Dynamic Interactions between Microtubules and Artificial Membranes†

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ABSTRACT: We report that extensive adsorption of microtubule protein to liposomes occurs above the transition temperature of the phospholipid bilayer, occurs to phosphatidylcholine (PC) or phosphatidylserine (PS) vesicles, and is not affected by preincubation of microtubule protein with colchicine. Most importantly, we show that 51–63% of the tubulin adsorbed onto neutral (PC) phospholipid vesicles can be desorbed to form microtubules when buffer conditions are adjusted to favor microtubule assembly. By contrast, no microtubule assembly occurred with preadsorption of microtubule protein onto acidic (PS) phospholipid vesicles, suggesting irreversible binding.

The presence of tubulin in biological membranes has been reported by several investigators (Bhattacharyya & Wolff, 1975; Kelly et al., 1983; Stephens, 1985; Zisapel et al., 1980), and recently tubulin has been shown to bind to membranes isolated from mammalian liver (Reaven & Azhar, 1981) and adrenal medulla (Bernier-Valentin et al., 1983). In order to better understand the mechanism and consequences of tubulin interactions with membranes, we initiated studies of the interaction of tubulin with simple phospholipid vesicles. We found that both microtubule protein and phosphocellulose-purified tubulin bind to unilamellar liposomes of dimyristoylphosphatidylcholine (DMPC)[1] (Caron & Berlin, 1979). These observations have been confirmed and extended by Klausner et al. (1981, 1982), who studied the interaction of purified tubulin protein with vesicles of dipalmitoylphosphatidylcholine (DPPC) by determining leakage of vesicle content.

Although microtubule protein and tubulin in particular have an affinity for phospholipid membranes, the physiological role of this interaction remains unclear. We report here that binding of microtubule protein to neutral phospholipid vesicles is reversed upon introduction of microtubule assembly conditions and that the desorbed protein is incorporated into microtubules. These observations demonstrate that binding is not associated with tubulin denaturation and further suggest new physiological mechanisms by which microtubules and membranes are mutually and dynamically active via the tubulin protein.

Materials and Methods

Protein Isolation and Labeling Procedures. Microtubule protein was prepared from bovine brain by three cycles of assembly–disassembly according to the procedure of Margolis and Wilson (1978) with slight modifications (Regula et al., 1981). Microtubule-associated proteins (MAPs) and tubulin represented approximately 20% and 80% of the protein, respectively, as determined by SDS–polyacrylamide gel electrophoresis and scanning densitometry. Protein concentrations were determined by the method of Lowry (1951). The critical concentration (Cc) for microtubule assembly was determined as previously described (Regula et al., 1981).

Microtubule protein was labeled with the fluorescent dye (dichlorotriazino)aminofluorescein (DTAF) (Molecular Probes, Plano, TX) after the second polymerization (Keith et al., 1981) and taken through two additional cycles of assembly–disassembly. Immediately before use, the disassembled DTAF-labeled microtubule protein was applied to a Sephadex G-25 column equilibrated with 20 mM sodium phosphate and 100 mM sodium glutamate, pH 6.75, to remove unconjugated dye. The final dye to protein ratio was approximately 0.34.

^{3}H-Tyrosinolated tubulin was prepared by incubating three times cycled microtubule protein with purified tubulin:tyrosine ligase (Preston et al., 1979), [^{3}H]tyrosine (specific activity 3 Ci/mmol; New England Nuclear), and other required cofactors for 1 h at 37 °C (Deanin et al., 1977). Microtubule protein containing the labeled tubulin was subjected to an additional cycle of assembly–disassembly. Free [^{3}H]tyrosine was removed by molecular sieve chromatography (see above). The specific activity was 1.41×10^5 cpm/mg of protein. Microtubule protein was stored for up to 4 weeks at −80 °C.

Liposomes. Unilamellar phospholipid vesicles were prepared under nitrogen in 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 6.75, according to the method of Rogers and Strittmatter (1975). Vesicles were formed from lipids (Sigma Chemical Co., St. Louis, MO) of the following compositions: pure egg lecithin, 30 mg/mL; dimyristoylphosphatidylcholine (DMPC), 30 mg/mL; DMPC/phosphatidylserine (DMPC/PS; 50% w/w), 30 mg/mL. For density gradient experiments, [^{14}C]dipalmitoylphosphatidylcholine ([^{14}C]DPPC; 0.005 μCi/mL; New England Nuclear) was added to lipids before vesicle formation. Phospholipids were routinely stored dark and dry in the presence of butylated hydroxytoluene (BHT). Their purity was checked by thin-layer chromatography of silica-coated glass plates (Whatman LHP) using a solvent system of chloroform/ethanol/triethylamine/H_2O (30:35:34:8). Phosphate was assayed by the method of Fiske and Subbarow (1925).

Density Gradient Separation of Liposome–Protein Complexes from Unbound (Soluble) Protein. Liposomes containing tracer amounts of [^{14}C]DPPC were incubated with DTAF-labeled or ^{3}H-tyrosinolated microtubule protein at phospholipid to protein molar ratios of either 200:1 or 4000:1. After 30 min at 30 °C, an aliquot (240 μL) of the sample was

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[1] Abbreviations: Apo A-I, apolipoprotein A-I; Cc, critical concentration; DTAF, (dichlorotriazino)aminofluorescein; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPs, microtubule-associated proteins; PC, phosphatidylcholine; PS, phosphatidylserine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

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mixed with 1 mL of Ficoll of density 1.16. A discontinuous Ficoll gradient was formed by sequentially layering the following densities over the Ficoll–sample mixture: 1.13 (sample), 1.10, 1.08, 1.06, and buffer (50 mM Pipes, pH 6.75). Liposomes were separated from nonadsorbed proteins and washed by centrifugation upward through the gradient at 100000*g* for 16 h at 30 °C. Aliquots (approximately 0.33 mL) were removed sequentially from the gradient. Radioactivity of [^{14}C]DPPC was measured in a Beckman LS-7500 scintillation counter. Fluorescence of DTAF-labeled microtubule protein was assayed by using a Perkin-Elmer MPF-4 spectrofluorometer with excitation and emission set at 492 and 512 nm, respectively. In some experiments, DTAF-labeled microtubule protein was preincubated with a 10-fold molar excess of colchicine for 90 min at 30 °C before incubation with liposomes. Unbound colchicine was removed by molecular sieve chromatography. Although the stoichiomery of colchicine binding was not determined, sufficient binding to prevent assembly was inferred by the absence of microtubules, as judged by negative-staining electron microscopy, after introduction of assembly conditions.

