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Posttranslational Modification of Tubulin by Palmitoylation: I. In Vivo and Cell-Free Studies

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It is well established that microtubules interact with intracellular membranes of eukaryotic cells. There is also evidence that tubulin, the major subunit of microtubules, associates directly with membranes. In many cases, this association between tubulin and membranes involves hydrophobic interactions. However, neither primary sequence nor known posttranslational modifications of tubulin can account for such an interaction. The goal of this study was to determine the molecular nature of hydrophobic interactions between tubulin and membranes. Specifically, I sought to identify a posttranslational modification of tubulin that is found in membrane proteins but not in cytoplasmic proteins. One such modification is the covalent attachment of the long chain fatty acid palmitate. The possibility that tubulin is a substrate for palmitoylation was investigated. First, I found that tubulin was palmitoylated in resting platelets and that the level of palmitoylation of tubulin decreased upon activation of platelets with thrombin. Second, to obtain quantities of palmitoylated tubulin required for protein structure analysis, a cell-free system for palmitoylation of tubulin was developed and characterized. The substrates for palmitoylation were nonpolymerized tubulin and tubulin in microtubules assembled with the slowly hydrolyzable GTP analogue guanylyl-(α,β)-methylenediphosphonate. However, tubulin in Taxol-assembled microtubules was not a substrate for palmitoylation. Likewise, palmitoylation of tubulin in the cell-free system was specifically inhibited by the antimicrotubule drugs Colcemid, podophyllotoxin, nocodazole, and vinblastine. These experiments identify a previously unknown posttranslational modification of tubulin that can account for at least one type of hydrophobic interaction with intracellular membranes.

INTRODUCTION

Microtubules interact with membranes of intracellular vesicles and organelles in a dynamic manner (for review, see Kelly, 1990; Cole and Lippincott-Schwartz, 1995). To better understand the regulation of microtubule-membrane interactions, it is important to identify proteins that link microtubules to membranes. The microtubule motor proteins kinesin and dynein mediate vesicle and organelle transport along microtubules (for review, see Brady, 1991; Schroer and Sheetz, 1991; Skonfias and Scholey, 1993). The microtubule-binding protein CLIP-70 is essential for binding endocytic vesicles to microtubules in vitro and colocalizes with endocytic vesicles in vivo (Pierre *et al.*, 1992). Recently, the microtubule-associated protein tau was localized to growth cone membranes of neuronal cells suggesting that membrane-associated tau mediates microtubule-plasma membrane interactions during neurite outgrowth (Brandt *et al.*, 1995). However, there is also evidence for a direct interaction between tubulin, the major subunit of microtubules, and membranes (for review, see Stephens, 1986). Tubulin has been identified in a variety of membranes including intracellular vesicles and plasma membrane (Babitch, 1981; Kelly *et al.*, 1983; Pfeffer *et al.*, 1983; Simkowitz *et al.*, 1989; Lacey and Haimo, 1992). The molecular nature of the association between tubulin and membranes is not clear. Biochemical analysis of membrane fractions indicates a hydrophobic interaction. However, tubulin, a highly conserved molecule, contains no significant hydrophobic domains (Cleveland and Sullivan, 1985). Furthermore, known posttranslational modifications

of tubulin [e.g., tyrosination (Raybin and Flavin, 1977; Deanin *et al.*, 1980), acetylation (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1991), glutamylation (Eddé *et al.*, 1990; Alexander *et al.*, 1991), and phosphorylation (Gard and Kirschner, 1985; Alexander *et al.*, 1991; Peters *et al.*, 1996)] cannot account for membrane association. This has led to the suggestion that the putative membrane tubulin may actually be contaminating cytoplasmic tubulin. Studies showing that cytoplasmic tubulin binds to artificial lipid membranes (Caron and Berlin, 1979; Klausner *et al.*, 1981; Kumar *et al.*, 1981) support this concern. Ideally, one would like to identify a membrane-associated form of tubulin that is biochemically distinct from cytoplasmic tubulin.

Palmitoylation is a posttranslational modification that is found in membrane-associated proteins but not in cytoplasmic proteins. In eukaryotes, this modification involves the covalent attachment of the long chain fatty acid palmitate primarily to cysteine residues (for review, see Schlesinger *et al.*, 1993; Bizzozero *et al.*, 1994; Casey, 1995; Milligan *et al.*, 1995). There is no specific type of protein that is palmitoylated. Examples include integral membrane proteins such as the β_2-adrenergic receptor (O'Dowd *et al.*, 1989) and water-soluble proteins such as the neuronal growth cone protein GAP-43 (Skene and Virág, 1989). Cells often contain both palmitoylated and nonpalmitoylated forms of a protein (Skene and Virág, 1989). Although palmitoylated proteins are membrane associated (Lui *et al.*, 1993; Bizzozero *et al.*, 1994; Wedegaertner and Bourne, 1994), the nonpalmitoylated forms can be membrane associated or cytosolic (Hancock *et al.*, 1989). Palmitoylation of proteins is reversible leading to the belief that this modification plays a regulatory role in a manner analogous to phosphorylation.

Several proteins that are palmitoylated in vivo are also palmitoylated in cell-free systems with crude membrane preparations (Berger and Schmidt, 1984; Gutierrez and Magee, 1991). In cases where it has been studied, sites of palmitoylation in vivo and in vitro are the same (Bizzozero *et al.*, 1987; Gutierrez and Magee, 1991; Stanley *et al.*, 1994). In most cell-free systems, palmitoylating activity is dramatically reduced if membranes are pretreated by boiling (Berger and Schmidt, 1984; Slomiany *et al.*, 1984), suggesting an enzymatic reaction. In fact, a palmitoyl transferase has recently been purified (Lui *et al.*, 1996). Nonenzymatic palmitoylation can also occur in vitro (O'Brien *et al.*, 1987; Ross and Braun, 1988; Sudo *et al.*, 1992; Duncan and Gilman, 1996). In studies of G_α protein (Duncan and Gilman, 1996) and proteolipid protein (Bizzozero *et al.*, 1987), the cysteine residues that are nonenzymatically palmitoylated in vitro are the same residues that are palmitoylated in vivo. Whether nonenzymatic palmitoylation occurs in vivo is not known.

To better understand the nature of the interaction between tubulin and membranes, I investigated the possibility that tubulin is a substrate for palmitoylation. I found that tubulin was palmitoylated in resting platelets and that the level of palmitoylated tubulin decreased when platelets were activated with thrombin. To generate quantities of palmitoylated tubulin required for biochemical analysis, a cell-free system for the enzymatic palmitoylation of porcine brain tubulin was developed and characterized. In the following article (Ozols and Caron, 1997), the primary palmitoylation site on α-tubulin was identified.

MATERIALS AND METHODS

Palmitoylation of Platelet Proteins

Human platelets were isolated from 72 ml of freshly collected blood (Fox, 1985). Alternatively, 1 U of outdated platelets was obtained from the American Red Cross. Platelet proteins were labeled with [^{3}H]palmitate as described previously (Huang, 1989). Briefly, 9,10-[^{3}H]palmitate (New England Nuclear, Boston MA; specific activity 52 Ci/mmol) (dried down using a Savant Speedvac evaporator and redissolved in ethanol) was added to platelets to a final concentration of 0.5 mCi/ml [^{3}H]palmitate and 1% ethanol. Platelets were incubated at room temperature for 2 h. In some cases, thrombin (1 U/ml) was added during the last 5 min of the incubation. Platelets were centrifuged at 4°C for 30 s at 14,000 × *g* and resuspended in ice-cold lysis buffer [25 mM Tris, pH 7.4, 0.4 M NaCl, 0.5% SDS, 1.0% Nonidet P-40, 0.1% deoxycholate, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mM phenanthroline, 0.5 mg/ml antipain, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin A, 0.5 mg/ml chymostatin, 0.5 mg/ml aprotonin] containing unlabeled palmitoyl-CoA at a 1000-fold molar excess to [^{3}H]palmitate. After 5 min on ice, samples were sonicated briefly on ice and centrifuged at 4°C for 5 min at 14,000 × *g*. A portion of the samples was saved for a DC protein assay (Bio-Rad, Richmond, CA), analysis by one-dimensional PAGE (1D-PAGE)[1] (Laemmli, 1970), and immunoblot analysis. Immunoblot analysis was performed with mouse anti-β-tubulin monoclonal antibody (Amersham, Arlington Heights, IL) using the enhanced chemiluminescence detection system (Amersham). For two-dimensional PAGE (2D-PAGE), platelet proteins were quantitatively precipitated with chloroform:methanol (1:2), as described by Wessel and Flügge (1984), and stored overnight in methanol at −20°C. Precipitated protein was collected by centrifugation (9000 × *g* for 10 min) and resuspended in isoelectric focusing sample buffer (as described by Hoefer Instruments, San Francisco, CA). 2D-PAGE was performed as described by O'Farrell (1975) using Pharmalyte ampholines, pH 4–6.5 (Pharmacia, Piscataway, NJ). Approximately 250 μg of platelet protein were loaded per isoelectric focusing gel (0.3 × 10.5 cm). Immediately before loading, porcine brain microtubule protein (20 μg) was added to each sample to identify the positions of α- and β-tubulin by Coomassie blue staining.

