Evidence for Tubulin-binding Sites on Cellular Membranes: Plasma Membranes, Mitochondrial Membranes, and Secretory Granule Membranes

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ABSTRACT We describe the interaction of pure brain tubulin with purified membranes specialized in different cell functions, i.e., plasma membranes and mitochondrial membranes from liver and secretory granule membranes from adrenal medulla. We studied the tubulin-binding activity of cellular membranes using a radiolabeled ligand-receptor assay and an antibody retention assay. The tubulin-membrane interaction was time- and temperature-dependent, reversible, specific, and saturable. The binding of tubulin to membranes appears to be specific since acidic proteins such as serum albumin or actin did not interfere in the binding process. The apparent overall affinity constant of the tubulin-membrane interaction ranged between 1.5 and 3.0 $\times$ 10^7 M^{-1}; similar values were obtained for the three types of membranes. Tubulin bound to membranes was not entrapped into vesicles since it reacted quantitatively with antitubulin antibodies. At saturation of the tubulin-binding sites, the amount of reversibly bound tubulin represents 5–10% by weight of membrane protein (0.4–0.9 nmol tubulin/mg membrane protein). The high tubulin-binding capacity of membranes seems to be inconsistent with a 1:1 stoichiometry between tubulin and a membrane component but could be relevant to a kind of tubulin assembly. Indeed, tubulin-membrane interaction had some properties in common with microtubule formation: (*a*) the association of tubulin to membranes increased with the temperature, whereas the dissociation of tubulin-membrane complexes increased by decreasing temperature; (*b*) the binding of tubulin to membranes was prevented by phosphate buffer. However, the tubulin-membrane interaction differed from tubulin polymerization in several aspects: (*a*) it occurred at concentrations far below the critical concentration for polymerization; (*b*) it was not inhibited at low ionic strength and (c) it was colchicine-insensitive.

Plasma membranes, mitochondrial membranes, and secretory granule membranes contained tubulin as an integral component. This was demonstrated on intact membrane and on Nonidet P-40 solubilized membrane protein using antitubulin antibodies in antibody retention and radioimmune assays. Membrane tubulin content varied from 2.2 to 4.4 μg/mg protein. The involvement of membrane tubulin in tubulin-membrane interactions remains questionable since erythrocyte membranes devoid of membrane tubulin exhibited a low (one-tenth of that of rat liver plasma membranes) but significant tubulin-binding activity.

These results show that membranes specialized in different cell functions possess high-affinity, large-capacity tubulin-binding sites. We proposed that tubulin-membrane interaction involves the assembly of a limited number of tubulin dimers in a "micropolymerization" process initiated by a membrane component present in various cellular membranes or by different membrane components exhibiting a common property.

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In addition to containing free tubulin and tubulin assembled into microtubules, neural, and nonneural cells do contain membrane-bound tubulin as an integral component (1–5). Recent reports suggest that cellular membranes of different origins could also bind tubulin. This hypothesis arises from the fact that microsomes (6), plasma membranes, and mitochondria (7) were shown to inhibit microtubule assembly. This inhibition would result from a binding of tubulin to the membranes, leading to a decrease of free tubulin available for microtubule polymerization. To demonstrate the interaction of tubulin with membranes by direct experimental approaches, we looked for the existence of a saturable and reversible tubulin-binding activity in purified preparations of various cellular membranes. The interaction of tubulin with plasma membranes or mitochondrial membranes from liver and secretory vesicle membranes from adrenal medulla has been studied by competitive binding experiments using labeled and unlabeled pure brain tubulin and by an antibody retention assay based on the activity of tubulin bound to membranes to adsorb specifically and quantitatively antitubulin antibodies. High-affinity binding sites for tubulin have been found on the three subclasses of membranes. The study of the temperature dependency, colchicine sensitivity, tubulin concentration requirement of the tubulin-membrane interaction led us to propose the existence of a "micropolymerization" process of tubulin initiated by membrane component(s).

MATERIALS AND METHODS

Purification and Labeling of Rat Brain Tubulin: Rat brains were homogenized in buffer A: 100 mM 2-(*N*-morpholinoethane)sulfonic acid (MES), 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, 0.5 mM GTP, pH 6.4. The homogenate was centrifuged at 100,000 *g* for 60 min at 4°C. Microtubule protein (MTP) was purified by the temperature-dependent assembly-disassembly procedure according to Shelanski et al. (8). Tubulin was purified by phosphocellulose chromatography (9). The purity of tubulin was ≥98% as judged by PAGE in the presence of SDS (10). Pure tubulin was labeled by conjugation with the Bolton-Hunter reagent: *N*-succinimidyl 3-(4-hydroxy[5-^{125}I]iodophenyl) propionate (2,000 Ci/mmol, Amersham, England) as previously described (10). The specific radioactivity of ^{125}I-labeled tubulin was 300–400 Ci/mmol.

Purification of Rat Brain Actin: Actin was purified according to Ruscha and Himes (11). An acetone powder was prepared from crude pellets of brain homogenates obtained in the preparation of MTP. Actin was separated from other acetone-insoluble proteins by gel filtration on Sephadex G-200. Actin was ~90% pure as analyzed by PAGE in the presence of SDS.

