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Posttranslational Modification of Tubulin by Palmitoylation: II. Identification of Sites of Palmitoylation

Juris Ozols* and Joan M. Caron†‡

Departments of *Biochemistry and †Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030

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As shown in the companion article, tubulin is posttranslationally modified in vivo by palmitoylation. Our goal in this study was to identify the palmitoylation sites by protein structure analysis. To obtain quantities of palmitoylated tubulin required for this analysis, a cell-free system for enzymatic [^{3}H]palmitoylation was developed and characterized in our companion article. We then developed a methodology to examine directly the palmitoylation of all 451 amino acids of α-tubulin. ^{3}H-labeled palmitoylated α-tubulin was cleaved with cyanogen bromide (CNBr). The CNBr digest was resolved according to peptide size by gel filtration on Sephadex LH60 in formic acid:ethanol. The position of ^{3}H-labeled palmitoylated amino acids in peptides could not be identified by analysis of the Edman degradation sequencer product because the palmitoylated sequencer products were lost during the final derivatization step to phenylthiohydantoin derivatives. Modification of the gas/liquid-phase sequencer to deliver the intermediate anilinothiozolinone derivative, rather than the phenylthiohydantoin derivative, identified the cycle containing the ^{3}H-labeled palmitoylated residue. Therefore, structure analysis of peptides obtained from gel filtration necessitated dual sequencer runs of radioactive peptides, one for sequence analysis and one to identify ^{3}H-labeled palmitoylated amino acids. Further cleavage of the CNBr peptides by trypsin and Lys-C protease, followed by gel filtration on Sephadex LH60 and dual sequencer runs, positioned the ^{3}H-labeled palmitoylated amino acid residues in peptides. Integration of all the available structural information led to the assignment of the palmitoyl moiety to specific residues in α-tubulin. The palmitoylated residues in α-tubulin were confined to cysteine residues only. The major site for palmitoylation was cysteine residue 376.

INTRODUCTION

Tubulin, the major subunit of microtubules, is palmitoylated (Caron, 1997). Other studies show that palmitoylated proteins interact with membranes (for review, see Bizzozero *et al.*, 1994b). Thus, it is possible that palmitoylation of tubulin creates a novel species that is capable of direct interaction with membranes. To begin to understand the mechanism of this interaction and the function of palmitoylated tubulin, we first sought to identify the sites of palmitoylation on the α-tubulin subunit.

Most palmitoylated proteins contain one to three palmitoyl residues, although in some cases much higher levels occur (for review, see Huang *et al.*, 1988; Schlesinger *et al.*, 1993; Gundersen *et al.*, 1994). Cysteine residues are the primary sites of palmitoylation. However, serine, threonine, and lysine residues may also be palmitoylated (for review, see Bizzozero *et al.*, 1994b; Hackett *et al.*, 1994; Stanley *et al.*, 1994).

The most widely used method for determining sites of palmitoylation is site-directed mutagenesis. Although this is a reasonable approach for proteins that

‡ Corresponding author.

contain only a few cysteines, porcine brain α-tubulin contains 12 cysteine residues (Ponstingl *et al.*, 1981). In addition, chemical cleavage studies of palmitoylated tubulin demonstrate that at least some of the palmitate is covalently linked to cysteine residues, but the possibility of linkage to serine, threonine, or lysine residues could not be eliminated (Caron, 1997).

Direct protein structure analysis is the only approach to unequivocally identify sites of palmitoylation. Although there have been some successes, this approach has been problematic. First, it can be difficult to purify quantities of palmitoylated proteins required for protein structure analysis. This is due, at least in part, to the fact that palmitoylated proteins are susceptible to depalmitoylation during purification from cell extracts (Wedegaertner and Bourne, 1994). Second, protein structure analysis of palmitoylated proteins is often difficult because their increased hydrophobicity can lead to irreversible association with the column supports used in high-performance liquid chromatography (HPLC;[1] Ovchinnikov *et al.*, 1988).

The companion article (Caron, 1997) describes a cell-free system for the enzymatic palmitoylation of tubulin. By using this cell-free system, we first developed a method for the purification of palmitoylated tubulin in quantities amenable for protein structure analysis. Second, we developed a method for the isolation and sequence analysis of α-tubulin fragments by means of gel filtration in organic solvents.

MATERIALS AND METHODS

Materials

Detergents, enzyme substrates, cofactors, chromatographic media, and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise. Iodoacetic acid was obtained from Fluka (Buchs, Switzerland). Trypsin was obtained from Worthington (Freehold, NJ). *Achromobacter* Lys-C protease was obtained from Biochemical Diagnostics (Edgewood, NY). Cyanogen bromide was obtained from Pierce Chemicals (Rockford, IL). Solvents for HPLC and gel filtrations were from Burdick & Jackson (Muskegon, MI).