Purification of Microtubule Protein

Porcine brain microtubule protein was prepared by three or four cycles of assembly-disassembly (Margolis and Wilson, 1978). After the final step of cold-induced disassembly, samples were centrifuged at 39,000 × *g* for 30 min at 4°C. The resulting supernatant,

[1] Abbreviations: GMPCPP, guanylyl-(α,β)-methylene-diphosphonate; mem, membrane extract; MTP, microtubule protein; 1D-PAGE, one-dimensional PAGE.

containing microtubule protein at approximately 8 mg/ml, was stored at −70°C. The storage buffer was 20 mM phosphate (pH 6.75) and 100 mM glutamate.

Purification of a Rat Liver Membrane Fraction Containing Tubulin-palmitoylating Activity

All procedures were performed at 4°C. The membrane fractionation methods of Jones and Matus (1974) and Fleischer and Kervina (1978) were combined and modified as follows. Liver from adult male Sprague Dawley rats was removed, washed twice with 10% sucrose, 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), pH 7.4, and homogenized in 5 volumes of the sucrose-HEPES buffer. The homogenate was centrifuged (700 × *g*, 10 min) to remove unbroken cells and nuclei. A portion (1 ml) of the 700 × *g* supernatant was centrifuged at 100,000 × *g* for 30 min; the resulting pellet (whole membrane fraction) was resuspended in incubation buffer (20 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EGTA, 10 μg/ml leupeptin, 0.15% Triton X-100) and stored at −70°C. The remaining 700 × *g* supernatant was centrifuged at 9000 × *g* for 20 min. The 9000 × *g* supernatant was centrifuged at 100,000 × *g* for 30 min and the resulting pellet (crude microsomal membrane) was resuspended in incubation buffer and stored at −70°C. The 9000 × *g* pellet was resuspended in 25 ml of a hypotonic solution (1% sucrose, 1 mM HEPES, pH 7.4) and incubated on ice. After 1 h, 2 volumes of 48% sucrose and 10 mM HEPES (pH 7.4) were added to give a final sucrose concentration of 34%. This was covered with 10 ml of 28.5% sucrose and 10 mM HEPES (pH 7.4), and the sample was centrifuged at 60,000 × *g* for 2 h. The membrane fraction at the 28.5/34% sucrose interface was diluted in 10% sucrose and 10 mM HEPES (pH 7.4) and centrifuged at 100,000 × *g* for 30 min; the resulting pellet (crude plasma membrane) was resuspended in incubation buffer and stored at −70°C. The pellet from the 60,000 × *g* gradient spin (crude mitochondrial-peroxisomal membrane) was resuspended in 10 ml of high salt buffer (20 mM Tris, pH 7.4, 0.5 M NaCl, 1 mM EGTA, 10 μg/ml leupeptin) and incubated on ice. After 1 h, the sample was centrifuged at 100,000 × *g* for 30 min. This high salt extraction eliminated protease activity from the membranes. The pellet was resuspended in 5 ml of incubation buffer and kept on ice for 1 h. The sample was centrifuged at 100,000 × *g* for 30 min and the supernatant (Triton X-100-soluble membrane extract), which contained tubulin-palmitoylating activity, was stored at −70°C for up to 4 mo.

Cell-Free Palmitoylation of Tubulin

Microtubule protein (1.0–2.4 mg/ml) was centrifuged at 100,000 × *g* for 10 min at room temperature immediately before use. This step was included to ensure that nonpolymerized tubulin (and not polymers or aggregates of the protein) was the substrate for cell-free palmitoylation. The resulting supernatant (containing >85% of the tubulin) was incubated in incubation buffer with the rat liver membrane extract (0.35 mg/ml), 200 μM CoA, 2 mM ATP, and [9,10-^{3}H]palmitate (45–50 μCi/ml, New England Nuclear, Boston, MA; specific activity, 52 Ci/mmol). In some cases, [1-^{14}C]palmitoyl-CoA (3 μCi/ml; Amersham; specific activity, 57 mCi/mmol) was substituted for [^{3}H]palmitate, CoA, and ATP. The reaction was stopped by addition of sample buffer (Laemmli, 1970). Proteins were subjected to 1D-PAGE (Laemmli, 1970) using 10 mM dithiothreitol as the reducing agent. Gels were stained with Coomassie blue, photographed with an α Innotech IS-1000 Digital Imaging System, fluorographed with Amplify (Amersham), dried, and autoradiographed with Kodak X-OMAT AR x-ray film at −70°C. Band intensities were measured using an α Innotech IS-1000 Digital Imaging System.

Microtubule Assembly In Vitro

To form microtubules, microtubule protein (1.6–2.4 mg/ml) was centrifuged at 100,000 × *g* for 10 min at room temperature to pellet any aggregates from the starting material. The supernatant, containing approximately 85% of the protein, was incubated with either 1.4 mM guanylyl-(α,β)-methylene-diphosphonate (GMPCPP; a gift from Dr. Michael Caplow, University of North Carolina, Chapel Hill), 1.0 mM EGTA, 0.5 mM $MgCl_2$, or 20 μM Taxol (a gift from the National Cancer Institute, National Institutes of Health, Bethesda, MD), 1 mM GTP, 1 mM EGTA, and 0.5 mM $MgCl_2$, in incubation buffer for 1 h at 30°C.

Negative Stain Electron Microscopy

Carbon-coated Formvar grids were routinely glow discharged for 15 min within 2 h of use to ensure even spreading of samples on grids. A drop of sample was placed on the grid. After 30 s, protein was fixed for 1 min with 2% gluteraldehyde and 0.1 M cacodylate (pH 7.4), stained with filtered 2% uranyl acetate, and examined on a JEOL 100 CX electron microscope.

RESULTS

Platelet Tubulin Is Palmitoylated In Vivo

Palmitoylation of tubulin in platelets was examined because platelets have been reported to contain membrane-associated tubulin (Steiner, 1983). The effect of activation of platelets was also investigated because activation alters the extent of palmitoylation of some platelet proteins (Cieniewski *et al.*, 1989; Huang, 1989; Muszbek and Laposata, 1989; Fujimoto *et al.*, 1993). Initial examination of palmitoylated proteins was performed by 1D-PAGE (Figure 1A). Several proteins were palmitoylated, with resting and activated platelets displaying similar patterns. These patterns of ^{3}H-labeled palmitoylated proteins were different from the Coomassie blue-stained pattern. As shown previously by Huang (1989), a protein of 38 kDa was heavily labeled with [^{3}H]palmitate, and the extent of palmitoylation of this protein was increased upon activation of platelets with thrombin. A comparison of palmitoylated proteins by 2D-PAGE is shown in Figure 1B. Multiple palmitoylated isoforms of the 38-kDa protein were revealed with, as expected, an increase in labeling upon activation.

Analysis by 2D-PAGE also demonstrated that α- and β-tubulin were palmitoylated in resting platelets (Figure 1B). However, in contrast to the 38-kDa protein, levels of palmitoylated α- and β-tubulin were reduced upon activation of platelets with thrombin. Immunoblot analysis demonstrated that extracts from resting and activated platelets contained similar amounts of tubulin (Figure 1C). Therefore, activation with thrombin decreased palmitoylation of tubulin but did not alter the amount of tubulin protein. Treatment of the 2D gels with methanolic KOH released >90% of the palmitate from α- and β-tubulin, demonstrating that palmitate was attached to tubulin by ester linkages.

