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Tubulin–G Protein Interactions Involve Microtubule Polymerization Domains†

Nan Wang‡ and Mark M. Rasenick*

Department of Physiology and Biophysics and the Committee on Neuroscience, University of Illinois, College of Medicine, Chicago, Illinois 60680-6998

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ABSTRACT: It has been suggested that elements of the cytoskeleton contribute to the signal transduction process and that they do so in association with one or more members of the signal-transducing G protein family. Relatively high-affinity binding between dimeric tubulin and the α subunits of G_s and G_{i1} has also been reported. Tubulin molecules, which exist in solution as $\alpha\beta$ dimers, have binding domains for microtubule-associated proteins as well as for other tubulin dimers. This study represents an attempt to ascertain whether the association between G proteins and tubulin occurs at one of these sites. Removal of the binding site for MAP2 and tau from tubulin by subtilisin proteolysis did not influence the association of tubulin with G protein, as demonstrated in overlay studies with [^{125}I]tubulin. A functional consequence of that association, the stable inhibition of synaptic membrane adenylyl cyclase, was also unaffected by subtilisin treatment of tubulin. However, ring structures formed from subtilisin-treated tubulin were incapable of effecting such inhibition. Stable G protein–tubulin complexes were formed, and these were separated from free tubulin by Octyl-Sepharose chromatography. Using this methodology, it was demonstrated that assembled microtubules bound G protein quite weakly compared with tubulin dimers. The α subunit of G_{i1} and, to a lesser extent, that of G_o were demonstrated to inhibit microtubule polymerization. In aggregate, these data suggest that dimeric tubulin binds to the α subunits of G protein at the sites where it binds to other tubulin dimers during microtubule polymerization. Interaction with signal-transducing G proteins, thus, might represent a role for tubulin dimers which is independent of microtubule formation.

Guanine nucleotide binding regulatory proteins (G proteins) are linked to a number of surface membrane receptors and they mediate the regulation of a variety of effectors by hormones and neurotransmitters. Functional reconstitution studies with purified hormone receptors, G proteins, and adenylyl cyclase catalytic unit have shown that this signal transduction cascade can be modeled by incorporating the purified components into phospholipid vesicles (Cerione et al., 1986). These studies suggest that coexistence of these three components meet the minimum requirements for hormonal signal transduction. The regulation of adenylyl cyclase in vivo, however, is likely to be much more complicated. This and other laboratories have suggested that the cytoskeleton, particularly the microtubule–tubulin system, may be involved in adenylyl cyclase regulation [see Rasenick et al. (1985, 1989) and Zor (1983) for reviews], although the mechanism remains elusive.

Recent studies suggest that one locus of interactions between cytoskeletal components and the adenylyl cyclase system is between tubulin and G proteins which are involved in the regulation of that enzyme. In those experiments, a brief incubation of the tubulin–Gpp(NH)p[1] with synaptic membranes caused inhibition of adenylyl cyclase which was persistent to

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* Address correspondence to M.M.R. at the Department of Physiology and Biophysics, University of Illinois College of Medicine, m/c 901, P.O. Box 6998, Chicago, IL 60680. U20133@UICVM.Bitnet.

‡ Present address: Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD 20892.

[1] Abbreviations: tubulin-S, tubulin dimer with the carboxyl terminus of both α and β subunit cleaved by subtilisin, and equivalent to $\alpha s\beta s$ tubulin; $\alpha\beta s$ tubulin, tubulin dimer with the carboxyl terminus of β subunit cleaved by subtilisin; PC-tubulin, tubulin deprived of MAPs with phosphocellulose chromatography; tubulin–Gpp(NH)p, tubulin liganded with Gpp(NH)p; tubulin-S–Gpp(NH)p, tubulin-S liganded with Gpp(NH)p; tubulin-S–GTP, tubulin-S liganded with GTP; G_α, the α subunit of G protein; AAGTP, P^3-(4-azidoanilido)-P^1-5′-GTP; Gpp(NH)p, 5′-guanylylimidodiphosphate; G_s, stimulatory GTP-binding regulatory protein of adenylyl cyclase; G_i, inhibitory GTP-binding regulatory protein of adenylyl cyclase; G_o, a G protein abundant in brain with unknown functions; MAPs, high molecular weight microtubule-associated proteins; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, 1.5 sodium); DTT, dithiothreitol.

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membrane washing (Rasenick & Wang, 1988). In these experiments, tubulin was without effect unless an hydrolysis-resistant GTP analogue was bound to the exchangeable nucleotide binding site. Despite the similarities between tubulin and G proteins [reviewed in Rasenick et al. (1989)], inhibition of adenylyl cyclase in synaptic membranes by tubulin did not appear to be induced by substitution of tubulin for G proteins, since tubulin had no effect on the partially purified adenylyl cyclase catalytic moiety (Rasenick & Wang, 1988). Experiments wherein tubulin liganded with [^{32}P]AAGTP (a hydrolysis-resistant photoaffinity GTP analogue) was substituted for tubulin–GppNHp suggested that a direct nucleotide transfer between tubulin and G_i represented the mechanism for inhibition of adenylyl cyclase by exogenous tubulin. These experiments raised the possibility that physical interaction between tubulin and G proteins occurs (Rasenick & Wang, 1988).

Further studies with purified tubulin and G proteins, utilizing solid-phase protein blotting and immunoprecipitation, demonstrated that tubulin binds to G_α, but much less, if at all, to $G_{\beta\gamma}$ (Wang et al., 1990). Binding of tubulin is G_α-subspecies distinctive, with much higher affinity ($K_d \sim 130$ nM) to $G_{\alpha s}$ and $G_{\alpha i1}$ than to $G_{\alpha o}$, $G_{\alpha i2}$, $G_{\alpha i3}$, and $G_{\alpha r}$ (Wang et al., 1990). Since tubulin binds equally well to G protein heterotrimers ($\alpha\beta\gamma$) as it does to the G_α subunits, it is unlikely that the $G_{\beta\gamma}$ attachment site on G_α is involved in the binding of these species to tubulin. The conformational change in $G_{\alpha s}$ or $G_{\alpha i1}$ brought about by the binding of an hydrolysis-resistant GTP analogue did not alter the ability of these G_α subunits to bind tubulin.

This study represents an attempt to discern the binding site on tubulin for G protein α subunits. Since our previous studies have shown that $\alpha\beta$ tubulin dimers bind well to $G_{\alpha s}$ and $G_{\alpha i1}$, it appeared unlikely that the $\alpha\beta$ dimerization site of tubulin was involved in tubulin–G protein association (Wang et al., 1990). Two regions on the tubulin molecule have been implicated in the association with other proteins. Microtubule-associated proteins (MAPs) appear to bind to distinct regions of tubulin (Yang et al., 1989), and other (as yet unidentified) regions of the tubulin molecule represent association domains for microtubule polymerization. In this study, we suggest that the interaction site between tubulin dimers and $G_{\alpha s}$ or $G_{\alpha i1}$ is similar or identical to at least one site involved in the polymerization of microtubules.

