Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25

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Abstract Synaptotagmin, a likely calcium sensor for synaptic transmission, and SNAP-25, a t-SNARE of the presynaptic plasma membrane, are key proteins for the docking and fusion of synaptic and other vesicles. We report that both synaptotagmin and SNAP-25 are palmitoylated with their fatty acids attached in a labile thioester-type bond. A SNAP-25 mutant with deleted palmitoylation sites is found exclusively in the cytosol after cell fractionation, whereas the palmitoylated form of SNAP-25 is membrane-bound, establishing that SNAP-25 is membrane-anchored via covalently linked palmitate.

Key words: Neurotransmission; SNARE; SNAP-25; Synaptotagmin; Palmitoylation

1. Introduction

Palmitoylation is the post-translational attachment of fatty acids (mainly palmitic acid) to cysteine residues and is common among integral and peripheral membrane proteins. The amino acid sequence requirements for palmitoylation are not well defined; however, in integral membrane proteins the acylated cysteine residues are always located at the boundary between the transmembrane region and the cytoplasmic tail of the respective protein (for recent reviews see [1,2].

Palmitoylation has been suggested to play a role in vesicular transport based on a requirement for palmitoyl-CoA [3], the acyl-donor in the palmitoylation reaction [4]. More specifically, fatty acyl-CoA is required for the budding of transport vesicles [5,6] as well as for the fusion of the vesicles with their target membrane in vitro [7]. The relevant targets for acylation are not known.

Here we analysed whether known components of the docking and fusion machinery, v-SNAREs and t-SNAREs [8], might be fatty acylated. In neurons, the t-SNAREs of the presynaptic plasma membrane have been identified as syntaxin I and SNAP-25. Syntaxin is an integral membrane protein [9], whereas SNAP-25 is believed to be anchored by covalently linked fatty acids to the presynaptic membrane [10,11], but this has not been proven experimentally.

The fusion of neurosecretory vesicles is not a constitutive event, but is triggered by an increase in the calcium concentration. Synaptotagmin is a calcium-binding protein thought to function in the regulation of synaptic vesicle fusion [12–14] and is a v-SNARE specialized for exocytosis [15]. Its possible fatty acylation has not been reported.

Two yeast v-SNAREs without sequence homology to synaptotagmin have been shown to contain cysteine-linked palmitate [16]. Here, we explore whether synaptotagmin and the t-SNARE syntaxin are also potential targets for palmitoylation: each contains at least one cysteine residue that is a candidate for acylation [9,12]. Furthermore, we analyse the palmitoylation of SNAP-25 [11] in more detail and present evidence that this intrinsically hydrophilic protein is anchored by fatty acids.

2. Materials and methods

2.1. Cells

PC 12 cells were cultured in Dulbecco's medium supplemented with 10% fetal calf serum (FCS) and 5% horse serum. Neuronal phenotype was induced by treatment with nerve growth factor (NGF) at a concentration of 200 ng/ml for at least 4 days. CV 1 (kidney) cells were grown in Dulbecco's medium containing 10% FCS.

2.2. Recombinant DNA

The nucleotide sequence of rat SNAP-25 (isoform B) encoding the cysteine cluster was removed from the cloned gene in pQE9 by digestion with *Avr*II and *Hin*dIII. Protruding DNA ends were filled with dNTPs using Klenow enzyme. The resulting three DNA fragments were separated by electrophoresis. The large and middle fragments were isolated and religated. In doing so, nucleotides encoding 12 amino acids were deleted and one nucleotide triplet was changed. This results in the amino acid sequence L^{81}, D^{82}, L^{95}, whereas the wild-type SNAP-25 contains in this region the sequence L^{81}, G, K, F, C, G, L, C, V, C, P, C, N, K, L^{95}. Wild-type and deleted SNAP-25 were then subcloned with *Bam*HI into a modified version of the expression plasmid pTM1 [17], where the *Nco*I site is deleted. The rat synaptotagmin I c-DNA was subcloned into pTM1 by a limited digestion with *Pst*I. In these constructs start and stop of translation was initiated by the respective codons of SNAP-25 or synaptotagmin, resulting in authentic, full-length proteins.