In contrast to thrombin, activation of platelets with calcium ionophore A23187 did not alter the level of palmitoylated tubulin. Similar results with the 38-kDa protein were reported by Huang (1989). Thus, alterations in the level of palmitoylation of some proteins,

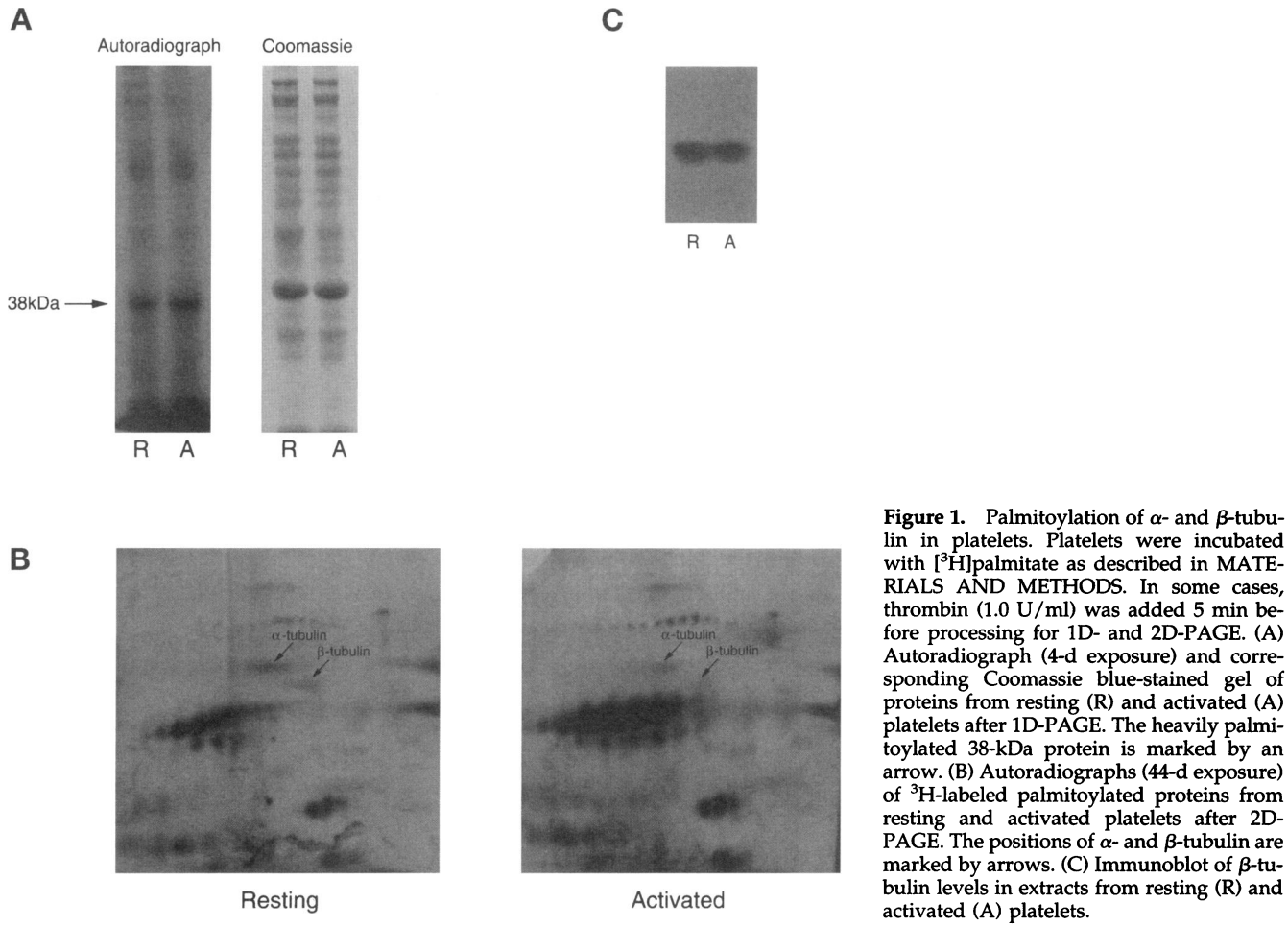

Figure 1. Palmitoylation of α- and β-tubulin in platelets. Platelets were incubated with [^{3}H]palmitate as described in MATERIALS AND METHODS. In some cases, thrombin (1.0 U/ml) was added 5 min before processing for 1D- and 2D-PAGE. (A) Autoradiograph (4-d exposure) and corresponding Coomassie blue-stained gel of proteins from resting (R) and activated (A) platelets after 1D-PAGE. The heavily palmitoylated 38-kDa protein is marked by an arrow. (B) Autoradiographs (44-d exposure) of ^{3}H-labeled palmitoylated proteins from resting and activated platelets after 2D-PAGE. The positions of α- and β-tubulin are marked by arrows. (C) Immunoblot of β-tubulin levels in extracts from resting (R) and activated (A) platelets.

including tubulin, were dependent on the mode of activation.

A Cell-Free System for Palmitoylation of Tubulin

To obtain quantities of palmitoylated tubulin required for protein structure analysis (Ozols and Caron, 1997), a cell-free system for palmitoylation of porcine brain tubulin was developed. Since rat liver membranes contain enzymes for palmitoylating proteins (Riendeau and Guertin, 1986), I chose this as a starting material. Rat livers were homogenized and fractionated by centrifugation using standard procedures (see MATERIALS AND METHODS). Transmission electron microscopy was used to confirm the predominant types of membrane in each fraction. Crude fractions of microsomal membrane, plasma membrane, and mitochondrial-peroxisomal membrane were obtained. Porcine brain microtubule protein was incubated with the different membrane fractions along with CoA, ATP, and [^{3}H]palmitate as described in MATERIALS AND METHODS. After 1 h at 30°C, the reaction was stopped by addition of Laemmli sample buffer. Samples were analyzed by 1D-PAGE and fluorography (Figure 2). As shown in lane 4, Figure 2, the mitochondrial-peroxisomal membrane fraction contained substantial tubulin palmitoylating activity. Both α- and β-tubulin were radiolabeled. Identity of the labeled bands as α- and β-tubulin was confirmed by immunoprecipitation. Lower levels of tubulin palmitoylating activity were found in the microsomal membrane and plasma membrane fractions. It remains to be determined whether these membrane fractions lack substantial tubulin palmitoylating activity or contain thioesterases that remove palmitate from tubulin or both.

In some preparations, the crude mitochondrial-peroxisomal membrane fraction contained a protease activity to which high molecular weight microtubule-associated proteins were sensitive. To eliminate this protease activity, the membrane fraction was incubated with 0.5 M NaCl followed by centrifugation. The resulting membrane pellet was then incubated with 0.15% Triton X-100 and again centrifuged. The Triton X-100-soluble extract (termed the membrane extract) was found to contain tubulin-palmitoylating activity

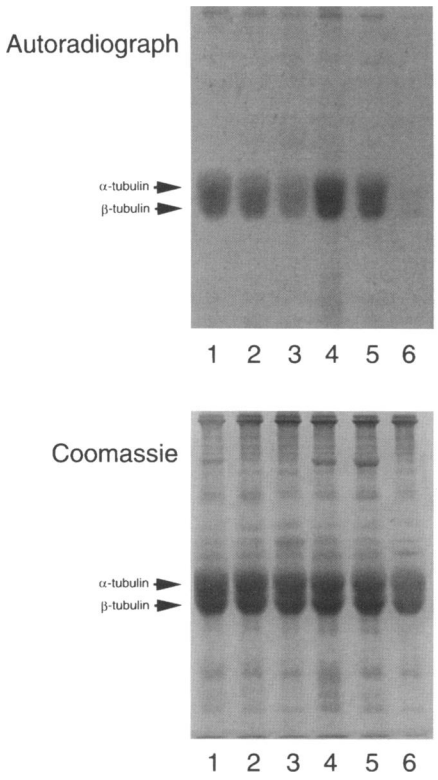

Figure 2. Cell-free palmitoylation of tubulin with membrane fractions from rat liver. Microtubule protein (1.5 mg/ml) was incubated with [^{3}H]palmitate, CoA, ATP, and various membrane fractions (0.35 mg/ml) isolated from rat liver as described in MATERIALS AND METHODS. After 1 h at 30°C, the reaction was stopped by addition of Laemmli sample buffer. Samples were analyzed by 1D-PAGE and fluorography. An autoradiograph of palmitoylated tubulin (1-d exposure) and the corresponding Coomassie blue-stained gel are shown. Lane 1, whole membrane fraction; lane 2, crude microsomal membrane fraction; lane 3, crude plasma membrane fraction; lane 4, crude mitochondrial-peroxisomal membrane fraction; lane 5, Triton X-100-soluble extract of the mitochondrial-peroxisomal membrane fraction; lane 6, no membrane.

(Figure 2, lane 5). Immunoblot analysis demonstrated that there was no endogenous tubulin in the membrane extract.

The optimal protein concentration of the membrane extract for palmitoylation of tubulin was determined. The membrane extract (0–2.0 mg/ml) was incubated with CoA, ATP, and [^{3}H]palmitate in the presence and absence of microtubule protein (1.5 mg/ml). After 1 h, samples were subjected to 1D-PAGE followed by Coomassie blue staining and fluorography. As shown in Figure 3, membrane protein concentrations of 0.3–0.4 mg/ml resulted in substantial palmitoylation of tubulin with only low levels of palmitoylation of membrane proteins. In the absence of microtubule protein, two proteins from the membrane extract were the primary targets for palmitoylation: one protein with a slightly slower electrophoretic mobility than α-tubulin and another protein that ran slightly faster than β-tubulin during SDS-PAGE. However, the relative levels of palmitoylation of these membrane proteins varied with different preparations of the rat liver membrane extract.

