Palmitoylation of the 25-kDa synaptosomal protein (SNAP-25) *in vitro* occurs in the absence of an enzyme, but is stimulated by binding to syntaxin

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The neuronal *N*-ethylmaleimide-sensitive-factor-attachment-protein receptor (SNARE) proteins 25-kDa synaptosomal protein (SNAP-25), syntaxin 1 and synaptobrevin 2 interact to form the intermembrane SNARE complex, which mediates docking and fusion of synaptic vesicles with the plasma membrane. Assembly of the SNARE complex is accompanied by conformational changes, especially in SNAP-25. SNAP-25 is palmitoylated *in vivo* at cysteine residues located in the middle of the molecule. Acylation is required for membrane binding or membrane targeting of this intrinsically hydrophilic protein. Palmitoylation of recombinant SNAP-25 was studied *in vitro* in the absence of an enzyme source with [³H]palmitoyl-CoA as the lipid donor. [³H]Palmitate incorporation into unbound SNAP-25 was negligible, but was stimulated 100-fold when SNAP-25 was present in the SNARE complex. SNAP-25 in a binary

complex with syntaxin 1 was palmitoylated with almost the same efficiency. A mutant of SNAP-25, which was not acylated *in vivo*, did not incorporate [³H]palmitate in this assay. [³H]Palmitate incorporation into wild-type SNAP-25 was blocked by chemical blocking of free SH groups, but slightly stimulated by reduction of disulfide-bonds. This shows that palmitoylation of SNAP-25 *in vitro* occurs at the same cysteine residues that are palmitoylated *in vivo*. This demonstrates that efficient palmitoylation of SNAP-25 depends on an interaction with a physiological binding partner. It suggests further that palmitoylation of SNAP-25 requires the α -helical conformation of the protein, which is induced by binding to syntaxin 1.

Key words: membrane fusion, neurosecretion, palmitoyl-coenzyme A, SNARE complex, synaptobrevin.

INTRODUCTION

Neuronal *N*-ethylmaleimide-sensitive-factor-attachment-protein receptor (SNARE) proteins have been identified as key players in neurosecretion. They are located on synaptic vesicles (synaptobrevin, also called vesicle-associated membrane protein, or VAMP) or on the presynaptic plasma membrane (syntaxin and 25-kDa synaptosomal protein, or SNAP-25). Intimate protein–protein interactions between the SNARE proteins might be responsible for the attachment and fusion of vesicles with the plasma membrane [1–3].

The crystal structure of the minimal core of the SNARE complex was resolved recently [4,5]. It consists of a four-helix bundle, with one helix from synaptobrevin, one from syntaxin and two from SNAP-25. In contrast to the form present in the complex, unbound SNAP-25 is unstructured. Thus a conformational change occurs in SNAP-25 during formation of the SNARE complex. The binary interaction between syntaxin and SNAP-25, which precedes the formation of the ternary SNARE complex, already induces the α -helical structure of SNAP-25 [6–8].

The two domains of SNAP-25 present in the minimal core complex are connected by a linker sequence, whose structure remains unknown. Its N-terminal part contains a cluster of four cysteine residues, which are palmitoylated *in vivo*. Fatty acids are required for membrane binding or membrane targeting of this intrinsically hydrophilic protein [9–13]. In contrast to other hydrophobic modifications, i.e. myristoylation or isoprenylation,

palmitoylation often is a dynamic event with cycles of acylation and deacylation [14].

The enzymology of protein palmitoylation is poorly understood. Two putative palmitoyl transferases have been purified recently [15,16]. The enzyme purified by Liu et al. [15] subsequently turned out to be a thiolase, an enzyme involved in β -oxidation of fatty acids in peroxisomes [17]. Furthermore, several enzymic activities extracted from microsomal membranes have been characterized, but none of them was purified to homogeneity [18–21].

