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Plasma Membrane Localization of Palmitoylated Tubulin

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PC12 pheochromocytoma cells incorporate [^{3}H]-palmitic acid into tubulin in a time- and cell-density-dependent manner. The plasma membrane-enriched fraction contains most of the radioactivity of the membrane pellet. While palmitoylated tubulin is found in both the cytoplasm and particulate fraction, the bulk of [^{3}H]palmitic acid bound to tubulin is present in the crude membrane pellet and the tubulin extracted from the plasma membrane is more heavily palmitoylated than that extracted from endoplasmic reticulum. Detergent-extracted tubulin from plasma membrane is, to a large extent, polymerization competent; a substantial fraction, increasing as a function of labeling time, is not hydroxylamine-labile. The requirement for detergent extraction, the accompanying changes in tubulin properties and the present findings of preferential incorporation of labeled tubulin into plasma membranes, make it clear that direct incorporation of tubulin into the plasma membrane can occur.

Key Words: **tubulin; palmitoylation; plasma membrane; endoplasmic reticulum; idroxylamine.**

Despite the fact that tubulin is a hydrophilic, very water soluble (>100 mg/ml) cytoplasmic protein, a significant fraction is firmly embedded in cellular membranes such that, e.g., its thermal stability is markedly enhanced and detergents are required to dislodge it. Such association is not due to artifactual tubulin binding during membrane isolation (1–3). Membrane tubulin can be visualized protruding intact red cell by the binding of Sepharose-immobilized colchicine, leading to rosette formation (3) and it has been identified as a firmly embedded constituent of membranes from coated vesicles (4), synaptic plasma membranes (5), and lymphoid cell membranes (6). Moreover, it has been suggested that tubulin extracted from membranes is more hydrophobic, but is not otherwise different from cytoplasmic tubulin, and is readily associated with lipids and membranes (7, 8).

Most of the currently described posttranslational modifications of tubulin do not provide sufficient hydrophobicity to promote membrane association. However, other hydrophilic proteins have been found to undergo various lipid modifications in order to associate with membranes. As the structural requirements for N-terminal myristoylation or C-terminal prenylation are absent in tubulin, palmitoylation seemed to be the most probable candidate for lipid modification. This was all the more likely because there are 20 free SH groups in tubulin, 12 in the α and 8 in the β monomer. Two previous studies have dealt with palmitoylation of tubulin. In one, platelet tubulin was labeled with [^{3}H]palmitate, presumably largely on the marginal band tubulin as particulate tubulin was not mentioned (9). In the other report it was shown that tubulin in crude pellet preparations from PC12 pheochromocytoma cells was metabolically labeled with [^{3}H]palmitic acid (10). It was important to learn which cellular compartment contained the bulk of the palmitoylated tubulin, and what kind of bond is formed between palmitic acid and tubulin. In the present study we examine the distribution of palmitoylated tubulin in cellular compartments, its partition between α and β monomers, and the nature of the bonds formed between palmitate and tubulin.

MATERIALS AND METHODS

Dulbecco's modified Eagle medium (DMEM), sera, L-glutamine and penicillin–streptomycin solution were from Biofluids, chymostatin was from Mega Biochemicals, [^{3}H]palmitate (specific activity 60 Ci/mmol) was from American Radiolabeled Chemicals Inc., palmitic acid, bestatin, aprotinin, leupeptin, protein A Sepharose, ATPase and glucose 6-phosphatase assay reagents, and NP-40 were from Sigma, the bicinchoninic acid assay kit was from Pierce, gel electrophoresis reagents, Tween 20 and SM2 Bio-Beads were from Bio-Rad, anti-α and anti-β tubulin monoclonal antibodies (clones DM1A and

Abbreviations used: Mes a.b., Mes assembly buffer; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum.