Palmitoylation of tubulin was markedly reduced if the membrane extract, CoA, or ATP was omitted (Figure 4A). The requirement for CoA and ATP demonstrates that palmitoyl-CoA, and not free palmitate, was the acyl donor. As expected, palmitoylation of tubulin also occurred when [^{14}C]palmitoyl-CoA was substituted for [^{3}H]palmitate, CoA, and ATP (Figure 4B). If the membrane extract was preheated at 65°C for 20 min before incubation with microtubule protein and [^{14}C]palmitoyl-CoA, palmitoylating activity was reduced by 76%. These data indicate that the membrane extract contained a thiokinase activity that generated palmitoyl-CoA from palmitate and CoA as well as enzymes (acyl transferases) to palmitoylate tubulin with palmitoyl-CoA.

In the absence of the membrane extract, an apparent nonenzymatic reaction between tubulin and [^{14}C]palmitoyl-CoA was observed, albeit at a much reduced level (approximately eightfold) than in the presence of membrane extract (Figure 4B). To eliminate the possibility that the microtubule protein preparation contained acyltransferase activity, phosphocellulose-purified tubulin (1 mg/ml; Weingarten *et al.*, 1975; Williams and Detrich, 1979), devoid of microtubule-associated proteins, was subjected to cell-free palmitoylation with [^{14}C]palmitoyl-CoA for 1 h in the presence and absence of the membrane extract. In either case, palmitoylation of phosphocellulose-purified tubulin was similar to that of tubulin from microtubule protein. These results demonstrate two points. First, palmitoylation of tubulin was not affected by the presence or absence of microtubule-associated proteins. Second, tubulin was, in fact, nonenzymatically palmitoylated, albeit at much reduced levels when compared with the enzymatic reaction.

No differences were found after examining the acylation of tubulin with either [^{3}H]palmitate or [^{3}H]myristate in the presence of the membrane extract, CoA, and ATP. Thin layer chromatography (Skene and Virág, 1989) was used to confirm that the ^{3}H-labeled fatty acids covalently attached to tubulin after incubation with either [^{3}H]palmitate or [^{3}H]myristate were indeed palmitate and myristate, respectively. Therefore, [^{3}H]myristate was not converted in the cell-free system to [^{3}H]palmitate before acylation of tubulin. Similar results have been reported for other cell-free acylation systems (Berger and Schmidt, 1984).

Palmitate-Tubulin Linkage Is Probably a Thioester

The linkage between tubulin and palmitate, generated in either the presence or absence of the membrane

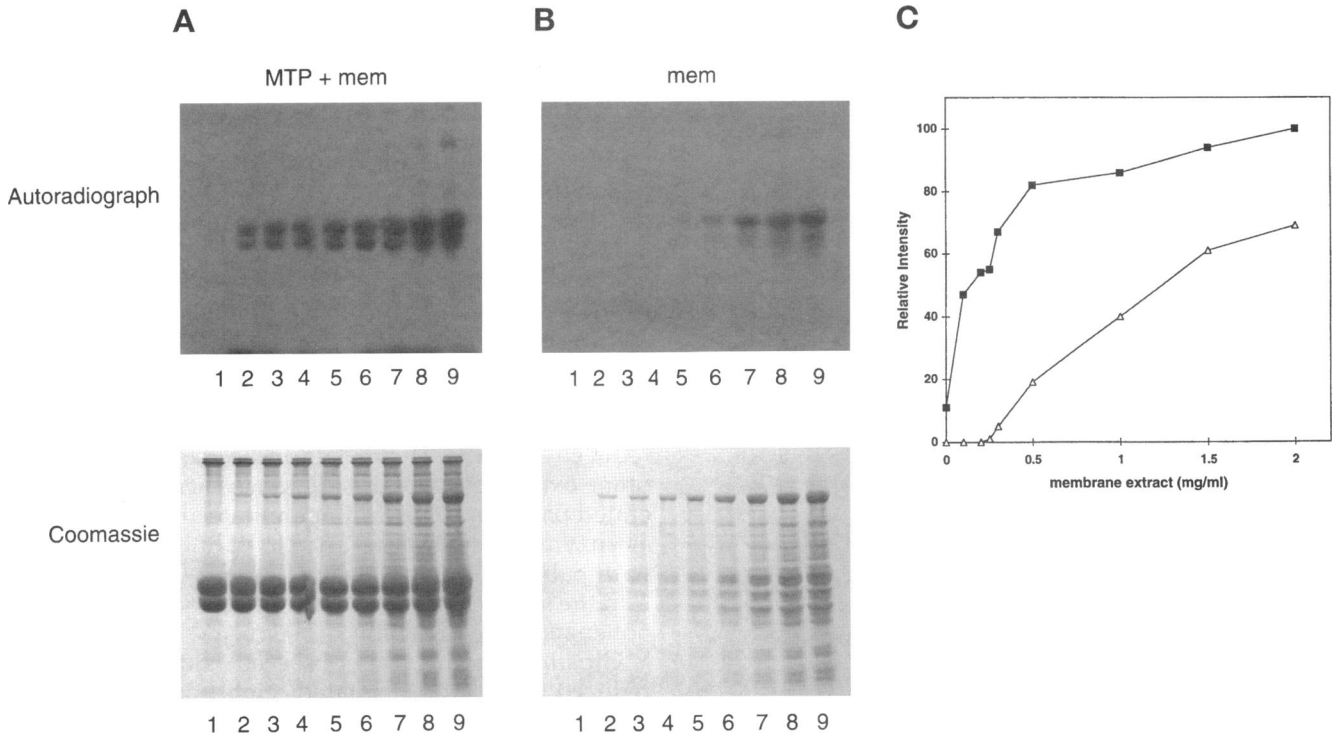

Figure 3. Effect of concentration of the membrane extract on cell-free palmitoylation of tubulin. Membrane extract (mem, 0–2.0 mg/ml) was incubated with [^{3}H]palmitate, CoA, and ATP in the presence (A) and absence (B) of microtubule protein (MTP, 1.5 mg/ml). After 1 h at 30°C, samples were processed for 1D-PAGE and fluorography. Autoradiographs of palmitoylated proteins (1-d exposure) and corresponding Coomassie blue-stained gels are shown. Concentrations of membrane extract were 0 (lane 1), 0.10 mg/ml (lane 2), 0.20 mg/ml (lane 3), 0.25 mg/ml (lane 4), 0.30 mg/ml (lane 5), 0.50 mg/ml (lane 6), 1.00 mg/ml (lane 7), 1.50 mg/ml (lane 8), and 2.00 mg/ml (lane 9). (C) Comparison of levels of palmitoylated tubulin with levels of palmitoylated proteins from the membrane extract. Band intensities from autoradiographs shown in A (■) and B (△) were measured by scanning densitometry. The highest value was set at 100%.

extract, was not sensitive to boiling in 1% SDS, extraction with chloroform:methanol (1:2), or incubation with 9.5 M urea or 6 M guanidine hydrochloride, consistent with covalent bonding. The linkage was sensitive to methanolic 0.2 M KOH; after enzymatic palmitoylation, approximately 90% of the palmitate was released from α- and β-tubulin with no selectivity for either. The identity of the KOH-released ^{3}H-labeled fatty acids was determined by thin layer chromatography (Skene and Virág, 1989). All of the counts comigrated with the palmitate-methyl ester standard, demonstrating that palmitate was covalently linked to tubulin through ester bonds. Quantitation of the release of palmitate by methanolic KOH was not performed with nonenzymatically labeled tubulin because there were too few counts for analysis.

Cleavage of palmitate from proteins by treatment with neutral hydroxylamine suggests thioester linkages (for review, see Bizzozero *et al.*, 1994). Incubation of palmitoylated tubulin with 1 M neutral hydroxylamine for 2 h at room temperature released approximately 40% of the palmitate. The residual label may be due to incomplete cleavage with hydroxylamine as found with other proteins that are palmitoylated on cysteines (Alvarez *et al.*, 1990; Degtyarev *et al.*, 1993). During longer incubations with neutral hydroxylamine, tubulin protein itself was cleaved. Other proteins show a similar sensitivity to hydroxylamine (Deselnicu *et al.*, 1973; Zeng and Weigel, 1995).

Additional evidence that cysteine residues were sites of palmitoylation came from the following competition experiment. Cysteamine, the decarboxylated form of cysteine, was added to the cell-free reaction in the presence of the membrane extract at a 10-fold molar excess to cysteines in α- and β-tubulin. After 2 h at 30°C, there was a 60% inhibition of palmitoylation of tubulin. In the absence of the membrane extract, a similar inhibition was observed. These data indicate that cysteine residues were sites of both enzymatic and nonenzymatic palmitoylation of tubulin.

