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# Tissue Distribution of SNAP-23 and Its Subcellular Localization in 3T3-L1 Cells

Peggy P. C. Wong,* Nicholas Daneman,* Allen Volchuk,* Norman Lassam,†
Michael C. Wilson,‡ Amira Klip,*,[1] and William S. Trimble*

**Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada; †Toronto-Bayview Regional Cancer Centre, Toronto, Ontario, Canada; and ‡Department of Biochemistry, University of New Mexico, School of Medicine, Albuquerque, New Mexico*

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**The SNARE hypothesis of vesicular traffic proposes that three proteins, VAMP/synaptobrevin, syntaxin, and SNAP-25, constitute a complex that docks the vesicle at the target membrane. VAMP and syntaxin isoforms have been identified outside the nervous system, and a cDNA to a SNAP-25 related protein, SNAP-23, was recently identified in human lymphocytes. Here we report the generation of isoform-specific antibodies to SNAP-23 cloned from human melanoma cells, and their use in detecting the expression and localization of the endogenous SNAP-23 protein in several tissues and cell lines. SNAP-23 was readily detected in liver, lung, kidney, and spleen, to a lesser extent in muscle and heart, and was almost undetectable in brain. The protein was also abundant in fibroblast, muscle, and fat cell lines, but relatively less enriched in neuroendocrine PC12 cells. SNAP-23 abundance did not change during differentiation of 3T3-L1 fibroblasts into adipocytes. In both, SNAP-23 was membrane-bound and below detectable levels in the cytosolic fraction. Subcellular fractionation of 3T3-L1 adipocytes revealed that the majority of the protein was associated with plasma membranes. These findings support the conclusion that a tripartite SNARE complex exists outside of the nervous system, and suggest that SNAP-23 may play a role in vesicle traffic in most cell types.** 

The binding of vesicles to, and their fusion with, target membranes is a key step in intracellular traffic in all eukaryotic cells. The SNARE hypothesis predicts that a combination of vesicular (v-SNARE) and target (t-SNARE) membrane proteins interact to form the SNARE (SNAP-receptor) complex which serves as the target for the binding and action of soluble fusion components NSF and SNAP (1). Furthermore, the specificity of vesicle targeting is thought to be mediated by the correct association of v- and t-SNARE isoforms. In the nervous system the vesicle-associated membrane protein VAMP acts as a v-SNARE and binds to the t-SNARE membrane proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) to form a stable tripartite complex. Multiple isoforms of VAMP and syntaxin have been identified, and several of these are known to be expressed outside the nervous system. However, until very recently, only two alternatively spliced forms of SNAP-25 were known to exist, and their expression was found to be restricted to the nervous system (2, 3) and neuroendocrine cells such as pancreatic islets of Langerhans (4-6), adrenal chromaffin cells (7), and anterior pituitary cells (8). If the basic tenets of the SNARE hypothesis are correct, and if this ternary cassette of proteins also functions to mediate membrane fusion in other cell types, then SNAP-25-like molecules should be present in non-neuronal cells undergoing regulated membrane traffic events.

Recently, Ravichandran et al. (9) described the cDNA cloning of a novel isoform of SNAP-25, named SNAP-23, and observed expression of its mRNA in several non-neuronal tissues. To examine the presence and distribution of SNAP-23 at the protein level, we have isolated a full length cDNA clone from human melanoma cDNA library and produced antibodies specific for SNAP-23. Using these reagents we demonstrate the presence of this protein in a variety of tissues and cell lines. In contrast, we were unable to detect SNAP-25 outside neuronal/neuroendocrine cells. Further, we show that SNAP-23 is localized at the plasma membrane in the 3T3-L1 adipocyte cell line, where it may likely act as a t-SNARE along with syntaxin 4. These results support the generality of the SNARE hypothesis and suggest that the v-/t-SNAREs ternary complex may be found in most cell types.

