Role of the Cysteine-rich Domain of the t-SNARE Component, SYNDET, in Membrane Binding and Subcellular Localization*

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Wild-type syndet is efficiently recruited at the plasma membrane in transfected AtT-20 cells. A deletion at the cysteine-rich domain abolishes palmitoylation, membrane binding, and plasma membrane distribution of syndet. Syndet, SNAP-25A, and SNAP-25B share four cysteine residues, of which three, Cys2, Cys4, and Cys5, are absolutely conserved in all three homologs. Mutations at any pair of cysteines within cysteines 2, 4, and 5 shift syndet from the cell surface into the cytoplasm. Thus, at least two cysteines within the conserved triplet are necessary for plasma membrane localization. Syndet C1S/C3S, with substitutions at the pair Cys1 and Cys3, are necessary for plasma membrane localization. Syndet Mutagenesis—The original template for polymerase chain reactions (PCR) of syndet was pcDNA1-syndet plasmid (11). Syndet and SNAP-23 are expressed in many tissues (11–14). Syndet and syntaxin-4 are localized at the plasma membrane of adipocytes (11, 12, 14) and VAMP-2 is localized in glucose transporter-containing vesicles (14–16). It has been proposed that the SNARE complex, syndet-syntaxin-4-VAMP-2 mediates insulin-dependent exocytosis of glucose transporter-containing vesicles to the plasma membrane of adipocytes (17). Thus, syndet in adipocytes, like SNAP-25 in neurons, seems to be involved in exocytosis of intracellular vesicles at the cell surface. Syndet and SNAP-25 are integral membrane proteins, but do not have any predicted signal sequence or transmembrane domains (1, 11). Post-translational modifications may provide these proteins with a hydrophobic anchor to membranes. It has been shown that SNAP-25 is palmitoylated through a thioester bond to some or all of its four cysteines clustered at a region near the center of the protein sequence (18, 19). Deletion of this region abolishes palmitoylation of SNAP-25 and its binding to membranes (20). Also point mutations at single cysteines of SNAP-25 largely inhibit palmitoylation and membrane binding (19). This study was designed to investigate which domain of syndet is critical for its intracellular localization. In this paper we show that the number and configuration of cysteines at the cysteine-rich domain act as major determinants for the subcellular distribution of syndet.

EXPERIMENTAL PROCEDURES

Syndet Mutagenesis—The original template for polymerase chain reactions (PCR) of syndet was pcDNA1-syndet plasmid (11). Mutations were encoded in the oligonucleotide primers synthesized by Life Technologies, Inc. (Gaithersburg, MD). The enzyme used for PCR was Pwo DNA Polymerase (3 units/reaction, Boehringer Mannheim Corp.) with its supplied reaction buffer. The primers were used at a final concentration of 2 μM. The deoxynucleotide triphosphates (Promega, Madison, WI) used at a final concentration of 200 μM. The PCR was carried out on a "GeneAmp PCR System 2400" (Perkin-Elmer, Applied Biosystems, Division, Foster City, CA). All restriction enzymes were purchased from Promega.

Syndet cDNA was amplified from pcDNA1-syndet using primers: TGGAACTGTAACAGCTTGTGAACACTGGTGGTGGAGGAGAGGTG (number 1) which encodes a HindIII site, and TCAGGTACCTCTAGACTCTTATAAGACGTTCT (number 2) which encodes an XbaI site. It was then subcloned into the HindIII-XbaI sites of vector pcCAT (21) to obtain syndet-pCAT.

Delta-syndet mutant was also made from pcDNA1-syndet in two separate PCR reactions, using primers 1 and CGCATTTACCCGGGCTTGTGAGTTCTGTTAAAGTCCT, which encodes an XmaI site, for the first reaction, and primers 2 and AGTGTACGTTATCCCGGGAATTAGGACAAAGACCTTTGAG, which also encodes an XmaI site, for the second. The products of the first PCR reaction were digested with the

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restriction enzymes HindIII and XmaI and the products of the second PCR reaction with XmaI and XbaI. These two products were ligated into pCB7 vector to obtain delta-syndet-pCB7.