On the other hand, some proteins are palmitoylated *in vitro* in the absence of an enzyme source when incubated with palmitoyl-CoA (Pal-CoA), which serves as an acyl donor in the palmitoylation reaction. This autocatalytic or non-enzymic palmitoylation often shows all the characteristics of authentic palmitoylation [22–24]. Pal-CoA is furthermore of interest because it has a stimulatory effect on transport of vesicles *in vitro*. More specifically, fatty-acyl-CoA is required for budding as well as for fusion of transport vesicles. This stimulatory effect might be due to protein palmitoylation, but the relevant targets for acylation are not known [25–27].

EXPERIMENTAL

Buffers

Buffers used in the purification of proteins were as follows: sonication buffer, 20 mM Tris/HCl/150 mM NaCl/1 mM

Abbreviations used: SNAP-25, 25-kDa synaptosomal protein; VAMP, vesicle-associated membrane protein; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF-attachment protein; SNARE, SNAP receptor; GST, glutathione S-transferase; DTT, dithiothreitol; TNE, Tris/NaCl/EDTA; TN, Tris/NaCl; Pal-CoA, palmitoyl-CoA.

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PMSF. Wash buffer for His-tagged proteins, 20 mM Tris/HCl/150 mM NaCl/0.5% Triton X-100/20 mM imidazole; elution buffer for His-tagged proteins, as for wash buffer but with 250 mM imidazole. Wash buffer for glutathione S-transferase (GST)-syntaxin, 20 mM Tris/HCl, 150 mM NaCl, 0.5% Triton X-100; elution buffer for GST-syntaxin, 20 mM Tris/HCl/10 mM reduced glutathione/0.5% Triton X-100. All buffers are adjusted to a pH of 7.4.

Buffers for the palmitoylation assay were as follows: TNE (Tris/NaCl/EDTA) buffer, 20 mM Tris/HCl (pH 7.4)/150 mM NaCl/5 mM EDTA; TNE/Triton buffer, TNE with 0.1% Triton X-100; TN (Tris/NaCl) buffer, TNE buffer without EDTA. SDS/PAGE sample buffer (non-reducing), 62.5 mM Tris/HCl (pH 6.8)/2% SDS/10% glycerol. The reducing sample buffer contained 5% β -mercaptoethanol.

DNA constructs and bacteria

The full-length version of each protein was used and contained an N-terminal tag, either GST (syntaxin) or 6 $\times$ histidine (SNAP-25, synaptobrevin). Thomas Söllner (Memorial Sloan Kettering Cancer Center, New York, U.S.A.) cloned rat SNAP-25, isoform B, into the pQE 9 vector (Qiagen, Hilden, Germany). Its predicted amino acid sequence is identical to the clone described by Oylar et al. [28]. The construction of a SNAP-25 deletion mutant has been described elsewhere [11]. It is cloned into the pQE 9 vector and has had 13 amino acids deleted and one (glutamic acid) inserted between Leu-81 and Leu-95. Most of the deleted region is located downstream of the SN1 fragment of SNAP-25, which is present in the minimal SNARE complex [5,8]. Only two amino acids present in the minimal core complex are deleted in the mutant (Gly-82 and Lys-83). Rat syntaxin, isoform 1B, is cloned in pGEX-KG vector (Pharmacia, Freiburg, Germany). Rat synaptobrevin, isoform 2, is cloned in the pQE 30 plasmid (Qiagen).

The constructs were transformed into M15 (pREP4) *Escherichia coli* cells (Qiagen). For induction of expression of foreign genes, isopropyl β -D-thiogalactoside was added until a final concentration of 1 mM was reached. The cells were grown either for 5 h at 37 °C (His6-SNAP-25 and His6-synaptobrevin) or overnight at 22 °C (GST-syntaxin). Cells were pelleted (10 min at 5000 g) and frozen at -80 °C or processed immediately.