Materials and Methods

Tissue Preparations. Synaptic membrane-enriched fractions were prepared from 21-day-old male Sprague-Dawley rats as described by Rasenick and Bitensky (1980) and stored under liquid nitrogen until use.

Tubulin Preparation. Microtubule proteins were prepared by the method of Shelanski et al. (1973). Briefly, microtubules were polymerized and pelleted by incubation of the supernatant of rat or chicken brain homogenates with 2.5 M glycerol, 1 mM GTP, 2 mM EGTA, and 1 mM $MgCl_2$ in 100 mM PIPES, pH 6.9, at 37 °C followed by centrifugation at 100000g. The microtubule pellet was depolymerized on ice for 1 h. Nucleotides were removed from tubulin by charcoal treatment as described in Rasenick and Wang (1988). A second polymerization step was performed in the presence of GppNHp or azidoanilido-GTP (AAGTP) (150 μM), or GTP (1 mM). This tubulin preparation contained microtubule-associated proteins (MAPs), which were removed (where indicated) by phosphocellulose chromatography with the eluting buffer of 100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM DTT. The resulting preparations (PC-tubulin) were comprised of over 97% tubulin as estimated by Coomassie blue staining. As specified in text or legend, in certain experiments, the tubulin preparation was made by two assembly–disassembly cycles in a buffer composed of 50 mM MES, pH 6.9, 1 mM $MgCl_2$, 1 mM GTP, 2 mM EGTA, and 2.5 M glycerol. Tubulin preparations were stored in aliquots at −80 °C and used less than 4 weeks after preparation. No differences were seen between chicken and rat microtubule preparations in the experiments presented in this paper.

Tubulin Iodination. Purified PC-tubulin was iodinated as described by Wang et al. (1990) with the following modifications. Four hundred micrograms of PC-tubulin in 100 μL of PIPES buffer containing 1 mM GTP and 2.3 M glycerol were polymerized for 20 min at 37 °C and then transferred to incubate with Na^{125}I and Iodogen (Pierce, Rockford, IL) at 37 °C for 45 min. The iodinated microtubules were pelleted by centrifugation at 100000g for 15 min, and the supernatant was decanted and discarded. The microtubules were depolymerized by resuspension in 300 μL of PIPES buffer with 1 mM GTP and incubation on ice for 1 h with intermittent vortexing. The preparation was recentrifuged at 100000g for 20 min, and the free iodide was removed from the supernatant by ultrafiltration desalting. Aliquots of 50 μL of the preparation were stored under liquid nitrogen until use. The specific activity was approximately 360 Ci/mmol of tubulin. Iodination of subtilisin-treated tubulin (tubulin-S) was performed similarly, after subtilisin treatment. The specific activity for the tubulin-S preparations was approximately 290 Ci/mmol.

Overlay Studies. Tubulin binding to $G_{i\alpha 1}$ immobilized on nitrocellulose sheets was estimated as described by Wang et al. (1990). Where it was needed, unlabeled native tubulin was added to adjust the specific activity of native [^{125}I]tubulin to the same level as [^{125}I]tubulin-S (see below).

Proteolysis of Tubulin with Subtilisin. PC-tubulin (2 mg/mL) in PIPES buffer containing 1 mM GTP was incubated with subtilisin Carlsberg (Sigma) in a 1% w/w ratio at 30 °C for the time as noted in the text. The reaction was terminated by addition of 1 M PMSF dissolved in dimethyl sulfoxide to a final concentration of 300 μM. The proteolyzed tubulin preparation (tubulin-S) was either polymerized at 30 °C for 20 min and collected by ultracentrifugation or subjected to analysis by 8.5% SDS–polyacrylamide electrophoresis. In experiments where subtilisin-treated tubulin with Gpp(NH)p bound was used, tubulin-S–Gpp(NH)p was prepared similarly as tubulin-S–GTP except that PC-tubulin–Gpp(NH)p was used for subtilisin digestion and the polymerization of the digested tubulin was supported by 50 μM Gpp(NH)p.

For use in adenylyl cyclase assays, subtilisin-modified tubulin preparations (2 mg in 1 mL of PIPES buffer) were pelleted by warm centrifugation, washed twice with warm PIPES buffer without nucleotide, and resuspended in 200 μL of HEPES buffer. For groups with $\alpha\beta$s and $\alpha s\beta s$ tubulin polymers, the polymers were subjected immediately to incubation with synaptic membranes and, subsequently, to membrane washing and adenylyl cyclase assay. For groups with $\alpha s\beta s$ tubulin dimers, the $\alpha s\beta s$ tubulin polymers were first diluted 10 times with HEPES buffer and placed on ice for 20 min followed by centrifugation. The $\alpha s\beta s$ tubulin dimer concentration in the supernatant was determined, and the preparation was utilized in adenylyl cyclase assays as described for polymers. For the control, IgG was added in place of tubulin on a w/w basis. For other groups, final concentration of tubulin was 1 μM.

Adenylyl Cyclase Assay. Synaptic membranes were thawed and resuspended in a buffer containing 20 mM HEPES, pH

7.5, 1 mM $MgCl_2$, 1 mM DTT, and 0.3 mM PMSF and were incubated with the indicated protein (tubulin or IgG) at 23 °C for 3 min. After incubation, the membranes were washed twice to remove unbound protein or nucleotide and resuspended in the same buffer. Washed membranes (10–20 μg) were incubated at 23 °C in 100 μL of medium containing 15 mM HEPES, pH 7.5, 0.05 mM ATP, [α-^{32}P]ATP (~5 × 10^5 cpm/tube), 1 mM $MgCl_2$, 1 mM DTT, 0.05 mM cyclic AMP, 60 mM NaCl, 0.25 mg/mL of bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 unit/mL of adenosine deaminase, a nucleoside triphosphate-regenerating system consisting of 0.5 mg of creatine phosphate, 0.14 mg of creatine phosphokinase, 15 units of myokinase/mL, and other reagents as indicated in the text. The reaction was stopped by addition of 0.1 mL of a solution containing 2% sodium dodecyl sulfate (SDS), 1.4 mM cyclic AMP, and 40 mM ATP, and the [^{32}P]cAMP formed was isolated by the method of Salomon (1979) and was measured by liquid scintillation counting. [^{3}H]cAMP was used to monitor the recovery.

Electron Microscopy. Electron microscopy of tubulin polymers assembled from native or subtilisin-treated tubulin was performed with samples taken from polymerization reactions. Aliquots of 50 μL were mixed 1:1 with prewarmed 4% glutaraldehyde in assembly buffer. Approximately 10 μL of this solution was applied to a carbon/Formvar-coated grid for about 30 s. Grids were then stained with several drops of 1% uranyl acetate. Excess liquid was drawn off with a filter paper, and the grids were air dried. Samples were examined with transmission electron microscopy on a JEM 100CX (JEOL).