Reversible Binding of Microtubule Protein to Liposomes. Microtubule protein was added to liposomes to a final protein concentration of 1 mg/mL and a final phospholipid to protein molar ratio of 4000:1. After 30 min at 30 °C, the mixture was made 1.5 mM GTP, 1 mM EGTA, and 0.5 mM $MgCl_2$, and incubated for an additional 40 min at 30 °C. Immediately before addition of protein, liposomes were preincubated with 0.5 mM dithiothreitol for 5 min at 30 °C to prevent intermolecular disulfide bond formation between lipsome-bound microtubule protein molecules (Caron & Berlin, 1979).

Quantitation of Microtubule Assembly. To quantitate the concentration of microtubules formed after introduction of assembly conditions to protein–liposome mixtures, we first attempted to separate liposomes from microtubules by various sedimentation procedures. Unlike the clean separation of liposomes and free protein achieved in the Ficoll gradient, procedures could not be found that gave reliably complete separations of liposomes and microtubules. Consequently, we developed a morphological assay for microtubule assembly that did not require liposome removal. By this method, microtubule lengths were measured on negatively stained grids and compared with samples of microtubules of known concentration. Specifically, samples were diluted 1/10 with a microtubule stabilizing buffer [0.1 M Pipes, pH 6.75, 1 mM EGTA, 0.5 mm GTP, 4% poly(ethylene glycol) 6000], and immediately, a drop of sample was placed on a carbon-coated Formvar grid. After 30 s, the protein was fixed for 1 min with 2% glutaraldehyde and 0.1 M cacodylate, pH 7.4, stained with filtered 2% aqueous uranyl acetate, and examined on a JEOL 100 CX electron microscope. The carbon-coated, Formvar grids were routinely glow discharged for 15 min within 6 h of use to ensure even spreading on the grid. Micrographs of five predetermined positions per grid were enlarged to a final magnification of 17820×. Microtubule assembly was quantitated by determining total microtubule length per photograph as measured by a sonic digitizer (Graf/Pen, Science Accessories Corp.).

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis, using gradient slab gels (13 × 16 × 0.009 cm) of 5–15% acrylamide with a 3% stacking gel, was performed according to the method of Laemmli (1970). After electrophoresis, gels were fixed and stained in 0.025% Coomassie blue, 10% glacial acetic acid, and 15% 2-propanol and destained in 20% methanol and 10% glacial acetic acid.

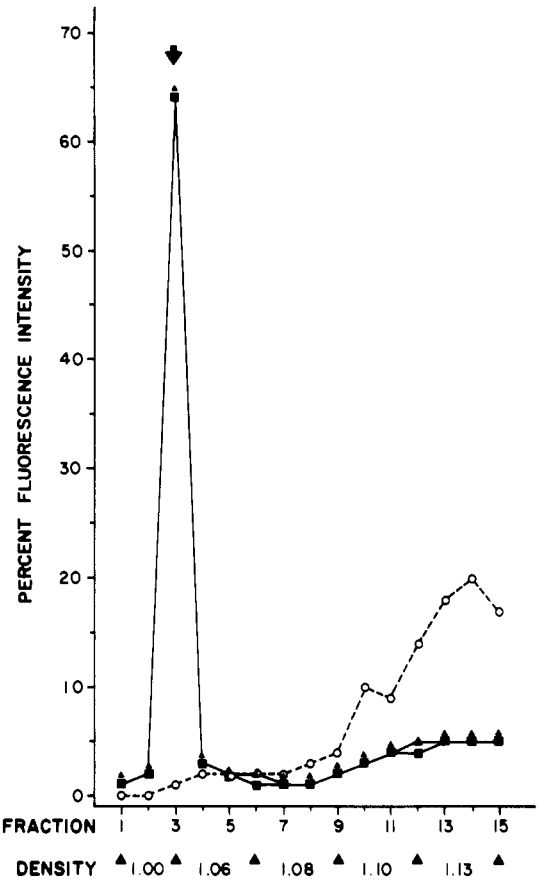

FIGURE 1: Binding of microtubule protein to liposomes. Microtubule protein, labeled with the fluorescent dye DTAF, was incubated with unilamellar vesicles of egg lecithin containing [^{14}C]DPPC (0.005 μCi/mL) at a phospholipid to protein molar ratio of 200:1 for 30 min at 30 °C. Liposome-bound microtubule protein was separated from nonadsorbed or soluble protein by centrifugation on a Ficoll gradient (see Materials and Methods). Density 1.13 represents the sample loading zone. The solid arrow indicates the position of 99% of the lipid after centrifugation. Symbols: (■) microtubule protein following incubation with liposomes, (▲) microtubule protein preincubated with colchicine before incubation with liposomes, and (○) microtubule protein alone (no liposomes).

