Tubulin–G Protein Association Stabilizes GTP Binding and Activates GTPase: Cytoskeletal Participation in Neuronal Signal Transduction†

Sukla Roychowdhury and Mark M. Rasenick*

Department of Physiology and Biophysics and the Committee on Neuroscience, University of Illinois, College of Medicine, Chicago, Illinois 60612-7340

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ABSTRACT: It has been suggested that dimeric tubulin can participate in the signal transduction process through its association with the GTP-binding (G) proteins G_s and G_{i1}. Using the photoaffinity GTP analog, azidoanilido-GTP, it has been shown that the transfer of nucleotide from tubulin to $G_{\alpha s}$ and $G_{\alpha i1}$ is the key step of this activation. The binding sites between tubulin and G_s or $G_{\alpha i1}$ appear to involve microtubule polymerization domains, since G protein α subunits were demonstrated to inhibit microtubule assembly [Wang, N., & Rasenick, M. M. (1991) *Biochemistry* 30, 10957–10965]. In order to understand tubulin–G protein interaction and the nucleotide transfer process in detail, tubulin was labeled with [α-^{32}P]GTP or [^{35}S]GTPγS and was incubated with recombinant $G_{\alpha i1}$ at increasing molar ratios. Rapid filtration through nitrocellulose was used to determine nucleotide binding in the protein complex. A substantial amount of bound nucleotide was lost from tubulin during the filtration assay. However, the addition of $G_{\alpha i1}$ to [α-^{32}P]-GTP–tubulin protected the nucleotide binding in a dose-dependent manner, suggesting a stabilization of GTP binding in the tubulin–$G_{\alpha i1}$ complex. $G_{\beta\gamma}$ mitigated this effect, and this was not dependent upon the presence of G_α, suggesting a direct interation between $\beta\gamma$ and tubulin. The retinal G protein, transducin, which displayed a much lower affinity for tubulin, did not elicit similar stabilization of GTP binding, and transducin $\beta\gamma$ did not release GTP from tubulin. The [α-^{32}P]GTP bound to tubulin was displaced completely by a 10-fold excess of free GTP but only by ~60% when GTP binding was stabilized in presence of $G_{\alpha i1}$. The residual nucleotide stayed bound to the tubulin–$G_{\alpha i}$ complex even in the presence of 1 mM GTP, suggesting a partial nonexchangeability of nucleotide in the complex. Thin layer chromatography was used to identify the nucleotide bound to the protein. When tubulin and G_i were present, 60–80% of the GTP was hydrolyzed. Thus $G_{\alpha i1}$ may evoke some change in tubulin similar to that seen during the formation of a microtubule. The transfer and hydrolysis of GTP from synaptic membrane tubulin to $G_{\alpha s}$ and $G_{\alpha i1}$ could provide a mechanism for cross-talk among signal transduction pathways in the brain and might represent a function of tubulin dimers which is independent of microtubule formation.

Receptors for a variety of hormones or neurotransmitters are coupled to their intracellular effectors via GTP-binding (G)[1] proteins. Current models of G proteins favor a heterotrimeric structure composed of the guanine nucleotide binding α, plus β and γ subunits, the latter two forming a tight association under nondenaturing conditions. Agonist bound receptors activate G proteins by allowing GTP to bind to the α subunit of the heterotrimer (Simon et al., 1991). Subsequently, activated G_α changes its association with $G_{\beta\gamma}$ in a manner which permits activation of intracellular effector molecules. Although receptor–G protein–effector complexes can reconstitute hormone-sensitive signaling systems in vitro, it is likely that the regulation of receptor–G protein signaling is substantially more complex in the cell.