Spectrophotometric Monitoring of Tubulin Polymerization. To start tubulin polymerization, aliquots (200 μL) of PC-tubulin at concentrations as indicated in the text or legend in PIPES buffer with 2.3 M glycerol were added to a 200-μL quartz cuvette which was placed in the cuvette holder of SLM AMINCO DW-2000 double beam spectrophotometer and prewarmed to 37 °C. Microtubule assembly was assessed by monitoring turbidity at 350 nm and, in some experiments, verified by 37 °C centrifugation of aliquots from the assembly reaction.

For experiments designed to test the effects of G proteins on tubulin polymerization, 80-μL aliquots of PC-tubulin in PIPES buffer/2 mM GTP/2.3 M glycerol were mixed with 20 μL of correspondent G proteins in 50 mM NaCl, 10 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, and 0.1% Lubrol-PX. The mixture was incubated on ice for 120 min followed by tubulin polymerization and spectrophotometric monitoring as described above.

Purification of G Proteins. This was performed according to the procedures of Katada et al. (1986) with the following modifications: ^{32}P-labeled azidoanilido-GTP (AAGTP) photoaffinity labeling of G proteins was used to detect the G proteins. An aliquot of 10 μL from each fraction was diluted with 20 μL of 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM EGTA, and then 10 μL of [^{32}P]AAGTP (~0.1 μCi) was added to the mixture. The solution was incubated at 30 °C for 20 min followed by UV photolysis, quenching, SDS–PAGE electrophoresis and autoradiography as described by Gordon and Rasenick (1988).

After DEAE-Toyopearl 650(S) chromatography, an Octyl-Sepharose chromatography step was added to remove minor contaminating proteins. An Octyl-Sepharose column 1.6 × 25 was equilibrated with 150 mL of 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.3% cholate. G_i and G_o fractions from DEAE-Toyopearl 650(S) were pooled and diluted with three volumes of the Tris buffer (to ~250 mL) and then loaded onto the column. The column was washed with the equilibration buffer until no protein was eluted (~100 mL). Proteins were subsequently eluted at a flow rate of 20 mL/h with a reverse gradient of NaCl and cholate (total volume 300 mL), starting with Tris buffer plus 250 mM NaCl and 0.3% cholate and ending with Tris buffer plus 50 mM NaCl and 1.2% cholate. Fractions of 5 mL were collected. For some experiments, G_i and G_o were separated by application of Mono-Q FPLC (Pharmacia) as described by Katada et al. (1986).

Octyl-Sepharose Chromatography of Tubulin–G Protein Complexes. Purified PC-tubulin (amounts noted in the text) in 60 μL of PIPES buffer and 1 mM GTP was mixed with G proteins (amounts given in text) in 20 μL of 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol-PX, and 50 mM NaCl or 20 μL of the same buffer without G proteins (control). The mixture was incubated for 120 min at 4 °C and then loaded on a 1-mL Octyl-Sepharose column which had been equilibrated with 25 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.3% cholate, and 100 mM NaCl. Three milliliters of the above buffer was added to the column followed by 3 mL of 25 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 1.2% cholate, and 50 mM NaCl. The eluate was collected in fractions of 0.2 mL.

Materials. [α-^{32}P]ATP (800 Ci/mmol, 1 Ci = 37 GBq) was purchased from New England Nuclear. [α-^{32}P]GTP was purchased from Amersham. GTP and Gpp(NH)p were from Boehringer Mannheim. *p*-Azidoaniline was synthesized by George L. Wheeler. Cholic acid (free acid) and Lubrol-PX were from Sigma. All other reagents used were of analytical grade.

Results

Characterization of Limited Proteolysis of Tubulin–Gpp(NH)p by Subtilisin. Subtilisin yields a distinct cleavage pattern at the carboxy terminal of both α and β tubulin (Bhattacharyya et al., 1985; Serrano et al., 1984). Unlike other limited tubulin digestions, however, carboxy-terminal fragments generated by subtilisin are separated from the rest of the tubulin dimer in the absence of denaturing agents. Thus, subtilisin treatment allows a determination of the importance of the carboxy terminal of tubulin in associating with $G_{i\alpha 1}$ and transferring GTP to that species. Furthermore, it is possible to form stable polymers with subtilisin-treated tubulin. Such polymers are useful in determining whether the dimer–dimer association sites are required for the interaction between tubulin and G proteins. The SDS–PAGE and Coomassie blue staining analysis of digestion of tubulin by subtilisin (Figure 1) shows that approximately 75% of β tubulin was cleaved after 5 min of incubation, while only about 15% of α tubulin was cleaved at this time. Fifteen minutes of incubation was required for complete digestion of β tubulin, and cleavage of α tubulin was not completed until approximately 40 min of incubation. The time course and SDS–PAGE pattern of the digestion for tubulin–Gpp(NH)p and tubulin–AAGTP were similar to that for tubulin–GTP (data not shown), suggesting that the nucleotide species bound to β tubulin did not alter subtilisin sensitivity or the cleavage site.

Formation of Tubulin-S Rings. As noted above, tubulin-S–Gpp(NH)p, like tubulin-S–GTP, is capable of polymerization. Under conditions used (i.e., low tubulin concentrations, no microtubule-associated proteins, and without polymerization-promoting agents such as glycerol or taxol), tubulin–Gpp(NH)p did not undergo any significant polymerization without subtilisin treatment. Electron microscopy indicated

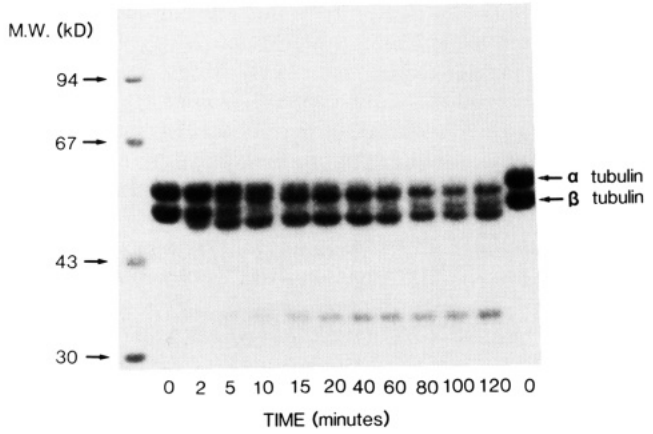

FIGURE 1: Time course of proteolysis of tubulin by subtilisin. Digestion of PC-tubulin–Gpp(NH)p (2.0 mg/mL) by subtilisin was analyzed by Coomassie blue staining of SDS–PAGE gels, as described under Materials and Methods.

that the tubulin polymers taken after 5 min of digestion were comprised mainly of rings (Figure 2). These ring structures formed were morphologically different from rings assembled from native tubulin (Erickson, 1974; Kirschner & Williams, 1974). However, they were similar to the ring structures formed by tubulin-S–GTP (Bhattacharyya et al., 1985). Although $\alpha s\beta s$ tubulin-S–GTP dimers formed sheets, tubulin-S–GppNHp $\alpha s\beta s$ dimers (40-min digestion) formed rings similar to $\alpha\beta s$ tubulin.