Optimal Conditions for Enzymatic Palmitoylation of Tubulin

For protein chemistry analysis, I sought to identify conditions for cell-free palmitoylation of tubulin that maximized the enzymatic reaction without denaturing tubulin protein. The first parameter examined was the

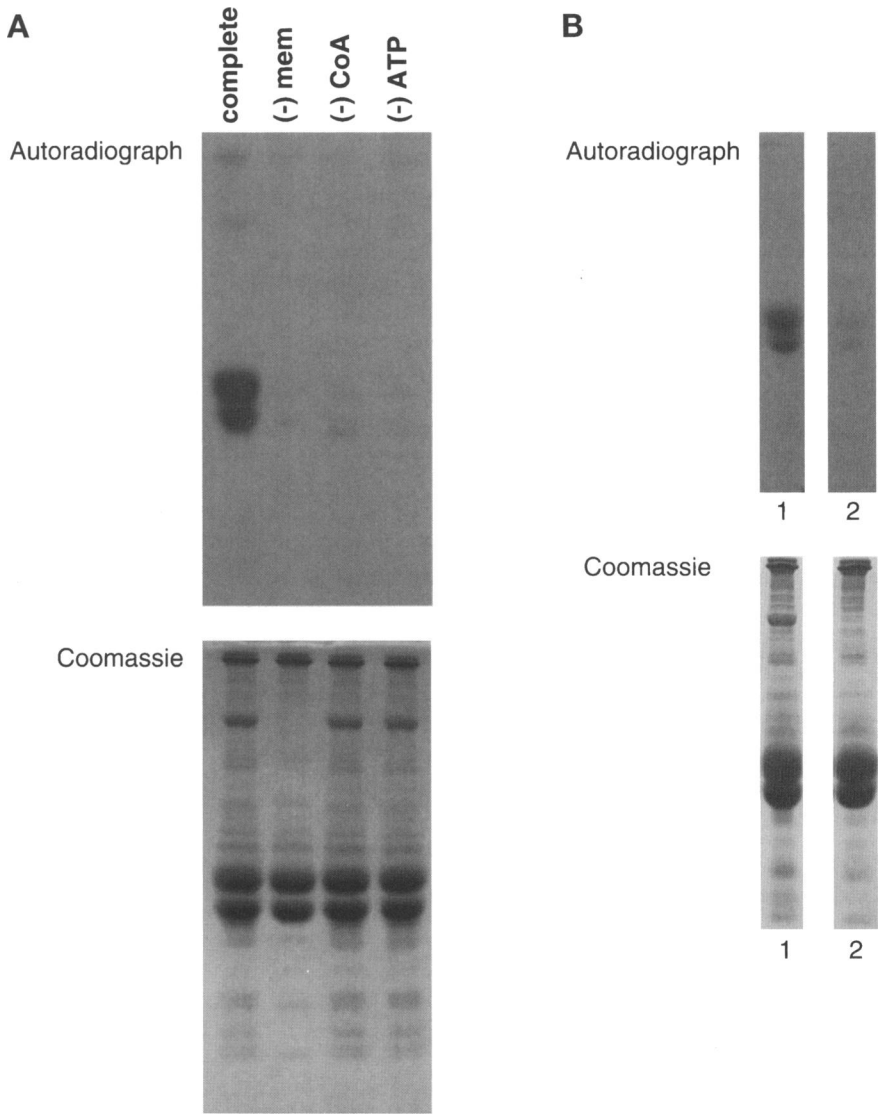

Figure 4. Palmitoyl-CoA is the acyl donor for palmitoylation of tubulin. (A) In the complete reaction, microtubule protein (1.2 mg/ml) was incubated with [^{3}H]palmitate, CoA, ATP, and membrane extract (0.35 mg/ml). In other samples, the membrane extract, CoA, or ATP was omitted. After 1 h at 30°C, samples were processed for 1D-PAGE and fluorography. An autoradiograph (1-d exposure) and corresponding Coomassie blue-stained gel are shown. mem, membrane extract. (B) Microtubule protein (1 mg/ml) was incubated with [^{14}C]palmitoyl-CoA in the presence (lane 1) and absence (lane 2) of membrane extract (0.35 mg/ml). After 1 h at 30°C, samples were processed for 1D-PAGE and fluorography. An autoradiograph (4-d exposure) and corresponding Coomassie blue-stained gel are shown.

effect of ions. Palmitoylation of tubulin was not affected by Ca^{2+}, Mg^{2+}, or Li^{2+} at concentrations of up to 5 mM. However, palmitoylation of tubulin and membrane proteins from the membrane extract was inhibited by 5 mM Mn^{2+}. This suggests that Mn^{2+} inhibited the acyltransferase activity as shown in other cell-free palmitoylation systems (Berger and Schmidt, 1984; Poulio and Bélireau, 1995).

The time course for cell-free palmitoylation of tubulin with [^{14}C]palmitoyl-CoA was examined. In the presence of the membrane extract, palmitoylation of tubulin was detected at 15 min with levels continuing to rise for at least 6 h. When microtubule protein was omitted from the reaction, a low level of palmitoylation of membrane proteins was detected that also increased over time. In the absence of the membrane extract, there was overall much less palmitoylation of tubulin and these levels increased at a slower rate than in the presence of the membrane extract. In the presence of the membrane extract, no lag was found when [^{3}H]palmitate, CoA, and ATP were substituted for [^{14}C]palmitoyl-CoA, demonstrating that the synthesis of [^{3}H]palmitoyl-CoA was rapid. Since the ability of tubulin to assemble into microtubules is sensitive to long incubations at 37°C, a sensitivity that denotes denaturation of the protein, all reactions with tubulin were performed within 2 h. During this period, tubulin did not lose its ability to assemble into microtubules.

Palmitoylation of tubulin was dependent on the concentration of Triton X-100. When microtubule protein was incubated with the membrane extract and [^{14}C]palmitoyl-CoA, little palmitoylation of tubulin occurred at or below 0.02% Triton X-100; palmitoyl-

ation was maximal at 0.15% Triton X-100. Higher concentrations resulted in an inhibition of palmitoylation of tubulin. Palmitoylation of membrane proteins, in the absence of microtubule protein, was maximal at 0.15–0.5% Triton X-100, as was palmitoylation of microtubule protein alone. A concentration of 0.02% is close to the critical micelle concentration of Triton X-100 (Helenius *et al.*, 1979), suggesting that micellar formation is important for both enzymatic and nonenzymatic palmitoylation of proteins.

The possibility that 1% ethanol from the [^{3}H]palmitate stock solution caused some unfolding of tubulin molecules, thus exposing additional cysteine residues for palmitoylation, was examined. Microtubule protein was incubated with [^{14}C]palmitoyl-CoA and ethanol (0–5%) in the presence and absence of the membrane extract. After 2 h, samples were prepared for 1D-PAGE and fluorography. In the presence of the membrane extract, there was a slight inhibition of palmitoylation of tubulin with increasing ethanol concentrations, suggesting that the enzymatic reaction was sensitive to ethanol. In the absence of the membrane extract, there was a small increase (1.7-fold) in the level of palmitoylation of tubulin at an ethanol concentration of 5%. It was concluded that ethanol at concentrations of up to 1.0% did not significantly affect the reaction.

Microtubules Assembled with GMPCPP, but Not Taxol, Are a Substrate for Palmitoylation of Tubulin

In experiments described above, nonpolymerized tubulin was palmitoylated. I next examined whether microtubule polymer was also a substrate for palmitoylation. These experiments required the presence of stable microtubules before palmitoylation was initiated. To induce the formation of stable microtubules, either GMPCPP, a slowly hydrolyzable GTP analogue (Hyman *et al.*, 1992), or Taxol/GTP was added to promote microtubule assembly under conditions optimal for palmitoylation of tubulin. These reagents inhibit dynamic instability of microtubules (Hyman *et al.*, 1992; Derry *et al.*, 1995). After microtubule assembly, the membrane extract and [^{14}C]palmitoyl-CoA were added and the palmitoylation reaction was continued for 1 h. By negative stain electron microscopy (Figure 5A), numerous microtubules were present in both the GMPCPP and Taxol/GTP samples. In the absence of assembly conditions (control), no microtubules were found although amorphous aggregates were sometimes observed. These aggregates were present in the absence of membrane extract, suggesting that tubulin may have interacted with micelles of Triton X-100 as reported by others (Andreu, 1982). Centrifugation was used to separate microtubule polymer (pellet) and nonpolymerized tubulin (supernatant). Fractions were analyzed by 1D-PAGE followed by Coomassie blue staining and fluorography (Figure 5B). By Coomassie blue staining, 71 and 88% of the tubulin was pelleted in the presence of GMPCPP or Taxol/GTP, respectively. In the absence of assembly conditions (control), 25% of the tubulin was pelleted.