2.3. Vaccinia virus T7 polymerase expression system

Approx. 80% confluent CV 1 cells grown in 2.5 cm diameter plates were infected at a multiplicity of infection of 10 with recombinant Vaccinia virus (VTF7-3), which expresses the RNA-polymerase of the bacteriophage T7 [17]. 2 h after infection the cells were transfected with the plasmid pTM1 containing the desired gene under control of the T7 promotor. Each cell monolayer was transfected with 3 μg DNA using 10 μg lipofectin as described by the manufacturer (Gibco/BRL, NY). Metabolic labelling was performed at 3–4 h after transfection.

2.4. Metabolic labelling, immunoprecipitation, endoglycosidase treatment

Cells were labelled with Express-^{35}S^{35}S-protein labelling mix (50 μCi/ml medium without methionine, 1200 Ci/mmol, DuPont, Boston, MA) or [^{3}H]palmitic acid (1 mCi/ml medium, 50–60 Ci/mmol, DuPont) for the periods of time indicated in the figure legends. For pulse-chase experiments with [^{3}H]palmitic acid, the chase was initiated by washing the cell monolayer three times with medium containing 0.1% fatty acid free BSA and then adding medium containing 100 mM unlabelled palmitic acid. Proteins were immunoprecipitated from cellular extracts as described [18]. For the chloroform/methanol extraction, antigen-antibody complexes were eluted from protein A-Sepharose by heating (50°C, 15 min) in phosphate buffer (10 mM, pH 7.4) containing 0.1% SDS. Equal aliquots were either extracted with chloroform/methanol (2:1) for 20 min on ice or left unextracted. Extracted proteins were pelleted (14000 rpm, 30 min) in an Eppendorf centrifuge and processed for SDS-PAGE. Fluorography was carried out with Enlightning as described by the manufacturer (DuPont).

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2.5. Hydroxylamine treatment and fatty acid analysis

Hydroxylamine treatment and fatty acid analysis were conducted as described [19]. Radioactivity on the thin-layer plate was made visible by spraying with En^{3}Hance (Amersham, Arlington Heights, IL).

2.6. Cell fractionation

CV 1 cells expressing SNAP-25 using the Vac T7 system were labelled for 60 min with [^{35}S]methionine or [^{3}H]palmitic acid. Cells were then scraped into ice-cold PBS, pelleted (2 min, 5000 rpm, Eppendorf centrifuge) and resuspended in buffer A (20 mM HEPES-NaOH, pH 7.4; 50 mM NaCl; 1 mM EDTA; 1 mM PMSF, 1 mg/ml leupeptin and 1 mM pepstatin). Cells were disintegrated with 40 strokes of a tight-fitting douncer and insoluble material was pelleted (2 min, 5000 rpm). The supernatant was centrifuged for 30 min at 100 000 rpm in a TLA 100.2 rotor. The resulting pellets were used either directly for immunoprecipitation (membranes) or resuspended in buffer A containing 1 M NaCl (salt washed membranes), incubated on ice for 30 min and again centrifuged for 30 min at 100 000 rpm. The pellets were then solubilized in RIPA buffer [18] and equal amounts of 2×RIPA buffer were added to the respective supernatants of the first (cytosol) and second centrifugation (salt-wash). Samples were clarified at 14 000 rpm for 15 min and the resulting supernatant was subjected to immunoprecipitation.

3. Results

3.1. Synaptotagmin is palmitoylated but syntaxin is not

To analyze possible palmitoylation of endogenous syntaxin and synaptotagmin present in a 'neuron-like' cell line, PC 12 cells induced with nerve growth factor (NGF) were labelled with [^{3}H]palmitic acid. Both proteins were then immunoprecipitated from cellular extracts. SNAP-25, which is already known to be palmitoylated [11], was subject to the same procedure. SDS-PAGE and fluorography of the respective immunoprecipitates shows [^{3}H]palmitic acid incorporation, as expected, into SNAP-25, but also into synaptotagmin (Fig. 1A, ^{3}H-PAL). In contrast, palmitoylation of syntaxin was not observed, although western blotting showed that the protein was immunoprecipitated from PC 12 cells (Fig. 1A, BLOT). Furthermore, [^{3}H]palmitic acid labelling of syntaxin 1b was also not detectable after overexpression of its cloned gene with the baculovirus system in insect cells (not shown). Thus, despite the presence of a cysteine residue in the palmitoylation region, syntaxin is not acylated.