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DM1B) were from Calbiochem, rabbit anti-mouse antibody was from Cappel, sheep anti-mouse IgG horseradish peroxidase conjugated, monkey anti-rabbit IgG horseradish peroxidase conjugated and ECL Western blotting detection reagent were from Amersham, GelBond was from FMC Bioproducts (Rockland, ME), Immobilon-P transfer membrane was from Millipore, Ultima Gold scintillation fluid was from Packard.

Cell culture. PC12 cells, derived from a rat pheochromocytoma (11), were kindly provided by Dr. Gordon Guroff. Cells were plated at $100 \times 10^3/cm^2$ and grown in DMEM containing 2 mM L-glutamine, 7% fetal calf serum, 7% horse serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin at 37°C in 5.5% CO_2. The time course and optimum cell density for [^{3}H]palmitate incorporation were monitored for 1–24 h, and for 25–150 $\times$ $10^3/cm^2$.

Cell labeling and processing. Cells were grown for 12 h, washed twice with DMEM supplemented with 1% fetal calf serum, and incubated with 150 μCi of [^{3}H]palmitate/17 $\times$ 10^6 cells in DMEM with 1% fetal calf serum. After incubation cells were washed once with DMEM containing 1% fetal calf serum and 15 μM unlabeled palmitate or 15 μM unlabeled palmitoyl CoA, harvested by shaking in phosphate-buffered saline, and centrifuged at 200*g* for 10 min at 4°C. No difference was noticed in the [^{3}H]palmitate incorporation when unlabeled palmitate or unlabeled palmitoyl CoA was used in the washing media, so unlabeled palmitate was used routinely. The cell pellet was then washed twice with 20 ml of phosphate-buffered saline and sonicated in ice-cold Mes assembly buffer (Mes a.b. = 0.1 M Mes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) containing 0.25% sucrose, 0.3 mM β-mercaptoethanol and a cocktail of protease inhibitors (200 nM aprotinin, 10 μM leupeptin, 10 μM bestatin, 10 μM chymostatin and 10 μg/ml phenylmethylsulfonyl fluoride) until >95% of cells were broken. Homogenates were centrifuged at 200*g* for 10 min at 4°C to remove nuclei and debris. The supernatant solution was centrifuged at 130,000*g* for 30 min at 4°C, and radioactivity from supernatant and pellet was measured.

Protein extraction and tubulin purification. (a) From cytoplasm. Labeled tubulin was collected from the crude 130,000*g* supernatant solution at 37°C for 45 min in the presence of 10% dimethyl sulfoxide and 1 mM GTP by copolymerization with 500 μg of pure rat brain tubulin prepared as previously described (12). The microtubules were collected by centrifugation at 130,000*g* for 30 min at 33°C, resuspended in cold Mes a.b. and sonicated. Protein was measured by the bicinchoninic acid assay using bovine serum albumin as standard.

(b) From membrane. The 130,000*g* pellet from crude homogenate was washed repeatedly with 3 ml of Mes a.b. until no radioactivity was detected in the washing solution. The pellet was resuspended by sonication and incubated for 5 h with Mes a.b., 1% NP-40 and protease inhibitors at 4°C. The resultant suspension was centrifuged at 130,000*g* for 30 min at 4°C and the supernatant solution was used to purify tubulin, as described for cytoplasm, after detergent removal for 2 h at 4°C with an equal volume of Bio-Beads. The extend of polymerization (39%) for labeled tubulin extracted from membrane was almost the same, under these conditions, as that of rat brain tubulin (44%). Only a small fraction was not polymerization competent.

Electrophoresis and bond analysis. Samples of purified membrane tubulin were loaded on 1-mm-thick 10% acrylamide gels cast on GelBond and run at 40 mA for 20 min past dye expulsion, stained with Coomassie brilliant blue, and photographed. Bands were processed as previously described (10). To study the nature of the palmitate bonds, gels were fixed for 30 min in 25% (v/v) isopropanol in water at room temperature and washed several times with 10 vol of distilled water. Separate gels were incubated for 4 h with 10 gel volumes of either 1.0 M Tris–HCl, pH 7.5, 1.0 M hydroxylamine–HCl, pH 7.5 freshly prepared, or 0.1 M KOH in methanol, washed several times with 10 gel volumes of distilled water, stained and counted (10). For fluorography, stained gels were treated for 6 h in 1 M sodium salicylate, dried, and exposed to Biomax MR Kodak film for 60 days at −80°C.