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**Fig. 1. Double immunofluorescence localization of endogenous SNAP-25 and exogenous syndet.** Confocal immunofluorescence microscopy of stably transfected AtT-20 cells expressing wild-type syndet. Cells were double-stained with mouse monoclonal antibodies against SNAP-25 and fluorescein isothiocyanate-conjugated antibodies against mouse IgG (A) and with rabbit polyclonal antibodies against syndet and Cy3-conjugated antibodies against rabbit IgG (B). The cell body is indicated by arrowheads, the tips by arrows.

**Fig. 2. Immunogold staining of broken cells expressing wild-type syndet.** Syndet (12 nm gold) and SNAP-25 (6 nm gold) are detected at the plasma membrane. There is no staining of mitochondria (m), endogenous murine leukemia virus (v), or nuclear membrane (not shown). Bar, 100 nm.
iovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM410 laser scanning confocal attachment was used to obtain the images. The cells were excited with an Argon-Krypton laser using the standard wavelengths for rhodamine and fluorescein isothiocyanate. The images were collected as 1-μm thick optical sections. They were processed using Zeiss LSM 3.95 software and Adobe Photoshop 3.0 (Adobe Systems).

**Immunoelectron Microscopy—**Immunogold labeling of broken and agarose-embedded AtT-20 cells was done as described elsewhere (25). Rabbit polyclonal anti-syndet antibodies were used at 1:20 dilution and mouse monoclonal anti-SNAP-25 antibodies were used at 1:100 dilution. Colloidal gold donkey anti-rabbit IgG (12 nm) and colloidal gold donkey anti-mouse IgG (6 nm) were used for immunolabeling at a concentration of 1:100. Immunolabeled cells were osmicated (0.5% for 30 min), dehydrated, and embedded in tEPON (Tousimis Research Corp., Rockville MD). Thin sections (70 nm) were cut on an AO Ultracut microtome (Reichert-Jung, Vienna, Austria), counterstained with uranyl acetate and lead citrate, and viewed and photographed on a JEOL 1200EX electron microscope (JEOL USA, Inc., Peabody MA).

**Cell Fractionation and Western Blots—**Protease inhibitors, leupeptin (6 μg/ml) and aprotinin (1.5 μg/ml), were purchased from Boehringer Mannheim, Phenylmethylsulfonyl fluoride (2 mM) was from Sigma. Cells were grown on 6-cm plates for at least 72 h and homogenized in 400 μl of buffer A (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and protease inhibitors) by passing the cells through an insulin needle six times. The homogenates were mixed with an equal volume of SDS sample buffer, boiled, and loaded into the SDS-PAGE gel. In some experiments, a fraction of the homogenate (200 μl) was spun at 400g for 30 min. The supernatant fraction was recovered, and the pellet was resuspended in 200 μl of buffer A. Each fraction (40 μl each) was mixed with an equal volume of SDS sample buffer, boiled, and loaded into the SDS-PAGE gel. Further steps including electrophoresis and Western blotting were carried out as described previously (11).

**Metabolic Labeling and Immunoprecipitation of Syndet—**Cells expressing syndet, delta-syndet, and syndet C1S/C3S were labeled with [35S]Met (10 μCi/ml, NEN Life Science Products, Peabody MA) for 2 h, as described. (19). Cells grown in a 3.5-cm plate were incubated at 4 °C with gentle agitation for 1 h. Protein A-Sepharose beads (40 l dry beads, Boehringer Mannheim) were added to the samples, which were further incubated at 4 °C for 45 min while being agitated. After centrifugation, pelleted immunobeads were washed three times with lysis buffer A and mixed with SDS-PAGE sample buffer. Samples were boiled and analyzed by SDS-PAGE electrophoresis and fluorography as described (20). Preliminary experiments were run to determine that syndet, delta-syndet, and syndet C1S/C3S were immunoprecipitated to the same extent by affinity purified syndet antibodies. To visualize immunoprecipitated wild-type and mutated syndet by Western blot, immunobeads were mixed with sample buffer and loaded onto SDS-PAGE. Blots were probed with affinity purified syndet antibody (1:400). Bands were visualized by enhanced chemiluminescence.