Purification of recombinant proteins

All subsequent steps were performed on ice or at 4 °C. *E. coli* cell pellets were resuspended in sonication buffer (5 ml per 100 ml of bacterial culture medium) and cells were broken by sonication with an ultrasonic cell disrupter (three bursts at 5 W). Lysozyme (1 mg/ml) and DNase I (2 μ g/ml) were added and samples were incubated for 30 min prior to an additional sonication step. Triton X-100 was added to a final concentration of 0.5% and the solution was stirred for 20 min. Following another sonication step, cell debris was pelleted (20 min at 10000 g) and the supernatant was incubated for 1 h with Ni-NTA (nitrilotriacetic acid)-agarose (Qiagen) or glutathione-Sepharose (Sigma, Deisenhofen, Germany): 100 μ l of a 50% slurry per 500 μ l of supernatant were used. Sepharose or agarose with bound proteins were put into a column, which was subsequently washed three times with wash buffer. Proteins were eluted with elution buffer, dialysed against TNE with 0.1% Triton X-100 and stored at -80 °C for further studies. Protein concentration was determined with the DC protein assay kit II from Bio-Rad (München, Germany), which is based on the Lowry method. This procedure yields 0.5–2.5 μ g of protein per ml of culture volume. Purity of

proteins was > 90% as analysed by SDS/PAGE and Coomassie Brilliant Blue staining (see Figure 1, below).

Assembly of the SNARE complex and palmitoylation assay

Preparation of [3 H]Pal-CoA

Acyl-CoA synthetase from *Pseudomonas fragi* (10 μ l, 10 mg/ml in phosphate buffer, 20 units in 2.8 mg of protein; Boehringer-Mannheim, Mannheim, Germany) was incubated for 10 min at 28 °C in glass vessels with CoA (15 μ l, 10 mM in distilled water), [3 H]palmitic acid (125 μ Ci, 50–60 Ci/mmol, dissolved in 25 μ l of ethanol), ATP (1 mM, final concentration) and MgSO₄ (4 mM) in a final volume of 250 μ l of phosphate buffer (pH 7.4). The reaction was stopped by adding 1 ml acetonitrile/1 M phosphoric acid (9:1) and precipitated proteins were pelleted (10 min, 5000 g). The supernatant was extracted with 1 ml of toluene. The upper phase, which contained [3 H]palmitic acid, was discarded and the toluene extraction was repeated twice. The lower phase, which contained [3 H]Pal-CoA, but also traces of toluene, was removed. The glass vessels were then washed with 200 μ l of TNE/Triton buffer. This wash contained the highest concentration of [3 H]Pal-CoA and was used in the palmitoylation assay. Purity of the preparation was checked using TLC with Silica 60 plates and butanol/acetic acid/distilled water (8:3:3) as a solvent system.

Palmitoylation assay for unbound SNAP-25

His6-SNAP-25 (1 μ l = 5 μ g in TNE/Triton buffer) was added to 95 μ l of TNE buffer and the reaction was started with 5 μ l of [3 H]Pal-CoA. Proteins were precipitated with chloroform/methanol (1:1) and pelleted (15 min, 14000 g). Pellets were washed twice with methanol and resuspended in 30 μ l of non-reducing SDS/PAGE sample buffer and boiled for 5 min. Aliquots of the samples were analysed by fluorography for incorporation of [3 H]palmitate (20 μ l) and by Western blotting for bound SNAP-25 (5 μ l).

Assembly of the SNARE complex

GST-Syntaxin (10 μ g per assay) was incubated with 20 μ l of packed glutathione-Agarose for 3–5 h in TNE buffer containing 0.1% Triton X-100. The beads were washed twice with TNE/Triton buffer and incubated overnight at 4 °C with His6-SNAP-25 (20 μ g, wild-type or deletion mutant) and/or His6-VAMP (20 μ g) in a final volume of 700 μ l of TNE/Triton buffer. SNARE complexes were washed three times with TNE buffer without Triton and were resuspended in 95 μ l of TNE without Triton.

Disassembly of the SNARE complex

SNARE complexes were incubated for 1 h with purified His6-soluble N-ethylmaleimide-sensitive-factor (NSF)-attachment protein (α -SNAP; 14 μ g) and His6-NSF (13 μ g) in TN buffer supplemented with MgCl₂ (10 mM) and ATP (1 mM) or ATP γ S (1 mM). Beads with bound proteins were pelleted and supernatant and pellet were analysed by Western blotting with antiserum against SNAP-25.