Cell-Free Palmitoylation of Tubulin

Porcine brain microtubule protein and the rat liver membrane extract were prepared as described in the companion article (Caron, 1997). Microtubule protein (1 mg/ml) was incubated with 200 μM CoA, 2 mM ATP, [9,10-^{3}H]palmitate (50 μCi/ml, specific activity, 52 Ci/mmol, New England Nuclear, Boston, MA), and the rat liver membrane extract (0.35 mg/ml) in incubation buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.15% Triton X-100) in a volume of 2 ml. After 2 h at 30°C, protein was precipitated with chloroform:methanol (1:2) as described by Wessel and Flügge (1984). Precipitated protein was stored overnight in methanol at −20°C.

[1] Abbreviations used: ATZ, anilinothiozolinone; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

Purification of α-Tubulin

Precipitated protein was solubilized in sample buffer (0.25 M Tris, pH 6.8, 10% glycerol, 2% SDS, 10 mM dithiothreitol, 0.2 mg/ml bromphenol blue) and heated at 100°C for 5 min. Proteins were purified by SDS-PAGE and electroelution using a Bio-Rad Model 491 Prep Cell. Briefly, SDS-PAGE was carried out with the 28-mm ID Prep Cell column using a running gel (8.5% acrylamide/0.75% bisacrylamide) of 7 cm and a stacking gel (2% acrylamide/0.18% bisacrylamide) of 0.5 cm. The running buffer contained 50 mM Tris base, 0.38 M glycine, 0.1% SDS, and 1 mM thioglycolate. Electrophoresis was performed at 150 V. When the dye front eluted from the gel, fractions were collected (80 drops/tube) at a rate of 1 ml/2.5 min. These conditions gave optimal separation of α- and β-tubulin without excessive dilution of proteins. Effluent fractions were monitored at 280 nm and samples were subjected to SDS-PAGE (Laemmli, 1970), followed by silver staining and fluorography to identify fractions containing ^{3}H-labeled palmitoylated α-tubulin. These fractions were pooled and protein was concentrated by means of Sephadex G-200 beads and precipitated with chloroform:methanol (1:2; Wessel and Flügge, 1984).

Cyanogen Bromide (CNBr) Cleavage

Precipitated protein was reduced and alkylated as follows. The sample was solubilized in 0.25 ml of 0.2 M Tris (pH 8.5), 6 M guanidine hydrochloride, 3 mM EGTA, and 60 mM dithiothreitol and flushed with N_2. After incubation for 60 min at 60°C, iodoacetic acid (6.5 mg in 25 μl of 50 mM Tris, pH 8.5) was added, and the sample was incubated in the dark at room temperature. After 30 min, the sample was dialyzed against 20 mM Tris (pH 7.4) overnight at room temperature and in the dark. Protein was precipitated with 10% trichloroacetic acid and then solubilized in 70% formic acid. CNBr was added to a final concentration of 100 μg/ml. Cleavage at methionyl residues proceeded under N_2 for 24 h at 4°C.

Enzymatic Cleavages

Enzymatic cleavages with trypsin and Lys-C protease were performed as described previously (Ozols, 1990a).

Sequence Analysis

Protein and peptide hydrolysis for amino acid analysis was performed with 6 M HCl in the gas phase at 150°C for 1 h (Ozols, 1990b). Peptide mixtures were separated by size on a 1.5 × 100-cm column of Sephadex LH60 equilibrated with formic acid:ethanol, 3:7 (vol/vol), as the solvent. Sequence analysis was carried out on an Applied Biosystems model 470A sequencer equipped with a model 120A phenylthiohydantoin (PTH) analyzer. Fifty percent of the sample was subjected to PTH-amino acid analysis and 50% was used for ^{3}H-labeled palmitoylated (anilinothiozolinone, ATZ)-derivative analysis. The latter was achieved by modification of the sequencer to deliver the ATZ derivatives directly to the sequencer fraction collector without conversion to PTH derivatives. Radioactivity of ^{3}H-labeled palmitoylated ATZ derivatives, released after each Edman degradation cycle, was counted with a Packard scintillation counter.

RESULTS

Cell-Free Palmitoylation of Tubulin

To establish a methodology for protein structure analysis of palmitoylated tubulin, we decided to focus on α-tubulin. This decision was based on the fact that a putative consensus sequence for palmitoylation of several G protein-linked receptors and the growth cone protein, GAP-43, (Strittmatter *et al.*, 1990) is