**Results**

**Targeting of Exogenous Syndet in Transfected AtT-20 Cells—**The endogenous level of syndet protein in AtT-20 cells is undetectable by Western blot and immunofluorescence (see below). We determined the distribution of exogenous syndet in stably transfected AtT-20 cells by immunofluorescence (Fig. 1). Syndet immunoreactivity was seen along the plasma membrane of the cell body, the neurite-like processes and the tips (Fig. 1B). Staining for SNAP-25, like syndet, was predominant along the entire plasma membrane (Fig. 1A). However, SNAP-25 antibodies also stained a region near the nucleus and the cytoplasm within the tip. We have shown elsewhere by immunoelectron microscopy that endogenous syndet in adult mouse kidney is found predominantly at the plasma membrane (11). At the ultrastructural level, we confirmed that exogenous syndet in AtT-20 cells was indeed localized at the plasma membrane. Fig. 2 shows that exogenous syndet (12-nm gold particles) and endogenous SNAP-25 (6-nm gold particles) are localized at the plasmalemma in agarose-embedded broken cells. There was no syndet or SNAP-25 labeling associated with the nuclear membrane, or mitochondria or endogenous murine leukemia virus particles. These results indicate that exogenous syndet expressed in AtT-20 cells is able to localize at the plasmalemma. We conclude that AtT-20 cells can be used as a model to study the role of various syndet domains in plasma membrane localization.

**Domains of Syndet Involved in Plasma Membrane Localization—**We prepared three deletion mutants of syndet to find domains responsible for targeting syndet to the plasma membrane (Fig. 3, *upper panel*). The deletion mutant delta-syndet lacks the syndet amino acid sequence from Cys79 to Cys85. Pro86 was left unchanged, and Cys87 was mutated to a glycine. Thus, delta-syndet does not have any cysteines. Syndet δα1 lacks the amino-terminal region which has a high propensity to form coiled-coil structures (11) and a short sequence after the cysteine-rich domain. The cysteine-rich domain was entirely preserved in the syndet δα1 mutant. Syndet δα1BoNT/E is the expected proteolysis product of mouse syndet protein treated with the botulinum toxin E (BoNT/E) (26). Syndet delta-BoNT/E lacks approximately 40% of the carboxyl-terminal region with high probability to form coiled-coil structures (11). Western blots of homogenates from AtT-20 cells expressing wild-type syndet (A-C), delta-syndet (A), syndet δα1 (B), and syndet-delta-BoNT/E (C), were mixed with sample buffer and loaded onto SDS-PAGE. Blots were probed with affinity purified syndet antibody (1:400). Bands were visualized by enhanced chemiluminescence.

![Fig. 3. Deletion mutants of syndet.](image)

The *upper part* of Fig. 3 shows the deletion mutants of syndet used in this work. The cysteine-rich domain and the regions predicted to form coiled-coil conformation are indicated. The *lower part* of the figure illustrates Western blots of wild-type and mutant syndet proteins. Homogenates of AtT-20 cells expressing wild-type syndet (A-C), delta-syndet (A), syndet δα1 (B), and syndet-delta-BoNT/E (C), were mixed with sample buffer and loaded onto SDS-PAGE. Blots were probed with affinity purified syndet antibody (1:400). Bands were visualized by enhanced chemiluminescence.
ing the protein to the cell surface. Syndet δα1 and syndet delta-BoNT/E immunofluorescence staining accumulated at the plasma membrane of the cell body (Fig. 4, C and E), of the neurite-like processes (not clearly visible in the optical sections shown in Fig. 4) and of the tips (Fig. 4, C and D). Thus, the subcellular distribution of syndet δα1 and syndet delta-BoNT/E was similar to the distribution of exogenous wild-type syndet (compare with Fig. 1). These experiments indicate that the entire predicted α-helix at the amino terminus of syndet and at least 40% of the helix at the carboxyl terminus are not required for targeting syndet to the plasma membrane. Unlike these regions, the cysteine-rich domain is necessary for plasma membrane targeting.