Inhibition of Adenylyl Cyclase by Subtilisin-Modified Tubulin Dimers but Not Polymers. When 0.5 μM tubulin–Gpp(NH)p was incubated briefly with rat cerebral cortex synaptic membranes, adenylyl cyclase (basal activity = 132 ± 4 pmol cAMP/(min·mg of protein) was inhibited by approximately 62%, and this effect persisted after membrane washing. Adenylyl cyclase inhibition by tubulin–Gpp(NH)p appears to result from a direct transfer of the nucleotide from tubulin to $G_{\alpha i}$ (Rasenick & Wang, 1988). When tubulin-S–Gpp(NH)p ($\alpha s\beta s$ dimers) were incubated with the membranes, a similar (61%) inhibition of adenylyl cyclase was observed. Although a small (8%) inhibition of adenylyl cyclase by tubulin-S–Gpp(NH)p rings was observed, this was likely due to the dissociation of a small percentage of rings into tubulin-S–GppNHp dimers.

Inhibition of adenylyl cyclase by tubulin-S–Gpp(NH)p dimers was dose dependent with an EC_{50} of 7.8×10^{-8} M, comparable to the EC_{50} of tubulin–Gpp(NH)p [2.6×10^{-7} M, Rasenick and Wang (1988)]. Tubulin-S–GppNHp rings had only a small effect on adenylyl cyclase at concentrations below 10^{-5} M. When tubulin-S–Gpp(NH)p was treated with charcoal to extract the nucleotide from the exchangeable site of β tubulin, the "empty" tubulin-S did not inhibit adenylyl cyclase.

Iodinated Tubulin-S Binding to $G_{\alpha i1}$. This laboratory has demonstrated that [^{125}I]tubulin, when incubated with $G_{\alpha i1}$ immobilized on nitrocellulose, binds to that G_α subunit, specifically, with a K_d of ~130 nM (Wang et al., 1990). The observation that dimeric tubulin-S–Gpp(NH)p inhibited adenylyl cyclase suggested that tubulin-S may interact with G_i to induce such inhibition. [^{125}I]Tubulin-S dimers were prepared and compared with iodinated native tubulin dimers for binding to purified $G_{\alpha i1}$ immobilized on nitrocellulose. As shown in Figure 3, the dimeric [^{125}I]tubulin-S bound to $G_{\alpha i1}$ with an affinity comparable to the dimeric [^{125}I]tubulin.[2]

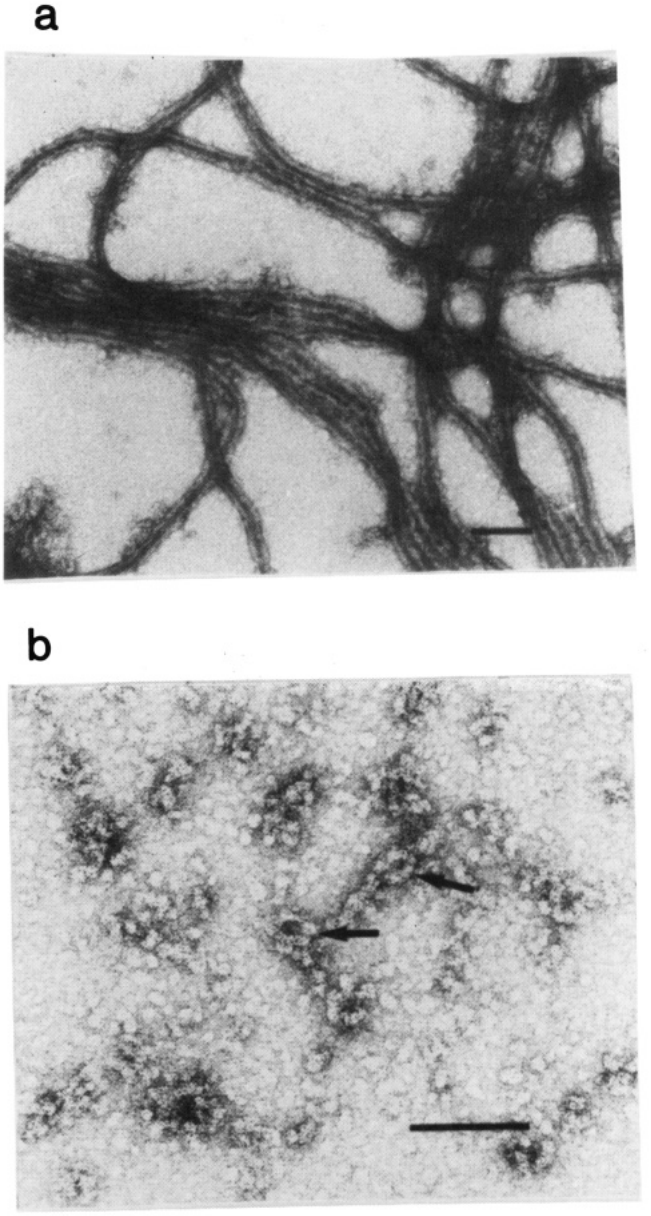

FIGURE 2: Electron microscopy of polymers formed by tubulin–Gpp(NH)p. (a) Microtubules formed by second cycle tubulin–Gpp(NH)p in the presence of MAPs. The bar denotes 100 nm. (b) Morphology of the polymers formed by PC-tubulin–Gpp(NH)p subsequent to subtilisin treatment. After a 5-min digestion by subtilisin, aliquots were removed, fixed, and processed for electron microscopy as described under Materials and Methods. Samples taken after a 40-min digestion showed a similar morphology.

Binding of [^{125}I]tubulin and [^{125}I]tubulin-S to $G_{\alpha i1}$ was blocked completely by excess (200 times) cold tubulin.

Longevity of Tubulin–G Protein Complexes. When purified PC-tubulin was applied to Octyl-Sepharose in the presence of 0.3% cholate, tubulin was not retained by the column

[2] When [^{125}I]tubulin-S polymers were compared with [^{125}I]tubulin-S dimers in binding to $G_{\alpha i1}$, a significant binding of tubulin-S polymers (~50% of dimeric tubulin-S binding) was observed (data not shown). Since the critical concentration for tubulin-S polymerization estimated under optimal conditions is approximately 400 nM (Bhattacharyya et al., 1985) and much greater than the estimated K_d (~130 nM) for tubulin–$G_{\alpha i1}$ interaction, it is assumed that there still existed significant amounts of free dimeric tubulin-S in tubulin-S polymer groups and these tubulin-S dimers could bind to $G_{\alpha i1}$. Quantitation of tubulin-S polymer binding to G protein with this method, therefore, was ambiguous and unfeasible.

