

Palmitoylation sites and processing of synaptotagmin I, the putative calcium sensor for neurosecretion

Ulrich Heindel, Michael F.G. Schmidt, Michael Veit*

Department of Immunology and Molecular Biology, Veterinary-Medical Faculty, Free University Berlin, Philipstraße 13, 10115 Berlin, Germany

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Abstract Synaptotagmin I, the calcium sensor for neurotransmission, is palmitoylated. We have identified the palmitoylation sites as five cysteine residues located between the transmembrane and cytoplasmic regions. In contrast to wild-type synaptotagmin, the non-acylated mutant is not converted to the endoglycosidase-H-resistant form after expression in CV-1 cells. This indicates a block in transport through the Golgi complex. However, when expressed in PC-12 and RBL cells non-acylated synaptotagmin is targeted to the plasma membrane and to secretory granules. No significant cleavage of [³H]palmitate from synaptotagmin was observed in pulse-chase experiments. This indicates that the majority of fatty acids are structural rather than dynamic components.

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1. Introduction

Synaptotagmin I, an abundant protein of synaptic vesicles, is the putative Ca²⁺ sensor for neurotransmission. It consists of a small luminal N-terminal segment, a single transmembrane region (TMR), and a large cytoplasmic domain, which contains the two calcium-binding C₂ domains. A cysteine-rich region and a highly charged domain connect the TMR to the C₂ domains [1–3].

Intracellular processing and protein modifications of synaptotagmin are not well understood. The TMR including neighbouring amino acids is required for targeting to the endoplasmic reticulum and translocation of the N-terminal domain [4]. The luminal domain contains a single site for N-glycosylation and two sites for O-glycosylation [5,6]. The latter are utilised only when the neuronal synaptosomal protein receptor (SNARE) protein vesicle-associated membrane protein 2 is expressed in the same cell. Two oligomerisation steps, Ca²⁺-dependent and Ca²⁺-independent, have also been described [3].

A further modification of synaptotagmin is palmitoylation

of cysteine residues, which are probably located in the cysteine-rich region [7,8]. Interestingly, mutagenic exchange of these cysteine residues blocked dimerisation of synaptotagmin, thus linking fatty acylation to Ca²⁺-independent oligomerisation [9,10]. This replacement reduced the molecular weight of the mutated protein [9], but it was not directly demonstrated that cysteine mutants are not acylated. Furthermore, the role of palmitoylation for processing and intracellular transport of synaptotagmin has not yet been analysed.

2. Materials and methods

2.1. Recombinant DNA methods

Construction of synaptotagmin mutants was done with overlap extension polymerase chain reaction as described [11]. The following primers were used: pTM sense GGG AAT TCC CCG GGG AGC TCA G; pTM antisense TTA GGC CTC TCG AGC TCG ACC CT; Tag C1 sense TCC TGC TTT TGT GTC TGT AAG AAA TGT TTG TTC AAG AAA TG; Tag C1 antisense CTT ACA GAC ACA AAA GCA GGA GGT TAC GAC TAA AAG GAC; Tag C1/2 sense TCC TCC TTT TGT GTC TGT AAG AAA TGT TTG TTC AAG AAA TG; Tag C1/2 antisense CTT ACA GAC ACA AAA GGA GGA GGT TAC GAC TAA AAG GAC; Tag C3/4 sense TCT GTC TCT AAG AAA TGT TTG TTC AAA AAG AAA AAC AAG AAG GAG GGG; Tag C3/4 antisense CAT TTC TTA GAG ACA GAA AAG CAG CAG GTT ACG AC; Tag C3-5 sense TCT GTC TCT AAG AAA TCT TTG TTC AAA AAG AAA AAC AAG AAG AAG GGG; Tag C3-5 antisense GAT TTC TTA GAG ACA GAA AAG GAG GAG GTT ACG AC; Tag C5 sense TGT GTC TGT AAG AAA TCT TTG TTC AAA AAG AAA AAC AAG AAG GGG; Tag C5 antisense GAT TTC TTA CAG ACA CAA AAG CAG CAG GTT ACG AC; Tag C1/2/5 sense TGT CTG TGT AAG AAA TCT TTG TTC AAA AAG AAA AAC AAG AAG AAG GGG; Tag C1/2/5 antisense GAT TTC TTA CAG ACA CAA AAC CAC CAG GTT ACG AC.