Purification of plasma membranes. Crude homogenate from PC12 cells incubated for 12 h with 600 μCi of [^{3}H]palmitate/17 $\times$ 10^6 cells was layered onto a discontinuous sucrose gradient of 30, 40, and 45% sucrose in Mes a.b. and centrifuged in a Beckman SW 27 rotor at 27,000 rpm for 180 min at 4°C. Bands at the interfaces were withdrawn, diluted with phosphate buffered saline and pelleted for 10 min at 130,000*g*. Pellets were resuspended in Mes a.b. and tested for glucose 6-phosphatase (13) and ouabain-sensitive Na/K ATPase (14). The radioactivity from each band was measured.

Immunoprecipitation of tubulin. The plasma membrane and endoplasmic reticulum fractions were extracted as described above. After detergent removal the samples were precleared for 1 h at 4°C with 100 μl of protein A-Sepharose to remove nonspecific IgG and centrifuged at 1500*g* for 2 min at 4°C. Tubulin immunoprecipitation was carried out with 5 μg of anti-α tubulin (clone DM1A) and 5 μg of anti-β tubulin (clone DM1B) incubated with gentle rotation at 4°C for 1 h, then 100 μl of protein A-Sepharose conjugated with anti-mouse IgG was added and incubated for one more hour. Immunoprecipitated proteins were washed 6 times with Mes a.b., centrifuged at 1500*g* for 2 min at 4°C, resuspended in loading buffer containing 10 mM dithiothreitol, boiled for 1 min and analyzed by SDS–PAGE or Western blotting. The immunoprecipitable tubulin from plasma membrane was equal to 0.1% of the total protein extracted by 1% NP-40 from plasma membrane. For Western blotting the proteins were transferred on an Immobilon-P transfer membrane for 2 h at 0.8 mA/cm^2. After blocking by incubation for 2 h in TNE 5% nonfat milk (TNE = 200 mM NaCl, 50 mM Tris, 25 mM EDTA) the transfer membrane was probed with anti-α and anti-β tubulin monoclonal antibodies (diluted 1:1000 in TNE) for 1 h at room temperature washed four times for 5 min with TNE 0.5% Tween 20, incubated with the second antibody, anti-mouse IgG conjugated with horseradish peroxidase, diluted 1:3000 in TNE for 1 h at room temperature, washed as above, and detected by ECL Western blotting detection reagents. When the same transfer membrane was used to visualize IgG, it was first stripped by incubation with 0.2 M NaOH for 5 min, then washed with distilled water four times, blocked as above, and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:3000 in TNE) for 1 h at room temperature, washed as above and exposed to ECL.

RESULTS

Variables Affecting the Uptake of [^{3}H]Palmitate by Membrane Tubulin

Several variables affecting the total uptake of [^{3}H]palmitate by PC12 cells were investigated. Cell density was varied from $25 \times 10^3/cm^2$ to $150 \times 10^3/cm^2$. Maximum [^{3}H]palmitate uptake by cells occurred between 70–100 $\times$ $10^3/cm^2$ (Fig. 1A). The cell density effect was primarily due to the particulate fraction, whereas [^{3}H]palmitate incorporation into the cytosol was independent of cell density. [^{3}H]palmitate uptake into PC12 cells increased as a function of time for the first 12 h after which it decreased (Fig. 1B). To test whether the conditions for total [^{3}H]palmitate incorporation were reflected in palmitoylation of tubulin, the crude pellet was extracted with 1% NP-40 and tubulin was purified and analyzed by gel electrophoresis. Both α and β tubulin labeling increased as a function of time up to 6 h, after which time the level remained stable