Purification and Characterization of Cellular Membranes: Mitochondria and plasma membranes were purified from rat liver according to the procedure of Fleischer and Kervina (12). Mitochondrial membranes were obtained by sonication (2 KHz, one 3-s pulse) of purified mitochondria; ~90% of the resulting mitochondrial protein were sedimentable at 10,000 *g*. Membranes from chromaffin granules were prepared from bovine adrenal medulla according to Aunis et al. (13). Chromaffin granules and mitochondria sedimented between 1.8 and 2.0 M and between 1.4 and 1.6 M sucrose, respectively, on a 1.0–2.2 M sucrose gradient centrifuged at 90,000 *g* for 180 min. The purity of the various membrane fractions was determined by the enrichment of key enzymes. Plasma membranes and mitochondrial membranes from liver were assayed for 5′-nucleotidase and cytochrome *c* oxidase using [^{3}H]AMP (14) and reduced cytochrome *c* (15) as substrate. Secretory granule membranes and mitochondrial membranes from adrenal medulla were assayed for monoamine oxidase and dopamine β hydroxylase using [^{14}C]-tyramine (16) and tyramine (17), respectively. Rat erythrocytes were purified by filtration on a cellulose column composed of equal amounts of alpha-cellulose and cellulose-type 50 (Sigmacell) according to Beutler et al. (18). Sealed ghosts were obtained as described by Steck and Kant (19).

Tubulin-binding Assay: Secretory granule membranes or mitochondrial membranes or plasma membranes (6–12 μg protein) were incubated with ^{125}I-labeled tubulin (≈50,000 cpm, 15 ng) in a total volume of 250 μl of buffer A supplemented with 5 mg/ml BSA (binding buffer). Incubations were carried out at 37°C for 45 min. Bound ^{125}I-labeled tubulin was separated from free ^{125}I-labeled tubulin by centrifugation at 10,000 *g* for 4 min as previously described (10). Pellets were counted for radioactivity in a Packard scintillation gamma counter (Packard Instrument Co., Inc., Downers Grove, IL) (30% efficiency). ^{125}I-tubulin binding values were corrected for nonspecific binding measured in the presence of an excess of unlabled tubulin (250 μg/ml). For competitive binding studies, unlabeled tubulin (0–65 μg) was added at the beginning of the incubation period.

Antitubulin Antibody Retention Assay: A three-step incubation procedure was used:

First step: membranes were incubated with or without unlabeled tubulin as described for the tubulin-binding assay. At the end of the incubation period, membranes or tubulin-membrane complexes were pelleted by centrifugation at 10,000 *g* for 4 min, washed in the binding buffer, and resuspended in 250 μl of the same buffer.

Second step: Membranes or tubulin-membranes complexes were incubated for 60 min at 37°C with 0.4 μl of an antitubulin antiserum (final dilution: 1/1,250). The incubation mixture was then centrifuged at 10,000 *g* for 4 min.

Third step: The supernatant was assayed for its antitubulin antibody activity by incubation with ^{125}I-labeled tubulin for 60 min at 37°C. 1 mg of protein A adsorbent (*Staphylococcus aureus*, Pansorbin™; Calbiochem-Behring Corp., San Diego, CA) was added and the incubation continued for 45 min at room temperature. Immune complexes were collected by centrifugation at 1,500 *g* for 20 min and the pellets were counted for radioactivity. The residual or free antitubulin antibody activity was expressed as the percentage of total antitubulin antibody activity of the same amount of antiserum (0.4 μl) which has been incubated neither with membranes nor with tubulin-membrane complexes.

Radioimmunoassay: The tubulin content of various cellular membranes was determined by radioimmunoassay after solubilization of membranes using 0.2% Nonidet P-40 (1-h treatment at 20°C). Radioimmunoassay was performed as previously described (20).

Other Methods: Protein was determined according to Lowry et al. (21) using BSA as standard. PAGE was performed as previously described using a mini-slab apparatus from Idea Scientific (Corvallis, OR) (9). Tubulin polymerization was followed by turbidity measurements at 350 nm using a Beckman 25K recording spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA).

RESULTS

Characterization of Purified Membrane Fractions

Specific activities of marker enzymes of the purified membrane fractions are given in Table I. Liver plasma membranes, liver mitochondrial membranes, adrenal medulla secretory granule membranes were enriched 5.9-, 4.4-, and 9-fold in 5′-nucleotidase, cytochrome *c* oxidase, and dopamine β hydroxylase, respectively, as compared to the corresponding tissue homogenate. Contamination of a given membrane preparation A by an other cell fraction B was calculated as the ratio between the specific activity of a marker enzyme of B in the fraction A and the specific activity of this enzyme in the purified preparation of B. For liver fractions, the contamination of purified plasma membranes by mitochondria and the contamination of purified mitochondria by plasma membranes were estimated at ~5%. Contamination of secretory granule membranes from adrenal medulla by mitochondria was 1.6%. Purified blood cell membranes analyzed by PAGE (see Fig. 6) exhibited protein profiles similar to those reported by Bennett and Davis (22) or Davis and Bennett (23).