Cytoskeletal proteins have been demonstrated to interact with several proteins which mediate neuronal signal transduction [see Yan and Rasenick (1990) for review]. It has been observed that the α subunits of certain G proteins form complexes with synaptic membrane tubulin and undergo a directed transfer of nucleotide from the exchangeable GTP-binding site of tubulin (Rasenick &Wang, 1988; Roychowdhury et al., 1993). This appears to be a highly specific process, as tubulin has been shown to bind with high affinity to the α subunit of only two G proteins, G_s and G_{i1} (Wang et al., 1990). Other G protein α subunits (αi2, αi3, αo, and αr) display a much lower affinity for tubulin despite a much greater amino acid sequence identity of these proteins to αi1 than to αs. Tubulin forms a complex with the G_α subunits, but it does not block the binding of $G_{\beta\gamma}$ to G_α under those conditions (Wang et al., 1990). Tubulin–G protein complex formation appears to occur at regions of tubulin which are likely to be involved in binding to other tubulin dimers during the process of microtubule polymerization (Wang & Rasenick, 1991).

In a recent study, using the photoaffinity GTP analog AAGTP, it was shown that tubulin–AAGTP was capable of transferring AAGTP to $G_{\alpha i1}$ in a resolved system (Roychowdhury et al., 1993). In that study, about 25% of the

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* Address correspondence to M.M.R. at the Department of Physiology and Biophysics, University of Illinois College of Medicine, 901 S. Wolcott m/c 901, Chicago, IL 60612-7342. Phone: (312) 996-6641. Fax: (312) 996-1414. E-mail: raz@uic.edu.

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[1] Abbreviations: PC-tubulin, tubulin deprived of MAPs with phosphocellulose chromatography; tubulin-GTP, tubulin with GTP bound; tubulin-GTPγS, tubulin with GTPγS bound; tubulin-AAGTP, tubulin with AAGTP bound; G_α, the α subunit of G protein; GTPγS, guanosine 5′-*O*-thiotriphosphate; 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; G_T, retinal rod transducin; MAPs, high molecular weight microtubule associated proteins; PEI cellulose, polyethyleneimine cellulose; TLC, thin layer chromatography; 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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AAGTP bound to tubulin was transferred to αi when tubulin–AAGTP was incubated in equimolar amounts with purified G protein (or recombinant αi1). However, the physical interaction between tubulin and G protein was disrupted prior to SDS–PAGE analysis. In the present study, rapid filtration and thin layer chromatography (TLC) were used to follow the consequences of tubulin–G protein complex formation for GTP binding and hydrolysis. The results of this study suggest that the tubulin–$G_{\alpha i1}$ interaction stabilizes nucleotide binding in the complex and activates GTPase.

EXPERIMENTAL PROCEDURES

Tubulin Preparation. Microtubule proteins (MTP) were prepared by the method of Shelanski et al. (1973). Briefly, microtubules were polymerized and pelleted by incubation of supernatant from 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 100 000*g*. The microtubule pellet was resuspended in the above buffer and depolymerized on ice. This allowed incorporation of 0.82–0.84 mol of GTP or GTP analog per mole of tubulin (Rasenick & Wang, 1988). The tubulin preparation made by two assembly–disassembly cycles (MTP) contains microtubule-associated proteins (MAPs). These MAPs were removed by phosphocellulose chromatography in a buffer of 100 mM Pipes, pH 6.9, 2 mM EGTA, and 1 mM $MgCl_2$ (PIPES buffer). The resulting preparations (PC-tubulin) were greater than 97% tubulin as estimated by Coomassie blue staining. Tubulin preparations were stored under liquid nitrogen and used in less than 4 weeks. Tubulin–[α-^{32}P]GTP or tubulin–[^{35}S]GTPγS was prepared by removing exchangeable nucleotide from PC-tubulin by charcoal treatment followed by incubation with 0.1 mM [α-^{32}P]GTP or [^{35}S]GTPγS. The samples were then desalted twice on centrifugal gel filtration columns using P6-DG resin (Bio-Rad) as described previously (Rasenick & Wang, 1988). After desalting, 0.4–0.6 mol of GTP or GTPγs was bound/mol of tubulin. To prepare tubulin–[α-^{32}P]GDP, 0.5 mM [α-^{32}P]-GTP was added to charcoal treated tubulin and allowed to polymerize in the presence of 30% glycerol in PIPES buffer containing 5 mM $MgCl_2$ at 37 °C. The microtubule pellet was then resuspended in PIPES buffer. Thin layer chromatography indicated 90% of the labeled nucleotide had been converted to GDP. The binding of nucleotide is entirely on the β subunit (Geahlen & Haley, 1979; Rasenick & Wang, 1988). Protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