found in every known sequence of α-tubulin. Microtubule protein was enzymatically ^{3}H-labeled palmitoylated in the cell-free system as described in MATERIALS AND METHODS. After 2 h at 30°C, the reaction was stopped by the addition of chloroform:methanol (1:2). The following experiment indicated that palmitoylation of tubulin did not continue after denaturation of protein with chloroform:methanol. Microtubule protein was palmitoylated with [^{3}H]palmitate, CoA, and ATP both in the presence and absence of membrane extract. A third sample contained [^{3}H]palmitate, CoA, ATP, and membrane extract to generate [^{3}H]palmitoyl-CoA, but no microtubule protein. After incubation for 2 h at 30°C, the three samples were processed under conditions identical to those used for preparative electrophoresis. Specifically, 9 volumes of chloroform:methanol (1:2) were added to each of the three samples. At this point, microtubule protein, incubated for 2 h at 30°C, was added to the third sample containing [^{3}H]palmitate, membrane extract, and chloroform:methanol. Precipitated proteins from all three samples were washed (Wessel and Flügge, 1984), stored overnight at −20°C, dried down with N_2, and processed for SDS-PAGE and fluorography. From Coomassie blue staining, all three samples contained similar amounts of tubulin protein (Figure 1). The corresponding autoradiograph was overexposed to detect low levels of ^{3}H-labeled palmitoylated tubulin. In the complete reaction, significant palmitoylation of tubulin occurred (Figure 1, lane 1). When the membrane extract was omitted, a low level of nonenzymatic palmitoylation of tubulin was detected (Figure 1, lane 2) as described previously (Caron, 1997). In the third sample, palmitoylated membrane proteins were observed as expected (Figure 1, lane 3). As described in the preceding article, two membrane proteins were enzymatically palmitoylated: one protein that ran slightly slower than α-tubulin (termed mem1) and one that ran slightly faster than β-tubulin (termed mem2) upon SDS-PAGE. This is seen by comparing the positions of ^{3}H-labeled palmitoylated α- and β-tubulin in lane 2 with ^{3}H-labeled palmitoylated membrane proteins in lane 3. However, palmitoylation of tubulin was not detected after denaturation of the protein by addition of chloroform:methanol (Figure 1, lane 3).

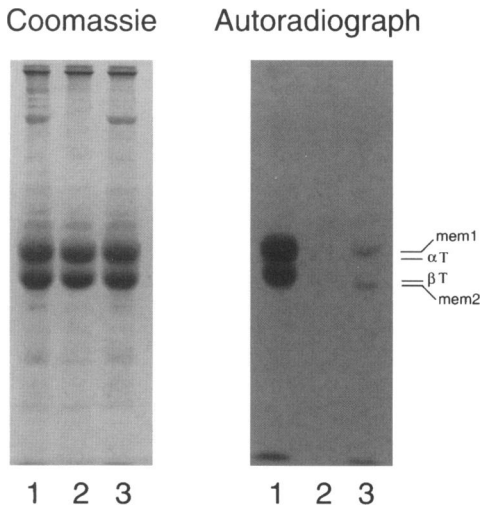

Figure 1. Tubulin is not palmitoylated after denaturation of the protein. In lane 1, microtubule protein (1 mg/ml) was incubated with [^{3}H]palmitate, CoA, ATP, and membrane extract (0.35 mg/ml) for 2 h at 30°C. The reaction was stopped by the addition of chloroform:methanol (1:2) and processing for SDS-PAGE and fluorography as described in MATERIALS AND METHODS. In lane 2, microtubule protein (1 mg/ml) was incubated with [^{3}H]palmitate, CoA, and ATP (but no membrane extract) for 2 h at 30°C before addition of chloroform:methanol and processing for SDS-PAGE and fluorography. In lane 3, membrane extract (0.35 mg/ml) was incubated with [^{3}H]palmitate, CoA, and ATP. After 2 h at 30°C, chloroform:methanol was added followed by addition of microtubule protein (1 mg/ml) that had been preincubated for 2 h at 30°C. The sample was then processed for SDS-PAGE and fluorography. A Coomassie blue-stained gel and corresponding autoradiograph (7-d exposure) are shown. Positions of ^{3}H-labeled palmitoylated α- and β-tubulin from lane 2 and ^{3}H-labeled palmitoylated membrane proteins (mem1 and mem2) from lane 3 are indicated.

Purification of α-Tubulin

After cell-free palmitoylation, α-tubulin was purified by SDS-PAGE and electroeluted using a Bio-Rad Prep Cell as described in MATERIALS AND METHODS. Samples from eluted fractions were analyzed by SDS-PAGE. Figure 2A is a silver-stained gel of fractions, and Figure 2B is the corresponding autoradiograph showing ^{3}H-labeled palmitoylated α- and β-tubulin. Fractions containing α-tubulin were pooled, concentrated, reduced, and alkylated as described in MATERIALS AND METHODS. Reduction with 60 mM dithiothreitol and subsequent alkylation did not cause a loss of radioactivity from ^{3}H-labeled palmitoylated tubulin; this was determined in a separate set of experiments in which SDS-PAGE and fluorography was used to compare the levels of ^{3}H-labeled palmitoylated tubulin before and after reduction and carboxymethylation. The yield and stoichiometry of palmitate labeling of α-tubulin was determined. Starting with 2 mg of microtubule protein, we obtained 150–200 μg of purified α-tubulin. There was approximately one [^{3}H]palmitate per three α-tubulin molecules.