Results

Quantitation of Binding of Microtubule Protein to Phospholipid Vesicles. Binding of DTAF-labeled microtubule protein to neutral phospholipid vesicles was quantitated after separation of the liposome–protein complex from free microtubule protein by Ficoll gradient centrifugation. This gradient provided a clean separation between liposome-bound and free protein with 99% of the lipid washing up and concentrating near the top of the gradient, leaving unbound protein in the sample loading zone. As shown in Figure 1, when DTAF-labeled microtubule protein was incubated with liposomes of egg lecithin, 72% of the protein moved up through the gradient along with the liposomes, leaving behind the nonadsorbed protein (approximately 23%). In contrast, 88% of the microtubule protein remained in the loading zone of gradients containing protein without liposomes. Data from six experiments, using a phospholipid to protein molar ratio of either 200:1 or 4000:1, demonstrated that an average of 73.1 ± 1.2% (SE) of the microtubule protein was adsorbed onto liposomes. By use of microtubule protein with ^{3}H-tyrosinolated tubulin as a marker, more than 95% of the protein was recovered in the liposome zone (data not shown). Control experiments demonstrated that tyrosinolated and nontyrosinolated tubulin bound equally to liposomes (Berlin and Deanin, unpublished observations), as shown by others (Klausner et al., 1981).

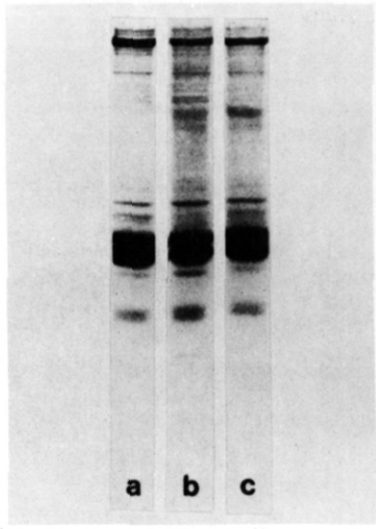

FIGURE 2: Adsorption of microtubule protein into neutral (DMPC) and acidic (DMPC/PS) phospholipid vesicles. Microtubule protein (1 mg/mL) was incubated with either DMPC or DMPC/PS vesicles (30 mg/mL) for 30 min at 30 °C. Liposome-bound protein was separated from nonadsorbed protein by centrifugation on a Ficoll gradient (see Materials and Methods). Proteins from the liposome-bound fraction were analyzed by SDS–polyacrylamide gel electrophoresis. (a) Three times cycled microtubule protein; (b) DMPC-adsorbed proteins; (c) DMPC/PS-adsorbed proteins.

Substituting dimyristoylphosphatidylcholine for egg lecithin produced identical results.

We note that there was a higher efficiency of adsorption of microtubule protein containing ^{3}H-tyrosinolated tubulin than that with DTAF-labeled protein. Since only α-tubulin was labeled with [^{3}H]tyrosine, while all proteins [microtubule associated proteins (MAPs) and α- and β-tubulins] were labeled with DTAF, it was possible that the different efficiencies were due to differential adsorption of MAPs and tubulin onto liposomes. Specifically, the observed differences in efficiency of adsorption would be predicted if only tubulin, and no MAPs, adsorbed onto liposomes. To examine this possibility, SDS–polyacrylamide gel electrophoresis was used to compare three times cycled microtubule protein with proteins adsorbed onto neutral and acidic phospholipid vesicles (Figure 2). No major differences in the electrophoretic pattern of proteins was found. The content of microtubule-associated proteins in liposome-adsorbed samples was approximately 20%, as was found in three times cycled microtubule protein. These results indicate that preferential adsorption of MAPs and tubulin, onto either type of vesicle, did not occur. Alternatively, the higher efficiency of adsorption of microtubule protein with ^{3}H-tyrosinolated tubulin may be related to a decreased stability of DTAF-labeled microtubule protein as determined by its progressive loss in ability to copolymerize with unlabeled microtubule protein (Berlin, unpublished observations). In any event, these results demonstrate that a majority of microtuble protein remained bound to liposomes even after their washing through the gradient, implying that highly stable microtubule protein–liposome complexes were formed.

When microtubule protein was preincubated with the antimitotic drug colchicine (see Materials and Methods) and then incubated with liposomes, again a total of 72% of the DTAF-labeled microtubule protein was adsorbed onto the vesicles (Figure 1). Preincubation of microtubule protein containing ^{3}H-tyrosinolated tubulin with colchicine or podophyllotoxin resulted in 95% adsorption onto liposomes (data not shown). One-dimensional gel electrophoretic analysis showed that the pattern of proteins bound to liposomes was not altered by preincubation of microtubule protein with these drugs.

Reversibility of Binding of Microtubule Protein to Neutral Phospholipid Vesicles. When microtubule protein at 1 mg/mL was first adsorbed onto DMPC vesicles for 30 min at 30 °C, the concentration of nonadsorbed protein was determined to be 0.05 (from data using microtubule protein containing ^{3}H-tyrosinolated tubulin)–0.28 mg/mL (as determined with DTAF-labeled microtubule protein) by the Ficoll gradient method described above. This range of concentration of nonadsorbed proteins (0.05–0.28 mg/mL) is at or below the critical concentration for assembly (Cc) of the microtubule protein preparations used in these experiments (Cc = 0.10–0.27 mg/mL). Yet, when the liposome–protein mixture was further incubated under assembly conditions for an additional 40 min at 30 °C, numerous microtubules were seen in negatively stained preparations (data not shown). These microtubules were morphologically indistinguishable from microtubules assembled in the absence of liposomes. However, if liposome–protein complexes was first centrifuged out of solution and microtubule assembly conditions were then introduced to the liposome-free supernate containing nonadsorbed proteins, no microtubules were seen by negative-staining electron microscopy. These results suggest that the concentration of nonadsorbed protein was below the Cc for microtubule assembly and that desorption of microtubule protein from liposomes must have occurred for microtubule assembly to take place.