Palmitoylation assay for SNARE complexes

Palmitoylation was started with the addition of 5 μ l of [3 H]Pal-CoA (50 nM final concentration, 0.25 μ Ci). The assay contained Triton X-100 at a final concentration of 0.005% due to the presence of this detergent in the Pal-CoA buffer. Samples were

incubated with gentle agitation for 30 min at 28 °C, if not indicated otherwise in the Figure legends. After the palmitoylation assay, unbound [3 H]Pal-CoA was removed by washing the beads three times with TNE/Triton buffer. Triton X-100 at a concentration of 0.1 % also stops palmitoylation (see Figure 5, below). Beads with bound proteins were then resuspended in 30 μ l of non-reducing sample buffer and boiled for 5 min. Aliquots of the samples were analysed by fluorography for incorporation of [3 H]palmitate (20 μ l) and by Western blotting for bound SNAP-25 (5 μ l).

Inhibition experiments shown below in Figure 3: SNARE complexes or monomeric SNAP-25 were incubated with *N*-ethylmaleimide (NEM) or dithiothreitol (DTT; both at a final concentration of 10 mM) for 20 min at 28 °C or heated at 60 °C for 20 min prior to addition of [3 H]Pal-CoA.

Palmitoylation of rhodopsin with or without the enzyme preparation *in vitro* was done exactly as described in [21]. Rhodopsin was also incubated with purified GST-syntaxin (0.1, 1 and 2.5 μ g) at a final concentrations of Triton X-100 of 0.001, 0.01 and 0.025 %, respectively.

SDS/PAGE, fluorography and Western blotting

Proteins were separated by SDS/PAGE in a 12 % gel using standard procedures. After SDS/PAGE was complete, the gels were fixed with 10 % methanol and 10 % acetic acid, washed with distilled water, and treated with 1 M sodium salicylate. The gels were then dried and exposed to X-ray film. Film-exposure times were between 5 and 15 days, if not indicated otherwise. Proteins were blotted to PVDF transfer membranes (Hybond-P, Amersham, Braunschweig, Germany) using a semi-dry blotter at 5 mA per cm². Membranes were probed for SNAP-25 with a polyclonal rabbit serum (1:2000 dilution) and anti-rabbit IgG coupled to peroxidase (1:5000 dilution; Sigma catalogue no. A 0545). Detection was done with the enhanced chemiluminescence kit (ECL Plus, Amersham). Film exposure times were between 1 and 30 s. Quantification of bands was carried out with an Epson scanner GT 9000 and Scan-Pack 3.0 software (Biometra, Göttingen, Germany). The amount of [3 H]palmitate incorporated into SNAP-25 as determined from the fluorogram was calculated relative to the amount of SNAP-25 present in the assay, as determined by Western blotting. X-Ray films with different exposure times were analysed for each experiment. The stoichiometry of SNAP-25 palmitoylation was estimated by liquid scintillation counting of gel slices as described in [21].

RESULTS

Recombinant His6-SNAP-25 was purified from *E. coli* cells (see Figure 1, upper panel), which synthesize proteins in a non-palmitoylated form, and incubated with [3 H]Pal-CoA. Samples were then precipitated and analysed by Western blotting with antibodies against SNAP-25 and by fluorography (see Figure 2). Incorporation of [3 H]palmitate into SNAP-25 was negligible; a faint band appeared only after 2 months of film exposure. This is in agreement with published results describing very low levels of SNAP-25 auto-acylation compared with that of other proteins [23].