[1] To whom correspondence should be addressed. Fax: (416) 813-5028.

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## MATERIAL AND METHODS

*Materials.* IMAGE Consortium expressed-sequence tag (EST) cDNA #384468 (GenBank™ accession number H82169) was obtained from Research Genetics. SNAP-25 monoclonal antibody SMI 81 (here called αSN25mAb) was obtained from Sternberger Monoclonals Inc. Anti α1 $Na^+/K^+$ATPase monoclonal antibody 6H was kindly provided by Dr. M. Caplan (Yale University, New Haven, CT). GLUT4 polyclonal antibody was from East Acres Biologicals. An affinity purified antiserum (αSN25pAb) was raised against residues 33-206 of SNAP-25b isoform (10).

*Cloning and bacterial expression of SNAP-23 and SNAP-25.* An EST clone (see Results) labeled with [α-$^{32}$P]dCTP was used to screen approximately 500,000 plaques from a UNI-ZAP XR human melanoma cDNA library. Twelve positive plaques were identified and their cDNA inserts were subcloned into Bluescript plasmids (Stratagene) for single-stranded DNA sequencing using the Sequenase version 2.0 kit (United States Biochemical). Sequences of the 5′ end of the twelve clones revealed one which began at nucleotide 50, 43 base pairs upstream from the putative initiation codon reported for the recently published SNAP-23 cDNA (9). The coding sequence of this clone was amplified by polymerase chain reaction (PCR) using primers (nucleotides 93-110, 714-731) which flanked the coding region. This amplified fragment was then subcloned into pGEX-2T vector. The entire sequence was confirmed by automated sequence analysis (Biotechnology Service Centre, Toronto, ON) and found to be identical to human SNAP-23 cDNA (9). GST fusion proteins of SNAP-23 and SNAP-25 were expressed and purified from *Escherichia coli* (11). The cDNA insert of the EST clone was isolated and subcloned into the pET-32a expression vector (Novagen).

*Generation of SNAP-23 antisera.* Two rabbit antisera specific to SNAP-23 were raised. Antibody αSN23.C116 was produced against a recombinant protein encoding carboxy-terminal 116 residues of SNAP-23 (amino acids 96-211) that was liberated from thioredoxin by thrombin cleavage. The serum was affinity purified on a column of Affi-gel 15 to which the GST SNAP-23 fusion protein described above was coupled. Antibody αSN23.C12 was raised against a peptide corresponding to carboxy-terminal residues 200-211 of SNAP-23 ($H_2$N-IANARAKKLIDS-COOH) coupled to keyhole limpet hemacyanin (Biotechnology Service Centre, Toronto, ON). An affinity column was produced by coupling the same peptide to SulfoLink Gel (Pierce Chemical Company). Antibodies bound to affinity columns were eluted with 20 mM glycine and 0.2 M NaCl, pH 2.5 and the eluates were neutralized with 0.1 M Tris, pH 8.5.

*Cell culture.* The following cell lines were grown: rat adrenal pheochromocytoma PC12, murine melanocyte melan-b, and human melanoma MeWo. Myoblasts of the rat muscle cell line, L6, were allowed to fuse and differentiate into myotubes upon fusion (12, 13). Mouse 3T3-L1 fibroblasts were differentiated into adipocytes as described previously (14).