For expression of tagmin in PC-12 cells the plasmid pcDNA3.1-myc-his(+) was used (Invitrogen). The tagmin gene was amplified from pTM1 with an N-terminal primer equipped with an *Eco*RI site and a C-terminal primer (without the stop codon) and an *Xba*I site. These enzymes were used to clone the tagmin gene (wt and mutant 1-5) into pcDNA3.1-myc-his resulting in full-length protein with a myc tag at the C-terminus. For expression of tagmin in RBL-2H3 cells, tagmin (wt and mutant 1-5) was excised from pTM1 with *Eco*RI and *Bam*HI and subcloned into the corresponding sites of the plasmid pEGFP-C1 (Clontech). This results in full-length proteins fused at the N-terminus to the green fluorescent protein (GFP).

2.2. Expression, metabolic labelling and immunoprecipitation

Expression, metabolic labelling and immunoprecipitation of tagmin with the M49 monoclonal antibody were done as described [8]. The immunoprecipitated protein was digested with 1 µl endoglycosidase-H (Endo-H) as described by the manufacturer (NEB).

2.3. Indirect immunofluorescence

PC-12 cells (5 × 10⁴) were seeded into 24-well plates covered with poly-D-lysine and cultured in Dulbecco's medium supplemented with

*Corresponding author. Fax: (49)-30-20936171.

E-mail address: mveit@city.vetmed.fu-berlin.de (M. Veit).

Abbreviations: SNARE, SNAP receptor; SNAP-25, synaptosomal protein with a molecular weight of 25 kDa; NGF, nerve growth factor; Endo-H, endoglycosidase-H

10% foetal calf serum, 5% horse serum and 50 ng/ml nerve growth factor (NGF) for 3 days. Cells were then transfected for 4 h or overnight with lipofectin (10 µl) and 3 µg pcDNA3.1-myc-his(+)-synaptotagmin plasmid. Cells were then removed from the plates, pelleted and seeded onto coverslips treated with poly-D-lysine, and were further cultured for 2 days in the presence of NGF. Cells were then fixed for 15 min with paraformaldehyde (3%) and permeabilised with Triton X-100 (0.1%). Synaptotagmin was stained with anti-myc monoclonal antibody 9E10 (1:200) and FITC-conjugated anti-mouse IgGs (1:50, Sigma). Coverslips were mounted in 90% glycerol and visualised with a confocal microscope (Leica TCS SP2 system with Leica DM IRBE). RBL-2H3 cells, a cell line usually used as a model for mast cells, were seeded on coverslips and cultured in Dulbecco's medium supplemented with 10% foetal calf serum. Cells (80% confluent) were transfected for 4 h with 2 µg pEGFC1-synaptotagmin plasmids and lipofectin (10 µl). Two days later coverslips were washed in phosphate-buffered saline and distilled water and mounted in 90% glycerol. Results were visualised with a Zeiss AxioScope microscope and recorded with a Nikon Coolpix 995 camera.