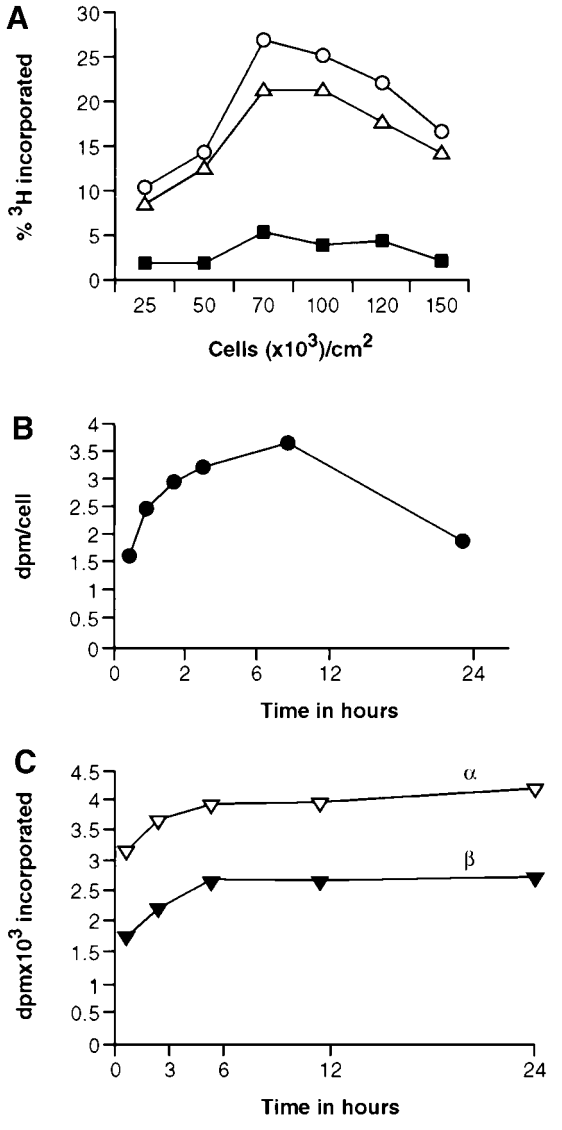

FIG. 1. Optimization of procedures. (A) Cell density. PC12 cells were plated at different densities and exposed to [^{3}H]palmitate for 24 h. Cells were sonicated and crude homogenate was centrifuged at 130,000*g* to separate pellet from cytosol. Radioactivity from all three fractions was measured and expressed as % of total radioactivity. ○, crude homogenate; △, pellet; ■, supernatant. (B) Time course of [^{3}H]palmitate incorporation. PC12 cells were plated at 100×10^3/cm^2 and exposed to [^{3}H]palmitate for 24 h. (C) Time course of [^{3}H]palmitate incorporation in α and β tubulin of the crude pellet. PC12 cells were exposed to 800 μCi [^{3}H]palmitate/17×10^6 cell for 1, 3, 6, 12, or 24 h. The dpm incorporated into α tubulin (▽) and β tubulin (▼) are indicated after background subtraction.

with $\alpha > \beta$ (Fig. 1C). Consequently we used 12-h incubation times.

Localization of Palmitoylated Tubulin to the Plasma Membrane

The crude homogenate from sonicated PC12 cells was centrifuged at 130,000*g* for 30 min and tubulin was purified from both supernatant (cytoplasm) and pellet (membrane) as described under Materials and Methods. Both cytoplasmic and membrane tubulin were labeled with [^{3}H]palmitate: 23% of the total labeled tubulin was present in cytoplasmic tubulin with 4.6×10^3 dpm in α tubulin and 3.7×10^3 dpm in β tubulin; 77% of the label was in membrane tubulin with 11×10^3 dpm in α tubulin and 10×10^3 dpm in β tubulin (Table 1). The pellet from the 130,000*g* centrifugation was partitioned in a discontinuous sucrose gradient that favored separation of the plasma membrane. The band at the 30/40% interface contained the bulk of the oubain-sensitive Na/K ATPase, while the band at the 40/45% interface contained the unfractionated endoplasmic reticulum as identified by the distribution of glucose 6-phosphatase. As shown in Table 2, the plasma membrane-enriched fraction contained most of the radioactivity of the membrane; this was also equal to 81% of the total radioactivity of the crude pellet. Only 5.7% of the total radioactivity, was associated with the endoplasmic reticulum fraction whereas the remaining 13.3% of radioactivity was found in the density gradient pellet.