G Protein Purification. G proteins were purified from bovine brain (Wang & Rasenick, 1991). Alternately, $G_{\alpha i1}$ was purified from *Escherichia coli* expressing the recombinant gene for that protein (supplied by Maurine Linder) (Linder & Gilman, 1991). *E. coli* were grown and harvested as described and protein was purified by a modification of the method of Linder and Gilman (1991). Briefly, proteins eluted from a DEAE-Sephacel column (instead of batch elution) were subjected to phenyl-Sepharose, hydroxylapatite, and Resource Q (Pharmacia) chromatography succesively. If necesary for purification, this was followed by a second hydroxylapatite chromatographic step. G proteins were detected by [α-^{32}P]AAGTP photoaffinity labeling as described previously (Wang & Rasenick, 1991). G proteins were purified from bovine brain according to the procedure of Katada et al. (1986) with the following modification. After DEAE-Sephacel and AcA-34 chromatography, phenyl-Sepharose chromatography (in the presence of 1 mM GDP) was included. This was followed by DEAE-Toyopearl and hydroxylapatite chromatography. The final purification step was a second DEAE-Sephacel chromatography using a 25–300 mM NaCl gradient in Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT. βγ fractions came off at the very beginning of the gradient and were identified by anti-βγ antibody. Fractions containing a mixture of G_i and G_o were eluted at about 125 and 250 mM NaCl and were identified by [α-^{32}P]-AAGTP labeling and immunoblotting.

Nucleotide Binding Assay. A rapid filtration technique was used to determine the change in nucleotide binding in tubulin–G protein complexes. The procedure described by Northup et al. (1982) was followed with a few minor modifications. Tubulin labeled with [α-^{32}P]GTP, [^{35}S]-GTPγS, or [α-^{32}P]GDP was incubated with or without the indicated additions at 30 °C for 20 min in a 100-μL volume. The reaction mixture (25 μL, triplicates) was then applied directly to nitrocellulose filters (HA, 0.45 μm, Millipore, Walthan, MA) which were previously saturated with 3 mL of 100 μM GTP in wash buffer (20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 5 mM $MgCl_2$ or 100 mM PIPES, pH 6.8, 1 mM $MgCl_2$ and 2 mM EDTA). The filters were then washed three times with 2 mL of the ice-cold washing buffer, air dried, and dissolved in 6 mL of scintillation fluid (Universol, ICN) for determination of bound radioactivity. Alternatively, the reaction mixtures (25 μL, triplicates) were diluted with 0.5 mL wash buffer and immediately poured onto nitrocelluse filters and treated as above. Both procedures using Tris and PIPES buffer produced similar binding data. However, all experiments described in this paper used PIPES buffer for the binding assay. The ability of nitrocellulose membrane to retain tubulin and G proteins under various experimental conditions was tested by incubating filters which had been subjected to these procedures with antitubulin or anti-G-protein antibodies, followed by detection of antigen–antibody complexes as described previously (Wang et al., 1990). Association of tubulin with G proteins or their subunits did not alter the binding of either tubulin or G proteins to nitrocelluose membranes.

Analysis of Nucleotide Bound to Protein. To determine the species of nucleotide bound to tubulin and/or G protein as well as the extent of GTP hydrolysis, samples were treated with 1% SDS at room temperature for 15 min. Nucleotide analysis was done by thin layer chromatography (TLC) on PEI cellulose. Two microliters of a 10 mM solution of GTP, GDP, or GTPγS was spotted on PEI-cellulose plates at 1.5-cm intervals, followed by 2–5 μL of samples. The chromatograms were developed in 0.35 M NH_4HCO_3. The spots containing GTP, GDP, or GTPγS were visualized with a UV lamp, and the plate was exposed to a film for autoradiography. The autoradiogram shows the composition of nucleotide bound to the protein which was further quantified by cutting out the nucleotide spots and desorbing them by addition of 1 M $MgCl_2$ and 0.02 M Tris, pH 8.0. Following a 15-min incubation, water miscible liquid scintillation fluid (6 mL of universol from ICN) was added, and the radioactivity was determined in a liquid scintillation counter.