CNBr Cleavage

Purified α-tubulin was precipitated with trichloroacetic acid, solubilized with formic acid, and cleaved with CNBr (see MATERIALS AND METHODS). None of these procedures resulted in a loss of radioactivity from α-tubulin. Attempts to isolate CNBr peptides by HPLC were unsuccessful, most likely because of the

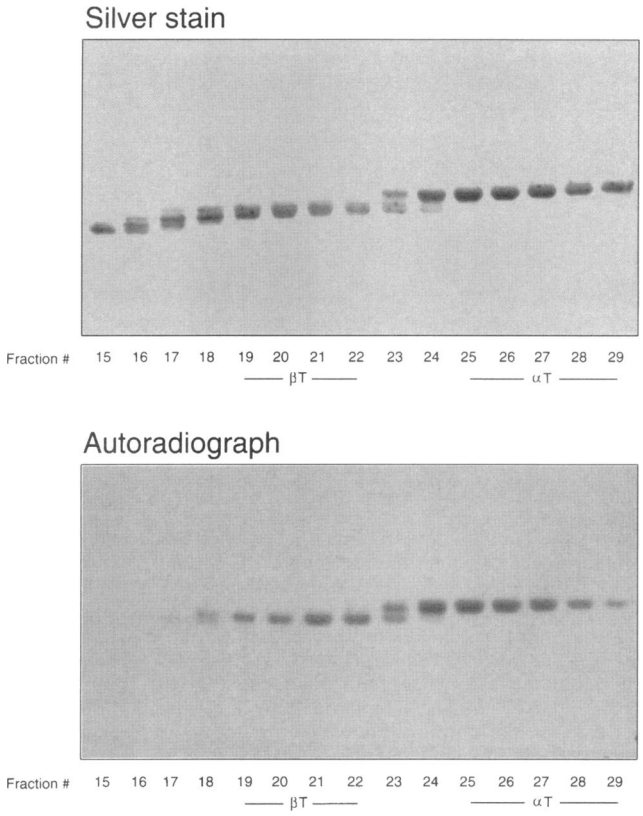

Figure 2. Purification of α-tubulin. After cell-free ^{3}H-labeled palmitoylation, α-tubulin was purified by SDS-PAGE and electroelution as described in MATERIALS AND METHODS. (A) Silver-stained gel of fractions after electroelution. (B) Corresponding autoradiograph (11-d exposure). Fraction numbers of eluted samples are indicated, as are the fractions containing purified α-tubulin (αT) and β-tubulin (βT).

hydrophobic nature of palmitoylated peptides. Therefore, peptides were separated by size with gel filtration on Sephadex LH60 (Figure 3A). Automated Edman degradation was used to confirm the identity of eluted peptides. All 10 of the expected CNBr peptides from α-tubulin were recovered as well as 67% of the radioactivity. As expected, peptides were eluted from the column according to size with one exception: the 11-residue peptide from residues 303–313 was eluted before the 12-residue peptide from residues 414–425. As shown in Figure 3B, there was one major peak of radioactivity corresponding to peptide 314–377. Three minor peaks of radioactivity contained all but one of the remaining CNBr peptides; peptide 414–425 was not radioactive.

Identification of Palmitoylated Amino Acids

We next sought to determine whether cysteines, serines, threonines, or lysines in the CNBr peptides contained covalently bound [^{3}H]palmitate by means of automated Edman degradation. This, however, could not be accomplished by conventional means, that is, by analysis of the final sequence products (PTH derivatives): palmitoylated amino acids were presumably lost during the final conversion step of the automated Edman degradation. To circumvent this problem, the gas/liquid-phase sequencer was modified to deliver the intermediate sequencing products (ATZ derivatives) to the sequencer fraction collector for tritium content determination. Therefore, analysis of CNBr peptides required dual sequencer runs: one to identify amino acids (PTH derivatives) and one to detect radioactivity (ATZ derivatives).

To identify palmitoylated amino acids, dual sequencer runs were performed on the CNBr peptides. Sequencer analysis of radiolabeled ATZ derivatives from the short (three or four residues) palmitoylated peptides was not successful; these peptides were not sufficiently retained on the Polybrene-coated fiberglass matrix due to their increased hydrophobicity. This resulted in a continuous release of radioactivity due to washout of the labeled peptides during degradation cycles. Coupling of labeled peptides to aryl membranes via the carboxyl terminus was not useful because the solid-phase sequencer could not be modified to collect the ATZ derivatives, nor could it be modified to extract the very hydrophobic palmitoyl-PTH derivatives for identification or counting purposes. Thus, the specific activity of a particular residue depended on peptide length and the position of the labeled residue in the peptide. Nevertheless, data obtained from dual sequencer runs demonstrated that only cysteine residues were labeled; there was no evidence of palmitoylation of serines, threonines, or lysines. This eliminated the possibility that peptides 378–398, 399–413, or 426–451 contained palmitoylated residues.