Next it was investigated whether palmitoylation can be stimulated if SNAP-25 is bound to syntaxin 1 and synaptobrevin 2 prior to addition of [3 H]Pal-CoA. Purified GST-syntaxin was fixed on glutathione beads and SNARE complexes were assembled by adding purified His6-SNAP-25 and His6-synaptobrevin. The SNARE complexes assembled under these conditions could be disassembled with α -SNAP, NSF and ATP (see Figure 1, lower panel), which provides evidence that they are functional

Figure 1 Purification of functional SNARE proteins

Upper panel, Coomassie Brilliant Blue-stained gel showing the recombinant SNARE proteins purified from *E. coli*. Lower panel, assembly and disassembly of the ternary SNARE complex shows that the recombinant SNARE proteins are functional. Ternary SNARE complexes were assembled and incubated for 30 min at 37 °C with or without α -SNAP, NSF, ATP or ATP γ S as indicated. Beads with bound proteins were pelleted. Pellet and supernatant were analysed with antibodies against SNAP-25 by Western blotting. The SNARE complex is disassembled only in the presence of α -SNAP, NSF and ATP [29]. SNAP-25 is not dissociated completely from GST-Syntaxin under these conditions [40].

[29]. Assembled SNARE complexes were then incubated with [3 H]Pal-CoA under assay conditions identical to those for unbound SNAP-25. After the palmitoylation reaction, unincorporated [3 H]Pal-CoA was washed off and SNARE complexes were analysed for bound SNAP-25 and for [3 H]palmitate labelling. Fluorography showed significant [3 H]palmitoylation of SNAP-25 present in the SNARE complex with a signal appearing after a 5-day exposure (see Figure 2). The experiment was performed three times and the results were quantified by densitometer scanning, which revealed that [3 H]palmitoylation of SNAP-25 bound to synaptobrevin and syntaxin is approximately 100-fold greater than for unbound SNAP-25. The stoichiometry of palmitoylation of SNAP-25 was also calculated. Approx. 1–3 % of the SNAP-25 molecules present in the SNARE complex were modified with [3 H]palmitate. Published work on protein palmitoylation *in vitro* often reveals substoichiometric acylation, regardless of whether an enzyme preparation is used or if acylation proceeds non-enzymically [18–22]. Most probably, efficient palmitoylation of some proteins *in vitro* requires additional cofactors or certain conditions, which are not known. However, there are also several reports where stoichiometric palmitoylation is described in the absence of an enzyme source [23,24].

Binding of SNAP-25 to syntaxin precedes the formation of the ternary SNARE complex and is accompanied by a structural change in SNAP-25. The next experiment investigated whether palmitoylation of SNAP-25 requires binding to both syntaxin and synaptobrevin or if binding to syntaxin alone is sufficient for stimulation. The fluorogram in Figure 3 shows that [3 H]palmitoylation of SNAP-25 bound to syntaxin was almost as

Figure 2 Binding to syntaxin and synaptobrevin stimulates palmitoylation of SNAP-25 *in vitro*

Ternary SNARE complexes were assembled using glutathione beads, GST-syntaxin 1, His6-synaptobrevin 2 and His6-SNAP-25. Unbound SNAP-25 and bound SNAP-25 (SNAP-25 present in the SNARE complex) were incubated with [³H]Pal-CoA in the absence of an enzyme source. After the assay, samples were precipitated and resuspended in non-reducing sample buffer. Aliquots were analysed by Western blotting with antiserum against SNAP-25 and by fluorography. Exposures of 5 days and 2 months of the same gel are shown. Positions of molecular-mass markers are indicated.

Figure 3 Binding to syntaxin 1 is sufficient to stimulate palmitoylation of SNAP-25, but a mutant of SNAP-25 with the palmitoylation domain deleted is not acylated

Binary SNARE complexes (syntaxin plus SNAP-25 or syntaxin plus synaptobrevin) and the ternary SNARE complex (SNAP-25, syntaxin plus synaptobrevin) were assembled and incubated with [³H]Pal-CoA. Instead of the wild-type protein (WT), a SNAP-25 mutant (M) with a 13-amino acid deletion of the palmitoylation domain [11] was used in some samples. Aliquots were analysed by Western blotting and fluorography.

effective as palmitoylation of SNAP-25 present in the ternary complex. Compared with the approx. 100-fold stimulation by binding to syntaxin alone, only a moderate, but variable, increase (from 1.1- to 1.8-fold; 1.3-fold was the mean of three experiments) in the [³H]palmitate incorporation was observed with SNAP-25 bound to syntaxin and synaptobrevin.