Time Course of the Binding of ^{125}I-labeled Tubulin to Membranes

The association of ^{125}I-labeled tubulin with each type of membranes was time- and temperature-dependent. This is illustrated for liver plasma membranes in Fig. 1 *a*. An equilibrium was reached after 45 min of incubation at 37°C. Incubation at 0°C reduced the binding of ^{125}I-labeled tubulin by 85%. It was verified by PAGE that the radioactivity associated with membranes actually represented ^{125}I-labeled tubulin and

TABLE I

Enzyme Activities in Purified Preparations of Plasma Membranes and Mitochondrial Membranes from Rat Liver and Secretory Granule Membranes from Adrenal Medulla

Tissue	Plasma membrane		Mitochondrial membrane		Secretory granule membrane	
Marker enzyme	SA*	RSA‡	SA	RSA	SA	RSA
Liver						
5′ nucleotidase	0.19	5.9	0.01	0.31	-	-
Cytochrome c oxidase	0.03	0.27	0.48	4.4	-	-
Adrenal medulla						
Dopamine β hydroxylase	-	-	0.008	0.26	0.287	9.0
Monamine oxidase	-	-	4.50	2.3	0.07	0.035

Each value represents the mean of assays performed on three different membrane preparations.
* Specific activities (*SA*) are expressed as μmol/min/mg protein excepted for that of monoamine oxidase which is expressed as nmol/min/mg protein.
‡ Relative specific activity (RSA): ratio between the specific activities of an enzyme in a given fraction and in the homogenate.

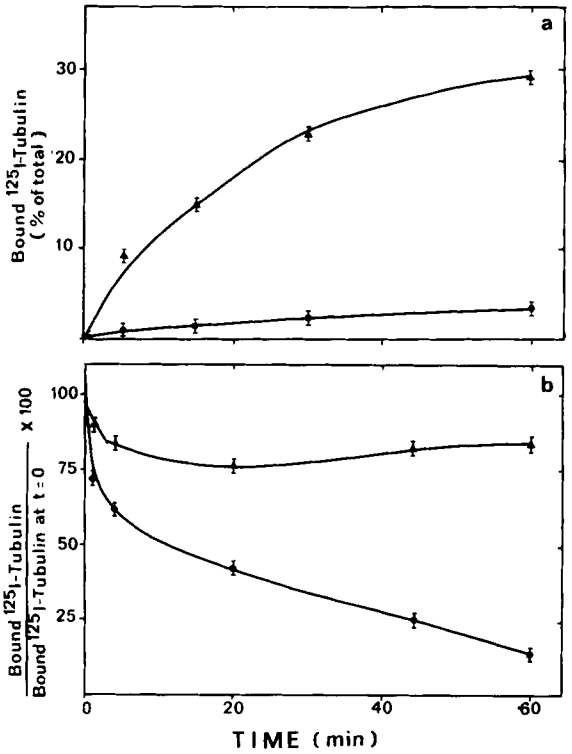

FIGURE 1 Time course studies of the interaction of ^{125}I-labeled tubulin with plasma membranes. (*a*) Binding of ^{125}I-labeled-tubulin to purified liver plasma membranes. ^{125}I-labeled tubulin (50,000 cpm, 0.2 pmol) was incubated with plasma membrane (10 μg protein) at 37°C (▲) or at 0°C (●), in a total volume of 250 μl. Specifically bound ^{125}I-labeled tubulin was expressed as the percentage of total ^{125}I-labeled tubulin. (*b*) Time course of the dissociation of ^{125}I-labeled tubulin-plasma membrane complexes. ^{125}I-labeled tubulin (≈600,000 cpm) was incubated with plasma membranes (600 μg protein) for 45 min at 37°C in a total volume of 1 ml of the binding buffer. The incubation mixture was centrifuged at 10,000 *g* for 4 min and the pellet was resuspended in 1 ml of buffer at 37°C. Aliquots of 375 μl were transferred to tubes containing 30 ml of buffer at 37°C or 0°C (t = 0); at various time intervals, three aliquots of 1 ml were taken out and centrifuged at 10,000 *g* for 4 min. The radioactivity of the pellet was expressed as the percentage of the amount of bound ^{125}I-labeled tubulin at the time t = 0 min. Symbols and vertical bars represent the mean and SEM of triplicate incubations.

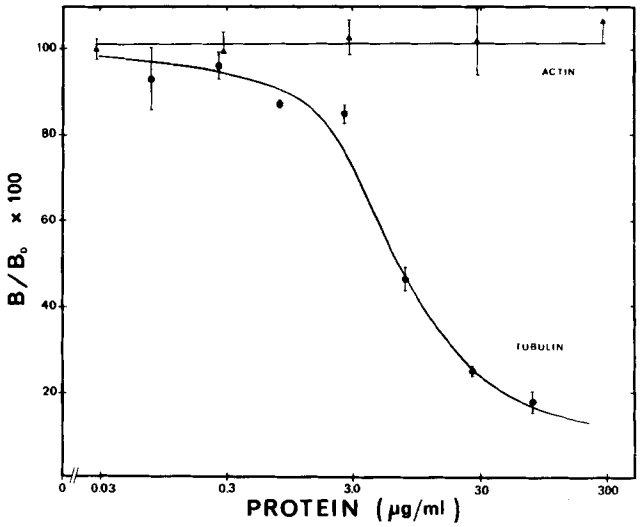

FIGURE 2 Specificity of the binding of tubulin to membranes. Plasma membranes (10 μg) were incubated with ^{125}I-labeled tubulin (50,000 cpm) and various concentrations of unlabeled tubulin or actin. Specifically bound ^{125}I-labeled tubulin measured in the presence of a given concentration of tubulin or actin (*B*) was expressed as a percentage of the maximum binding (B_0) measured in the absence of the competitor. Symbols and vertical bars represent the mean and SEM of values obtained from triplicate incubations.