To measure the [^{3}H]palmitoylated tubulin in plasma membranes or endoplasmic reticulum, proteins were extracted with 1% NP-40 from both purified membranes for 5 h. Using this procedure nearly all (>90%) of the tubulin present in the membrane was released despite the fact that a number of other proteins remained unextracted, as revealed by gel electrophoresis. After removal of the detergent with Bio-Beads, tubulin was purified by immunoprecipitation with anti-α and anti-β tubulin monoclonal antibodies. The immunoprecipitated proteins were electrophoresed in SDS–polyacrylamide gels and stained; both membrane fractions contained α and β tubulin. The bands corresponding to α and β tubulin were cut out and counted. Even though we started with the same amount of protein from both fractions and extracted comparable fractions of tubulin as judged by SDS gels, the labeling of the tubulin from plasma membrane was substantially

TABLE 1

Distribution of Radioactivity in PC12 Cells

	[^{3}H]Palmitate	α Tubulin (dpm $\times 10^3$)	β Tubulin (dpm $\times 10^3$)
Cytoplasm	23%	4.6	3.7
Membranes	77%	11	10

Note. Crude homogenate from sonicated PC12 cells was centrifuged at 130,000*g* for 30 min and tubulin was purified by copolymerization from both supernatant solution (cytoplasm) and NP-40-extracted pellet (membranes). The radioactivity incorporated into tubulin of each fraction is expressed as % of the total polymerizable tubulin of both fractions. α and β tubulin were separated by gel electrophoresis and the incorporated radioactivity in both subunits was counted.

TABLE 2

Membrane Identification

	Na/K ATPase (nmole P/mg)	G6Pase (mg P/mg)	dpm × 10^6/mg	α Tubulin dpm	β Tubulin dpm
Plasma membrane	61	0.37	81	1735	1143
Endoplasmic reticulum	7	0.75	9.5	581	198

Note. Fractions from discontinuous sucrose gradients were isolated and characterized enzymatically for 20 min; radioactivity was counted. α and β tubulin were immunoprecipitated from purified membrane fractions and separated by electrophoreses; dpm are expressed after background correction. All results are expressed per mg of protein and are the average of three different experiments. G6Pase, glucose 6-phosphatase; P, inorganic phosphate.

greater than that of endoplasmic reticulum tubulin (Table 2). The α/β ratio of radioactivity was >1 in both membrane fractions and was similar to that seen in the crude pellet fraction (see Fig. 1C). It is interesting to note that the electrophoretic mobility in the gel of both tubulin subunits purified from plasma membrane is slightly faster than that of pure rat brain tubulin used as a control (Fig. 2). Because the molecular weights of the heavy subunit of IgG and the α and β subunits of tubulin are similar (~50 kDa), it was necessary to rule out comigration of IgG with α or β tubulin. Figure 3 compares immunoprecipitated plasma membrane tubulin of PC12 cells with untreated rat brain tubulin. The faster mobility of IgG and the lack of contamination of the tubulin bands with IgG are clearly demonstrated by incubation with antibody anti-IgG (Fig. 3C). Superimposition of Figs. 3B and 3C confirms this difference as shown in Fig. 3D.