*Membrane isolation and immunoblotting.* Total membranes (TM) were prepared from confluent cells. All procedures were carried out at 4 °C. Monolayers were washed with homogenization buffer (20 mM Hepes, 255 mM sucrose, and 1 mM EDTA, pH 7.4), scraped with a rubber policeman in homogenization buffer containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 10 μM E-64) and homogenized with 10 strokes using a cell cracker. Homogenates were centrifuged at 1,000 xg for 3 min. The resultant postnuclear supernatants were centrifuged at 245,000 xg for 90 min to yield total membranes. The supernatants represented the cytosolic fraction. Confluent 3T3-L1 adipocytes (7-10 days post-differentiation) were sub-fractionated as described previously (7). Organs were dissected from mice and microsomes were prepared according to the protocol described (15). Protein samples were solubilized in Laemmli sample buffer (16) and separated by SDS-PAGE (17). Proteins were detected with the following primary antibodies: anti α1 $Na^+/K^+$ ATPase monoclonal (1:250), anti SNAP-25 monoclonal αSN25mAb (SMI 81, 1:2000), anti GLUT4 polyclonal (1:1000), anti SNAP-25 polyclonal (αSN25pAb, 1:200), affinity-purified SNAP-23 polyclonal antisera αSN23.C116 and αSN23.C12 were used at 4 ng/ml and 0.12 mg/ml respectively. αSN25pAb was preincubated with bacterial GST protein followed by incubation with glutathione linked to agarose beads to remove any antibodies that may cross-react with the GST portion of SNAP-23 or SNAP-25 fusion proteins before immunoblotting. Enhanced chemiluminescence was used as the method for detection.

## RESULTS AND DISCUSSION

In preliminary experiments using a full-length SNAP-25 cDNA probe, we were unable to detect signals corresponding to SNAP-25 in total RNA from 3T3-L1 fibroblasts or adipocytes under high or low stringency conditions (18). In addition, immunoblotting of proteins from 3T3-L1 adipocytes with the monoclonal antibody specific to SNAP-25 (αSN25mAb) also failed to give a positive result. However, when plasma membranes prepared from 3T3-L1 adipocytes were probed with a polyclonal SNAP-25 antiserum (αSN25pAb) raised to near full-length of the protein (10), a polypeptide that migrated at 29 kDa was detected. The detection was specific since it was prevented upon preincubation of the antiserum with recombinant SNAP-25 protein (18). These results implied that an immunoreactive species related to SNAP-25 existed in 3T3-L1 cells and prompted us to search for such a gene product.

Through a search of the Genbank™ database we identified a human EST clone which encoded a protein predicted to share 64% sequence identity with the C-terminal 78 amino acids of SNAP-25. Using this cDNA as probe, we screened approximately 500,000 plaques of a human melanoma cDNA library. Twelve positive plaques were isolated, the longest of which contained a cDNA of approximately 2.0 Kb. Sequence analysis of this clone revealed that it was identical to the recently described SNAP-25-related gene product SNAP-23 (9), and contained the entire coding sequence.

We have generated two antisera to the C-terminal portion of SNAP-23. Antibody αSN23.C116 was raised against a truncated portion of the protein (amino acids 96-211) and antibody αSN23.C12 was directed against a peptide corresponding to residues 200-211 of SNAP-23. Equal amounts (1 μg) of GST SNAP-23 and GST SNAP-25 fusion proteins were immunoblotted to test the specificity of the two affinity-purified antisera. Both of these antisera detected only SNAP-23 fusion protein but not SNAP-25 (Fig. 1, top two panels). Moreover, no cross-reactivity to the GST portion of the recombinant proteins was detected and no immunoreactivity was detected with the preimmune sera (data not shown). Further characterization of the two antibodies used in preliminary studies (18) is shown in the bottom two panels of Fig. 1. The polyclonal SNAP-25 antiserum, αSN25pAb, cross-reacts to both SNAP-23 and SNAP-25, whereas the monoclonal SNAP-25 antibody, αSN2-

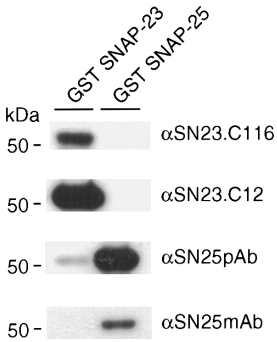


**FIG. 1.** Characterization of SNAP-23 and SNAP-25 antibodies. Immunoblot analysis was used to test the specificity of four antibodies to either SNAP-23 or SNAP-25 GST fusion proteins. One microgram of each fusion protein was subjected to SDS-PAGE and immunoblotted with the following antibodies: αSN23.C116 affinity purified antiserum raised to the C-terminal 116 amino acids of SNAP-23; αSN23.C12 affinity purified antiserum raised to the C-terminal 12 amino acids of SNAP-23; αSN25pAb polyclonal antiserum raised to residues 33-206 of SNAP-25; and αSN25mAb (SMI 81) monoclonal antibody to SNAP-25.