This apparent desorption of microtubule protein was quantitated from the appearance of microtubules after introduction of assembly conditions to protein–liposome mixtures. To quantitate the concentration of microtubules, we initially attempted to separate microtubules and liposomes by gradient centrifugation. However, unlike the clean separation of liposome-adsorbed and nonadsorbed proteins obtained by Ficoll gradient centrifugation in the absence of assembly conditions (Figure 1), no procedure were found that cleanly separated assembled microtubules and liposomes. Therefore, an electron microscopic morphometry assay, which did not require separation of microtubules and liposomes, was used to quantitate the concentration of microtubules (see Materials and Methods). Briefly, microtubule lengths were measured on negatively stained grids and compared to samples of microtubules of known concentration. To ensure accurate measurement of microtubule lengths, a 1/10 dilution of the sample in microtubule stabilizing buffer was used before negative staining. Since dilution is known to cause disassembly of microtubules (Karr et al., 1980; Mitchison & Kirschner, 1985), it was first necessary to show that the microtubule stabilization buffer employed was, indeed, preventing disassembly. Microtubule protein, assembled at 1 mg/mL for 40 min at 30 °C, was diluted 1/10, 1/20, and 1/50 in stabilizing buffer and processed for electron microscopy as described under Materials and Methods. A plot of total microtubule length per photograph vs. microtubule protein yielded a straight line with a correlation coefficient of 1.00, indicating that dilution in the range tested did not cause measurable disassembly of microtubules.

We next set up a standard curve to establish a correlation between total microtubule length measured in micrographs and the concentration of assembled microtubules. Microtubule protein was assembled at three or four concentrations to steady state (40 min at 30 °C). Half of each sample was processed for electron microscopy, and microtubule lengths were measured. With the remaining sample, the concentration of as-

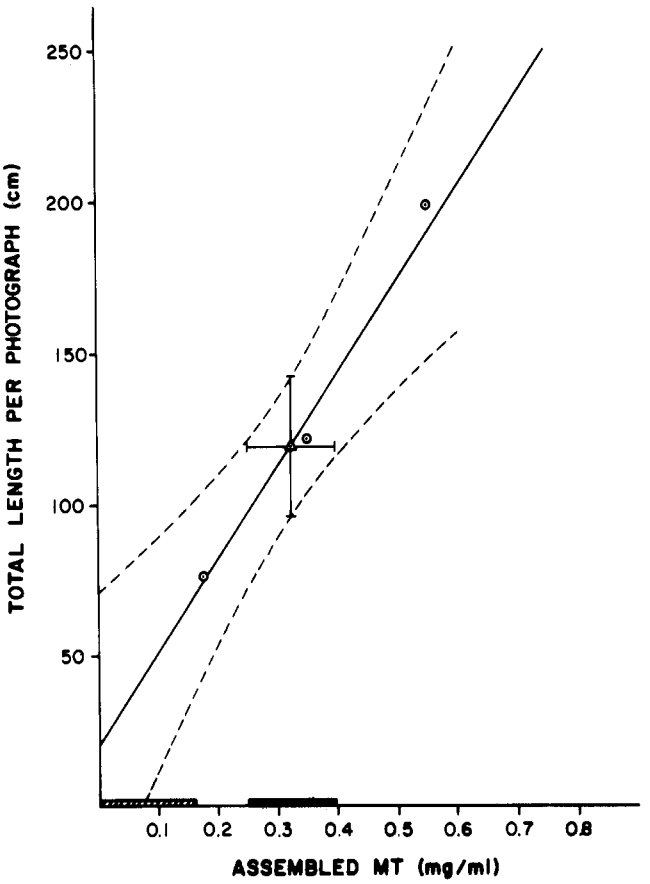

FIGURE 3: Concentration of assembled microtubules that formed after preincubation of microtubule protein with DMPC vesicles. A standard curve (⊙) was established as follows. Microtubule protein was assembled at three concentrations (1.00, 0.75, 0.50 mg/mL) to steady state (40 min at 30 °C). Half of each sample was processed for morphometric analysis to measure microtubule length, while the remaining sample was used to determine the concentration of assembled microtubules. A standard curve was established by plotting total microtubule length per photograph against the concentration of assembled microtubules. The 95% confidence limit (correlation curve; broken lines) was determined along the regression line (solid line). In the experiment (▲), microtubule protein (1 mg/mL) was adsorbed onto DMPC vesicles for 30 min at 30 °C, assembly conditions were introduced, and the incubation was continued for 40 min at 30 °C. Samples were processed for morphometric analysis, and microtubule lengths were measured. The concentration of assembled microtubules was determined from the standard curve: (■) the concentration of assembled microtubules after introducing assembly conditions to DMPC liposomes preadsorbed with microtubule protein and (⧄) the estimated concentration of assembled microtubules that would be present in the absence of desorption of protein from liposomes (zero point of assembled microtubules).

sembled microtubule protein was determined from the difference between total protein and nonpelletable (or nonassembled) microtubule protein. These same data were used to calculate the critical concentration for assembly of the particular preparation of the microtubule protein in use (Regula et al., 1981). Figure 3 shows the regression line (solid line) for one experiment, relating microtubule length to the concentration of assembled microtubules with its 95% confidence limit (correlation curve; broken lines). Once this standard curve was established, the concentration of assembled microtubules in an unknown sample could be readily determined from measured microtubule lengths by interpolation on the regression line.