The level of palmitoylated tubulin was determined from the corresponding autoradiograph (Figure 5B). As expected, in the absence of microtubule assembly (control), palmitoylated tubulin was present. Similar levels of palmitoylated tubulin were found in the GMPCPP sample. After centrifugation of both samples, 78% of the palmitoylated tubulin in the GMPCPP sample was found in the pellet fraction, whereas 35% was pelleted in the control sample. These percentages correlate well with the amount of tubulin found in each fraction by Coomassie blue staining. The data demonstrate that both nonpolymerized tubulin and tubulin in GMPCPP-assembled microtubules were substrates for palmitoylation. In contrast, tubulin in Taxol/GTP-assembled microtubules was not a substrate for palmitoylation. Likewise, no palmitoylation of tubulin occurred after Taxol/GMPCPP-induced assembly of microtubules. Control experiments showed that dimethyl sulfoxide, the solvent for Taxol, had no effect on the palmitoylation of tubulin.

The small amount of labeling found in the Taxol/GTP samples was due to palmitoylation of proteins from the membrane extract (as shown in Figure 3). This suggests that the palmitoylating enzyme machinery was functional in the presence of Taxol. Nevertheless, the following experiment was performed to further examine the possibility that Taxol interfered directly with palmitoylating enzymes and that this interaction blocked palmitoylation of microtubules. Under conditions required for palmitoylation, in particular, the presence of 0.15% Triton X-100, both Taxol and GTP were required for microtubule assembly; little if any microtubule assembly occurred with Taxol alone. This requirement for GTP during Taxol-induced microtubule assembly contrasts the work of Schiff and Horwitz (1981) and others (Kumar, 1981; Thompson *et al.*, 1981) who found that Taxol alone induces microtubule polymerization. However, the in vitro assembly conditions used in these other studies were different from those described herein. More recently, Collins and Vallee (1987) showed that combinations of microtubule depolymerizing conditions cause the destabilization of Taxol-treated microtubules. Thus, Taxol alone does not stabilize microtubules under all conditions. In in vitro assembly experiments, Andreu (1982) showed that Triton X-100, at a concentration similar to that used herein, decreased the rate and extent of microtubule assembly; however, the structure of microtubules formed in the presence of Triton X-100 was normal as judged by negative

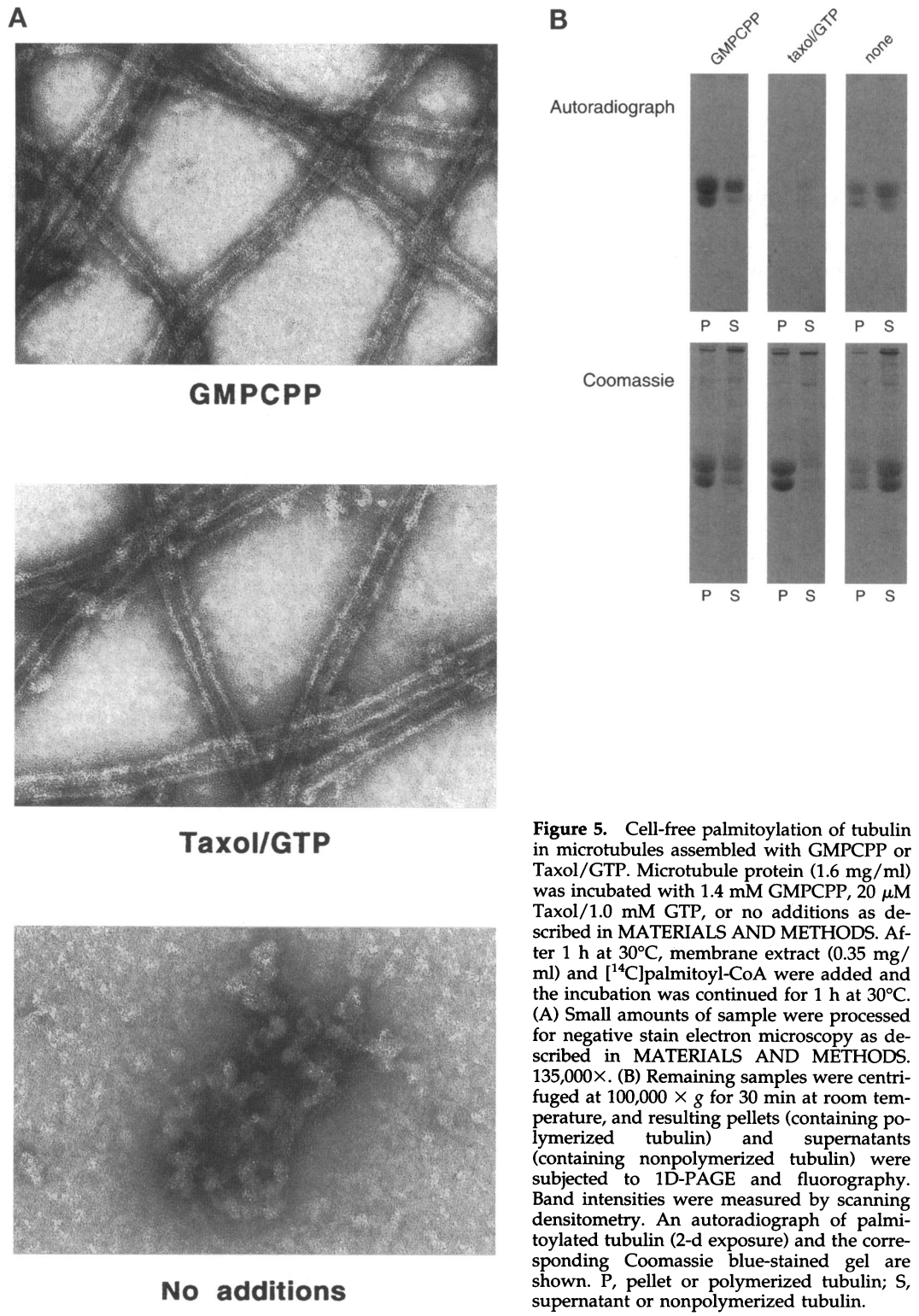

Figure 5. Cell-free palmitoylation of tubulin in microtubules assembled with GMPCPP or Taxol/GTP. Microtubule protein (1.6 mg/ml) was incubated with 1.4 mM GMPCPP, 20 μM Taxol/1.0 mM GTP, or no additions as described in MATERIALS AND METHODS. After 1 h at 30°C, membrane extract (0.35 mg/ml) and [^{14}C]palmitoyl-CoA were added and the incubation was continued for 1 h at 30°C. (A) Small amounts of sample were processed for negative stain electron microscopy as described in MATERIALS AND METHODS. 135,000×. (B) Remaining samples were centrifuged at 100,000 × *g* for 30 min at room temperature, and resulting pellets (containing polymerized tubulin) and supernatants (containing nonpolymerized tubulin) were subjected to 1D-PAGE and fluorography. Band intensities were measured by scanning densitometry. An autoradiograph of palmitoylated tubulin (2-d exposure) and the corresponding Coomassie blue-stained gel are shown. P, pellet or polymerized tubulin; S, supernatant or nonpolymerized tubulin.

stain electron microscopy. In any event, the fact that Taxol alone did not lead to formation of stable microtubules under palmitoylating conditions was exploited to determine whether Taxol interfered directly with the palmitoylating enzymes. Microtubule protein was incubated with Taxol alone or with no additions

(control) for 40 min at 30°C before initiating the palmitoylating reaction by addition of the membrane extract and [^{14}C]palmitoyl-CoA. After 1 h at 30°C, samples were subjected to SDS-PAGE and fluorography. The level of palmitoylated tubulin in the control was set at 100%. In the presence of Taxol alone, palmitoylation of tubulin was 88% of the control. Thus, Taxol did not significantly inhibit palmitoylation of tubulin under conditions in which microtubule assembly did not occur. These data suggest that Taxol did not interfere directly with palmitoylating enzymes. This leaves the possibility that Taxol, which binds with a 1:1 stoichiometry to tubulin dimers in microtubules, inhibited palmitoylation of tubulin by making palmitoylation sites inaccessible either directly through steric hindrance or indirectly through induction of a conformational change in the protein.