Identification of Palmitoylated Cysteine Residues

Because peptide 314–377 contained significantly higher levels of [^{3}H]palmitate than the other peptides (Figure 3), we decided to focus on it. This CNBr peptide contained four cysteine residues. To determine which of these four residues were palmitoylated, peptide 314–377 was subcleaved with trypsin. Peptides were resolved according to size by gel filtration on Sephadex LH60 (Figure 4). All nine of the expected fragments were recovered.

Because of the small size of the tryptic peptides, we expected those with covalently attached [^{3}H]palmitate to elute from the Sephadex LH60 column two fractions earlier than their nonpalmitoylated counterparts (as described above, approximately 30% of the α-tubulin molecules were ^{3}H-labeled palmitoylated). This was confirmed by dual sequencing runs described above: the first run identified the elution positions of peptides

A

PEPTIDE	RESIDUES	IDENTIFIED CNBr PEPTIDES	FRACTION #
CB-1	37-154	PSDKTIGGGDDSFNTFFSETGAGKH VPRAVFVDLEPTVIDEVRTGTYRQL FHPEQLITGKEDAANNYARGHYTIG KEIIDLVLDRIRKLADQCTGLQGFS VFHSFGGGTGSGFTSLLM	14
CB-2	204-302	VDNEAIYDICRRNLDIERPTYTNLNR LIGQIVSSITASLRFDGALNVDLTEF QTNLVPYPRAHFPLATYAPVISAEK AYHEQLSVAEITNACFEPANQM	14
CB-3	314-377	ACCLLYRGDVVPKDVNAAIATIKTK RTIQFVDWCPTGFKVGINYEPPTVV PGGDLAKVQRAVCM	17
CB-4	155-203	ERLSVDYGKKSKLEFSIYPAPQVST AVVEPYNSILTTHTTLEHSDCAFM	19
CB-5	1-36	M RECISIHVGQAGVQIGNACWELY CLEHGIQPDGQM	22
CB-6	426-451	AALEKDYEEVGVDSVEGEGEEEGE EY	24
CB-7	378-398	LSNTTAIAEAWARLDHKFDLM	24
CB-8	399-413	YAKRAFVHWYVGEGM	26
CB-9	303-313	VKCDPRHGKYM	26
CB-10	414-425	EEGEFSEAREDM	28

B

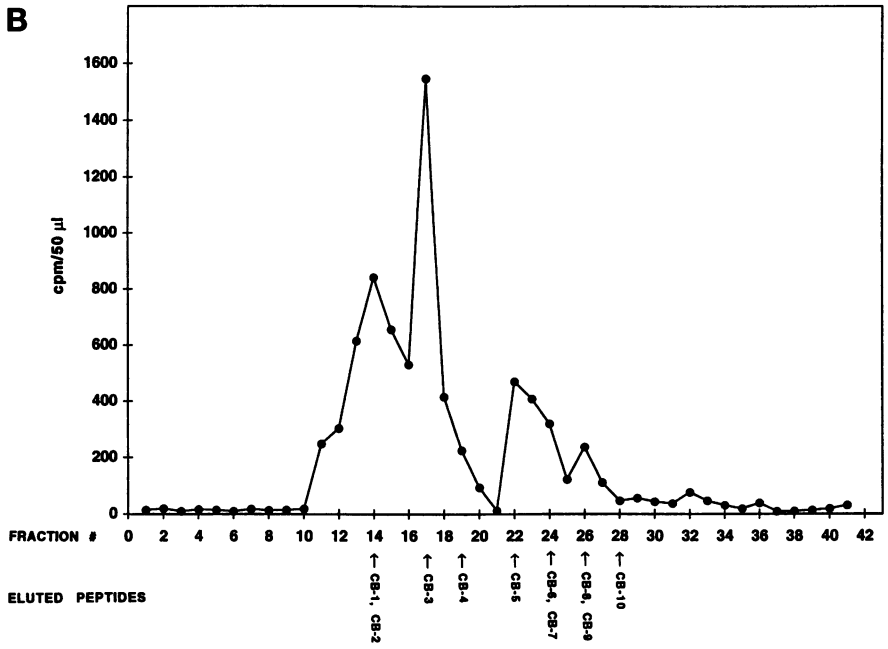

Figure 3. Gel filtration of CNBr peptides of α-tubulin on Sephadex LH60. A sample of purified α-tubulin, of which approximately 30% was ^{3}H-labeled palmitoylated, was cleaved with CNBr as described in MATERIALS AND METHODS. The CNBr digest (7 nmol containing 5.1×10^5 cpm) was applied to a Sephadex LH60 column equilibrated with formic acid:ethanol (3:7), and peptides were resolved according to size as described in MATERIALS AND METHODS. Fractions of 3.4 ml were collected at a flow rate of 3.5 ml/h. Fractionation was monitored by absorbance at 280 nm and by radioactivity. (A) Amino acid sequence of CNBr peptides recovered after gel filtration. (B) Radioactivity of eluted fractions after gel filtration. Positions of eluted CNBr peptides are indicated by arrows.