Microtubule protein at 1 mg/mL was first adsorbed onto DMPC vesicles for 30 min at 30 °C. Assembly conditions were then introduced, and the incubation was continued for

Table I: Desorption of Microtubule Protein[a]

Calculation of Adsorbed and Nonadsorbed Protein			
total concn of microtubule protein (mg/mL)			1
(A) microtubule protein adsorbed onto liposomes (mg/mL)			0.72–0.95
(A′) nonadsorbed protein (total − A) (mg/mL)			0.05–0.28
	experiment		
	1	2	3
Calculation of Protein Desorption			
(B) concn of assembled microtubules after introduction of assembly conditions (mg/mL)	0.62	0.47	0.34
(C) critical concn for assembly (Cc) (mg/mL)	0.10	0.15	0.27
(D) total nonadsorbed microtubule protein (B + C) (mg/mL)	0.72	0.62	0.61
(E) amt remaining adsorbed (1 mg/mL − D)	0.28	0.38	0.39
(F) % desorbed	61.1–70.5	47.2–60.0	45.8–58.9
(G) av % desorbed			
DTAF-labeled microtubule protein	51.4		
^{3}H-labeled microtubule protein	63.1		

[a] (A) Derived from Ficoll gradient separations of free and liposome-bound microtubule protein. Since Ficoll compromised direct protein assays, the concentration of protein was estimated from DTAF- or ^{3}H-labeled microtubule protein. The former yielded a lower estimate (0.72 mg/mL) than the latter (>0.95 mg/mL). (A′) The difference between total and adsorbed protein. (B) Estimated from morphometric assay. (C) Determined by extrapolation of the concentration of supernatant proteins obtained after sedimentation of microtubules from solutions of different total protein concentrations (Regula et al., 1981). (D) Assembled mass (B) + Cc (C). (E) Total (1 mg/mL) − D. (F) (A–E)/A × 100. (G) Average of three experiments with estimates of initially adsorbed tubulin obtained from DTAF- or ^{3}H-labeled microtubule protein.

an additional 40 min at 30 °C before negative staining. Microtubule lengths were then measured. This value, indicated by ▲, was placed on the regression line (Figure 3). From the correlation curve, the concentration of assembled microtubules was calculated to be 0.25–0.40 mg/mL, within the 95% confidence limit. Figure 3 also illustrates that, in this experiment, the statistical zero point of assembled microtubules (the amount of assembly expected in the absence of desorption) corresponded to 0–0.16 mg/mL. Thus, the concentration of assembled microtubules (0.25–0.40 mg/mL) following, first, liposome adsorption and, then, introduction of assembly conditions was significantly different from the zero point of assembled microtubules (0–0.16 mg/mL).

Results from three experiments, in which microtubule assembly was quantitated after introduction of assembly conditions to liposome–protein mixtures, are shown in Figure 4. These experiments, along with those that determined the concentration of adsorbed proteins before introduction of assembly conditions, were used to calculate protein desorption. This is summarized in Table I (lines A–G).

We have shown that adsorption is 72–95% complete, depending on whether DTAF-labeled microtubule protein (Figure 1; 72%) or protein containing ^{3}H-tyrosinolated tubulin (95%) is used as a marker. Starting with a concentration of 1 mg/mL microtubule protein, a range of 0.72–0.95 mg/mL was estimated to adsorb onto liposomes (line A). Therefore, the concentration of nonadsorbed protein was 0.05–0.28 mg/mL (line A′).

Upon introduction of assembly conditions, the concentration of assembled microtubules was determined to be 0.62, 0.47, and 0.34 mg/mL in three experiments (Figure 4; Table I, line B). However, the critical concentrations for assembly (Cc)

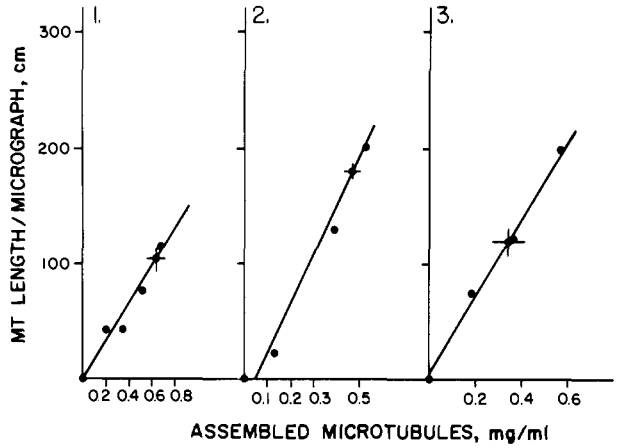

FIGURE 4: Quantitation of microtubule assembly after preincubation of microtubule protein with liposomes. Standard curves (●) for three experiments were established as described in Figure 2. Microtubule protein (1 mg/mL) was incubated with DMPC liposomes for 30 min at 30 °C, followed by incubation under microtubule assembly conditions for 40 min at 30 °C. Samples were processed for electron microscopy, and concentrations of assembled microtubules (⊕) were determined: experiment 1, 0.62 mg/mL; experiment 2, 0.47 mg/mL; experiment 3, 0.34 mg/mL. Experiment 3 is also shown in Figure 3.

of the three microtubule protein preparations used in these experiments were 0.10, 0.15, and 0.27 mg/mL, respectively (line C). Therefore, total nonadsorbed protein (line D) was calculated as the sum of Cc (line C) and microtubule polymer concentration (line B). It is readily seen that, after introduction of assembly conditions, the amount of microtubule protein remaining adsorbed onto liposomes (line E) was significantly reduced from the amount initially adsorbed (line A). Determination of the magnitude of desorption depended on two factors. First, we have assumed that the Cc did not change upon introduction of assembly conditions to liposome–protein complexes. Such changes might be expected if preferential adsorption or desorption of MAPs and tubulin occurred. We have shown in Figure 2 that preferential adsorption did not occur. Since we were unable to separate microtubules from liposomes, the possibility of preferential desorption could not be ruled out. Thus, the amount of desorption may be somewhat different than calculated. For example, if MAPs desorbed from liposomes less efficiently than did tubulin, the Cc may have increased, and more protein would have desorbed than calculated. Conversely, a lowering of the Cc would have resulted in less desorption than estimated. We note, however, that even if the Cc approached zero, the level of desorbed protein (line B) would be significantly higher than the level of nonadsorbed proteins before introduction of assembly conditions (line A). Second, determination of the amount of desorption also depended on the estimate of the amount of protein initially adsorbed, and as noted above, the estimate obtained with DTAF-labeled microtubule protein or microtubule protein containing ^{3}H-tyrosinolated tubulin was somewhat different. Because of the lability of the former, we favor the higher adsorption value obtained with ^{3}H-tyrosinolated tubulin. However, with either estimate, the calculated desorption was highly significant ($P < 0.001$) and averaged between 51% and 63%.