Figure 4 GST-syntaxin does not acylate rhodopsin

Deacylated rhodopsin was incubated with [3 H]Pal-CoA, either alone (—), or in the presence of a partially purified protein acyltransferase (PAT) or increasing amounts of purified GST-syntaxin. Samples were then subjected to SDS/PAGE and fluorography. A 7-day exposure is shown.

These results suggest that palmitoylation of SNAP-25 proceeds non-enzymically, but the formal possibility that syntaxin is an enzyme which palmitoylates SNAP-25 is not excluded completely. To analyse whether syntaxin might be an acyltransferase, the photoreceptor rhodopsin was used as an alternative substrate protein. Depalmitoylated rhodopsin is a good substrate for our 100-fold-enriched preparation of a protein acyltransferase, which is derived from DEAE chromatography of membrane extracts (see Figure 4 and also [21] for details). However, incubation of rhodopsin with increasing amounts of purified GST-syntaxin at different concentrations of Triton X-100 did not lead to [3 H]palmitate incorporation into the photoreceptor above background levels (see Figure 4). Thus it is unlikely that syntaxin acts as an acylating enzyme.

A mutant of SNAP-25 with a deletion of the palmitoylation domain [11] was used to analyse whether palmitoylation *in vitro* occurs at the same cysteine residues that are palmitoylated *in*

vivo. The affinity of the mutant SNAP-25 to syntaxin was only decreased marginally compared with the wild-type SNAP-25 (Figure 3). This reveals that the palmitoylation domain is not essential for this protein–protein interaction. This is not surprising, because only two amino acids present in the minimal SNARE complex are deleted in the mutant (Gly-82 and Lys-83). However, despite binding to other SNARE proteins, no palmitoylation was detectable in mutant SNAP-25.

Pre-incubation of the SNARE complex with NEM, an agent that blocks free SH groups, also blocked [3 H]palmitate incorporation into wild-type SNAP-25. In contrast, treatment with the reducing agent DTT at low concentrations slightly increased [3 H]palmitate labelling (see Figure 5, left-hand panel). The extent of this stimulation depended on the SNAP-25 preparation used and was probably a result of reduction of disulphide bonds and the availability of additional SH groups to incorporate [3 H]palmitate.

Figures 2 and 4 also show a band at 60 kDa. A band with a similar molecular mass has been reported to consist of a disulphide-linked dimer of SNAP-25 [30]. Since this band disappeared if samples were boiled with reducing buffer prior to SDS/PAGE (Figure 1 and results not shown), it was likely to be the dimer. SNAP-25 monomers incorporated more [3 H]palmitate compared with dimers. This again underscores the importance of free SH groups for palmitoylation *in vitro*. The physiological role of SNAP-25 dimers, if any, is not known.

Palmitoylation of SNAP-25 in the SNARE complex was reduced, but not completely blocked, when the assay was performed on ice and was slightly enhanced at 37 °C compared with 28 °C. This is consistent with the non-enzymic character of the reaction. Heating of SNARE complexes at 60 °C prior to the assay still permitted acylation of SNAP-25 with slightly reduced efficiency (Figure 5, left-hand panel). SNARE complexes are resistant to high temperatures and treatment with detergent [7]. Addition of Triton X-100 at a final concentration of 0.025 % reduced, and at 0.2 % completely inhibited, acylation of SNAP-25 (Figure 5, right-hand panel). We have observed a similar effect of Triton X-100 on the auto-acylation of rhodopsin [21], but Bano et al. [24] observed a 1.8-fold increase for non-enzymic

Figure 5 Palmitoylation of SNAP-25 is inhibited by NEM and Triton-X 100 and reduced at low temperatures

Left-hand panel: ternary SNARE complexes were assembled, treated with NEM or DTT for 20 min at 28 °C and incubated with [3 H]Pal-CoA at different temperatures as indicated. Samples were then analysed by Western blotting and fluorography. Middle panel: unbound SNAP-25 was treated (+ NEM) or not (– NEM) with 10 mM NEM for 20 min at 28 °C, NEM was quenched with DTT (20 mM) and SNARE complexes were assembled and incubated with [3 H]Pal-CoA. Samples were then analysed by Western blotting and fluorography. Right-hand panel: palmitoylation assay with ternary SNARE complexes was performed in the presence of increasing concentrations of Triton X-100. Only the fluorogram is shown. The Triton X-100 concentration in the standard assay was 0.005%.