3. Results and discussion

3.1. Five cysteines are involved in palmitoylation of synaptotagmin I

Cysteine residues located between the transmembrane and cytoplasmic regions have been identified as palmitoylation sites in numerous proteins [12]. With one exception (tagmin XII), all synaptotagmin isoforms and also their orthologues from lower eukaryotes have at least one cysteine in this region [9]. In synaptotagmin I five cysteines are present. Assuming that the first positively charged amino acid (lysine) marks the boundary, one cysteine is located in the cytoplasmic tail and four cysteines are in the transmembrane region. To prove that the cysteines are indeed the palmitoylation sites, one, two or three cysteines were replaced by serine residues. In one mutant (M1-5) all five cysteines were replaced simultaneously (see Fig. 1A). Wild-type and mutant tagmins were expressed with the T7 polymerase vaccinia virus system in non-neuronal CV-1 cells [11]. Labelling with [³⁵S]methionine and immunoprecipitation showed that wild-type and mutant forms of tagmin are

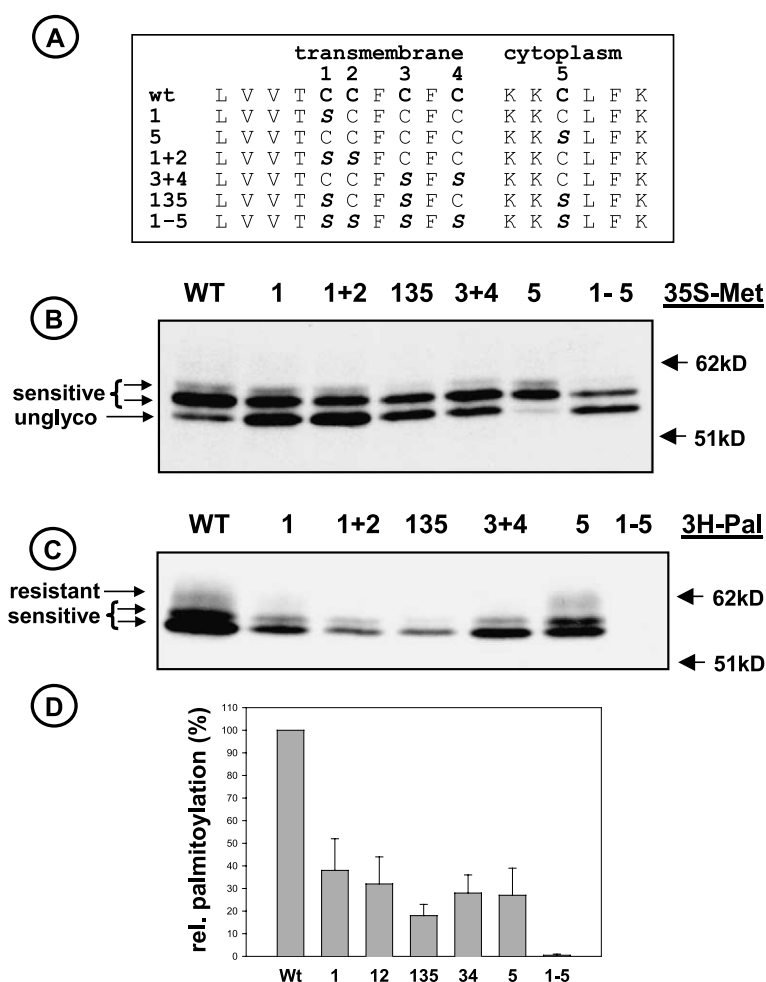


Fig. 1. Identification of palmitoylation sites in synaptotagmin I. A: Site-specific mutagenesis of cysteine residues. Amino acids at the boundary between the cytoplasmic tail and the TMR of synaptotagmin I (cloned from rat) are shown. Cysteine residues are in bold. One, two, three or all five cysteine residues were replaced by serines (bold and italics). B,C: Synaptotagmin I and the cysteine mutants were expressed with the vaccinia T7 polymerase system in CV-1 cells and labelled for 1 h with [³⁵S]methionine (B) or [³H]palmitate (C). Tagmin was then immunoprecipitated from cellular extracts and subjected to SDS-PAGE and fluorography. The sensitivity or resistance of protein bands against Endo-H digestion (analysed in Fig. 2) is also indicated. unglyco=unglycosylated protein. Bands with a higher molecular weight, which might represent dimers [9], were not detected under our experimental conditions. D: Densitometric quantification of bands shown in B and C and from four identical experiments. The mean of relative palmitoylation (wild-type protein = 100%) including the variance is shown.