not a labeled degradation product. Reduction of the concentration of the MES component of the incubation medium from 100 to 20 mM did not alter significantly ^{125}I-tubulin binding. Similar binding values were obtained at pH 6.4 and 6.9. In contrast, replacement of the MES component by phosphate (pH 6.9, 20–100 mM) in the incubation medium, abolished the binding of ^{125}I-tubulin to any kind of membranes. The reversibility of the tubulin-membrane interaction is documented in Fig. 1 *b*. The ^{125}I-labeled tubulin-membrane complexes dissociated after dilution and incubation at 0°C. At this temperature, the dissociation was time-dependent; 85% of initially bound (at t = 0) ^{125}I-labeled tubulin was found as free ^{125}I-labeled tubulin after 60 min of incubation. In contrast, there was almost no dissociation at 37°C.

Properties of the Tubulin-Membrane Interaction

Results of Fig. 2 show that binding of ^{125}I-labeled tubulin to membranes was decreased by increasing the concentration of unlabeled tubulin from 0.3 to 100 μg/ml. The specificity of this competitive inhibition is illustrated by the lack of effect of another cytoskeletal acidic protein:actin over the same range of concentration. That binding of ^{125}I-labeled tubulin at a concentration of 0.5 nM occurred in the presence of 80 μM serum albumin (included in the incubation medium) also documents the specificity of the tubulin-membrane interac-

tion. Colchicine (10^{-7} to 10^{-4} M) either preincubated with tubulin or added at the beginning of the incubation period did not alter the tubulin-membrane interaction measured at different tubulin concentration ranging from 0.06 to 100 μg/ml.

Saturation binding curves (illustrated for secretory granule membrane from adrenal medulla and plasma membranes from liver in Fig. 3, *a* and *c*) were generated from results of competitive binding between labeled tubulin and unlabeled tubulin (at concentration ranging from 0.1 to 200 μg/ml). Double reciprocal plots and Hill plots of binding data (Fig. 3, *b* and *d*) were used to determine the parameters of the interaction. Results obtained with the three subclasses of cellular membranes are reported in Table II. Values deriving from the two graphical representations are in good agreement. The values of the apparent dissociation constant (K_D) for the interaction between tubulin and plasma membranes or mitochondrial membranes or secretory granule membranes varied between 3.0 and 6.4 10^{-8} M. The maximum binding capacity expressed as nmoles of tubulin bound per milligram of protein varied from 0.4 to 0.9. The Hill coefficient was always close to 1. The criteria of saturation, reversibility, and