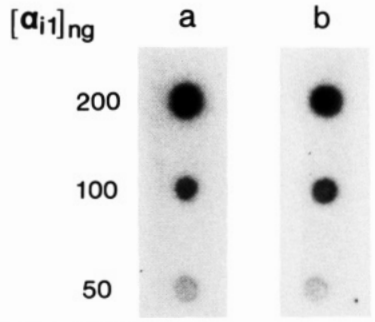

FIGURE 3: [^{125}I]Tubulin binding to $G_{\alpha i1}$. Purified $G_{\alpha i1}$ (from T. Katada and Y. Kaziro) were dotted on a nitrocellulose sheet in the dilution series indicated. The nitrocellulose sheet was air dried and then incubated with blocking buffer followed by incubation with native [^{125}I]tubulin (a) and [^{125}I]tubulin-S (b) as described under Materials and Methods. The final concentration of tubulin was 80 nM. The autoradiograph of the dried nitrocellulose is shown.

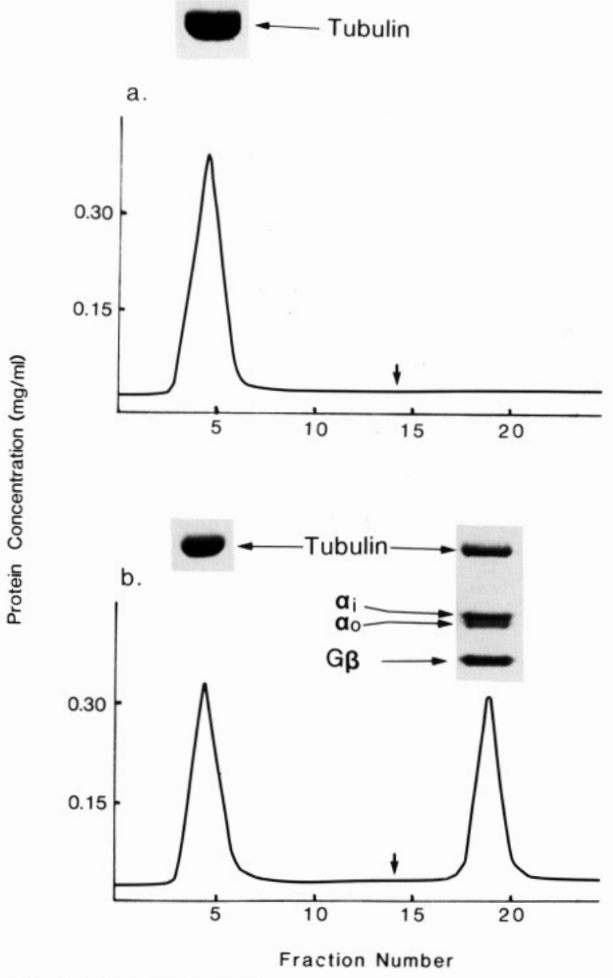

FIGURE 4: Octyl-Sepharose chromatography of tubulin–G protein complexes. (a) 100 μg of PC-tubulin in 60 μL of PIPES buffer and 1 mM GTP was added to 20 μL of 20 mM Tris, pH 7.4, 1 mM DTT, 0.1% Lubrol-PX, and 50 mM NaCl. This was loaded onto a 1-mL Octyl-Sepharose column which had been equilibrated with the Tris–3% cholate buffer as described under Materials and Methods. After a wash with 3 mL of the equilibration buffer, 3 mL of the Tris buffer containing 1.2% cholate and 50 mM NaCl (indicated by the arrow) was applied. Coomassie blue staining of the indicated fractions is shown. (b) Same as in panel a except that a mixture of 100 μg of PC-tubulin and 40 μg of G_i/G_o was applied to the column. Coomassie blue staining of the indicated fractions is shown.

(Figure 4a). If a mixture of PC-tubulin and G proteins was loaded onto the column, some of the tubulin molecules, as well as most (95%) of the G proteins, were retained by the Octyl-Sepharose. These retained tubulins were eluted along with G proteins by a high detergent/low salt buffer (Figure 4b). Since tubulin alone did not bind to Octyl-Sepharose, it is likely that the tubulins were retained on Octyl-Sepharose by forming complexes with G proteins which bound to the hydrophobic matrix. It is noteworthy that, prior to loading onto the Octyl-Sepharose columns, tubulin–G protein mixtures had been retained on ice for 24 h. Percentages of tubulin associated with G protein did not change during the course of storage.

Binding of G Proteins to Microtubules and to Dimeric Tubulin. The results above demonstrated that tubulin rings were relatively ineffective in inhibiting synaptic membrane adenylyl cyclase. Since the normal polymeric form of tubulin is the microtubule, an attempt was made to compare G protein binding to microtubules vs that to tubulin dimers. The experimental design, shown in Table I, indicates three different groups. Group 1 compares the distribution of tubulin and G_i in microtubule and soluble protein fractions, subsequent to microtubule polymerization. Group 2 allows tubulin–G protein complexes to form under conditions which allow the formation of tubulin–G protein complexes but do not support microtubule polymerization. Group 3 represents a control to determine whether the warming step required for microtubule polymerization interferes the tubulin–G_i complex formation or dissociation.

After incubation of tubulin and G_i under microtubule polymerizing conditions and separation of microtubules from soluble proteins by centrifugation (Table I, group 1), most of the G_i (~78%) remained in soluble protein fractions while ~76% of the tubulin formed microtubules and was pelleted by centrifugation (Figure 5, lanes a and b; Table II). When tubulin–G protein complexes, which formed under nonpolymerizing conditions, were separated from free tubulin by Octyl-Sepharose (Table I, group 2), the molar ratio of tubulin to G_i (Figure 5, lane d) in these complexes was approximately 0.91. The binding between tubulin and G protein appears to be a simple bimolecular interaction involving a single binding site (Wang et al., 1990). It is, therefore, assumed that 91% of the G_i was complexed with tubulin. There was a small amount of G_i, approximately 11%, which was not retained by the column (Figure 5, lane c). Increasing the bed volume of the Octyl-Sepharose column reduced the amount of G_i in the void volume fractions, with a proportional increase of tubulin retained by the matrix (data not shown). It is thus assumed that the small unretained fraction of G_i was no different from the G_i which was bound to Octyl-Sepharose, and this fraction of G_i was complexed by tubulin to a similar extent as the Octyl-Sepharose-bound G_i fraction.

Tubulin–G_i complex formation or dissociation did not appear to be disturbed dramatically by the warming step, since the molar ratio of tubulin to G_i in tubulin–G_i complex fractions from group 3 was similar to that in group 2 (compare Figure 5, lane e with Figure 5, lane d; see Table II).