Figure 6 Analysis of the parameters of the reaction

Upper left-hand panel: kinetics of the reaction. Palmitoylation assay of the ternary SNARE complex was performed for different periods of time at 37 °C. Upper middle panel: dependency of palmitoylation on the [^3H]Pal-CoA concentration. Palmitoylation assay of the ternary SNARE complex was performed at increasing concentrations of [^3H]Pal-CoA for 30 min at 37 °C. Upper right-hand panel: dependency of palmitoylation on the SNAP-25 concentration. The ternary SNARE complex was assembled with fixed amounts of GST-syntaxin (10 μg) and His6-synaptobrevin (20 μg), but with increasing amounts of His6-SNAP-25. SNARE-complexes were then incubated for 30 min at 37 °C with [^3H]Pal-CoA. Left-hand and middle graphs: relative palmitoylation (%) is plotted against incubation time and [^3H]Pal-CoA concentration (where a linear regression of the data is included in the graph), respectively. The data point at 90 min and 100 mM [^3H]Pal-CoA does not have an error bar because it was defined as 100% relative palmitoylation. Right-hand graph: Δ , binding of SNAP-25 to the SNARE complex (%); $\blacktriangle$, palmitoylation of SNAP-25 (%). Each experiment was performed at least three times. Representative examples are shown in the upper panels of the Figure. Results were quantified by densitometric scanning of blots and fluorograms. Note that the data points plotted on the y axes were taken from a variety of experiments, using different incubation times, [^3H]Pal-CoA concentrations and amounts of SNAP-25.

palmitoylation of myristoylated Yes-peptides in the presence of 0.1% Triton X-100.

[^3H]Palmitate labelling of SNAP-25 *in vitro* was sensitive to treatment with hydroxylamine at neutral pH and to boiling in buffer containing β -mercaptoethanol (results not shown). Thus palmitoylation of SNAP-25 *in vitro* shows identical characteristics to the acylation in cells [11].

It was also tested whether the cysteine residues are buried in monomeric SNAP-25 and therefore unavailable for palmitoylation. In order to do so, unbound SNAP-25 was incubated with 10 mM NEM. The reaction was then quenched by adding 20 mM DTT, and SNARE complexes were assembled and incubated with [^3H]Pal-CoA. However, adding NEM to monomeric SNAP-25 prevented subsequent palmitoylation, indicating that cysteine residues were already exposed in monomeric SNAP-25 (Figure 5, middle panel).

Finally, the parameters of the palmitoylation reaction were analysed in more detail. When the ternary SNARE complex was incubated with [^3H]Pal-CoA for different periods of time, labelling of SNAP-25 increased until saturation was reached after 60–90 min of incubation (see Figure 6, left-hand panel).

Using fixed SNAP-25 and Triton X-100 concentrations and increasing the amount of [^3H]Pal-CoA from 5 to 100 nM, the reaction was linear but did not reach saturation (Figure 6, middle panel). This is consistent with the data from Bano et al. [24]. Saturation of auto-acylation was observed by Duncan and Gilman [23] at a [^3H]Pal-CoA concentration of approx. 800 μM . In the SNARE-protein assay it was not possible to further

increase the [^3H]Pal-CoA concentration due to the inhibitory effect of Triton X-100 on the reaction.

Binding of His6-SNAP-25 to GST-syntaxin and His6-synaptobrevin reached saturation at approx. 20 μg of SNAP-25 added for assembly of the SNARE complex (Figure 6, right-hand panel, Δ). Palmitoylation of SNAP-25 also increased with increasing amounts of SNAP-25 bound to the SNARE complex (Figure 6, right-hand panel, $\blacktriangle$).