Synaptotagmin is known to bind phospholipids non-covalently at one of its C2 domains [12]. If the binding of only a small amount of phospholipids to synaptotagmin were to resist SDS-PAGE, this would simulate covalent fatty acid binding because the phospholipids are heavily labelled with [^{3}H]palmitic acid. To exclude possible non-covalent lipid binding, we extracted [^{3}H]palmitic acid labelled and immunoprecipitated synaptotagmin and SNAP-25 with chloroform/methanol prior to SDS-PAGE and fluorography. Chloroform/methanol completely removes the lipids, which run to the bottom of the gel in non-extracted samples, but the labelling of synaptotagmin and also that of SNAP-25 is not diminished after extraction with organic solvents (Fig. 1B). This further suggests a covalent linkage of fatty acid in synaptotagmin and SNAP-25.

To confirm palmitoylation of synaptotagmin in a non-neural cell line, its cloned gene was expressed in CV 1 kidney cells with the Vaccinia virus T7 polymerase (Vac T7) system. Palmitic acid labelling of synaptotagmin is easily detectable in CV 1 cells (Fig. 2A, left panel), which reveals that a neuron-specific enzyme is not required for acylation of synaptotagmin.

Next we determined the chemical nature of the fatty acid bond in synaptotagmin. To distinguish between a labile thioester-type fatty acid linkage and a stable amide bond, the previous gel was treated with neutral hydroxylamine prior to re-exposure for the same period of time (Fig. 2A, right panel). The [^{3}H]palmitate labelling of synaptotagmin is completely sensitive to hydroxylamine, implying a thioester bond [20].

It has been described that exogenously added ^{3}H-labelled fatty acids can be metabolized into species of different chain length before attachment to an acylated protein [19,21]. To analyse the actual protein-bound ^{3}H-labelled fatty acids of synaptotagmin and also of SNAP-25, the respective [^{3}H]palmitic acid labelled bands were cut out of the gel, the