If the affinity of tubulin for G_i was equal to that of microtubules, then the distribution of G_i bound to microtubules and soluble tubulin dimers should be concordant with the distribution of each tubulin fraction. Thus, if G_i bound to microtubules equally well as to soluble tubulin dimers and 100% of G_i were complexed by tubulin and microtubules, then 76% of G_i should appear in the microtubule fraction, as microtubule represented 76% of total tubulin under these conditions (Table II). Since only 91% of G_i was complexed by tubulin, the predicted percentage of G_i associated with microtubules would be approximately 69% (76% × 91%). The observed value of G_i associated with microtubules, however, was only ~22% of total G_i. Approximately 78% of the G_i, of which ~80% was complexed with soluble tubulin dimers

Table I: Comparison of G_i Binding to Tubulin Polymers and Tubulin Dimers (Figure 5)

Group 1	Group 2	Group 3
tubulin 200 μg + G_i 40 μg	tubulin 200 μg + G_i 40 μg	tubulin 200 μg + G_i 40 μg
↓	↓	↓
incubate on ice for 100 min	incubate on ice for 100 min	incubate on ice for 100 min
↓	↓	↓
37 °C, 20 min	load the mixture onto Octyl-Sepharose	37 °C, 20 min
↓	↓	↓
pellet microtubules	collect and measure protein content of tubulin and tubulin–G protein complex fractions	pellet microtubules
↓	↓	↓
measure protein content (supernatant and pellet)	SDS gels	measure protein content (supernatant and pellet)
↓	↓	↓
SDS gels		resuspend supernatant (100 μL) and load onto Octyl-Sepharose
↓		↓
		collect and measure protein content of tubulin and tubulin–G protein fractions
		↓
(lane a) pellet[a,b]; (lane b) supernatant	(lane c) tubulin, not bound to the column; (lane d) tubulin–G protein eluted from the column	(lane e) tubulin–G protein eluted from the column

[a] The distribution of tubulin and G_i in each fraction was determined and quantitated by densitometric scan of each band in SDS gels in combination with the values of protein content measured. [b] The amount of the soluble fraction trapped in the microtubule pellet was determined by adding 10^5 cpm of iodopindolol to the tubulin–G_i mixture in the ice incubation step of a parallel experiment. It accounts for approximately 6% of the total soluble fraction.

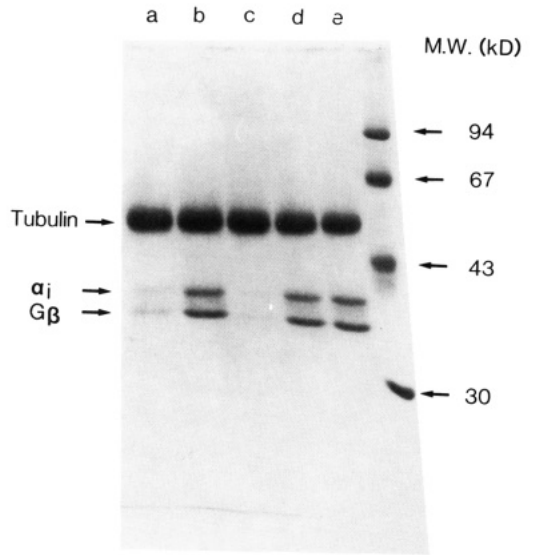

FIGURE 5: Binding of G_i to tubulin dimers and microtubules. The procedures are described in Table I and under Materials and Methods. Nine micrograms of protein from the microtubule pellet fraction of group 1 (lane a) and the protein fraction unbound to Octyl-Sepharose of group 2 (lane c), 18 μg of protein from the supernatant fraction of group 1 (lane b), the protein fraction eluted by 1.2% cholate buffer of group 2 (lane d), and the protein fraction eluted by 1.2% cholate buffer of group 3 (lane e) were subjected to SDS–PAGE (see Table I for details). Coomassie blue staining of the SDS gels is shown. The result presented is from one of two similar experiments.

Table II: Distribution of Tubulin and G_i in Microtubule Pellet and Supernatant Fractions (Figure 5)

fraction	tubulin	G_i
supernatant	24%[a]	78%
microtubule pellet	76%	22%

[a] The distribution of tubulin and G_i in microtubule pellet and supernatant fractions was quantitated as described for group 1 in Table II and under Materials and Methods. The soluble protein trapped in microtubule pellet fractions was estimated as described in Table I and accounts for ~6% of total soluble fraction. This fraction was subtracted from microtubule pellet fractions and added to supernatant fractions. After this calibration, ~67% of total protein (~160 μg) was determined to be in microtubule pellet fraction, and 33% of the total protein (~80 μg) was in supernatant fractions. The ratio of G_i vs tubulin staining was approximately 0.39:0.61 for the supernatant (Figure 7, lane b) and 0.07:0.93 for the microtubule pellet fraction (Figure 7, lane a). The density for G protein was corrected by adding the estimated density of G_γ subunit (molecular mass is approximated as 8 kDa), which is not seen in the PAGE system employed. Therefore, G_i in the supernatant was calculated to be 39% × 80 μg = 31.2 μg, which accounts for approximately 78% of total G_i. Using this calculation, 22% of G_i was in the microtubule pellet fraction. Another calculation scheme assumes that, if 7% of the microtubule pellet protein was G_i (see above), then 7% × 160 μg = 11.2 μg, which represents 28% of the total G_i, was in the pellet. Using this calculation, 72% of G_i was in the supernatant fraction. Tubulin distribution in supernatant and microtubule pellet was ~24% and 76%, respectively, by the first method of calculation and ~26% and 74%, respectively, by the second method of calculation. Data obtained by first method of calculation are shown. Data from one of two similar experiments are represented.

(Figure 5, lane e), remained in soluble protein fractions. Therefore, it appears that G_i binds weakly to microtubules.

G_i and G_o Inhibit Microtubule Polymerization. The above data demonstrate that polymerization of tubulin decreases binding of tubulin to G_i and diminishes the inhibitory effect of tubulin on adenylyl cyclase. These results suggest the possibility that G proteins might bind to domains on tubulin which associate with other tubulin molecules during microtubule formation and, in doing so, inhibit the polymerization of microtubules. When 15 μM PC-tubulin was incubated with 20 μM G_i or G_o, microtubule polymerization was inhibited 57.5% by G_i and 40.2% by G_o, as estimated by spectrophotometric monitoring (Figure 6). These G protein–tubulin ratios represented the maximum achievable inhibition of microtubule polymerization by G protein. The minimum G protein concentrations which caused a significant (10%) inhibition of microtubule polymerization were 4 μM G_i and 5

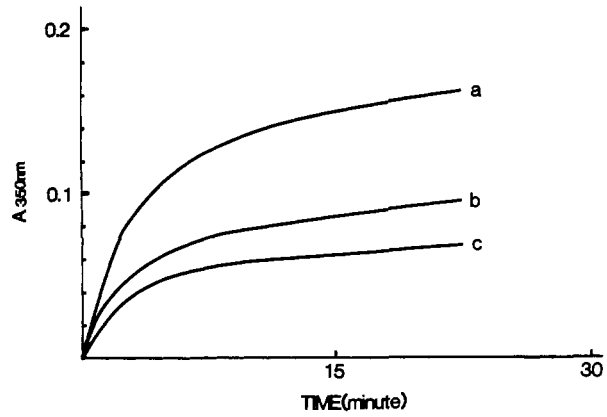

FIGURE 6: Inhibition of tubulin polymerization by G_i and G_o. Tubulin preincubation with G proteins or IgG and the spectrophotometric monitoring of tubulin polymerization was performed as described under Materials and Methods. The final G protein or IgG concentration was 20 μM. The final tubulin concentration was 15 μM. (Curve a) Tubulin + IgG (control); (curve b) tubulin + G_o; (curve c) tubulin + G_i.