Palmitoylated Tubulin Is Not Incorporated into Taxol/GTP-assembled Microtubules

To determine whether palmitoylated tubulin assembled into microtubules, microtubule protein was, first, partially palmitoylated in the cell-free system with [^{3}H]palmitate; under conditions used, it was estimated that 11–15% of both α- and β- tubulin were palmitoylated as judged from stoichiometry studies (Ozols and Caron, 1997). Microtubule assembly conditions were then introduced by addition of Taxol and GTP, and after 1 h, microtubule polymer (pellet) and nonpolymerized tubulin (supernatant) were separated by centrifugation. In some samples, Taxol and GTP were omitted. By Coomassie blue staining (Figure 6), 79% of the tubulin was pelleted in the presence of Taxol and GTP. In the absence of Taxol and GTP, 19% of the tubulin was pelleted. The corresponding autoradiograph demonstrates that in both the presence and absence of microtubule assembly, the majority of palmitoylated tubulin (84% and 81%, respectively) remained in the nonpolymerized fraction. These results show that palmitoylated tubulin did not assemble into microtubules in the presence of Taxol. The most likely explanation is that tubulin-bound palmitate blocked Taxol binding to microtubules and without Taxol binding, microtubules were not stable. However, it is also possible that tubulin-bound palmitate sterically blocked microtubule assembly. To differentiate between these two possibilities, similar studies were attempted in which GMPCPP was substituted for Taxol/GTP. However, these were not successful. As described above, GMPCPP-assembled microtubules were a substrate for palmitoylation, and I was not able to identify conditions that inhibited palmitoylation of GMPCPP-assembled microtubules without causing nonpolymerized palmitoylated tubulin to aggregate and pellet during separation of polymer and supernatant by centrifugation. Thus, it remains possible that palmitoylated tubulin can polymerize into GMPCPP-assembled microtubules.

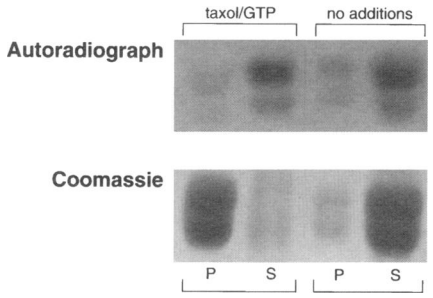

Figure 6. Palmitoylated tubulin is not incorporated into Taxol/GTP-assembled microtubules. Microtubule protein (2.4 mg/ml) was incubated with [^{3}H]palmitate, CoA, ATP, and membrane extract (0.35 mg/ml). After 1 h at 30°C, the sample was centrifuged at 100,000 × *g* for 10 min at room temperature and the supernatant (containing approximately 80% of the protein) was transferred to fresh tubes. Microtubule assembly was induced by addition of 20 μM Taxol/1.0 mM GTP. In some cases, Taxol/GTP was omitted. After 1 h at 30°C, samples were centrifuged at 100,000 × *g* for 30 min at room temperature. Pellet (containing polymerized tubulin) and supernatant (containing nonpolymerized tubulin) were subjected to 1D-PAGE and fluorography. Band intensities were measured by scanning densitometry. An autoradiograph (1-d exposure) and corresponding Coomassie blue-stained gel are shown. P, pellet or polymerized tubulin; S, supernatant or nonpolymerized tubulin.

Effect of Colcemid, Podophyllotoxin, Nocodazole, and Vinblastine on Palmitoylation of Tubulin

Studies of Taxol/GTP-assembled microtubules demonstrated that Taxol blocked palmitoylation of polymers by its binding along the lengths of microtubules. I next examined the effect of other drugs that bind to tubulin. Recent studies indicate that the tubulin-binding site for colchicine, podophyllotoxin, and nocodazole is in close proximity to or overlapping the binding site for Taxol (Hoebeke *et al.*, 1976; Cortese *et al.*, 1977; Uppuluri *et al.*, 1993; Rao *et al.*, 1994, 1995; Bai *et al.*, 1996). Therefore, the effect of Colcemid (a derivative of colchicine), podophyllotoxin, and nocodazole on cell-free palmitoylation of tubulin was examined. In the presence of the membrane extract, palmitoylation of both α- and β-tubulin was markedly reduced by all three drugs at stoichiometric concentrations (Figure 7A). Overexposure of the autoradiographs revealed that palmitoylation of proteins in the membrane extract was not affected by the drugs, indicating that the effect of these drugs on palmitoylation was specific for tubulin. Interestingly, nonenzymatic palmitoylation of tubulin was likewise inhibited by stoichiometric concentrations of Colcemid, podophyllotoxin, and nocodazole, suggesting that the sites of palmitoylation of tubulin by enzymatic and nonenzymatic reactions were the same.

The effect of vinblastine on palmitoylation of tubulin was examined because the binding site of this drug is

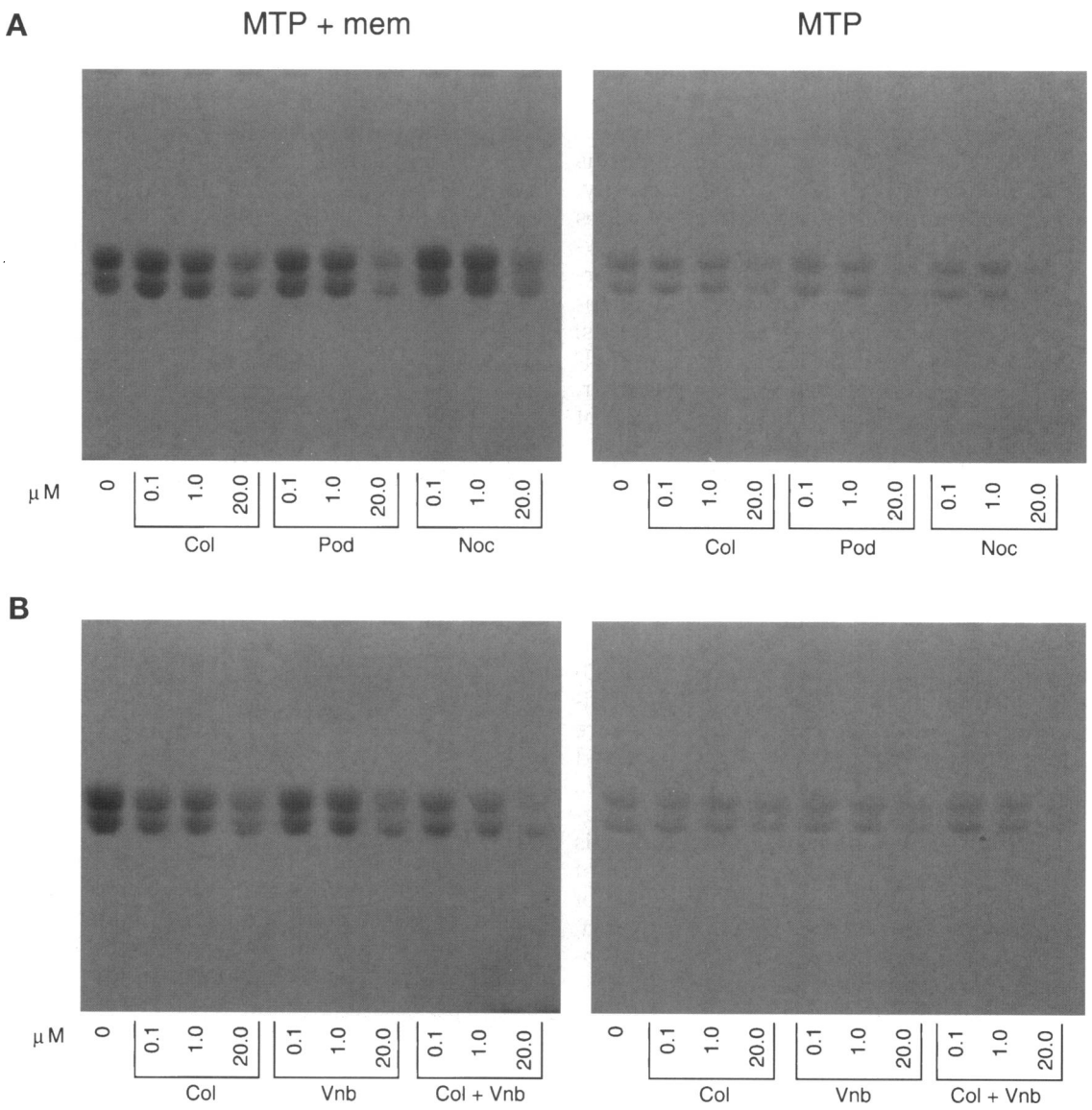

Figure 7. Effect of tubulin-specific drugs on cell-free palmitoylation of tubulin. (A) Microtubule protein (1 mg/ml) was incubated with Colcemid, podophyllotoxin, or nocodazole (0–20 μM) for 5 min at room temperature followed by the addition of membrane extract (0.35 mg/ml) and [^{14}C]palmitoyl-CoA. In duplicate samples, the membrane extract was omitted. After 2 h at 30°C, samples were processed for 1D-PAGE and fluorography. Resulting autoradiographs (2-d exposure) are shown. (B) Microtubule protein (1 mg/ml) was incubated with Colcemid, vinblastine, or Colcemid and vinblastine (0–20 μM) for 5 min at room temperature followed by the addition of membrane extract (0.35 mg/ml) and [^{14}C]palmitoyl-CoA. In duplicate samples, the membrane extract was omitted. After 2 h at 30°C, samples were processed for 1D-PAGE and fluorography. Resulting autoradiographs (2-d exposure) are shown. Col, Colcemid; Pod, podophyllotoxin; Noc, nocodazole; Vnb, vinblastine.

distinct from that of colchicine and Taxol (Burns and Surridge, 1994). In the presence of the membrane extract, stoichiometric concentrations of vinblastine reduced palmitoylation of both α- and β-tubulin to approximately the same level as did Colcemid (Figure 7B). Palmitoylation of tubulin was reduced even further if both Colcemid and vinblastine were added to the cell-free reaction.