Requirement of Cysteines for Syndet Localization at the Plasma Membrane—The cysteine-rich domains of wild-type syndet and two alternatively spliced isoforms of SNAP-25, SNAP-25 A and SNAP-25 B (27) are shown in Fig. 5. The five cysteines of syndet are designated as Cys1, Cys2, Cys3, Cys4, and Cys5 and correspond to Cys79, Cys80, Cys83, Cys85, and Cys87, respectively. Unlike syndet, SNAP-25 A and SNAP-25 B have four cysteines in their cysteine-rich domain. In SNAP-25 A, the cysteine corresponding to Cys3 of syndet is substituted by a phenylalanine. In SNAP-25 B, the cysteine corresponding to syndet Cys1 is substituted by a phenylalanine. The triplet of cysteines, -Cys2-Cys4-Cys5-, of syndet is conserved in both SNAP-25 A and SNAP-25 B. To study the role of individual cysteines at the cysteine-rich domain, we prepared a series of mutations. In the mutant syndet C2S/C4S/C5S, all the cysteines of the -Cys2-Cys4-Cys5- triplet were substituted with serines. Within the -Cys2-Cys4-Cys5- triplet, we also made pairwise substitutions, syndet C2S/C4S, syndet C2S/C5S, syndet C4S/C5S, of two cysteines with serines. In syndet C4S/C4S and syndet C5S, Cys4 and Cys5, respectively, were substituted with serines. In syndet C1S/C3S, both Cys1 and Cys3 of syndet were substituted with serines.

Immunofluorescence experiments were performed on AtT-20 cells transiently expressing either wild type or mutated syndet proteins. Plasma membrane staining of wild-type syndet in
transiently transfected AtT-20 cells was indistinguishable from that observed in the stable AtT-20 cell lines. Syndet C2S/C4S/C5S was found in the cytoplasm (Fig. 6A), and had a distribution similar to delta-syndet (Fig. 4B). This experiment indicates that the conserved -Cys²-Cys⁴-Cys⁵- triplet is critical for syndet localization at the plasma membrane. Syndet C4S/C5S (Fig. 6B), syndet C2S/C4S (Fig. 6C), and syndet C2S/C5S (Fig. 6D) were also found in the cytoplasm and did not localize at the plasma membrane. Syndet proteins with single point mutations at C5S (Fig. 6E) or C4S (Fig. 6F) were localized at the plasma membrane. Thus, a mutation of any pair of cysteines within the conserved triplet is sufficient to prevent plasma membrane localization. Single substitutions do not alter syndet distribution. We conclude that at least 2 cysteines within the -Cys²-Cys⁴-Cys⁵- triplet are necessary for localization of syndet at the plasma membrane.

Subcellular Localization of Syndet C1S/C3S—Syndet distribution in AtT-20 cell lines stably expressing the syndet C1S/C3S protein was examined. In contrast to wild-type syndet (Fig. 1B), the mutant, syndet C1S/C3S, was found at an intracellular site as well as at the plasma membrane (Fig. 7B). Double staining with SNAP-25 antibodies indicate that endogenous SNAP-25 is predominantly at the plasma membrane (Fig. 7A). We determined the relative distribution of ACTH and syndet immunoreactivities in AtT-20 cells expressing wild-type syndet and syndet C1S/C3S (not shown). ACTH immunoreactivity is concentrated in a Golgi-like region near the nucleus and at the tips (25) where ACTH-containing granules accumulate (28). Wild-type syndet did not co-localize with ACTH immunoreactivity either at the cell body or at the tips. Syndet C1S/C3S co-localized with ACTH immunoreactivity in the Golgi-like compartment, but not at the tips of the processes. These results show that mutations at Cys¹ and Cys³: (a) shift a fraction of syndet from its plasma membrane localization to a Golgi-like intracellular compartment; (b) do not induce re-distribution of syndet to dense core granules.