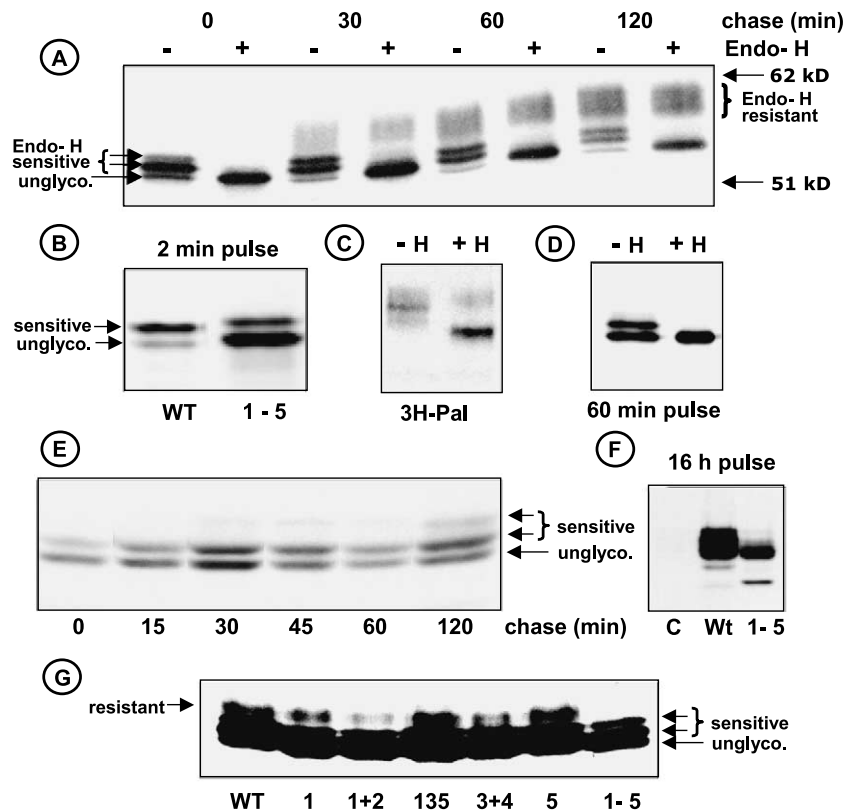


Fig. 2. Processing of non-acylated tagmin is impaired in CV-1 cells. A: Acylated tagmin is processed to an Endo-H-resistant form. Wild-type tagmin was expressed in CV-1 cells, labelled for 10 min with [35 S]methionine and then chased for 0, 30, 60 or 120 min. Tagmin was immunoprecipitated and equal aliquots were digested with Endo-H (+) or were mock-digested (–) prior to SDS-PAGE and fluorography. The position of molecular weight markers is shown on the right side of the fluorogram. B: Acylated (wt) and non-acylated (1-5) synaptotagmin were labelled for 2 min with [35 S]methionine. Tagmin was immunoprecipitated and subjected to SDS-PAGE and fluorography. C: Wild-type tagmin was expressed in CV-1 cells, labelled for 120 min with [3 H]palmitate, immunoprecipitated and equal aliquots were digested with Endo-H (+H) or were mock-digested (–H). D: Non-acylated tagmin does not acquire Endo-H resistance. Tagmin mutant 1-5 was expressed in CV-1 cells, labelled for 1 h with [35 S]methionine and an aliquot of the immunoprecipitated protein was digested with Endo-H (+H) or was mock-digested (–H). E: Tagmin mutant 1-5 was labelled for 15 min with [35 S]methionine and then chased for 0, 15, 30, 45, 60 or 120 min prior to immunoprecipitation. F: Acylated (wt) and non-acylated (1-5) tagmin were expressed in CV-1 cells, labelled for 16 h with [35 S]methionine and subjected to immunoprecipitation, SDS-PAGE and fluorography. C: non-transfected CV-1 cells. G: Acylated (wt) and non-acylated (1-5) and the other cysteine mutants (see Fig. 1A) of tagmin were labelled for 2 h with [35 S]methionine. The proteins were immunoprecipitated and subjected to SDS-PAGE and fluorography. The fluorogram is overexposed to show that the upper, fully processed band is detectable for all mutants except the completely non-acylated mutant 1-5.