5mAb, is specific for SNAP-25. These data indicate that the immunoreactive protein detected by αSN25pAb in 3T3-L1 adipocytes in previous studies was most likely SNAP-23 and not SNAP-25 (18).

The predicted amino acid sequence of SNAP-23 is 59% identical and 72% similar to SNAP-25b isoform (9). Northern blot analysis demonstrates a wide tissue distribution of SNAP-23 mRNA (9), however endogenous expression of the protein has not been examined. Here we report the expression of SNAP-23 protein in various cell lines and tissues. SNAP-23 protein was detected using αSN23.C12 in both non-neuroendocrine cell lines such as mouse 3T3-L1 fibroblasts, 3T3-L1 adipocytes, melan-b melanocytes, rat L6 myotubes, and human melanoma MeWo cells as well as lower levels in PC12 neuroendocrine cells (Fig. 2A, top panel). The protein detected by the αSN23.C12 antiserum migrates at approximately 29 kDa. The immunodetection of SNAP-23 was specific since it was blocked by preincubating the antiserum with excess amount of the peptide (Fig. 2A, middle panel). In contrast to the widespread distribution of SNAP-23, SNAP-25 showed a very restricted expression, limited only to the neuroendocrine PC12 cells (Fig. 2A, bottom panel).

In contrast to our inability to detect SNAP-25 in 3T3-L1 adipocytes and L6 muscle cells, Jagadish et al. (19) recently reported the expression of SNAP-25a in fat tissue and SNAP-25b in skeletal muscle. The detection of SNAP-25 in the fat and muscle tissues could potentially result from the presence of nerve terminals in the biological material. Alternatively, the amount of SNAP-25 protein in our fat and muscle cell lines may be below the detection level of our antibodies.

As shown above for cell lines, SNAP-23 was also widely distributed in different tissues from rats and mice (Fig. 2B). The protein was found to be abundant in lung, liver, spleen and kidney, but relatively less concentrated in rat skeletal muscle and mouse heart. Upon longer exposure of the immunoblot to autoradiography, a low level of SNAP-23 could also be detected in the mouse brain (result not shown).

The subcellular distribution of SNAP-23 is not known but, like SNAP-25, it lacks a stretch of hydrophobic residues necessary to form a transmembrane domain. In the case of SNAP-25, the majority of the protein is tightly associated with synaptosomal membranes (20) probably via palmitoylation of one or more of the four cysteine residues clustered in the middle of the protein (21). It has recently been shown that deletion of a stretch of 12 amino acids encompassing the 4 cysteine residues renders the deletion mutant of SNAP-25 completely soluble and resistant to [$^3$H] pal-