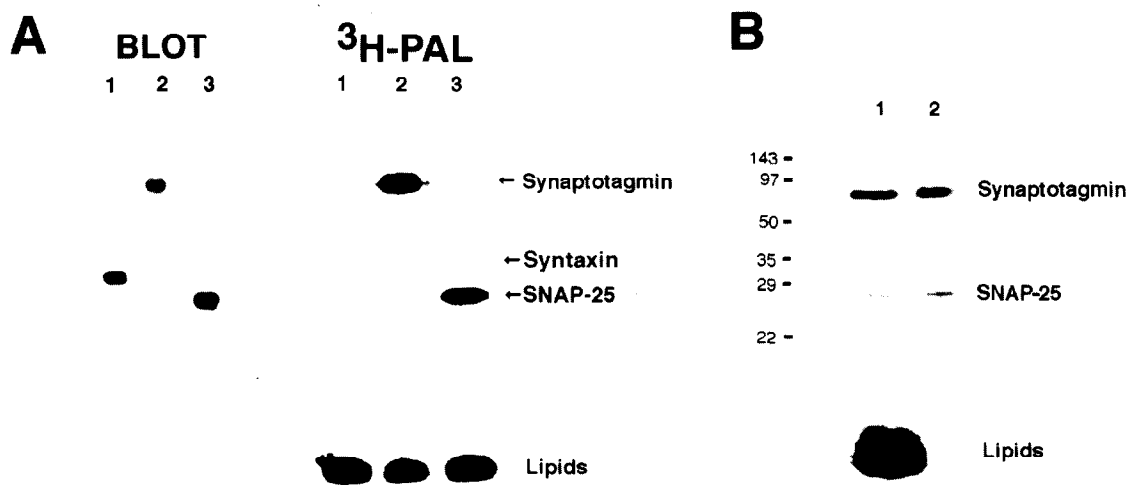

Fig. 1. Synaptotagmin is covalently acylated. (a) NGF-induced PC 12 cells were labelled with [^{3}H]palmitic acid (^{3}H-PAL) for 4 h. Cellular extracts were immunoprecipitated with anti-syntaxin I (lane 1), anti-synaptotagmin I (lane 2) or anti-SNAP-25 (lane 3) antibodies. Samples were then subjected to SDS-PAGE and fluorography for 10 days. BLOT: immunoprecipitated samples were probed with antibodies against syntaxin (lane 1), synaptotagmin (lane 2) or SNAP-25 (lane 3) in a Western blot. (b) Prior to SDS-PAGE and fluorography, [^{3}H]palmitic acid labelled and immunoprecipitated synaptotagmin and SNAP-25 were extracted with chloroform/methanol (lane 2) or left untreated (lane 1).

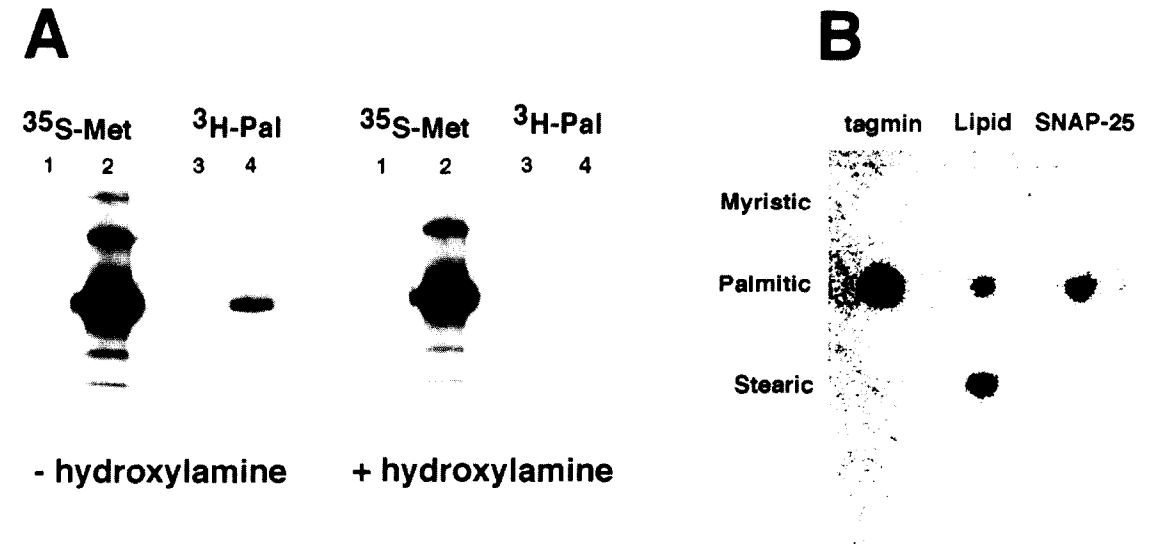

Fig. 2. (a) Fatty acids are bound in a thioester-type linkage to synaptotagmin. Synaptotagmin was expressed with the Vac T7 system in CV 1 cells and the cells were labelled with [^{35}S]methionine or [^{3}H]palmitic acid. Synaptotagmin was immunoprecipitated from cellular extracts and subjected to SDS-PAGE and fluorography for 2 days (left panel, −hydroxylamine). The gel was then rehydrated, treated with hydroxylamine and again processed for fluorography (right panel, +hydroxylamine). Lane 1+3, untransfected CV 1 cells; lane 2+4, transfected CV 1 cells. (b) Palmitate is the major fatty acid linked to synaptotagmin and SNAP-25. [^{3}H]Palmitate-labelled synaptotagmin and SNAP-25 were immunoprecipitated from PC 12 cells and purified by SDS-PAGE. The proteins were localizied by fluorography and cut out of the gel. Lipid-linked fatty acids, which run to the bottom of the gel (see Fig. 1) were analysed in parallel. Fatty acids were liberated by acid hydrolysis and extracted with hexane. Extracts were run on a TLC plate (RP 18) with acetonitrile/acetic acid (1:1) as solvent system. A fluorogram of the TLC plate is shown. Myristic, palmitic and stearic depict the locations of ^{3}H-labelled reference fatty acids.

fatty acids were liberated by acid treatment and extracted with hexane. The extracts, together with similarly prepared lipid-linked fatty acids, were separated into individual fatty acid species by thin layer chromatography (TLC). The fluorogram of the resulting TLC plate shows that metabolism of [^{3}H]palmitate indeed occurred, because the lipids contain both palmitate and stearate. However, only palmitic acid is released from synaptotagmin and SNAP-25 (Fig. 2B), affording further evidence against non-covalent attachment.