In contrast to studies with neutral phospholipid vesicles, there was no microtubule assembly when microtubule protein was first adsorbed onto vesicles containing the acidic phospholipid, phosphatidylserine (PS). When microtubule protein (1 mg/mL) was preincubated with liposomes of DMPC/PS (50% w/w) for 30 min at 30 °C (phospholipid to protein molar ratio of 4000:1) and assembly conditions were introduced, no microtubules were detected by negative-staining electron microscopy.

Discussion

Our data establish two major points: First, tubulin binds to either PC or PC/PS lipid vesicles above the lipid transition temperature. On the basis of their results showing leakage from DPPC vesicles, Klausner et al. (1981) concluded that tubulin interacts with liposomes only at the transition temperature. However, all of our experiments with egg lecithin were performed at 30 °C, well above its transition temperature of 0 °C. Thus, a unique temperature is not required for binding of microtubule protein to liposomes. Since lipid phase transitions are not widely accepted as present in mammalian biomembranes at physiological temperatures (McElhaney, 1982; Tanford, 1980), our results support the biological significance of tubulin–membrane interactions.

Second, we have established that 51–63% of microtubule protein bound to phospholipid vesicles can be desorbed to form microtubules and that this reversal of binding may be a function of the phospholipid composition. Desorption from egg lecithin or DMPC liposomes occurs; reversal from PS-containing vesicles was not apparent. Since we have not measured desorption directly, it remains possible that desorption of protein from acidic vesicles did occur but this protein did not assemble into microtubules. The mechanism by which microtubule assembly was inhibited by preadsorption onto acidic vesicles remains unclear. Gel electrophoretic analysis demonstrated that the pattern of proteins bound to either neutral or acidic liposomes was the same in the presence or absence of 1.0 M NaCl, suggesting that binding to either type of vesicle was not dependent on electrostatic interactions. Finally, we note that at the phospholipids to protein ratio used in these reversibility experiments, addition of microtubule protein to liposomes did not result in significant disruption of vesicle structure (Oliver et al., 1983). Distinct vesicles were visible by negative staining before and after introduction of microtubule assembly conditions. Thus, microtubule protein can desorb from phospholipid vesicles without obvious loss of their structure.

We emphasize that only introduction of microtubule assembly conditions reversed adsorption; there was no apparent reversal with colchicine, podophyllotoxin, cold, Ca^{2+}, or dilution. In contrast, Bernier-Valentin et al. (1983) have reported that binding of purified tubulin protein to cellular membranes is temperature-dependent: binding was prevented or reversed by incubation at 0 °C. As stated above, we have not observed such a temperature dependence of binding to phospholipid vesicles; binding occurred at 4 °C as well as at 30 °C (Caron, 1982). Since Bernier-Valetin et al. also demonstrated that the membranes employed contained endogenous tubulin (0.2–0.4% of membrane protein), one explanation for these apparently contradictory results is that binding to cellular membranes involved tubulin–tubulin interactions. Thus, binding of tubulin to membranes as demonstrated by Bernier-Valentin et al., unlike that described here, was readily reversible and inhibited by cold, these characteristics being shared by tubulin oligomers (Kravit et al., 1983). Therefore, the observations of Bernier-Valentine et al. may be a reflection of the association/dissociation of tubulin oligomers.

We have shown that microtubule protein binds to both neutral (PC) and acidic (PC/PS) phospholipid vesicles. This result appears to differ from that of Reaven and Azhar (1981). They reported that acidic, but not neutal, phospholipids inhibited microtubule assembly, from which they inferred

binding to the former, but not to the latter. However, they did not directly measure binding. Their data and our binding experiments can be reconciled since we show here that binding to vesicles is reversible from neutral phospholipids under conditions of microtubule assembly. Thus, under assembly conditions net binding to neutral phospholipids may indeed be small.

Our results, demonstrating that conditions of microtubule assembly cause tubulin desorption (i.e., prevent adsorption), can also explain the results of Carlin et al. (1982). They show that the amount of tubulin bound to postsynaptic densities undergoes progressive accumulation with time post-mortem, prior to tissue extraction. During this period there is an increase in cytosolic free calcium and a depletion of ATP. These conditions can be expected to lead to microtubule disassembly, which we predict would lead to an increase in tubulin adsorption to membranes.

Although we have shown that desorption of tubulin from liposomes occurs under conditions that allow microtubule assembly, it remains unclear what causes desorption and what initiates assembly. To address the first point, we originally considered that the binding of tubulin was freely reversible and that assembled microtubules served simply as a sink pulling the equilibrium toward desorption. However, several observations make this unlikely. First, more than 70% of the microtubules protein can be coisolated with liposomes that have been washed by centrifugation through a Ficoll gradient. A freely reversible reaction would probably lead to complete desorption on exposure to this high dilution. Second, the concentration of free microtubule protein after adsorption is at or below the Cc. Consequently, the addition of MgGTP could only be effective in promoting assembly after sufficient desorption had occurred to bring the free protein concentration above that required for assembly. From this, it might be predicted that MgGTP alone would cause desorption even if assembly were blocked. In preliminary experiments we have found that, in the presence of sufficient colchicine to block assembly, MgGTP did not cause detectable desorption. Specifically, the presence of MgGTP did not alter the amount or the pattern of microtubule protein adsorbed onto vesicles as determined by the Ficoll gradient method described in Figure 1. However, it must be acknowledged that a small degree of desorption would not be readily detected by the method employed. Thus, it remains possible that MgGTP causes a low level of desorption that allows microtubule assembly and further desorption.