Role of the Cysteine-rich Domain in Syndet Binding to Membranes—AtT-20 cells expressing wild-type syndet, delta-syndet, and syndet C1S/C3S were homogenized in a buffer containing either 150 mM NaCl or 100 mM Na₂CO₃ at pH 11.5. Buffers containing sodium carbonate at pH 11.5 are known to dissociate peripheral proteins from membranes (29). The homogenates were then centrifuged at 400,000 × g for 30 min to

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**Fig. 5. List of mutations at the cysteine-rich domain of syndet.**

|   | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| syndet | K C C G L C I C P C N R T |
| SNAP-25 A | K C C G L F I C P C N K L |
| SNAP-25 B | K F C G L C V C P C N K L |
| syndet Cys 2-4-5 Ser | C S G L C I S P S N R T |
| syndet Cys 2-4 Ser | K C G L C I S P C N R T |
| syndet Cys 2-5 Ser | K C G L C I S P C N R T |
| syndet Cys 4-5 Ser | K C G L C I S P C R T |
| syndet Cys 4 Ser | K C G L C I S P C N R T |
| syndet Cys 5 Ser | K C G L C I S P C N R T |
| syndet Cys 1-3 Ser | K S C G L S I C P C N R T |

**Fig. 6. Confocal microscopy of transiently transfected AtT-20 cells expressing wild-type and mutant syndet proteins.** Cells expressing syndet C2S/C4S/C5S (A), syndet C4S/C5S (B), syndet C2S/C4S (C), syndet C2S/C5S (D), syndet C5S (E), and syndet C4S (F) were stained with antibodies against syndet as indicated in Fig. 1.
obtain a membrane-containing pellet and a cytosol-containing supernatant. Wild type SNAP-25 and syndet were mostly or entirely recovered in the pellet fraction (Fig. 8). We conclude that exogenous syndet in AtT-20 cells is an integral membrane component, like endogenous syndet in adipocytes. Overexpression of syndet did not affect the binding of endogenous SNAP-25 to membranes (Fig. 8). Delta-syndet was almost entirely recovered in the supernatant fraction, indicating that deletion of the cysteine-rich domain makes the protein soluble (Fig. 8). Approximately 50% of syndet C1S/C3S was tightly associated to membranes (Fig. 8). We conclude that Cys1 and Cys3 are important, but not absolutely necessary, for syndet binding to membranes.

**DISCUSSION**

Pairing of individual SNAREs before fusion may be at least one of the mechanisms by which cells maintain different organelles as unique compartments. In this light, it is essential that individual SNARE components be targeted to their correct destination. In this paper, we have identified the cysteine-rich region of the t-SNARE component, syndet, as critical for subcellular localization of syndet. We find that deletion of the cysteine-rich domain of syndet abolishes membrane binding. This result is in agreement with the finding that a mutant SNAP-25, lacking its cysteine-rich domain, is soluble (20). SNAP-25 is palmitoylated in the central nervous system and in transfected cells (18, 19). Syndet, syndet C1S/C3S, but not the mutant lacking the cysteine-rich domain, are palmitoylated in the transfected AtT-20 cell model. Thus, it is likely that fatty acylation is necessary for the binding of syndet to membranes. Moreover, we find that the cysteine-rich domain of syndet is essential for targeting the protein to the plasma membrane. We conclude that the cysteine-rich domain of syndet has a dual role in membrane binding and plasma membrane targeting. Unlike the deletion of the cysteine-rich domain, deletions of 32 amino acids at the amino terminus domain or 25 amino acids at the carboxyl terminus domain of syndet do not have an appreciable effect on protein distribution.

Our data show that the distribution of syndet which has been mutated at three cysteines, syndet C2S/C4S/C5S, is shifted from the plasma membrane to a diffuse intracellular localization. We also find that syndet C4S/C5S, C2S/C4S, and C2S/C5S are unable to accumulate at the cell periphery and are found in the cytoplasm. Syndet C4S and syndet C5S are both localized at the plasma membrane. Our observations indicate that: 1) at least two cysteines within the -Cys2-Cys4-Cys5- triplet must be substituted to prevent syndet localization at the cell surface; 2) single and double mutations of cysteines within the -Cys2-Cys4-Cys5- triplet have the same effect on syndet distribution, irre-
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Syndet C1S/C3S is palmitoylated. We conclude that Cys1 and syndet is necessary for its binding to membranes, we find that at steady state. Consistent with the concept that acylation of syndet C1S/C3S remains as an integral membrane component mutated syndet proteins were immunoprecipitated using the affinity purified anti-syndet antibody. Immunoprecipitates were analyzed by SDS-PAGE electrophoresis and fluorography (see "Experimental Procedures"). The gel was exposed for 20 days. This experiment was repeated three times with similar results.