Finally, we estimated the molar extent of palmitoylation of synaptotagmin and SNAP-25 relative to the acylation of another, better characterized acylprotein, the hemagglutinin (HA) of influenza virus [18]. To this end, HA, SNAP-25 and synaptotagmin were expressed with the Vac T7 system in CV 1 cells, and their [^{3}H]palmitic acid incorporation was determined by liquid scintillation counting and related to their respective expression levels. The results revealed, that synaptotagmin and SNAP-25 are palmitoylated to a slightly greater extent than HA. Assuming that HA contains three covalently bound fatty acids per molecule [18], synaptotagmin and SNAP-25 are acylated with about 3–4 moles of palmitate.

Thioester-linked fatty acids can either be stably attached or they can be a dynamic modification, with cycles of fatty acid removal and reacylation. In such cases half-times for turnover of attached fatty acids between 20 min and 3 h have been reported [1,2]. Differentiated PC 12 cells were labelled for 2 h with [^{3}H]palmitic acid and then chased in the presence of excess unlabelled palmitate for up to 5 h. However, no loss of [^{3}H]palmitate was observed for either SNAP-25 and synaptotagmin during the chase (data not shown).

3.2. *SNAP-25 is anchored via covalently linked palmitate to membranes*

SNAP-25 has been shown to be fatty acylated, but the sites of acylation have not been determined [11]. SNAP-25 has four evolutionarily conserved cysteine residues, which are clustered in the middle of the molecule [10,22] and have been suggested to be acylation sites [11,23]. This cysteine-rich region was deleted from the cloned gene of SNAP-25, isoform B. Wild-type SNAP-25 and this mutant were then expressed with the Vac T7 system in CV 1 cells. [^{35}S]methionine labelling and immunoprecipitation revealed that both proteins are synthesized to a similar extent (Fig. 3). However, [^{3}H]palmitate labelling was observed only for the wild-type protein, but not for the cysteine deletion mutant. This provides evidence that the deleted cysteine residues of SNAP-25 are essential for the

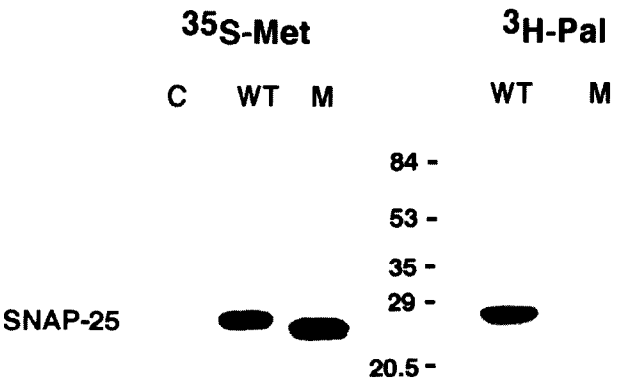

Fig. 3. SNAP-25 requires cysteine residues for palmitoylation. Wild-type SNAP-25 (WT) and a deletion mutant without the cysteine cluster (M) were expressed in CV 1 cells and labelled for 1 h with [^{35}S]methionine or [^{3}H]palmitic acid. The proteins were immunoprecipitated from cellular extracts and subjected to SDS-PAGE and fluorography. C, untransfected control cells.