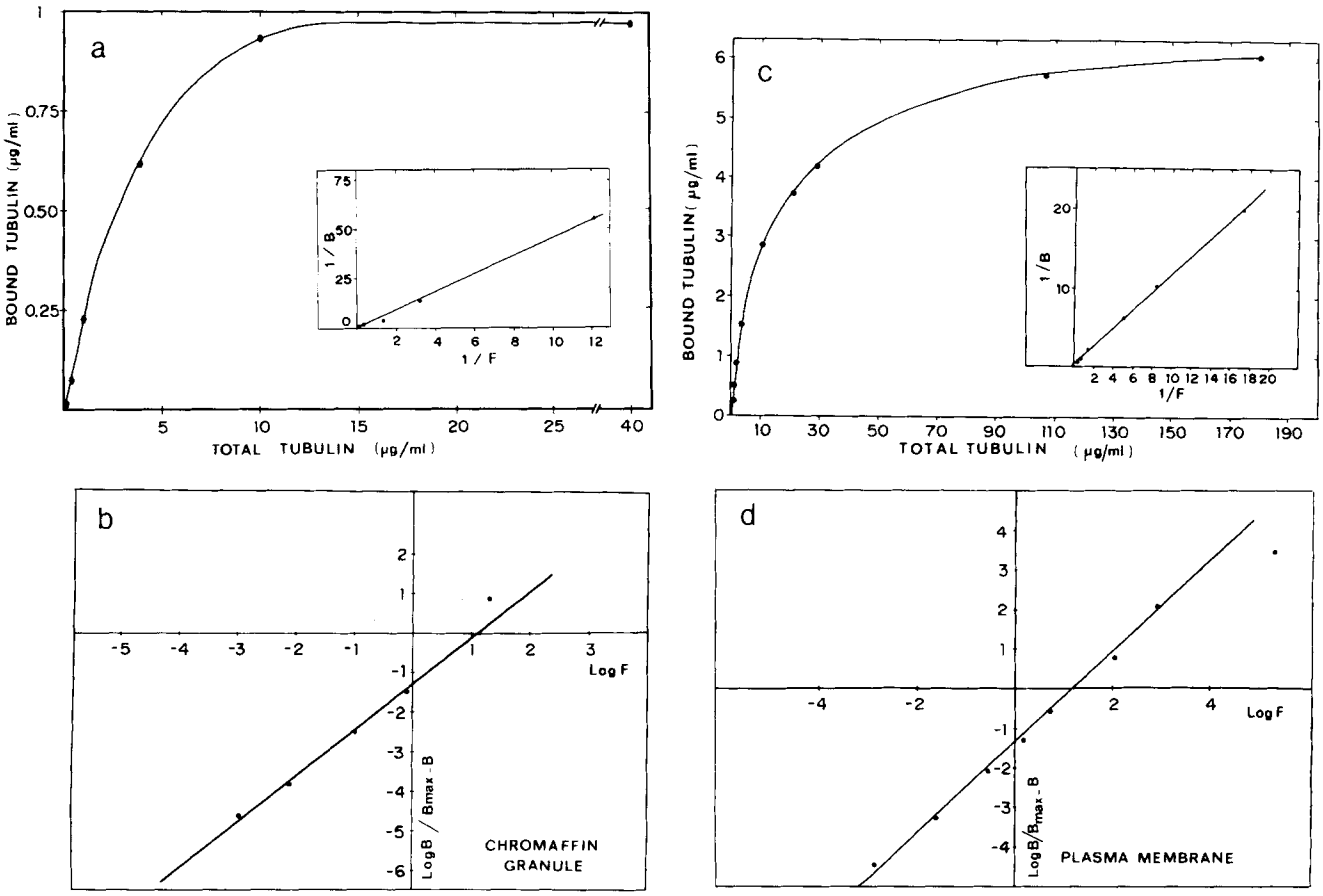

FIGURE 3 Equilibrium binding studies of ^{125}I-labeled tubulin to adrenal medulla chromaffin granule membranes and liver plasma membranes. (*a–c*) Specific binding of tubulin to adrenal medulla chromaffin granule membranes and liver plasma membranes as a function of tubulin concentration. Chromaffin granule membranes or plasma membranes (10 μg of protein) were incubated with ^{125}I-labeled tubulin (50,000 cpm) and various concentrations of unlabeled tubulin. Bound tubulin was calculated from the values of bound ^{125}I-labeled tubulin and the specific radioactivity of tubulin in the assay. (*Inset*) Double reciprocal plot of bound tubulin (*B*) versus free tubulin (*F*). (*b–d*) Hill plot of specific binding of tubulin to chromaffin granule membranes and plasma membranes. B_{max} represents the maximum binding capacity, *B* and *F* the bound and free tubulin concentrations. Each point represents the mean value obtained from incubations performed in triplicate of a typical experiment.

TABLE II

Parameters of Tubulin Binding to Membranes

Graphical representation	Binding parameters	Plasma membranes	Mitochondrial membranes	Secretory granule membranes
Hill	$K_D \times 10^8$ (M)	6.4 ± 2.8	3.7 ± 0.5	3.0 ± 1.3
	h	1.00 ± 0.10	0.95 ± 0.05	1.02 ± 0.01
Lineweaver-Burk	$K_D \times 10^8$ (M)	3.8 ± 0.5	4.9 ± 1.5	4.5 ± 1.3
	B_{max} (nmol tubulin/mg protein)	0.9 ± 0.2	0.6 ± 0.1	0.4 ± 0.1

Experiments were performed as described under Fig. 3. Results are the mean ± SE of six determinations obtained from three different membrane preparations. K_D: apparent dissociation constant; *h*: Hill coefficient; B_{max}: maximal binding capacity.

specificity of the interaction of tubulin with membranes appear to be fulfilled.

Antitubulin Antibody Retention Assay

To demonstrate that the interaction of tubulin with membranes actually corresponds to a binding of tubulin onto the membranes and not to a trapping of tubulin within membrane vesicles, we looked for the ability of tubulin-membrane complexes to react with specific antitubulin antibodies. Results of such an experiment conducted on mitochondrial membranes are reported in Fig. 4. Results of Fig. 4*a* show that purified mitochondrial membranes (which were not preincubated with tubulin) were able to bind antitubulin antibodies in a concentration-dependent manner. That a parallel binding curve was obtained when tubulin immobilized on Sepharose-4B was used instead of membranes strongly suggests that the binding of antitubulin antibodies to membranes was specifically related to the presence of tubulin. Tubulin responsible for the antitubulin antibody retention activity of untreated cellular membranes corresponds to intrinsic membrane tubulin (see below). Cellular membranes (in amounts exhibiting a low antitubulin antibody retention activity) pre-incubated with tubulin to form tubulin-membrane complexes were able to bind the antibody in a manner related to the amount of tubulin present in the membrane-tubulin complexes. As illustrated in Fig. 4*b*, membranes preincubated with tubulin 10 μg/ml (a concentration in the range of the K_D value) bound ~50% of the total antitubulin antibody activity. When membranes were preincubated in the presence of an excess of tubulin (leading to the saturation of tubulin binding sites on membranes according to Fig. 3), 80% of the antibody activity was associated with the tubulin-membrane complexes. The use of such an antibody retention assay was only possible because dissociation of tubulin-membrane complexes did not occur at 37°C.