expressed at similar levels (Fig. 1B). Labelling with [3 H]palmitate revealed that only the mutant with all five cysteines replaced lacked fatty acids completely. The other cysteine mutants showed [3 H]palmitoylation, albeit at reduced levels (Fig. 1C). The acylation efficiency of the tagmin mutants was quantified by densitometry of 35 S- and 3 H-labelled bands. The most significant result was that exchange of a single cysteine decreased palmitoylation more than half compared to the wild-type protein (Fig. 1D). Replacing two or three cysteines leads to a further reduction of palmitoylation. The drastic reduction in palmitoylation after replacement of a single cysteine is surprising. One likely explanation could be that it decreased the transport of tagmin to the intracellular site of palmitoylation (see below). Nevertheless, all five cysteine residues are, directly or indirectly, involved in the palmitoylation reaction. However, it cannot be inferred from these data that each cysteine is palmitoylated in each synaptotagmin molecule.

3.2. Processing of wild-type synaptotagmin in CV-1 cells

Synaptotagmin occurs in multiple bands in the SDS gel

(Fig. 1B). One obvious explanation would be that the bands represent different processing steps of the single *N*-linked carbohydrate side chain. To confirm this assumption we conducted a series of pulse-chase experiments with [35 S]methionine and digested an aliquot of the immunoprecipitated protein with Endo-H, a glycosidase which cleaves *N*-linked carbohydrates of the high-mannose type (Fig. 2A). Freshly synthesised tagmin (10 min labelling) occurs as three bands during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The upper two bands are sensitive to Endo-H and therefore must represent *N*-glycosylated molecules with sugars of the mannose-rich type [13]. The fastest-migrating band has the same size as the Endo-H-digested product and is therefore not glycosylated. After 30 min of chase a fuzzy band with the highest molecular weight appears, which is resistant to digestion with Endo-H thus representing the protein species containing complex carbohydrates.

Next we asked whether the two Endo-H-sensitive bands occur independently of each other or if one band is the precursor to the other. Short pulse-labelling (2 min) showed only

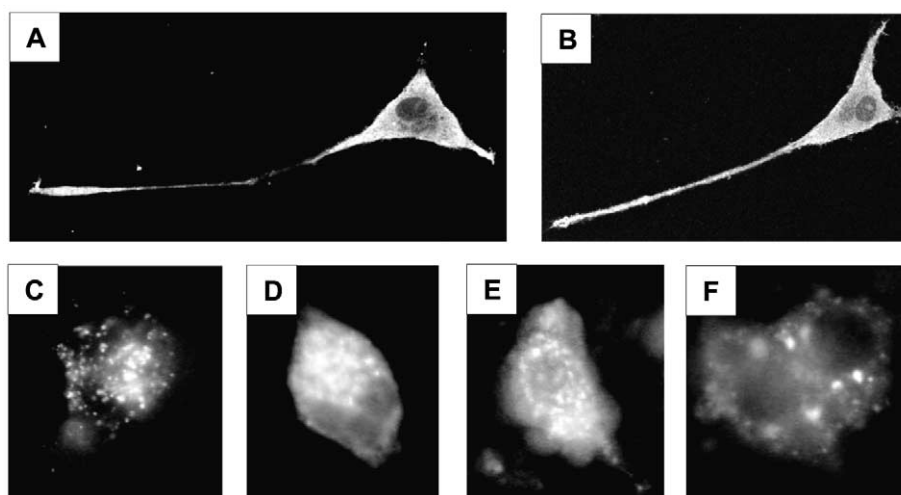


Fig. 3. Targeting of acylated and non-acylated tagmin in PC-12 and RBL cells. A,B: Differentiated PC-12 cells were transfected with wild-type (A) or non-acylated (mutant 1-5, B) tagmin-myc constructs and stained 2 days later with anti-myc 9E10 antibodies and FITC-conjugated anti-mouse IgGs. Results were recorded with confocal microscopy. C–F: RBL-2H3 cells were transfected with wild-type (C,D) or non-acylated (mutant 1-5, E,F) GFP-tagmin constructs. Two days later results were recorded with fluorescence microscopy.