with the PTH derivatives, and the second run identified the sequencing cycle that released radioactive ATZ derivatives. Since studies described above demonstrated that only cysteine residues were palmitoylated, this second run also confirmed the identity of the palmitoylated peptide.

Approximately 90% of the radioactivity was recovered after gel filtration. Most of the radioactivity was eluted from the Sephadex LH60 column in fraction 30, which corresponded to the 4-amino acid peptide AVCM (peptide 374–377). Radioactivity was released from peptides in fraction 30 after three sequencing cycles demonstrating that [^{3}H]palmitate was covalently bound to Cys-376. A similar analysis was performed with the remaining fractions. Little palmitoylation occurred at cysteine residues 315, 316, or 347.

A

Parent peptide CB-3: ACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDW
CPTGFKVGINYEPPTVVPGGDLAKVQRAVCM

Peptide	Residues	Identified tryptic peptides	Fraction # for nonpalmitoylated peptides	Fraction # for ^{3}H-palmitoylated peptides
T-1	353-370	VGINYEPPTVVPGGDLAK	25	-
T-2	340-352	TIQFVDWCPTGFK	27	-
T-3	327-336	DVNAAIATIK	28	-
T-4	314-320	ACCLLYR	29	-
T-5	321-326	GDVVPK	29	-
T-6	374-377	AVCM	32	30
T-7	371-373	VQR	32	-
T-8	337-338	TK	32	-
T-9	339	R	32	-

B

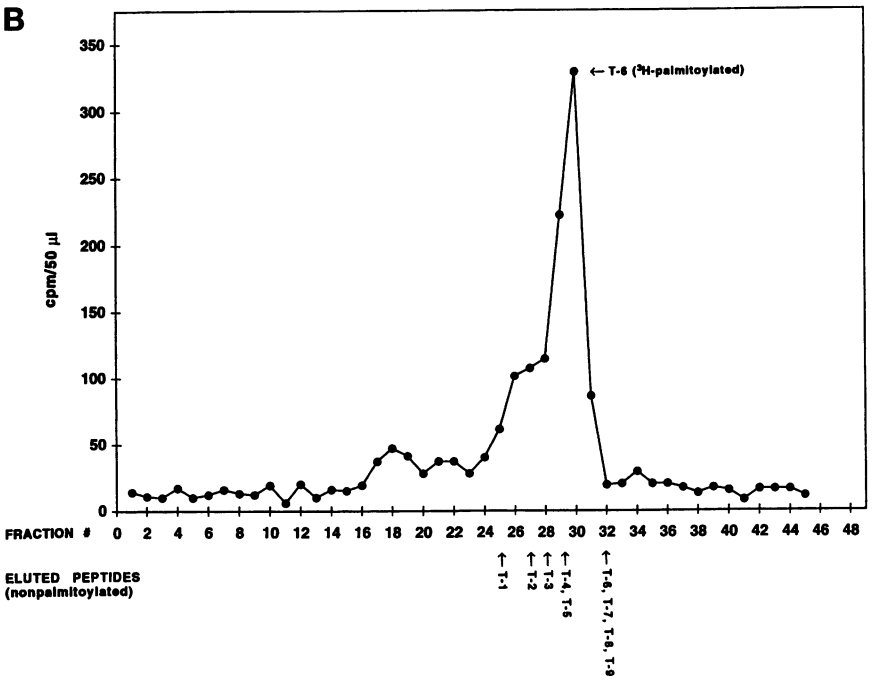

Figure 4. Gel filtration of tryptic peptides generated from the CNBr peptide 314–377. The purified CNBr peptide 314–377 was digested with trypsin as described in MATERIALS AND METHODS. The tryptic digest (approximately 1 nmol containing 7.6×10^4 cpm) was applied to a Sephadex LH60 column and peptides were resolved as described in Figure 3. Fractionation was monitored by absorbance at 280 nm and by radioactivity. Dual sequencing runs were used to confirm the identity of eluted peptides and to identify [^{3}H]palmitoylated amino acids. (A) The parent peptide, with cleavage sites underlined, and the tryptic peptides recovered after gel filtration. (B) Radioactivity of eluted peptides after gel filtration. Elution positions of nonpalmitoylated peptides are indicated by arrows. Peptides containing [^{3}H]palmitate were eluted two fractions earlier than their nonpalmitoylated counterparts. The peak of radioactivity (fraction 30) contained the palmitoylated form of peptide 374–377 (T-6); its elution position is indicated.