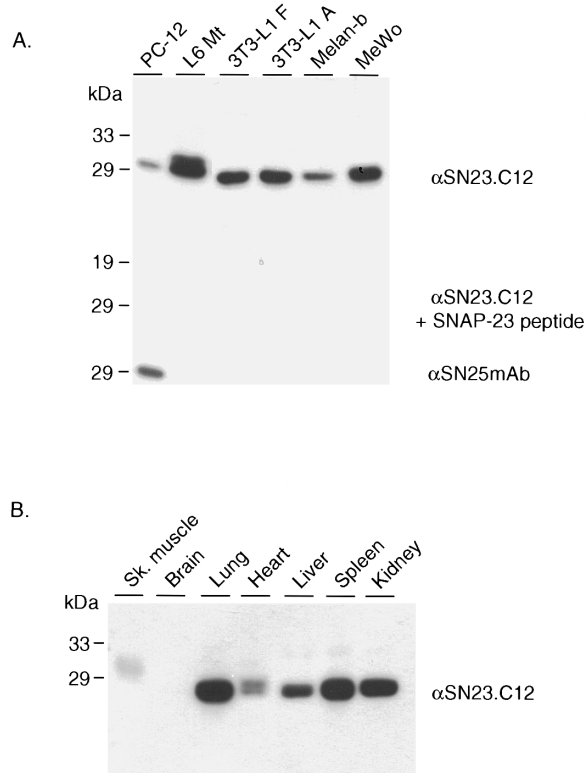


**FIG. 2.** Expression of SNAP-23 in cell lines and tissues. (**A**) Immunoblot of total membranes prepared from various cell lines of different species. Twenty micrograms of proteins from rat neuroendocrine PC12 cells, rat L6 myotubes (L6 Mt), mouse 3T3-L1 fibroblasts (3T3-L1 F) and 3T3-L1 adipocytes (3T3-L1 A), mouse melanocytes (melan-b), and human MeWo melanoma cell line were resolved by SDS-PAGE and probed for SNAP-23 with αSN23.C12 antiserum (0.12 μg/ml) (top). The specificity of immunodetection was confirmed by preincubation of αSN23.C12 antiserum with excess amount of peptide (0.2 μg/ml) (middle). Detection of SNAP-25 by the monoclonal antibody αSN25mAb (SMI 81) was restricted to neuroendocrine PC12 cells (bottom). (**B**) Immunoblotting of total membranes from rat skeletal muscle (Sk. muscle) and other tissues from mouse with αSN23.C12 antiserum.

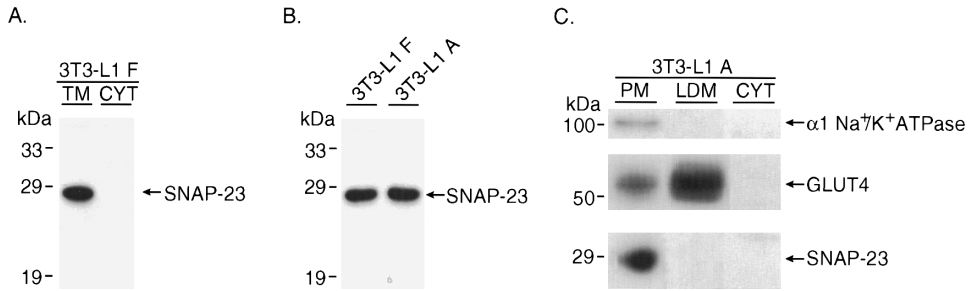


**FIG. 3.** Localization of SNAP-23 in 3T3-L1 cells. (**A**) Twenty micrograms of total membranes (TM) and cytosolic fraction (CYT) from 3T3-L1 fibroblasts (3T3-L1 F) were subjected to immunoblotting using the $\alpha$SN23.C12 antiserum. (**B**) Twenty micrograms of total membranes (TM) from 3T3-L1 fibroblasts (3T3-L1 F) and adipocytes (3T3-L1 A) were immunoblotted for SNAP-23 using $\alpha$SN23.C12 antiserum. (**C**) To study the subcellular distribution of SNAP-23 in 3T3-L1 adipocytes (3T3-L1 A), cells were subfractionated and equal amounts (15 $\mu$g) of plasma membrane (PM), light density microsomes (LDM), and cytosolic fraction (CYT) were separated by SDS-PAGE and probed for $\alpha$1 subunit of $Na^+/K^+$ ATPase with 6H monoclonal antibody, GLUT4 with a polyclonal antiserum, and SNAP-23 with $\alpha$SN23.C12 antiserum.