With regard to the initiation of microtubule assembly, there are two parameters that should be considered: the role of microtubule associated proteins, or MAPs, and nucleation of assembly on the liposome surface. We have shown that MAPs and tubulin adsorbed onto vesicles at a ratio that was virtually identical with that of three times cycled microtubule protein. Thus, preferential adsorption of proteins onto vesicles did not occur. However, since we were unable to separate microtubules from liposomes, it was not possible to determine what fraction of these MAPs desorbed with tubulin to form microtubules, i.e., if there was preferential desorption of MAPs and tubulin. Therefore, as stated earlier, it remains possible that introduction of assembly conditions caused desorption of proteins which selectively altered the capacity of nucleation. Finally, it has been suggested that membranous structures may nucleate microtubule assembly (Andreu, 1982). For example, concerted adsorption of MAPs and tubulin may have led to the formation of aggregates that, in turn, nucleated assembly of microtubules. However, we estimate that there was, on the average, one tubulin dimer per vesicle before introduction of microtubule assembly conditions. Therefore, in order to form such hypothetical aggregates, desorption would have to occur from some liposomes independently.

The reversible binding of microtubule protein to liposomes suggests that this is a potentially dynamic interaction. Other studies, however, indicate that a second, more static type of interaction also exists. Stephens (1978, 1985) has identified a membrane-associated tubulin from molluscan cilia that is distinct from that of the 9 + 2 axoneme in its amino acid composition and detergent binding properties. In addition, Regula et al. (1986) have recently demonstrated that synaptosomal membranes from bovine brain contain tubulin molecules that are more hyrophobic than cytoplasmic tubulin. Purification of these tubulins from cilia and synaptosomes requires detergent solubilization of the membranes, suggesting that they are firmly embedded in the bilayer. These data and our studies suggest that differences in the tubulin molecule can result in either a stable, static association between tubulin and the lipid bilayer or a dynamic interaction.

To unravel the apparent paradox of a soluble protein such as cytoplasmic tubulin which can interact with the lipid bilayer, it is interesting to note similarities between this protein and a bona fide membrane protein, apolipoprotein A-I (Apo A-I) of high-density lipoproteins. (1) Although both tubulin and Apo A-I bind to liposomes primarily through hydrophobic interactions, both contain approximately the same percentage of hydrophobic amino acids as do water-soluble proteins (25–30%). (2) Adsorption of these proteins occurs relatively fast (within 15 min) when compared to other membrane proteins such as cytochrome b_5, which requires hours of incubation with liposomes. (3) Adsorption does not require the presence of the proteins during liposome formation, again, unlike cytochrome b_5. (4) As with Apo A-I, as the protein to phospholipid ratio increases, the ability of tubulin to perturb vesicle bilayer structure also increases. (5) Once adsorbed, the association of either tubulin or Apo A-I with liposomes can be reversed under appropriate conditions (microtubule assembly conditions for tubulin).

Finally, by studying the interaction of purified calf brain tubulin with nondenaturing amphiphiles, Andreu (1982) has demonstrated that the soluble tubulin molecule has extensive areas capable of hydrophobic interactions. Tubulin bound anionic (deoxycholate) as well as nonionic (octyl glucoside and Triton X-100) detergents. These results support our earlier conclusions that binding of tubulin to liposomes involves hydrophobic interactions (Caron & Berlin, 1979). However, unlike results presented here, the interaction with detergents was largely reversible upon dilution. In addition, by analyzing changes in circular dichroism spectra, Andreu reported that interactions with detergents resulted in a modest decrease in the α-helical content of tubulin. This contradicts the results of Klausner et al. (1981), who concluded that adsorption of tubulin onto phospholipid vesicles caused an increase in α-helical content. One explanation for these conflicting results is that the tubulin molecule does, in fact, contain extensive areas capable of hydrophobic interactions but such interactions are limited or controlled by the liposome structure. Such limitations would not exist during incubation of the protein with a solution of detergent, which may, in fact, lead to denaturation of the tubulin molecule. Since phospholipid vesicles represent a reasonably accurate model for biological membranes, results with liposomes rather than detergents are more likely to be physiologically relevant to in vivo interactions between tubulin and membranes.

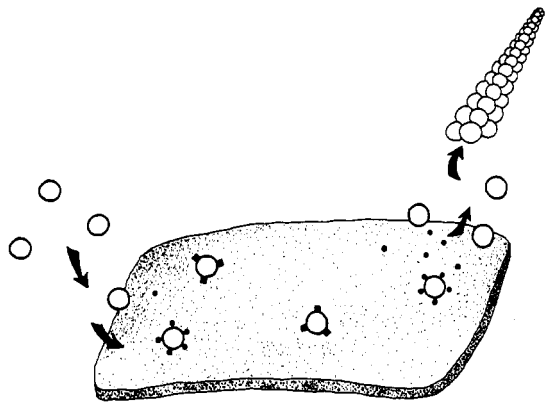

FIGURE 5: Adsorption of microtubule protein (○; left) onto membrane followed by selective desorption and assembly into microtubules (right). Protein may be adsorbed onto neutral (●) or acidic (■) phospholipids but can only be desorbed from the former. This leaves an excess of neutral phospholipid at the desorption site that is free to diffuse to the adsorption site and repeat the cycle. According to this model, microtubule protein bound to acidic phospholipids will be dispersed more or less uniformly throughout the membrane.