To confirm the site of palmitoylation, digestion of the CNBr peptide 314–377 with Lys-C protease was also performed (Figure 5). After gel filtration of peptides, approximately 72% of the radioactivity and five of the six expected peptides were recovered; the two-residue peptide, 337–338 (TK), was not recovered. The peak of radioactivity (fractions 25–28) was broader than after trypsin digestion. However, its position indicates that the 7-residue peptide VQRAVCM (residues 371–377) contained the majority of the counts. The only other cysteine-containing Lys-C peptides were peptides 319–352 and 314–326; if these peptides were palmitoylated, they would have eluted in fraction 24. Dual sequencing runs established that Cys-376 of the peptide 371–377 contained [^{3}H]palmitate.

A

Parent peptide CB-3: ACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDW
CPTGFKVGINYEPPTVVPGGDLAKVQRAVCM

Peptides	Residues	Identified Lys-C peptides	Fraction # for nonpalmitoylated peptides	Fraction # for ^{3}H-palmitoylated peptides
K-1	353-370	VGINYEPPTVVPGGDLAK	25	-
K-2	339-352	RTIQFVDWCPTGFK	26	-
K-3	314-326	ACCLLYRGDVVPK	26	-
K-4	327-336	DVNAAIATIK	27	-
K-5	371-377	VQRAVCM	29	27

B

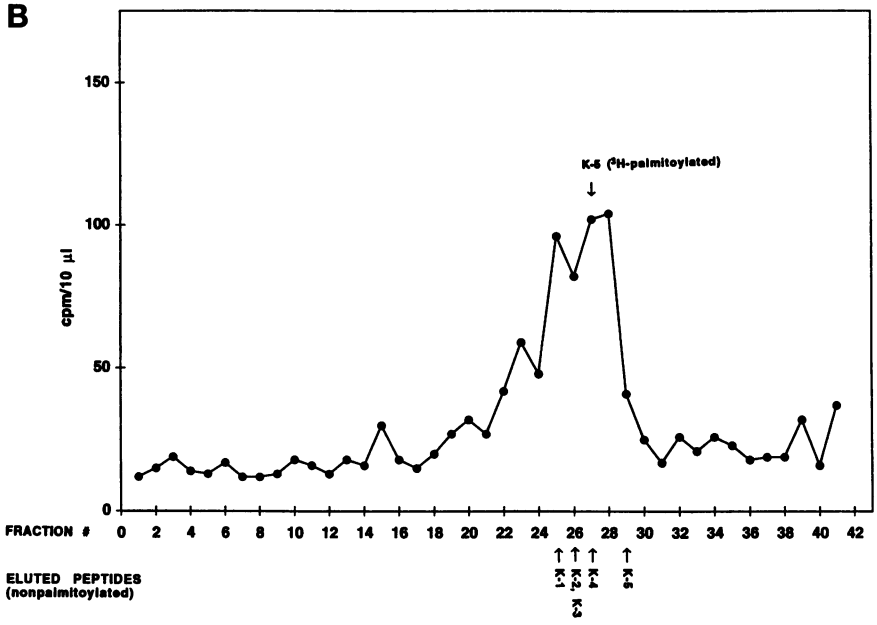

Figure 5. Gel filtration of Lys-C peptides generated from the CNBr peptide 314–377. The purified CNBr peptide 314–377 was digested with Lys-C protease as described in MATERIALS AND METHODS. The Lys-C digest (approximately 1 nmol containing 4.6×10^4 cpm) was applied to a Sephadex LH60 column and peptides were resolved as described in Figure 3. Fractionation was monitored by absorbance at 280 nm and by radioactivity. Dual sequencer runs were used to confirm the identity of eluted peptides and to identify [^{3}H]palmitoylated amino acids. (A) The parent peptide, with cleavage sites underlined, and the Lys-C peptides recovered after gel filtration. (B) Radioactivity of eluted peptides after gel filtration. Elution positions on nonpalmitoylated peptides are indicated by arrows. Peptides containing [^{3}H]palmitate was eluted two fractions earlier than their nonpalmitoylated counterparts. The peak of radioactivity (fraction 27) contained the palmitoylated form of peptide 371–377 (K-5); its elution position is indicated.