Quantitation of Tubulin in Cellular Membranes

Data obtained with the antitubulin antibody retention assay give evidence for the presence of tubulin in cellular membranes. For quantitative estimation of this fraction, membranes were treated by a nonionic detergent Nonidet P-40 and the tubulin content of the solubilized protein fraction was measured by radioimmunoassay. About 90% of protein from mitochondrial membrane, plasma membrane, and chromaffin granule membrane was recovered in the 26,000 *g* supernatant after detergent treatment. Serial dilutions of detergent-extracted material gave displacement curves parallel to the standard curve generated with pure tubulin (Fig. 5). Radioimmunoassayable tubulin represented between 0.2 and 0.4% of solubilized membrane protein (Table III).

Is Membrane Tubulin Involved in the Binding of Tubulin to Membranes?

We attempted to answer this question by measuring the tubulin-binding activity of erythrocyte membranes known to be devoid of membrane tubulin. Results are presented in Fig. 6. Membranes prepared from purified rat erythrocytes exhibited a low tubulin-binding activity when expressed in terms of membrane protein. The tubulin-binding activity of rat erythrocyte membranes represented about one-tenth of that of rat liver plasma membranes. Although low, tubulin binding

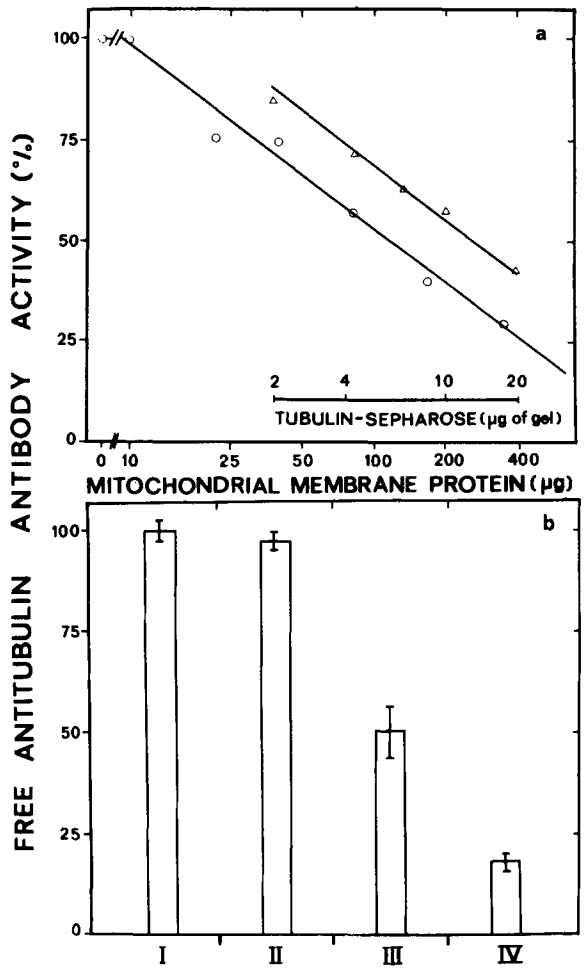

FIGURE 4 Binding of antitubulin antibodies to mitochondrial membranes. (a) Antitubulin antibody retention activity of untreated mitochondrial membranes. Various amounts of mitochondrial membranes were incubated with a given amount of an antitubulin immune serum (final dilution: 1:1,250) for 60 min at 37°C. The supernatant obtained after centrifugation at 10,000 *g* for 4 min was assayed for its free antitubulin antibody activity. Similar experiments were conducted with tubulin immobilized on CNBr-activated Sepharose 4B (3.8 mg tubulin coupled/g wet gel). Results are expressed as the percentage of the total antitubulin antibody activity (measured in the same conditions in the absence of membranes or immobilized tubulin). Symbols represent the mean of triplicate determination on mitochondrial membranes (○) or tubulin-Sepharose (△). (*b*) Antitubulin antibody retention activity of mitochondrial membranes preincubated with tubulin. Mitochondrial membranes preincubated with or without tubulin for 45 min at 37°C were pelleted by centrifugation at 10,000 *g* for 4 min and resuspended in the same volume of binding buffer at 37°C. Measurements of the antitubulin antibody retention activity were performed as described above. Each column and vertical bar represent the mean and SEM of triplicate incubation. Total antitubulin antibody activity (*I*); free antitubulin antibody activity after incubation with untreated membranes (*II*) or membranes preincubated with tubulin: 10 μg/ml (*III*) or 200 μg/ml (*IV*).

to erythrocyte membranes did not represent nonspecific binding, since the overall apparent affinity constant of this process was within the range of the K_A values obtained with other membranes. Similar binding values were obtained when sealed erythrocyte ghosts were used instead of unsealed ghosts, i.e., erythrocyte membranes.

