neurotoxin E, which cleaves SNAP25 but not SNAP23, does not markedly inhibit insulin-induced GLUT4 translocation in 3T3-L1 adipocytes (42) is consistent with the hypothesis that SNAP23, but not SNAP25, functions as a t-SNARE in the translocation of GLUT4 in these cells.

SNAP25 is hydrolyzed by botulinum neurotoxin E between Arg180 and Ile181 and is also cleaved by botulinum neurotoxin A between Gln197 and Arg198 (43). Removal of the nine COOH-terminal residues of SNAP25, which yields a fragment corresponding to that generated by neurotoxin A, reduces the extent of the interaction between SNAP25 and VAMP2 but not of that between SNAP25 and syntaxin1 (29). Neither of these neurotoxins hydrolyzes SNAP23 (31, 42). We therefore constructed two SNAP23 mutants. SNAP23-DC8 lacks the eight COOH-terminal amino acids of the full-length protein and corresponds to the fragment of SNAP25 generated by neurotoxin A, whereas SNAP23-DC49 lacks the COOH-terminal coiled-coil region. The observation that SNAP23-DC8 retained the ability to bind syntaxin4 but was not able to bind VAMP2 is consistent with the binding properties of the SNAP25 fragment lacking the nine COOH-terminal residues of the full-length protein. However, whereas the binding of SNAP23-DC49 to syntaxin4 was markedly reduced compared with that of the wild-type protein, the binding affinity of the NH2-terminal half of SNAP25 for syntaxin1 was similar to that of the full-length protein.

**FIG. 6.** Effects of overexpression of SNAP23-WT, SNAP23-ΔC8, or SNAP23-ΔC49 on insulin-induced translocation of GLUT1 or GLUT4 in 3T3-L1 adipocytes. Cells were infected with adenoviruses encoding SNAP23-WT, SNAP23-ΔC8, or SNAP23-ΔC49 at an MOI of 60 PFU/cell. After 48 h, the cells were incubated in the absence or presence of 100 nM insulin for 20 min, and plasma membrane fractions (40 μg of protein/lane) were then subjected to immunoblotting with specific rabbit antibodies to GLUT4 (upper panel) or GLUT1 (lower panel). These results are representative of independently performed experiments.

**FIG. 7.** Effects of overexpression of SNAP23-WT, SNAP23-ΔC8, or SNAP23-ΔC49 on endothelin-1-induced translocation of GLUT4 in 3T3-L1 adipocytes. Cells were infected with adenoviruses encoding SNAP23-WT, SNAP23-ΔC8, or SNAP23-ΔC49 at an MOI of 60 PFU/cell. After 48 h, the cells were incubated in the absence or presence of 10 nM endothelin-1 for 30 min, and plasma membrane fragments were then prepared for immunofluorescence microscopy with antibodies to GLUT4.
On the basis of these observations, it is likely that the coiled structure to which the v-SNARE (VAMP) appears to bind (44) t-SNARE complex (syntaxin and SNAP25) forms a cradle-like hold VAMP in position in the four-helix coiled coil; the binary syntaxin and two by SNAP25). The syntaxin and SNAP25 by the v-SNARE (VAMP) and three by the t-SNAREs (one by GLUT4 proteins, which were not inhibited by the SNAP23- to regulated exocytosis in these cells. Possible reasons why taxin4, SNAP23, and VAMP2 in 3T3-L1 adipocytes contributes GLUT1, suggest that the SNARE complex comprising syn- participate in the translocation of GLUT4, but not that of SNAP23 into 3T3-L1 adipocytes inhibited insulin-induced these data, introduction of antibodies to SNAP23 or a synthetic partment but not the endosomal compartment. Consistent with suggest that SNAP23 may regulate the post-endocytotic com- C8 inhibited translocation of GLUT4 but not that of GLUT1 cytes. A synthetic peptide corresponding to the 24 COOH- another study concerning SNAP23 reports that (Fig. 3) studies that SNAP23 potentiates the association between VAMP and syntaxin4. The syntaxin4-binding protein Munc18c inhibits the interaction between syntaxin4 and SNAP23 (16) as well as that between syntaxin4 and VAMP2 (50), suggesting that this protein may negatively regulate the association between syntaxin4 and SNAP23 in 3T3-L1 adipocytes. Dissociation of Munc18c from syntaxin4 may result in the formation of the syntaxin4-SNAP23-VAMP2 complex and thereby lead to fusion of GLUT4 vesicles with the plasma membrane.

In conclusion, we have shown that SNAP23, acting together with syntaxin4 and VAMP2, mediates the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes. The mechanism by which insulin regulates the formation of the syntaxin4-SNAP23-VAMP2 complex needs to be determined.

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Role of SNAP23 in Insulin-induced Translocation of GLUT4 in 3T3-L1 Adipocytes: MEDIATION OF COMPLEX FORMATION BETWEEN SYNTAXIN4 AND VAMP2

Masatoshi Kawanishi, Yoshikazu Tamori, Hideki Okazawa, Satoshi Araki, Hiroaki Shinoda and Masato Kasuga

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