The CNBr and subcleavage experiments described above demonstrate that Cys-376 of the CNBr peptide, peptide 314–377, was the primary site for palmitoylation of α-tubulin. However, three minor peaks of radioactivity contained five additional CNBr peptides (peptides 1–36, 37–154, 155–203, 204–302, and 303–313) that may have been ^{3}H-labeled palmitoylated at cysteine residues (see Figure 3). These additional peptides were analyzed by dual sequencer runs. Although the specific activity of each palmitoylated residue could not be determined, as discussed above, integration of this information led to the assignment of the palmitoylated moiety to specific residues. Cysteine residues 20 and 213 contained [^{3}H]palmitate. Low levels of radioactivity were found in cysteine residue 305.

DISCUSSION

We have developed a methodology to examine directly the palmitoylation of all 451 amino acids of porcine brain α-tubulin. To our knowledge, this is the first report of a complete sequence analysis of a protein for palmitoylation sites. Only cysteine residues were palmitoylated. Cys-376 was the primary site of palmitoylation. Secondary sites were identified at Cys-20 and Cys-213. These three cysteine residues are

conserved in all known sequences of α-tubulin. The fact that Cys-315 and Cys-316 were not major sites for palmitoylation is interesting because these residues are contained in a putative consensus sequence for palmitoylation of GAP-43 and G protein-linked receptors (Strittmatter *et al.*, 1990; Lui *et al.*, 1993).

Protein Structure Analysis of Palmitoylated Proteins

In the preceding article, palmitoylated tubulin was identified in human platelets. Unfortunately, it would take liters of blood to purify even a milligram of α-tubulin for protein structure analysis. As an alternative, we chose to study porcine brain tubulin that was palmitoylated in a cell-free system (Caron, 1997). In support of this decision, studies of other proteins have shown that sites of palmitoylation in vivo and in vitro are equivalent (Bizzozero *et al.*, 1987; Gutierrez and Magee, 1991; Stanley *et al.*, 1994).

Different methodologies for protein structure analysis have been used to identify palmitoylated amino acids (Papac *et al.*, 1992; Weimbs and Stoffel, 1992; Bizzozero *et al.*, 1994a; Hackett *et al.*, 1994). A direct approach is to label sites with radioactive palmitate and analyze palmitoylated peptides by sequence analysis as described in this article. In our studies, we found that HPLC isolation of palmitoylated peptides was unpredictable. Most of the palmitoylated peptides of tubulin simply failed to elute from HPLC columns under conditions used to resolve membrane protein digests (Ozols, 1984), necessitating the use of gel filtration on Sephadex LH60. Similar problems with recovery of palmitoylated peptides from HPLC columns have been described in other studies (Ovchinnikov *et al.*, 1988). Clearly, unique problems and advantages are associated with different techniques of protein structure analysis of palmitoylated proteins. Our approach can be labor intensive. The degree of difficulty for a particular palmitoylated protein depends on how easy it is to generate cysteine-containing peptides that can be resolved by gel filtration. Nevertheless, the methods presented herein lead to a complete and direct analysis of palmitoylated proteins and circumvent many of the problems associated with other techniques.

Palmitoylated Cysteine Residues of α-Tubulin

Ludueña and Roach (1981) determined that nondenatured α- and β-tubulin from brain, in the form of heterodimers, each contain three or four cysteine residues that are accessible for alkylation, implying that these cysteine residues are at the surface of the molecules. Through detailed alkylation studies, the accessible cysteine residues of β-tubulin were identified (for review, see Ludueña and Roach, 1991). Similar studies have not been performed with α-tubulin. Our studies demonstrate that cysteine residues 376, 20, and 213 of α-tubulin were accessible to palmitoylating enzymes. This indicates that these cysteine residues are at the surface of nondenatured α-tubulin while it is dimerized to β-tubulin.

Once α- and β-tubulin are assembled into microtubules, it is believed that there are no accessible cysteine residues (Ludueña and Roach, 1981). However, as shown in the preceding article, α- and β-tubulin in microtubules (assembled with the slowly hydrolyzable GTP analogue guanylyl-(α,β)-methylene-diphosphonate were substrates for palmitoylation. Thus, tubulin in microtubules, as well as nonpolymerized tubulin dimers, contains accessible free sulfhydryl groups at the surface of the protein. Whether the same cysteine residues were palmitoylated in microtubules and nonpolymerized tubulin is not yet known. However, in the preceding article, it is shown that palmitoylation of nonpolymerized tubulin was specifically blocked by Colcemid and that palmitoylation of tubulin in microtubules was specifically blocked by taxol. The fact that the binding sites for these two drugs on tubulin overlap (Uppuluri *et al.*, 1993; Rao *et al.*, 1994, 1995; Bai *et al.*, 1996) gives some support to the idea that nonpolymerized tubulin and tubulin in microtubules may be palmitoylated at the same sites. Since we have available a methodology for identification of palmitoylation sites in tubulin, this question can be addressed. Furthermore, it is now reasonable to turn to site-directed mutagenesis, first, to determine whether these in vitro sites of palmitoylation are the same as those found in vivo and, second, to begin an analysis of the function of palmitoylated tubulin.

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