The Nature of the Tubulin–Palmitic Acid Bond

Protein palmitoylation is generally considered to occur primarily on −SH groups of cysteine and this more labile thioester bond would be more likely to be readily reversible. Depalmitoylation by 1 M neutral hydroxylamine is considered to be evidence for the presence of the fatty acid in thioester bonds (17). However, we have showed previously that in both α and β tubulin subunit only a portion of [^{3}H]palmitate was hydroxylamine-labile. An additional part of palmitate was bound by methanolic KOH-labile bonds, and a remaining portion of the protein-bound palmitate was even KOH-resistant (10). Indeed, labeling experiments in other systems have shown that hydroxylamine resistant (15) and even amide (16) bonds with [^{3}H]palmitate may occur. In attempting to understand if this bond distribution was due to failure of slow hydroxylamine hydrolysis we used replicate gels, treated them with hydroxylamine or methanolic KOH for 4 to 24 h and stained to check for any loss of protein. No loss was found as a result of these treatments and the bond distribution was not changed after 24 h.

To understand whether these bonds show different dynamics, a time course of [^{3}H]palmitate incorporation was carried out followed by depalmitoylation as above. Cells were incubated in the presence of [^{3}H]palmitate

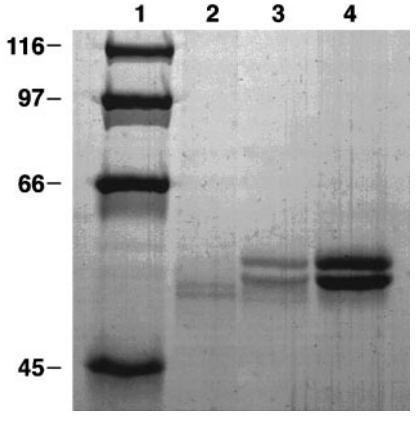

FIG. 2. Electrophoretic mobility of plasma membrane tubulin. Tubulin purified by immunoprecipitation from plasma membrane and electrophoresed in 10% polyacrylamide gel in SDS. Lane 1, molecular weight standards; Lane 2, α and β tubulin purified from plasma membrane; Lane 3, immunoprecipitated rat brain tubulin; Lane 4, rat brain tubulin.

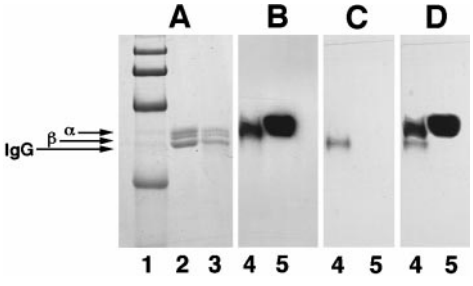

FIG. 3. Immunoprecipitation of plasma membrane tubulin. Proteins were extracted from plasma membrane sucrose fractions of 125 × 10^6 cells by incubation with 1% NP-40. Tubulin was purified by immunoprecipitation with monoclonal anti-α and anti-β tubulin antibodies and visualized by polyacrylamide gel electrophoreses in SDS and Western blotting. (A) Coomassie-stained gel. Lane 1, molecular weight standards; Lane 2, 5.5 μg rat brain tubulin + 2 μg IgG; Lane 3, immunoprecipitated rat brain tubulin. (B) Western blot probed with anti-α and anti-β tubulin; Lane 4, tubulin immunoprecipitated from plasma membrane; Lane 5, rat brain tubulin. (C) Western blot probed with anti IgG. Lanes as in B. (D) B and C superimposed. Lanes as in B. IgG and tubulin subunits migrate in different position.