Materials. [α-^{32}P]GTP and [^{35}S]GTPγS were purchased from ICN (Irvine, CA). [^{32}P]Azidoanilido GTP (AAGTP) was synthesized as described in Rasenick et al. (1994). All other nucleotides were from Boehringer Mannheim. Reagents used were of analytical grade. Bovine transducin (α) and transducin (αβγ) were provided by Drs. Yee Kin Ho and Heidi Hamm (UIC), respectively. The polyclonal anti-tubulin

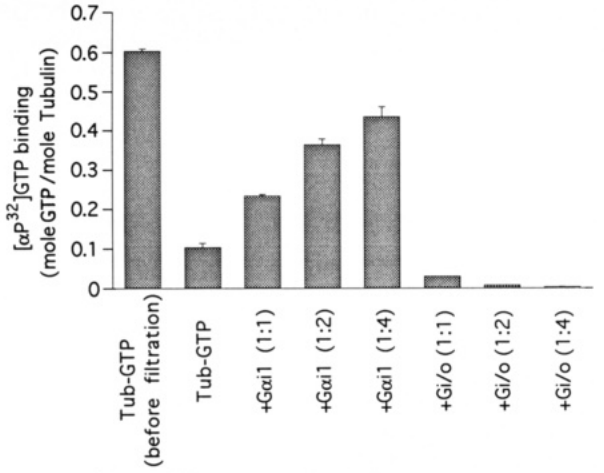

FIGURE 1: $G_{\alpha i1}$ stabilizes nucleotide binding when incubated with tubulin–[α-^{32}P]GTP. Tubulin–[α-^{32}P]GTP (1 μM) was incubated with $G_{\alpha i1}$ at the indicated concentration for 20 min at 30 °C. The mixtures were then filtered through nitrocellulose as described in the text, and the radioactivity bound to the filters was determined. Lane 1 shows the [α-^{32}P]GTP bound to tubulin before nitrocelluse filtration and was made by incubating tubulin with 0.1 mM [α-^{32}P]GTP which was followed by separating free nucleotide from that bound to tubulin by gel filtration as described under Experimental Procedures. One of three triplicate experiments are shown (±SEM).

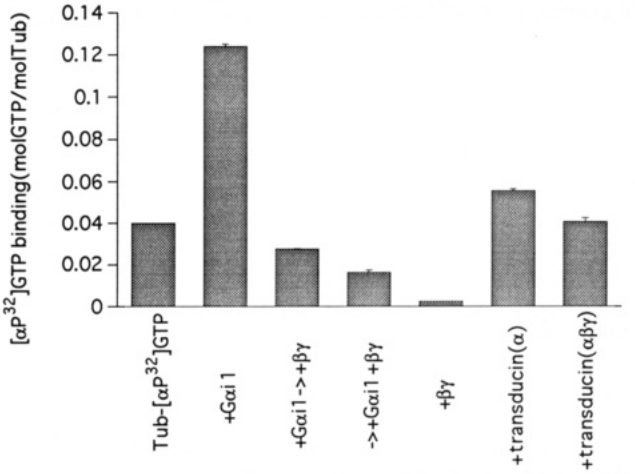

FIGURE 2: $G_{\beta\gamma}$ blocks the stabilization of nucleotide binding in presence of $G_{\alpha i1}$ through its association with tubulin. Tubulin–[α-^{32}P]GTP (0.6 μM) was incubated with or without $G_{\alpha i1}$ (1.5 μM), $G_{\beta\gamma}$ (0.8 μM), transducin α (1.5 μM), or transducin $\alpha\beta\gamma$ (0.78 μM) at 30 °C for 10 min followed by a postincubation with or without $\beta\gamma$ as indicated in the figure. In lane 4, tubulin–[α-^{32}P]GTP was added to $G_{\alpha i1}$ which was preincubated with $\beta\gamma$. [α-^{32}P]GTP binding was measured by filtration through nitrocellulose as in Figure 1. One of two similar experiments are shown.