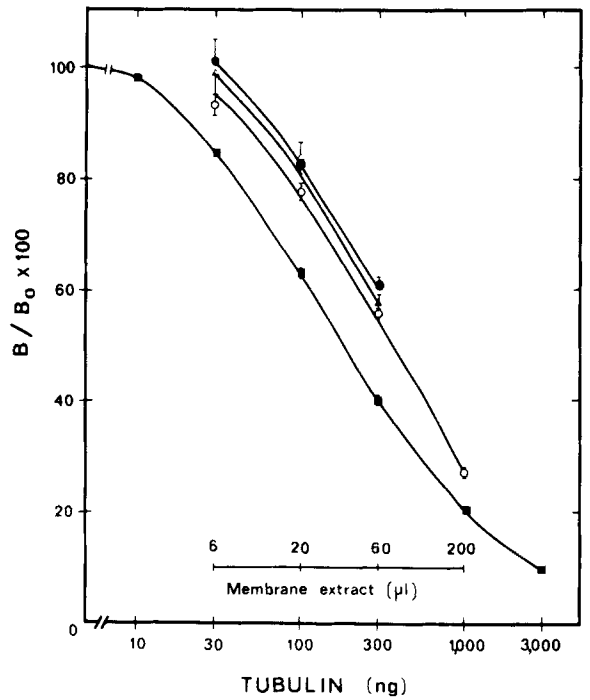

FIGURE 5 Radioimmunoassay of tubulin in soluble fractions of detergent-treated cellular membranes. Competition curves between ^{125}I-labeled tubulin and unlabeled pure tubulin (■) (standard curve) or solubilized mitochondrial membranes (○), plasma membranes (●), or chromaffin granule membranes (△). Cellular membrane fractions treated by 0.2% Nonidet P-40 for 60 min at 20°C were centrifuged at 26,000 *g* for 20 min. The soluble material (membrane extract) was assayed for its tubulin content. Protein concentration of membrane extracts was 0.5, 0.7, and 0.9 mg/ml for plasma membranes, mitochondrial membranes and chromaffin granule membranes, respectively. Values of ^{125}I-labeled tubulin binding to the antibodies obtained at the different amounts of competitor are expressed as the percentage of the maximum binding (measured without competitor). Symbols and vertical bars represent the mean and SEM of values obtained from triplicate incubations.

TABLE III

Radioimmunoassay of Tubulin in Solubilized Fractions of Cellular Membranes

Fraction	Total protein	Nonidet P-40 solubilized fraction		
		Protein	Tubulin	Tubulin/protein × 100
	mg	*mg*	*µg*	
Mitochondrial membrane	3.7	3.3	13.3 ± 1.2	0.40
Plasma membrane	2.5	2.4	8.4 ± 0.8	0.34
Chromaffin granule membrane	5.0	4.5	2.3 ± 1.2	0.20

Cellular membranes were treated with 0.2% Nonidet P-40 for 60 min at 20°C and centrifuged at 26,000 *g* for 20 min at 4°C. Tubulin was assayed in supernatants. Results are expressed as the mean ± SE of values obtained at three protein inputs.

DISCUSSION

Our data indicate that membranes specialized in different cell functions—plasma membranes, mitochondrial membranes, and secretory vesicle membranes—possess high-affinity binding sites for tubulin: apparent K_A values ranging from 1.5 to 3×10^7 M^{-1}. The existence of such binding sites has been documented by two different experimental approaches based on the use of radiolabeled tubulin or antitubulin antibodies. Antitubulin antibody data indicate that tubulin bound to the membranes is freely accessible and therefore not entrapped within membrane vesicles. A similar conclusion can be drawn from the dissociation experiments. Indeed, tubulin bound to membranes was rather rapidly recovered as free tubulin upon dilution and decreasing the incubation temperature.

Saturation of tubulin-binding sites corresponds to the binding of 50–100 µg of tubulin per milligram membrane protein. Assuming that tubulin binds in a 1:1 stoichiometry to a 100,000 mol wt membrane component, this component should represent 5–10% of membrane protein. The presence of a component in such a proportion in different cellular membranes seems very unlikely. Therefore, we postulate that a given molecular species of the membrane could bind or promote the binding of several molecules of exogenous tubulin. Such a process could correspond to a kind of tubulin assembly different, however, from that involved in microtubule formation. The similarities and differences between microtubule assembly and tubulin-membrane interaction are reported in Table IV. The most striking difference is the tubulin concentration requirement. Indeed, complete saturation of tubulin-binding sites in membranes was obtained using