μM G_o (both in the presence of 20 μM tubulin). G protein did not inhibit microtubule polymerization simply by acting as a sink for Gpp(NH)p, since PC-tubulin was polymerized in the presence of 0.5 mM Gpp(NH)p, a nucleotide concentration in excess over tubulin or G protein. Furthermore, G_i and G_o, presaturated with Gpp(NH)p prior to incubation with tubulin, inhibited microtubule polymerization in a manner similar to the data reported in Figure 6. Despite the apparent ability of G_i or G_o to inhibit microtubule polymerization to a limited extent, the low cytosolic concentrations of G protein relative to tubulin would appear to preclude G protein as a usual regulator of this process.

Discussion

Similarities among a variety of proteins which bind GTP have evoked the notion that tubulin belonged to this class as well. Evidence on the basis of structural (Linse & Mandelkow, 1989; Sternlicht et al., 1989) and biochemical [reviewed in Rasenick et al. (1989)] properties was consistent with such a taxonomy. Previous studies from this laboratory have suggested (Rasenick et al., 1981), and then demonstrated (Rasenick & Wang, 1988), a functional interaction between dimeric tubulin and G proteins, which modulates synaptic membrane adenylyl cyclase. Most recently, it has been demonstrated that dimeric tubulin binds to $G_{\alpha s}$ and $G_{\alpha il}$ with a relatively high affinity, whereas it binds to other G protein α subunits (αi2, αi3, αo, and αr) with a markedly lower affinity (Wang et al., 1990). The formation of these complexes suggests that similarities between tubulin and G proteins might extend to the formation of more elaborate molecular complexes. This report presents data consistent with the hypothesis that "polymerization interfaces" on tubulin are the regions which bind to G proteins. Further, it appears that regions involved in the binding of MAPs are unlikely to represent the portions of the tubulin molecule which bind to G protein.

Adenylyl cyclase inhibition by dimeric tubulin-S–Gpp(NH)p suggests that physical interaction between tubulin-S and G_i in membranes is no different than that observed between G_i and native tubulin dimers. Furthermore, AAGTP-labeled tubulin-S dimers transfer AAGTP to $G_{\alpha i}$ in a manner and extent which is indistinguishable from native tubulin–AAGTP dimers (data not shown). These data, together with the observation that iodinated tubulin-S dimers bind to $G_{i\alpha 1}$ with an affinity similar to that of native tubulin dimers (Figure 3), demonstrate that the carboxyl-terminal regions of tubulin subunits are not required for tubulin–$G_{\alpha il}$ interactions. This suggests that the MAP-binding site is not the site of G protein binding.

Adenylyl cyclase inhibition by polymers assembled from subtilisin-modified tubulin, in the form of either $\alpha\beta$s rings or αsβs rings, was much reduced compared with native tubulin dimers. Since the tubulin-S dimers were effective in inhibiting adenylyl cyclase, it appears that polymerization of these modified tubulin dimers masks the binding sites on tubulin for G_α.

Further efforts to characterize the binding of tubulin dimers and tubulin polymers to G proteins involved Octyl-Sepharose chromatography to separate tubulin–G protein complexes from uncomplexed tubulin dimers. In this regard, it is noteworthy that tubulin has been demonstrated to bind to Octyl-Sepharose (Prasad et al., 1987) and to Phenyl-Sepharose (Hanssens et al., 1990). In the latter, tubulin binding to Phenyl-Sepharose was reversible and guanine nucleotide dependent. In the presence of 0.3% cholate, however, tubulin did not bind to Octyl-Sepharose (Figure 4a) unless G protein was present.

Quantitation of tubulin–G_i and microtubule–G_i complexes indicates that, under our experimental conditions, approximately 91% of the added G_i is complexed with tubulin dimers or microtubules (Figure 5). The disparate distribution of tubulin and G_i in microtubule and supernatant fractions supports the conclusion that G_i binds to microtubules, but much less well than to dimeric tubulin.[3] It is possible that the G protein associated with microtubules in this experiment is bound to the ends of microtubules, which may have binding sites available.

Peraldi et al. (1989) demonstrated significant binding of G_o to cellular structures which were thought to be microtubules. These data are not necessarily contradictory to the poor binding of G protein to microtubules reported above. The microtubule fractions studies in this report were cold labile. A considerable portion of cellular microtubules are likely to be cold stable (Webb & Wilson, 1980), and these microtubules may have an entirely different interaction with G protein than those examined in this study.

Despite the efforts made over the past decades, the three-dimensional structure of tubulin still remains largely unknown. Although existing electron microscopy and X-ray diffraction studies have revealed a great deal about microtubules, structural aspects of the molecules which comprise those organelles are poorly understood. Tubulin crystals suitable for high-resolution X-ray analysis have not been reported. Although considerable data are available for the rate and nature of tubulin polymerization, the domains of the molecule involved in binding to other tubulin dimers in formation of polymers are, as yet, unidentified. It is assumed, however, that during tubulin polymerization into microtubules several points of contact must be made (Kirschner, 1974; Mandelkow et al., 1985). Specifically, α tubulin engages in some interaction with β tubulin from another heterodimer and vice versa. Finally, as the microtubule coils around, a vertical association among α and β heterodimers might occur. One hypothesis proposed by this laboratory is that the segments of G_α involved in interaction with tubulin may be sufficiently "tubulin-like" to interact with tubulin at the sites where dimer–dimer binding normally occurs (Rasenick et al., 1989). Given this, the po-

[3] It is suggested that the concentrations of G protein used in these experiments would inhibit microtubule polymerization by 10%. This has not been considered in the calculations because it is not clear which group (dimers or microtubule) would be more affected. Regardless, the net effect would be small.

lymerization of tubulin would be expected to render the binding sites of tubulin poorly accessible to G_α, and data in Figure 5 and Table II are consistent with that expectation.