DISCUSSION

Enzymatic and Nonenzymatic Palmitoylation of Tubulin

The results presented herein demonstrate that tubulin was posttranslationally modified by palmitoylation both in vivo and in vitro. In the cell-free system, tubulin was palmitoylated enzymatically. In addition,

a low level of nonenzymatic palmitoylation of tubulin occurred. The fact that tubulin-specific drugs blocked both enzymatic and nonenzymatic palmitoylation of tubulin suggests that the palmitoylation sites are the same.

In platelets, it is not yet known whether tubulin is palmitoylated enzymatically or nonenzymatically. However, the purification of palmitoylating enzymes (Dunphy *et al.*, 1996; Lui *et al.*, 1996) argues for enzymatic palmitoylation of proteins in vivo. More importantly, several lines of evidence demonstrate that tubulin was not palmitoylated after lysis of platelets. First, a 1000-fold molar excess of unlabeled palmitoyl-CoA to [^{3}H]palmitate was included in the lysis buffer. Second, differences were found between levels of palmitoylated tubulin in resting and activated platelets. Third, when the cell-free system was used to mimic conditions for lysis and processing of palmitoylated proteins from platelets, no palmitoylation of tubulin was observed after addition of the lysis buffer (my unpublished results).

The amount of palmitoylated tubulin in platelets is not known. Relative levels can be estimated from autoradiographs of [^{3}H]palmitoylated proteins after 2D-PAGE. The exposure time for detection of [^{3}H]palmitoylated tubulin was 44 d. Although this exposure time is close to those of other nontransfected [^{3}H]palmitoylated proteins (42–90 d; Parenti *et al.*, 1993; Mumby *et al.*, 1994), it is a long exposure, suggesting that either a small percentage of tubulin is palmitoylated in platelets or the rate of turnover of palmitate on tubulin is slow. However, detection of palmitoylated proteins in cells can be difficult, in part, because of the reversible nature of this posttranslational modification (Verkruyse and Hofman, 1996).

Assembly-competent Tubulin Was the Substrate for Palmitoylation

Several lines of evidence demonstrate that assembly-competent tubulin, and not denatured tubulin, was the substrate for palmitoylation in the cell-free system. First, studies of stepwise denaturation of tubulin demonstrate that the most sensitive property of the molecule is its ability to assemble into microtubules (Sackett *et al.*, 1994). Therefore, if tubulin assembles into microtubules, it can be assumed that these molecules are not denatured. As shown in Figure 5, tubulin in GMPCPP-assembled microtubules was a substrate for palmitoylation.

Second, experiments described above demonstrate that, at least at the beginning of the cell-free reaction, when microtubules were assembled, tubulin was not denatured. It was possible, however, that polymerization of tubulin into microtubules, as shown in Figure 5, actually protected tubulin from denaturation that may have occurred during the course of the experiment. Experiments described in Figure 6 demonstrate that this was not the explanation. In these experiments, one sample of microtubule protein was palmitoylated before addition of Taxol and GTP and subsequent microtubule assembly. In the control, no Taxol or GTP was added. If microtubules did protect tubulin against denaturation and if nonpolymerized tubulin did denature during the course of the experiment, then one would expect much higher levels of palmitoylated tubulin in the control (no microtubule assembly) than in the sample in which microtubule assembly occurred. This was not the case, suggesting that nonpolymerized tubulin was not denatured during the course of the experiment.

Finally, another property of tubulin that is sensitive to denaturation is binding of colchicine (Wiche *et al.*, 1977). Results shown in Figure 7 demonstrate that addition of the colchicine analogue, Colcemid, to the cell-free reaction caused a specific inhibition of the palmitoylation of tubulin without affecting the palmitoylation of proteins from the membrane extract. These data suggest that Colcemid blocked palmitoylation of tubulin by direct binding of the drug to tubulin protein. Such binding of the drug would not occur if tubulin was denatured.

Inhibition of Cell-Free Palmitoylation of Tubulin by Tubulin-specific Drugs

All of the tubulin-specific drugs tested (Taxol, Colcemid, podophyllotoxin, nocodazole, and vinblastine) blocked cell-free palmitoylation of tubulin. Colcemid (an analogue of colchicine), podophyllotoxin, and nocodazole share a binding site on β-tubulin (Hoebeke *et al.*, 1976; Cortese *et al.*, 1977; Uppuluri *et al.*, 1993; Bai *et al.*, 1996). Recent evidence indicates that this binding site is part of the binding site for Taxol (Rao *et al.*, 1994, 1995). Vinblastine, on the other hand, binds to a distinct region of β-tubulin (Burns and Surridge, 1994). Colchicine and vinblastine induce structural changes in both α- and β-tubulin that confer opposite effects on the tubulin dimer. For example, colchicine stimulates GTP hydrolysis while vinblastine inhibits hydrolysis (Sackett, 1995). This suggests that conformational changes induced by colchicine and vinblastine may involve at least overlapping regions of the tubulin molecule. In support of this conclusion, colchicine and vinblastine inhibit alkylation of α- and β-tubulin (Ludueña and Roach, 1981), suggesting that both of these drugs alter the accessibility or reactivity of sulfhydryl groups in α- and β-tubulin. Therefore, although the colchicine-binding site is distinct from that of vinblastine, it is possible that both drugs block palmitoylation of α- and β-tubulin at the same amino acid sites.

Possible Roles for Palmitoylated Tubulin

In the cell-free system, substrates for palmitoylation of tubulin were nonpolymerized tubulin (which contained GDP at the exchangeable site) and tubulin in microtubules assembled with the slowly hydrolyzable GTP analogue GMPCPP. In vivo, microtubules are composed of GDP-tubulin along microtubule lengths and GTP-tubulin at microtubule ends (Drechsel and Kirschner, 1994; Caplow and Shank, 1996). Since GMPCPP-assembled microtubules have subtle structural differences from microtubules assembled in vivo (Vale *et al.*, 1994; Hyman *et al.*, 1995), it remains to be determined whether GDP-tubulin, found along microtubule lengths, is also a substrate or whether palmitoylation is restricted to microtubule ends containing GTP-tubulin.

Palmitoylated proteins are membrane associated. Therefore, depending on the substrate for palmitoylation of tubulin in vivo, one would expect to find associations between membranes and tubulin along microtubule lengths or tubulin at microtubule tips or nonpolymerized tubulin. Interactions between membranes and microtubules have been clearly established. For example, intracellular vesicles and organelles, such as the endoplasmic reticulum, Golgi apparatus, mitochondria, and lysosomes, are transported and held in position along the sides of microtubules (for review, see Kelly, 1990; Cole and Lippincott-Schwartz, 1995). Motor proteins such as kinesin and dynein provide the force required for transport (for review, see Brady, 1991; Schroer and Scheetz, 1991; Skonfias and Scholey, 1993). However, maintenance of vesicles and organelles at particular positions may occur by palmitoylation of tubulin in microtubules and subsequent interaction of this modified protein with membranes. Since membranes are dynamic structures, repositioning could be accomplished by depalmitoylating tubulin and releasing its hold on the membrane.

Some intracellular membranes interact with the ends of microtubules. Waterman-Storer *et al.* (1995) presented in vitro studies showing that membranes from *Xenopus* eggs are moved and positioned after selective attachment to the growing ends of microtubules. Whether such events occur in vivo is not yet known. However, regulation of membrane-microtubule tip interactions may occur by cycles of palmitoylation and depalmitoylation of GTP-tubulin at microtubule ends.

Finally, interactions between membranes and nonpolymerized tubulin have also been described (Babitch, 1981; Kelly *et al.*, 1983; Pfeffer *et al.*, 1983; Simkowitz *et al.*, 1989; Lacey and Haimo, 1992). A role for membrane-associated tubulin in signal transduction has been proposed for a number of systems including lymphocyte activation (Offringa and Bierer, 1993) and regulation of G protein activities (Rasenick and Wang, 1988; Popova *et al.*, 1994). The fact that palmitoylated proteins are often involved in signal transduction (James and Olson, 1990; Casey, 1995; Milligan *et al.*, 1995; Ross, 1995) makes these studies even more compelling. The discovery that tubulin is a substrate for palmitoylation may provide a key to understanding some of the intriguing functions that have been attributed to a membrane-associated form of tubulin.

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