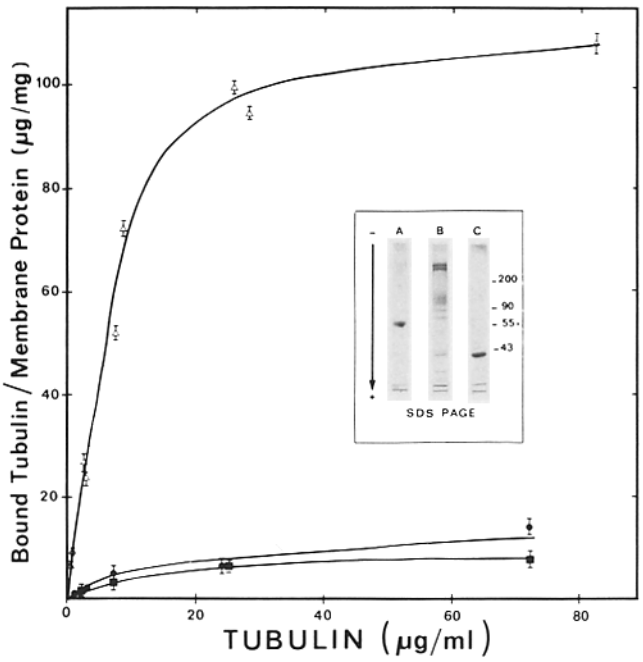

FIGURE 6 Comparison between tubulin binding activity of rat erythrocyte membranes and rat liver plasma membranes. Liver plasma membranes (8 µg) or erythrocyte membranes (10 µg) or sealed erythrocyte ghosts (50 µg containing 10 µg membrane protein) were incubated with ^{125}I-labeled tubulin (50,000 cpm) for 45 min at 37°C in the binding buffer. The amount of ^{125}I-labeled tubulin which binds to liver plasma membranes (△) or erythrocyte membranes (■) or sealed erythrocyte ghosts (●) is plotted as a function of tubulin concentration. Experiments were performed as indicated under Fig. 3. Symbols and vertical bars represent the mean and SEM of triplicate incubations. (*Insert*) Analysis of erythrocyte membrane proteins by PAGE in the presence of SDS. (Lane *A*) Rat brain tubulin. (Lane *B*) Rat erythrocyte membrane proteins. (Lane *C*) Rat brain actin.

TABLE IV

Similarities and Differences between Microtubule Assembly and Tubulin-Membrane Interaction

Parameter	Microtubule assembly	Tubulin-membrane interaction
Temperature		
37°C	Assembly	Association
0°C	Disassembly	Dissociation
Buffer composition		
MES 0.1 M	Induction	Induction
Phosphate 0.1 M	Inhibition	Inhibition
Tubulin concentration range (μg/ml)	≥300*	0.3–100
Decrease of buffer concentration (from 100 to 20 mM MES)	Progressive decrease	No effect
Effect of colchicine (10^{-4} M)	Inhibition	No effect

* Critical concentration for polymerization of tubulin in the presence of MAPs.

tubulin concentration below the critical concentration for polymerization (≈300 μg/ml for MTP:tubulin + MAPs; >2.0 mg/ml for phosphocellulose purified tubulin). Moreover, tubulin-membrane interaction did not appear to be colchicine-sensitive. On the contrary, the tubulin-membrane interaction exhibits the same temperature dependency as microtubule formation. Dissociation or disassembly was inversely proportional to the temperature. As observed for microtubule assembly (24), binding of tubulin to membranes did not occur in phosphate buffer. These data led us to think that binding of tubulin to membranes could involve the assembly of a limited number of tubulin molecules in a "micropolymerization" process. The fact that binding data yielded a Hill coefficient close to 1 is not in agreement with the hypothesis of a "micropolymerization" process which should introduce cooperativity.

Recent studies from Caron and Berlin (25), Klausner et al. (26), and Kumar et al. (27) have shown that tubulin strongly interacts with phospholipid vesicles. Similarly, Reaven and Azhar (7) have found that liposomes prepared from phospholipids extracted from liver fractions were able to inhibit microtubule formation. In a previous work, we found that mild trypsin treatment prevented the interaction of tubulin with intact mitochondria, suggesting that protein components could participate to the binding process. Taken together, these findings led us to consider that tubulin "receptor" site on cellular membranes could be composed of or could require the presence of both lipids and protein(s). The presence of tubulin in membranes as an intrinsic membrane component (0.2–0.4% of membrane protein) raised the question as to whether membrane tubulin could be involved in the binding of tubulin. If so, this would imply that a membrane tubulin molecule is able to bind or to promote the binding of about 20 molecules of exogenous tubulin; this would be in agreement with a tubulin assembly process. Although promising, measurements of tubulin binding to erythrocyte membranes devoid of membrane tubulin did not give a definite answer to the question. Indeed, the tubulin-binding activity of erythrocyte membranes was very much lower than that of rat liver plasma membranes. This result can be interpreted in several different ways. The difference in tubulin-binding capacity could indicate that membrane tubulin plays a significant role. Alternatively, the occurrence of a significant binding activity in absence of membrane tubulin could mean that membrane tubulin is not involved in the tubulin-membrane interaction. Another explanation would be that tubulin binding to erythrocyte and tubulin binding to liver membranes represent related but not identical processes. This is supported by recent reports from V. Bennett and J. Davis (22, 23) demonstrating that an erythrocyte protein, ankyrin, binds to tubulin and that immunoreactive forms of ankyrin are found in a variety of cells and tissues. They also found that the α subunit of spectrin was immunologically related to the microtubule-associated protein 2. The fact that sealed and unsealed erythrocyte ghosts exhibited the same tubulin-binding activity suggests that the process involves either a trans-membrane component or a component facing out of the cell. The question as to whether or not liver plasma membranes, mitochondrial membranes and secretory granule membrane bind tubulin on their cytoplasmic side has not been solved. The similarities between these different types of membranes with regard to tubulin-binding properties indicate that their interaction with tubulin could be mediated by a membrane component that is common to the various membrane fractions or by different membrane components exhibiting common properties.

The tubulin-binding sites identified in this study could have a biological significance since they are characterized by an apparent K_D within the expected intracellular free tubulin concentration range. The association of tubulin molecules in a "micropolymerization" process could be involved in a general cellular phenomenon such as the association between microtubules and organelles. It seems also reasonable to think that tubulin reversibly bound to membranes could constitute a tubulin compartment participating in a regulation process inside the cells.

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