two bands, which represent the unglycosylated and the first Endo-H-sensitive form of tagmin (Fig. 2B). Likewise, inspection of the pulse-chase experiment (Fig. 2A) showed that the faster-migrating form is converted into the slower-migrating form after 30 min of chase. Thus, processing of synaptotagmin expressed in CV-1 cells occurs in three steps: core glycosylation, rapid conversion to a slower-migrating, but still Endo-H-sensitive form, and finally slow processing to the Endo-H-resistant form. The reason for the difference in the molecular weight of the two Endo-H-sensitive forms is not known. The usual processing of carbohydrates before they acquire Endo-H resistance only involves cleavage of sugars and should therefore not result in an increase in molecular weight [13]. Palmitoylation is also not responsible for the shift in the molecular weight because (i) wild-type and non-acylated tagmin have the same molecular weight (Fig. 1A), (ii) the slower-migrating form is also labelled with [3 H]palmitate (Fig. 1C) and (iii) the Endo-H-sensitive band with the higher molecular weight is also detectable with non-acylated synaptotagmin (Fig. 2C, discussed below). Perhaps another, uniden-

tified protein modification is responsible for the increase in molecular weight.

Which of the described forms of synaptotagmin are palmitoylated in CV-1 cells? Cells expressing wild-type synaptotagmin were labelled for 120 min with [3 H]palmitate and the immunoprecipitated protein was treated with Endo-H prior to SDS-PAGE and fluorography. Inspection of the [3 H]palmitoylated protein showed three bands (Fig. 2C, see also Fig. 1C). The upper band is resistant to Endo-H, the lower two bands are sensitive to Endo-H. The unglycosylated form of tagmin is not palmitoylated. Thus, it is likely that palmitoylation occurs on the first, Endo-H-sensitive band, which is then processed to the later forms during the [3 H]palmitate labelling period. This is consistent with other studies showing that palmitoylation of transmembrane proteins occurs before acquisition of Endo-H resistance [14].

3.3. Processing of non-acylated synaptotagmin in CV-1 cells

Resistance of carbohydrate chains to Endo-H is due to the action of *N*-acetylglucosamine transferase, an enzyme located