mitic acid labeling (22). In order to determine the localization of SNAP-23 in 3T3-L1 fibroblasts we used subcellular fractionation. The results in Fig. 3A show that SNAP-23 is exclusively found in the membrane fraction (TM) with no detectable amount found in the cytosolic fraction (CYT). Since SNAP-23 also contains a cluster of cysteine residues, it may associate with the membrane by a similar mechanism. Interestingly, SNAP-23 contains 5 cysteine residues in this cluster, as is the case for *Torpedo* SNAP-25 (23). In contrast, the mammalian SNAP-25a and SNAP-25b have each a substitution of one of the cysteines, breaking the cluster arrangement. The extra cysteine residue found in SNAP-23 and *Torpedo* SNAP-25 may define an ability to associate with specific proteins.

3T3-L1 fibroblasts can be differentiated into adipocytes, hence allowing us to study the protein at two stages of differentiation. Proteins that are involved in intracellular traffic have been shown to be expressed at different levels following differentiation into adipocytes. For example, cellubrevin, a member of the synaptobrevin family, is expressed predominantly in differentiated 3T3-L1 adipocytes (24). In contrast, syntaxin 4 is already abundant at the fibroblast stage (Volchuk and Klip, unpublished). As can be seen in Fig. 3B, there was no significant change in the level of expression of SNAP-23 upon differentiation, suggesting that the protein is necessary in both fibroblasts and adipocytes.

The detection of SNAP-23 in 3T3-L1 adipocytes provided us with an opportunity to further study the subcellular distribution of the protein in plasma membrane (PM), light density microsomes (LDM), and cytosol (CYT). In contrast to fibroblasts, the fractionation protocol for 3T3-L1 adipocytes has been well established (25). The $\alpha$1 subunit of $Na^+/K^+$ ATPase is a useful marker for the PM. This protein was detected almost exclusively in the PM and not in the LDM or CYT of 3T3-L1 adipocytes (top panel of Fig. 3C). The LDM contains the majority of the GLUT4 glucose transporter which locates intracellularly in unstimulated cells (Fig.3C, middle panel). The majority of SNAP-23 was found to be associated with the PM (Fig. 3C, bottom panel). Occasionally, a small amount of SNAP-23 was also detected in the LDM (not shown).

The functional importance of SNAP-25 in synaptic vesicle traffic has been established by the discovery that it is the substrate for botulinum neurotoxins A and E (26, 27). These toxins are endopeptidases and potent inhibitors of neurotransmitter release. Botulinum neurotoxins A and E cleave SNAP-25 at distinct peptide bonds (10). Sequence alignment of SNAP-23 and SNAP-25 proteins reveals that the two amino acids which constitute the proteolytic cleavage site for botulinum neurotoxin type E, but not those for type A, are conserved in SNAP-23. Therefore we predict that SNAP-23 may be protected from proteolytic cleavage by botulinum neurotoxin A. Botulinum toxin E may be a useful tool to discern whether SNAP-23 plays a role in exocytosis. Indeed, we have shown that both SNAP-23 and SNAP-25 are present in PC12 cells; in these cells, exocytosis of large dense core vesicles is inhibited completely by botulinum neurotoxin E but not A, although both toxins effectively cleaved SNAP-25 (28). The presence of SNAP-23 and its potential insensitivity to proteolysis by botulinum neurotoxin A may explain the incomplete inhibition in the peptide hormone secretion observed in PC12 cells.

The detection of SNAP-23 in non-neuronal cells completes the tenet of the SNARE hypothesis, that functional homologues of SNAP-25, syntaxin-1 and VAMP exist at all steps of vesicle docking/fusion in non-neuronal cells. Since muscle and 3T3-L1 cells express syntaxin 4 (29, 30) and VAMP-2 (31), it is tempting to speculate that SNAP-23 could potentiate the binding of syntaxin 4 to VAMP-2 and in this way contribute to the formation of a stable complex that would facilitate exocytic activity in these tissues.

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