Biological Implications. The discovery of the affinity of tubulin for membranes may illuminate some difficult questions. For example, these observations may provide insight into the means by which tubulin bound to biological membranes could affect their structure and how antimicrotubule drugs could alter such mechanisms. Some possibilities are indicated in Figure 5. We propose that there is a steady state of tubulin bound to membranes reflecting both its adsorption and desorption. In some cases, microtubule assembly is limited to certain regions, for example, at the tips of flagella (Dentler & Rosenbaum, 1977; Mesland et al., 1980), suggesting that adsorption and desorption of tubulin are spatially separated. This would provide a diffusion gradient of PC-bound tubulin in the fluid membrane from adsorption to desorption sites. Further, since tubulin bound to phospholipids such as PS would *not* be available for desorption, there would be no net movement of PS-bound tubulin. Thus, a partial separation of phosphatidylcholine and phosphatidylserine within the membrane could result. We do not believe that these abundant phospholipid species would be partitioned to a large degree, but instead would lead to changes within "microenvironments" of the membrane. One effect of antimicrotubule drugs would be to block desorption, arresting tubulin diffusion in the membrane and the associated segregation of membrane constituents. It follows from this hypothetical mechanism that there may be regional differences in membrane composition and that these differences would be partially eliminated by agents that block microtubule assembly. Results consistent with this hypothesis have been reported. Phagocytosis in leukocytes results in an increase in membrane fluidity of plasma membrane, suggesting that there is a selective partioning of lipids (Smolen & Shotet, 1974; Berlin, 1975). This change in "microviscosity" induced by phagocytosis is blocked when cells are preincubated with colchicine.

Recent studies by Aszalos et al. (1985) have elegantly demonstrated that changes in membrane fluidity, which occur after incubation of Chinese hamster ovary cells with antimicrotubule drugs, are not due to a direct effect of the drugs on the plasma membrane. These authors concluded that microtubules interact directly or indirectly with the cell membrane. Since tubulin has been shown to bind to biological membranes (Reaven & Azhar, 1981; Bernier-Valentin et al., 1983), it seems reasonable that increasing levels of nonpolymerized tubulin, induced by treatment of cells with antimicrotubule drugs, would increase the level of membrane-bound tubulin. This, in turn, may have direct effects both on the physical properties of the bilayer (Aszalos et al., 1985) and on the activities of other membrane proteins. In support of this hypothesis, Simonin et al. (1981) have shown that purified tubulin, devoid of microtubule-associated proteins and of nucleoside diphosphate kinase activity, stimulated membrane-bound adenylate cyclase from murine plasmacytoma cells. Whether this stimulation occurred directly through protein–protein interactions or indirectly through alterations in membrane structure induced by tubulin adsorption is not clear. In any case, this work demonstrates the ability of tubulin to alter the activity of a membrane-associated enzyme.

The potential mechanisms by which membrane-bound tubulin affects membrane structure and function may be summarized as follows: storage of tubulin; transport of tubulin to sites of assembly; topographical segregation of membrane constituents; alterations in activities of other membrane proteins. Data presented here demonstrate that at least one type of tubulin–membrane interaction can be a reversible event. Since microtubules are in a dynamic equilibrium with their tubulin subunits, it is possible that changes in the level of nonpolymerized tubulin will affect the functions of both microtubules and membranes. We have recently shown that small changes in soluble tubulin levels in cultured cells had marked effects on microtubule physiology, i.e., the rate of tubulin synthesis and the processing of endocytosed ligands (Caron et al., 1985). These results suggest that such small variations in the level of soluble tubulin, which are thought to occur in vivo (Saxton et al., 1984), may also affect the interplay between microtubules and membranes.

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Two Classes of Binding Site for Hydrophobic Molecules on Bacterioopsin†

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ABSTRACT: Using environmentally sensitive fluorescent probes, we demonstrate the presence of two classes of binding site for hydrophobic compounds on the apoprotein of bacteriorhodopsin derived from the purple membrane of *Halobacterium halobium*. Dansyl fatty acids bind at two to four sites per monomer, and we suggest that these sites are located at protein–protein interfaces (nonannular sites). Dansylpropranolol, an amine, binds at six to eight sites, probably at the lipid–protein interface (annular sites). We find that binding of the fluorescent probes to the lipid component of the membrane, essentially all of which is closely associated with protein, is restricted relative to probe binding to simple lipid bilayers.

Purple membrane, a specialized region of the cytoplasmic membrane of *Halobacterium halobium*, contains a single type of protein, bacteriorhodopsin (Stoeckenius & Kunau, 1968; Oesterhelt & Stoeckenius, 1971), arranged in a regular pseudocrystalline lattice (Blaurock & Stoeckenius, 1971). The extensive two-dimensional order within the purple membrane has enabled the broad outline of the structure of the protein to be determined by a combination of electron diffraction and imaging (Henderson & Unwin, 1975; Hayward & Stroud, 1981; Agard & Stroud, 1982; Henderson et al., 1986). Until recently (Diesenhofer et al., 1985), bacteriorhodopsin was the only example of an intrinsic membrane protein to be so characterized and has therefore served as an archetype for many others (Stoeckenius & Bogomolni, 1982; Henderson, 1979). The observed cluster of α-helical segments traversing the membrane may be a common feature of all transmembrane proteins (Burres & Dunker, 1980; Engleman & Steitz, 1981); available sequence data for many membrane proteins contain stretches of polypeptide chain that could be folded into α-helices of the required length and arranged so as to present a hydrophobic face to the bilayer lipid (Engelman & Zaccai, 1980; Argos et al., 1982; Eisenberg, 1984; MacLennan et al., 1985).

Purple membrane assembles spontaneously in vivo and in vitro to form a rigid array of protein trimers, each surrounded by a single shell of phospholipid (Henderson & Unwin, 1975). This stable arrangement, which may or may not be required for physiological function, is evidently the result of a balance of lipid–protein, lipid–lipid, and protein–protein interactions. The forces between the membrane components, predominantly hydrophobic in nature, will be modulated by electrostatic and geometrical constraints, which may be particularly influential in determining the nature and degree of protein–protein associations. To gain an insight into the nature of these interactions, we have studied the binding of a number of environmentally sensitive fluorescent probes to purple membrane modified by extraction of the retinaldehyde chromophore. Bleaching of the purple membrane in this way serves a 3-fold

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