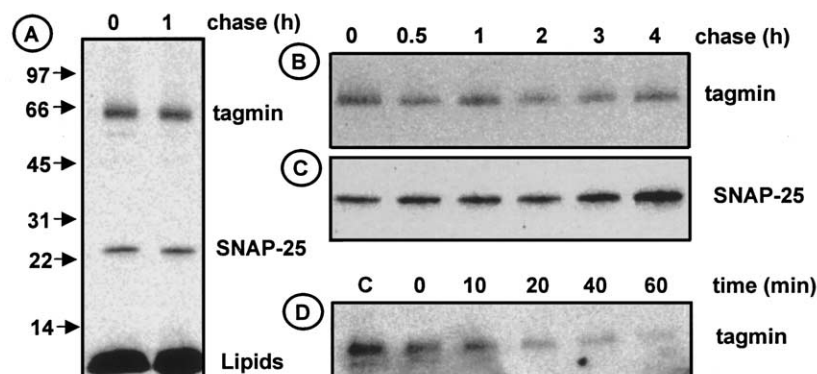


Fig. 4. Fatty acids are stably attached to tagmin and SNAP-25 in PC-12 cells. A: PC-12 cells containing endogenous synaptotagmin and SNAP-25 were labelled for 30 min with [3 H]palmitate and then chased for 0 or 1 h. SNAP-25 and tagmin were immunoprecipitated and subjected to SDS-PAGE and fluorography. B,C: PC-12 cells were labelled for 2 h and then chased for 0.5, 1, 2, 3 or 4 h. D: Protein synthesis in PC-12 cells was blocked with cycloheximide (50 μ g/ml) and cells were labelled simultaneously or 10, 20, 40 or 60 min later with [3 H]palmitate for 30 min. Synaptotagmin was then immunoprecipitated and subjected to SDS-PAGE and fluorography. C = no addition of cycloheximide.

in the medial Golgi apparatus [13]. Thus, palmitoylated synaptotagmin expressed in CV-1 cells passes the medial Golgi cisternae. This is consistent with other studies, where synaptotagmin expressed in CHO cells was transported to the plasma membrane [15].

Does the non-acylated mutant of tagmin follow the same intracellular pathway of processing? Because in Fig. 1B acylated and non-acylated tagmin show different band patterns, we conducted more detailed experiments. In contrast to the wild-type protein, unpalmitoylated tagmin is present after 1 h of labelling as only two bands, which represent the unglycosylated and Endo-H-sensitive form of the protein (Fig. 2D). In a pulse-chase experiment the second Endo-H-sensitive form of the protein is clearly visible only after 2 h of chase (Fig. 2E). However, with this mutant a fuzzy, Endo-H-resistant band fails to appear after 2 h of chase (Fig. 2E) and is only barely detectable after 16 h of labelling (Fig. 2F). Thus, transport of non-acylated synaptotagmin is impaired at a site prior to the medial Golgi complex.

Is the replacement of a single cysteine residue, which is accompanied by a drastic reduction in palmitoylation, sufficient to prevent processing of tagmin to the Endo-H-resistant form? Labelling with [³⁵S]methionine for 2 h showed the upper, fully processed band for every cysteine mutant except the completely non-acylated mutant 1-5 (Fig. 2G). Thus, only when all five cysteines are exchanged the intracellular transport of synaptotagmin is blocked.

Exchange of cysteine residues has a further effect on processing of synaptotagmin. It is apparent from Figs. 1B and 2B that the band with the lowest molecular weight, which we have identified as unglycosylated tagmin, is much more prominent in the cysteine mutants. Unglycosylated synaptotagmin most likely arises because the N-terminus of the protein is not translocated into the lumen of the endoplasmic reticulum, where core glycosylation occurs during translation. Palmitoylation is a post-translational event. Thus, increased occurrence of unglycosylated tagmin cannot be due to the loss of protein-bound fatty acids, but rather to exchange of the cysteine residues. Recently, it has been suggested that a helical hairpin is formed between the transmembrane domain and a N-terminal region that is required for efficient translocation [16]. Thus, exchange of cysteine residues in the transmembrane region might influence formation (or subsequent disassembly) of the helical hairpin and this might lead to inefficient translocation of the molecule. In support of this, mutant 5 shows only a very low amount of unglycosylated synaptotagmin, just like the wild-type protein. This particular mutant is the only one with an exchange of a cysteine not located in the TMR.

3.4. Targeting of wild-type and non-acylated synaptotagmin in PC-12 and RBL cells

The results so far indicate that non-acylated synaptotagmin is not properly processed in CV-1 cells. We therefore asked whether we could detect differences in intracellular targeting between palmitoylated and non-acylated tagmin when the proteins are expressed in PC-12 cells, a neurone-like cell line. Because transfection of PC-12 cells is inefficient and detailed metabolic labelling studies are difficult, we analysed subcellular targeting by fluorescence microscopy. Wild-type and non-acylated tagmin were equipped with a C-terminal myc tag and were transfected into NGF-differentiated PC-12 cells. Staining with myc antibodies and confocal laser micros-

copy clearly showed non-acylated tagmin at the plasma membrane, both in the cell body and at the tip of the neurites (Fig. 3A,B).