DISCUSSION

These results provide evidence that palmitoylation of SNAP-25 *in vitro* occurs in the absence of an enzyme source, but efficient acylation requires interaction with its physiological binding partner, the SNARE protein syntaxin 1. Non-enzymic palmitoylation dependent on protein–protein interactions has also been described for α -subunits of heterotrimeric G-proteins, which is stimulated 5-fold by binding to the β - and γ -subunits [23]. The most likely explanation for this observation would be that palmitoylation of SNAP-25 *in vitro* requires the α -helical conformation of the protein, which is induced by binding to syntaxin 1. The role of this observation remains to be defined, but its extent, a 100-fold stimulation, suggests that it is physiologically meaningful.

The requirements for palmitoylation of SNAP-25 and its homologues were analysed recently *in vivo*. Vogel and Roche [31] found a correlation between the extent of palmitoylation and the ability of SNAP-23 or SNAP-25 to bind to syntaxin. This is in

agreement with the *in vitro* data presented here. In contrast, using fragments of SNAP-25 fused to the green fluorescent protein, Gonzalo et al. [32] mapped the structural requirements for palmitoylation of SNAP-25 to residues 85 to 120, a region of the molecule that does not include its syntaxin-binding domain. This result suggests that palmitoylation of SNAP-25 *in vivo* does not require binding to syntaxin or other SNARE proteins. Instead, a short sequence (QPXRV) 24 amino acids distal to the palmitoylation region was identified as critical for palmitoylation. This sequence is thought to bind SNAP-25 to a hypothetical membrane protein, an interaction that might trigger a change in the molecule, allowing subsequent palmitoylation. Although the data presented here and by Gonzalo et al. [32] seem to be contradictory, the different molecular requirements for palmitoylation of SNAP-25 might reflect two different palmitoylation events occurring at different intracellular sites. It is possible that the requirement for the QPXXV sequence reflects palmitoylation of freshly synthesized SNAP-25 on internal membranes [13,33]. The here-described stimulation of SNAP-25 palmitoylation might occur in the synapse during assembly of the SNARE complex. This is consistent with recent data showing that palmitoylation of SNARE proteins is detectable in isolated synaptosomes and synaptic vesicles ([34] and M. Veit, A. Becher and G. Ahnert-Hilger, unpublished work).

The above-mentioned assumption implies that palmitoylation of SNAP-25 is dynamic. Indeed, turnover of SNAP-25-bound fatty acids was described recently [12]. The overall rate of fatty acid cleavage was low, but it might be higher if only the subpopulation of SNAP-25 molecules involved in a fusion event is considered. After depalmitoylation, SNAP-25 remains attached to the membrane by binding to other proteins, for example to syntaxin 1. This is consistent with the observation that SNAP-25 stays membrane-bound after chemical cleavage of the fatty acids [13].

It is tempting to speculate that palmitoylation of SNAP-25 occurs each time during assembly of a SNARE complex. This would imply that the fatty acids play a role during docking and fusion of synaptic vesicles. The acyl chains might perturb the lipid bilayer, which could facilitate opening of a fusion pore. Although non-palmitoylated SNARE proteins purified from bacteria are sufficient to cause membrane fusion *in vitro* [35], the rate of fusion is too low to account for the fast kinetics of neurosecretion. An alternative but not exclusive theory is that reversible palmitoylation may be a modification to charge the SNAP-25 molecule with energy. A thioester bond between a cysteine residue and an acyl chain stores almost the same amount of energy as a phosphodiester bond, e.g. in ATP. This energy might fuel the fusion reaction or might enable otherwise endothermic interactions with other proteins.

Recently the structural similarity between the SNARE complex and the active form of viral fusion proteins was pointed out, an observation which suggests a common mechanism for membrane fusion [36]. In this regard it is interesting to note that almost every viral fusion protein is palmitoylated [14] and that a modulatory function of the protein-bound fatty acids on the fusion process has been reported [37–39].

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