The association constant for tubulin dimers is similar to that (130 nM) for the binding of $G_{\alpha i1}$ to tubulin (Wang et al., 1990). This and the ability of tubulin–G protein complexes to form in solution would suggest that, given conditions under which microtubules would polymerize, G proteins complexed with tubulin could either incorporate into microtubules or prevent tubulin polymerization. The data in Figure 6 indicate that the latter appears to be the case. Further, since data in Wang et al. (1990) demonstrated that the tubulin–G protein interaction appears to be at a single site, incorporation of G proteins into microtubules, which would require multiple tubulin-binding sites on G proteins, is unlikely. These results suggest that a single site on a given G_α binds to tubulin and, in doing so, prevents the association among tubulin dimers necessary to form microtubules. The ability of a small amount of G protein to complex with microtubules is not necessarily contradictory to this. Conformational changes in the tubulin molecule brought about by microtubule polymerization might create new binding sites for G proteins, which behave differently from those on tubulin dimers (e.g., showing similar affinities for G_o and G_i). The reported association of G_o with microtubule-like structures (Peraldi et al., 1989) may reflect such a phenomenon.

It is noteworthy that, whereas previous data from this laboratory have demonstrated that only $G_{\alpha s}$ and $G_{\alpha i1}$ bind to tubulin with high affinity, G_o inhibited microtubule polymerization to a similar extent as G_i. Our estimate of the K_d of tubulin for G_o was 2–5 μM (Wang and Rasenick, unpublished), but due to high nonspecific binding incurred using the high [^{125}I]tubulin concentrations necessary to examine this, these estimates are crude. Conditions under which polymerization studies and complex-formation studies (Figures 4 and 5) were done employed micromolar concentrations of G_o and tubulin. Additionally, whereas previous affinity determinations were done with a large (50–300×) excess of tubulin to G protein, in these studies, the greatest excess of tubulin was 4×. Under these conditions, G_o, G_{i2}, G_{i3}, and transducin would all be expected to display some tubulin-binding activity, and they do just that (Wang and Rasenick, unpublished). Further, at these high tubulin concentrations, tubulin dimers might begin to associate. Since it is likely that the dimer association involved in the formation of a microtubule evokes some change in the conformation of tubulin, the G protein binding properties may be modified as well.

The data presented in this report do not pinpoint the binding domain on tubulin for G proteins. It is noteworthy that dynamin, a newly discovered microtubule-associated mechanochemical enzyme (Obar et al., 1990), is also without a known tubulin-binding sequence. The 18-residue repeats on MAP2 and tau, which are suggested to constitute the microtubule-binding site (Lee et al., 1989; Lewis et al., 1989), are not found in G proteins or dynamin. Amino acid sequence analysis of dynamin reveals three consensus sequence elements, which together are indicative of a "G-protein-like" GTP-binding motif (Obar et al., 1990). Comparison of dynamin with kinesin heavy chain, another microtubule-associated protein, showed considerable homology in a limited region which is within a domain proposed as the microtubule-binding site of kinesin (Yang et al., 1989). This region includes the GTP-binding element II sequence, and the amino acid sequence homology between dynamin and α subunits of G_i and G_s is limited to this region (Obar et al., 1990). The idea that this region is involved in tubulin-binding both for G_α and dynamin merits consideration.

Although this study is limited to an examination of the association between G protein and tubulin, the suggestion of interaction among G protein α subunits is inherent. If the G protein–tubulin association sites are "polymerization domains", this could explain the observations from this laboratory that $G_{\alpha s}$ and $G_{\alpha i1}$ form complexes and transfer GTP analogues from one to another (Hatta et al., 1986; Rasenick & Childers, 1989; Ozawa & Rasenick, 1991). Other evidence suggests the formation of polymers among G proteins (Nakamura & Rodbell, 1990; Vaillancourt et al., 1990; Hingorani et al., 1989), and these polymerization domains might play a role in such a process.

It is suggested that tubulin dimers might participate in the signal transduction process, and, in doing so, they acquire a role which is independent of microtubule formation. The observation that 6% of the protein in these synaptic membrane preparations is tubulin (Wang and Rasenick, unpublished observations) contributes to the possibility that this role is indeed significant. Modulation of neuronal signal transduction by components of the cytoskeleton and the various implications of that modulation are just beginning to be explored.

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Characterization of Lipid Insertion into Monomolecular Layers Mediated by Lung Surfactant Proteins SP-B and SP-C†

Marja A. Oosterlaken-Dijksterhuis,*,‡ Henk P. Haagsman,‡ Lambert M. G. van Golde,‡ and Rudy A. Demel§

Laboratory of Veterinary Biochemistry and Centre for Biomembranes and Lipid Enzymology, Utrecht University, P.O. Box 80.176, 3508 TD Utrecht, The Netherlands

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ABSTRACT: Pulmonary surfactant proteins, SP-B and SP-C, if present in preformed monolayers can induce lipid insertion from lipid vesicles into the monolayer after the addition of (divalent) cations [Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991) *Biochemistry 30*, 8276-8287]. This model system was used to study the mechanisms by which SP-B and SP-C induce monolayer formation from vesicles. Lipid insertion proceeds irrespectively of the molecular class, and PG is not required for this process. In addition to lipids that are immediately inserted from vesicles into the monolayer, large amounts of vesicles are bound to the monolayer and their lipids eventually inserted when the surface area is expanded. SP-B and SP-C are directly responsible for the binding of vesicles to the monolayer. By weight, the vesicle binding capacity of SP-B is approximately 4 times that of SP-C. For vesicle binding and insertion, the formation of close contacts between monolayer and vesicles is essential. SP-B and SP-C show very similar surface properties. Both proteins form extremely stable monolayers (collapse pressures 36-37 mN/m) of α-helical structures oriented parallel to the interface. In monolayers consisting of DPPC and SP-B or SP-C, an increase in mean molecular area is observed, which is mainly attributed to the phospholipid. This will greatly enhance the insertion of new lipid material into the monolayer. The results of this study suggest that the surface properties and the hydrophobic nature of SP-B and SP-C are important for the protein-mediated monolayer formation.

Pulmonary surfactant, a complex lipid/protein mixture, is synthesized in alveolar type II cells, assembled into lamellar bodies, and secreted into the alveolar space (Haagsman & van Golde, 1991). Within the alveolar space, surfactant lipids are found in a number of different structural forms, including lamellar bodies, tubular myelin, and various vesicular structures (Manabe, 1979). Tubular myelin is thought to be the precursor of the monomolecular surface film that stabilizes the alveoli at end expiration (Goerke, 1974; Clements, 1977). Lipids comprise the majority (approximately 90%) of this surface-active material. The most abundant phospholipid components are dipalmitoylphosphatidylcholine (DPPC),[1] unsaturated phosphatidylcholine (PC), and phosphatidyl-

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* Correspondence should be addressed to this author.
‡ Laboratory of Veterinary Biochemistry.
§ Centre for Biomembranes and Lipid Enzymology.

[1] Abbreviations: SP-A, SP-B, and SP-C, surfactant proteins A, B, and C, respectively; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; MLV, multilamellar vesicle(s); SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); CD, circular dichroism.

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