TABLE 3

Depalmitoylation of [^{3}H]Palmitic Acid-Labeled Tubulin as a Function of Labeling Time

	Time in hours	% NH_2OH sensitive	% KOH sensitive	% unhydrolyzed
α	1	57	30	13
	3	43	23	33
	6	38	30	30
	12	22	21	56
β	1	52	35	11
	3	40	25	33
	6	19	44	35
	12	14	43	41

Note. PC12 cells were incubated in the presence of [^{3}H]palmitic acid for 1, 3, 6, or 12 h. Samples of purified tubulin were electrophoresed on a 10% SDS acrylamide gel. Lanes were incubated for 4 h with either 1.0 M Tris–HCl, pH 7.5 (control), 1.0 M hydroxylamine–HCl, pH 7.5, or 0.1 M KOH in methanol. Bands were cut and residual radioactivity was measured.

for 1, 3, 6, and 12 h, tubulin was extracted and purified from the crude pellet as described above, electrophoresed on parallel SDS gels and treated as above. The fraction of these bonds was calculated and the results are reported in Table 3. The portion present as hydroxylamine labile bonds decreased in a time-dependent manner: these bonds decreased substantially in 12 h of incubation with compensatory increases in both KOH-labile and KOH-resistant bonds.

DISCUSSION

Several points have been brought to light in the present study, one technical and one functional. The former deals with the uncertain nature of protein-palmitic acid bond in some proteins. The current dogma for differentiating thioester bonds from presumptive oxyester bonds is largely based on hydrolysis of the former by 1 M neutral hydroxylamine whereas the latter require methanolic KOH to be hydrolyzed. In many proteins hydroxylamine indeed releases nearly all of the bound palmitic acid. However, hydroxylaminolysis is not entirely specific for thioesters, rates of depalmitoylation of viral glycoproteins vary widely (17), and rate constants for depalmitoyation of oxy- and thioesters may overlap. There are numerous reports in which hydroxylamine did not hydrolyze all the protein-bound palmitic acid (9, 10, 17–19) and the unhydrolyzed residue is usually ascribed to incomplete treatment with hydroxylamine or it is ignored. Tubulin from platelets was also incompletely depalmitoylated (40%) by hydroxylamine (9) and autopalmitoylated rat brain tubulin released only 70% of its palmitic acid with hydroxylamine (20). In the case of plasma membrane tubulin from PC12 cells, the fraction resistant to 1 M hydroxylamine is substantial even after 24 h of exposure. Moreover, the proportion of hydroxylamine-resistant ester bonds increases as a function of time. Since the more stable oxyester is not likely to form thioesters, the accumulation of hydroxylamine-resistant palmitate bonds as a function of time may imply that oxyesters of palmitate with tubulin may have been formed, but their identity has not been established. Regarding possible functional consequences of tubulin palmitoylation, it should be noted that microtubules have numerous and varied interactions with organelle and plasma membranes. This extends from very elaborate associations seen in lower species, e.g., *Distigma proteus* (21), *Paramecium* (22), *Platyhelminthes* (23), and many others, to higher eukaryotes where microtubules are often seen in contact with mitochondria (24–26), synaptic vesicles (27), endoplasmic reticulum (28), lysosomes (29), cilia (30), and plasma membranes (31). The nature of the microtubule/membrane attachment is often not well specified. In some cases there are linker proteins such as MAPs, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (32), or kinesins and dyneins (33) attaching vesicles. In other cases there is a very close contact between microtubule and membrane that presumably does not require a linker (30). Direct insertion into the membrane has been proposed and binding of extrinsic tubulin to membranes has been demonstrated (24, 29). We recently reported that both α and β tubulin monomers in a crude membrane pellet are posttranslationally modified by palmitic acid (10). We now show that tubulin modified by [^{3}H]palmitic acid is present in both the cytoplasmic and membrane fractions of PC12 cells with the bulk of [^{3}H]palmitoylated tubulin preferentially localized to the plasma membrane, with a smaller fraction in the endoplasmic reticulum and other membranes. Much of the membrane-derived tubulin is polymerization competent in the presence of pure rat brain tubulin, a smaller fraction is not. Which of these two moieties is more important for microtubule attachment and which subserves others functions is not known at present. Whereas indirect membrane association of tubulin via linker proteins (32) is in no way ruled out, the requirement for detergent extraction and the accompanying changes in tubulin properties, together with the present findings of preferential incorporation of labeled tubulin into plasma membranes, make it clear that direct incorporation of tubulin into the plasma membrane can occur.

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