Secretory vesicles are not clearly visible in our immunofluorescence pictures of PC-12 cells. To analyse whether acylated and non-acylated tagmins are also transported to secretory organelles, we fused both proteins at the N-terminus to the GFP and transfected the constructs into RBL-2H3 cells, a mast cell line with prominent secretory granules. Targeting of synaptotagmin I to these organelles has already been demonstrated by cell fractionation [20]. Fluorescence microscopy showed that wild-type synaptotagmin is mainly present in numerous vesicular structures likely to represent secretory granules (Fig. 3C,D). A similar staining pattern was observed for non-acylated tagmin indicating that palmitoylation is not essential for transport to secretory organelles (Fig. 3E,F).

In summary, we have shown that processing of non-acylated tagmin (and probably also intracellular transport through the Golgi complex) is retarded in CV-1 cells, but the non-acylated protein is nevertheless targeted to the plasma membrane and secretory organelles in PC-12 and RBL cells. One possible explanation might be that there are differences in intracellular transport of non-acylated tagmin in cells without (CV-1) and with (PC-12 and RBL cells) the protein machinery for regulated secretion. Numerous interactions of synaptotagmin with neurone-specific proteins have been described and it is possible that they compensate for the lack of the cysteine residues and/or palmitoylation. Alternatively, intracellular transport of non-acylated tagmin through the Golgi complex might also be retarded in PC-12 and RBL cells, but is not obvious when analysing the total pool of tagmin in fluorescence studies.

Similar to published results [17] we observed that expression of synaptotagmin in PC-12 promotes neurite outgrowth. However, a difference between palmitoylated and non-acylated tagmin was not detectable (data not shown).

3.5. The majority of fatty acids are stably attached to synaptotagmin

Protein palmitoylation is thought to be a reversible process with cycles of palmitoylation and depalmitoylation. Depalmitoylation of proteins involved in signal transduction is almost quantitative and half-times for the fatty acid turn-over as fast as 15 min have been described [18]. In contrast, for other proteins, for example viral glycoproteins, no cleavage of fatty acids was detectable [14]. To analyse whether the fatty acids bound to synaptotagmin and to another synaptosomal protein (SNAP-25) turn-over rapidly, a pulse-chase experiment with [³H]palmitate using PC-12 cells was performed. Although there is some variation in the intensity of the bands, a significant and continuous reduction of [³H]palmitate labelling of both proteins is not detectable during the chase (see Fig. 4A–C). This indicates that the majority of fatty acids bound to synaptotagmin and SNAP-25 are stably attached.

Palmitoylation of a protein in the absence of protein synthesis is usually taken as an indication for reacylation of a previously deacylated protein [11]. To analyse whether palmitoylation of tagmin requires ongoing protein synthesis, PC-12 cells were treated with cycloheximide at different time points prior to labelling with [³H]palmitate. The intensity of [³H]palmitoylation of tagmin continuously decreases with the duration of the cycloheximide preincubation. After 20

min of preincubation almost no [^3H]palmitate labelling is detectable (see Fig. 4D). The [^3H]palmitate-labelled bands detectable at earlier time points most likely represent synaptotagmin molecules synthesised immediately before the cycloheximide block and passing the intracellular site of palmitoylation during the labelling period. Thus, significant turn-over of the fatty acids of synaptotagmin does not occur in PC-12 cells.

These results are surprising, because we and others have found that synaptosomes and even synaptic vesicles can acylate endogenous tagmin [7,19]. However, it is feasible that only some of the fatty acids are removed from each molecule or that only a small subpopulation of all molecules present in PC-12 cells is depalmitoylated. Furthermore, turn-over of fatty acids might require activation of synaptotagmin, for example during neurosecretion. Nevertheless, it is clear that there is a difference in depalmitoylation of neuronal SNARE proteins and proteins involved in signal transduction. Only the latter are significantly de- and reacylated, even in unstimulated cells.

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