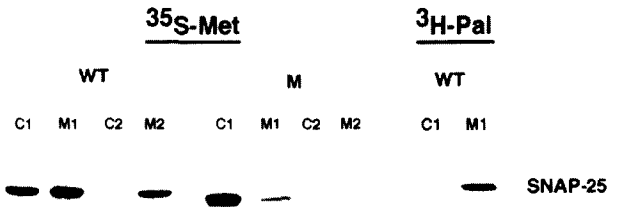

Fig. 4. Membrane anchoring of SNAP-25 requires palmitoylation. Wild-type (WT) and non-palmitoylated (M) SNAP-25 were expressed in CV 1 cells and labelled with [^{35}S]methionine or [^{3}H]palmitic acid for 1 h. Cells were disintegrated and fractionated into cytosol (C1), membranes (M1) and salt-extracted membranes (M2) by high-speed centrifugation. C2 is the supernatant of the salt-washed membranes. SNAP-25 was immunoprecipitated from each fraction and subjected to SDS-PAGE and fluorography.

attachment of palmitic acid, most likely because they are the sites for covalent attachment of palmitate.

SNAP-25 behaves as an integral membrane protein in subcellular fractionation, yet it lacks a distinct hydrophobic anchor in its amino acid sequence that could attach it to the presynaptic membrane [10]. To compare the subcellular distribution of wild-type SNAP-25 with its fatty acid free mutant, both proteins were again expressed with the Vac T7 system and labelled with [^{35}S]methionine, cells were homogenized and membranes were prepared by ultracentrifugation. Wild-type (acylated) SNAP-25 is found in the membrane fraction, to a lesser extent in the cytosolic fraction. In contrast, mutant (fatty acid free) SNAP-25 is exclusively present in the cytosol fraction. Membrane bound wild-type SNAP-25 is resistant to high salt extraction. Only the membrane bound pool of wild-type SNAP-25 can be labelled with [^{3}H]palmitic acid (Fig. 4). Together, these results clearly imply that SNAP-25 is anchored to membranes via covalently linked palmitate. A soluble pool of SNAP-25, evident here when SNAP 25 is overexpressed, was not reported for endogenous SNAP-25 in neurons under steady-state conditions [10].

4. Discussion

We show here, that synaptotagmin I is post-translationally modified by the attachment of palmitic acid, with its fatty acids attached in a thioester-type linkage. Synaptotagmin I has six cysteine residues. One is located in a C2 domain and the other five are clustered together at the border between the transmembrane segment and the cytoplasmic domain [12]. Palmitoylation in the latter region has been described for numerous transmembrane proteins [1]. Quantitation of the extent of palmitoylation of synaptotagmin relative to the palmitoylation of influenza virus hemagglutinin [18] suggests that one synaptotagmin molecule contains about three to four molecules of covalently bound fatty acids, consistent with acylation of most cysteine residues in this region as the most likely site(s) for acylation.

There are several known isoforms of synaptotagmin [24,25]. All have cysteine residues in the predicted palmitoylation region at the membrane interface, but their number varies from only one (rat synaptotagmin VI and *Drosophila* synaptotagmin) to up to seven (rat synaptotagmin VII). Palmitoylation of these synaptotagmin isoforms remains to be directly established. However, the conservation of at least one cysteine residue as potential palmitoylation site in synaptotagmins present in different organisms as well as in synaptotagmin isoforms in the same organism argues in favour of an important role for their covalently linked fatty acids. Palmitoylation may influence the local conformation of synaptotagmin in the transmembrane region and at the interface between the membrane and the cytoplasm. It is interesting in this regard that in model systems with hydrophobic peptides, protein bound palmitate can change the conformation of specific amino acid side chains in the transmembrane region [26] and even realign the whole transmembrane segment from its normal overall orientation perpendicular to the bilayer surface [27].

Although the functional role of the palmitate linked to synaptotagmin remains to be established, we clearly could demonstrate that the lipid modification of SNAP-25 is responsible for the membrane anchoring of this intrinsically hydrophilic protein. Membrane anchoring of SNAP-25 via palmitate is likely to be evolutionary conserved, as both *Drosophila* and animal SNAP-25s contain the palmitoylated cysteine cluster [10,22,23]. In contrast, Sec9p, the yeast homologue of SNAP-25, does not contain cysteines, but it is tightly associated with membranes [28], by what must be a distinct mechanism.

In summary, postranslational modification of SNAREs by lipophilic components – palmitoylation in the case of SNAP-25 [11], Snc proteins [16] and synaptotagmin, isoprenylation in the case of Ykt6p [29] – appears to be a common theme, and implies that such modifications have a functional role in vesicle docking and fusion.

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