Fig. 9. Syndet is palmitoylated at the cysteine-rich domain. Cells expressing syndet, delta-syndet, and syndet C1S/C3S were labeled with [3H]palmitate for 2 h, as described (19). Wild-type and doubly lipid modified peptides are stably anchored to membranes (30). Since syndet and SNAP-25 have multiple cysteines, some cysteines may target these proteins to intermediate locations along the biosynthetic route or to specialized domains at the plasma membrane. Indeed, it appears that SNAP-25 A, SNAP-25 B, and SNAP-23 accumulate in specific domains at the cell surface (5, 31–33). Modifications at cysteines may also modulate their binding to membranes (19) or the formation of SNARE complexes (34).

Because syndet Cys1 and Cys3 are not conserved in SNAP-25 B and SNAP-25 A, it is reasonable to conclude that modifications at these residues may account at least in part for differences in the localization and function of these proteins. We have generated a mutant syndet protein in which both cysteines are substituted with serines. We find that, unlike the double mutants within -Cys2-Cys4-Cys5- triplet, a fraction of syndet C1S/C3S remains as an integral membrane component at steady state. Consistent with the concept that acylation of syndet is necessary for its binding to membranes, we find that syndet C1S/C3S is palmitoylated. We conclude that Cys1 and Cys3, unlike Cys2, Cys4, and Cys5, are not absolutely required for membrane binding. We also find that mutations at Cys1 and Cys3 of syndet result in localization of the mutated protein to the plasma membrane, as well as a Golgi-like compartment and the cytosol. Interestingly, a mutation of a specific cysteine palmitoylation site in Src protein kinase p56Lck, can shift a fraction of the protein from the plasma membrane to a Golgi compartment (35). Our results support the concept that the cysteine-rich domain of syndet plays a major role in subcellular localization of the protein. We conclude that the distribution of syndet, like that of Src protein kinase p56Lck, may be influenced by the configuration of the cysteines.

SNAP-25 associates with the Golgi compartment at the cell body of the neurons and then undergoes fast axonal transport as an acylated protein (18, 36). Thus, SNAP-25 reaches the plasma membrane by association with transport vesicles. We find that syndet C1S/C3S is found in the cytosol, the Golgi, and the plasma membrane. The distribution of mutated syndet may mimic the steps of syndet or SNAP-25 biosynthetic routes. If this is the case, then acylations within the -Cys2-Cys4-Cys5- triplet may be sufficient to bind syndet to a Golgi localization. Acylation(s) at Cys1 and/or Cys3 may shift wild-type syndet from the trans-Golgi network to the plasma membrane. In the absence of Cys1 and Cys3 transit to the plasma membrane is slow and mutated syndet C1S/C3S accumulates at the Golgi localization. Alternatively, it is also possible for mutated syndet C1S/C3S to localize both at the Golgi and at the plasma membrane directly, from the cytosol. In mast cells, SNAP-23 relocates from the plasma membrane to dense core granules in response to stimulation (37). Our results indicate that modifications at the cysteine-rich motif may induce redistribution of syndet to an intracellular compartment. However, in AIT-20 cells, syndet C1S/C3S is redistributed to an intracellular compartment different from dense core granules. More work is necessary to understand whether modifications at the cysteine-rich motif play a role in changes of SNAP-23/syndet or SNAP-25 cell distribution under different conditions.

In conclusion, our paper shows that the cysteine-rich domain of syndet is a major determinant for the subcellular localization of the protein. We propose that modifications at the clustered cysteines may control syndet distribution and function in cells.

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