antibody (code no. 65-095-1) was from ICN. Antibodies against $G_{i\alpha1,2}$ and $G_{\beta\gamma}$ were provided by D. Manning (Philadelphia), and a monoclonal antibody against bovine rod transducin (4A) was provided by H. Hamm. Antibodies were supplied and used as serum or ascites fluid. Anti-tubulin antibody titre was measured by ELISA and adjusted so that equal amounts of antitubulin were present (unless indicated otherwise).

RESULTS

Tubulin–$G_{\alpha i1}$ Interaction Stabilizes GTP Binding. In order to investigate nucleotide binding and hydrolysis events involved in tubulin–$G_{\alpha i1}$ complex formation, tubulin–[α-^{32}P]GTP (0.6 mol/mol tubulin) was incubated with the indicated concentrations of recombinant $G_{\alpha i1}$ or brain $G_{i/o}$ at 30 °C for 20 min (Figure 1). The reaction mixture was filtered through nitrocellulose membranes, and radioactivity bound to the protein was determined. A substantial amount (50–96% depending upon nucleotide species) of bound nucleotide is lost from tubulin during the filtration assay. However, the addition of $G_{\alpha i1}$ to tubulin–[α-^{32}P]GTP stabilized nucleotide binding in a concentration-dependent manner. A 4.2-fold increase in nucleotide binding was observed when tubulin–[α-^{32}P]GTP was incubated with $G_{\alpha i1}$ at a 1:4 molar ratio. This increase in nucleotide binding occurring when both tubulin and $G_{i\alpha}$ were present suggests a stabilization of nucleotide bound to tubulin or a partial transfer of nucleotide from tubulin to G_{α}. The later has been observed for tubulin–AAGTP (Roychowdhury et al., 1993). It is noteworthy that free [α-^{32}P]-AAGTP, added at the same concentrations as tubulin–[α-^{32}P]AAGTP to $G_{\alpha i1}$, did not bind to the later protein (Roychowdhury et al., 1993). Thus, it is unlikely that nucleotide released from tubulin is simply rebound by G_{α}.

$G_{\beta\gamma}$ Attenuates Stabilization of Nucleotide Binding to Tubulin. Purified $G_{i/o}$ did not effect a stabilization of GTP binding similar to that observed with $G_{i\alpha1}$. In fact, $G_{i/o}$ reduced the nucleotide binding to tubulin (Figure 1). Different preparations used for these experiments varied in the αi1/αi2/αo content. In most of the preparations used, αi/αo was about 1:1 with αi2/αi1 being about 2:1 (Wang & Rasenick, 1991). The $\beta\gamma$ subunits were present in roughly equimolar concentration with the α subunits. Despite the limited ability of αo or αi2 to bind tubulin, sufficient αi1 was present in these preparations to modify GTP binding in a manner similar to that seen for the recombint αi1. This raised the possibility that $\beta\gamma$ might be having an independent effect. Addition of purified $G_{\beta\gamma}$ diminished the GTP binding to tubulin (by 94%), consistent with such a direct interaction of $\beta\gamma$ with tubulin. Furthermore, postincubation of the tubulin–$G_{\alpha i1}$ complex with $\beta\gamma$ was able to override the stabilizing effect of $G_{\alpha i1}$ (Figure 2). This result suggests that there may be distinct binding sites for $G_{\alpha i1}$ and $G_{\beta\gamma}$ on tubulin. This also suggests that the reduction in nucleotide binding in presence of $G_{i/o}$ is due to the presence of $\beta\gamma$ rather than α. The α subunit of the retinal G protein transducin, which displayed a much lower affinity for tubulin and was not a recipient for nucleotide transfer from tubulin, did not evoke a similar stabilization of GTP binding (Figure 2). Furthermore, the transducin heterotrimer ($\alpha\beta\gamma$) did not affect nucleotide binding to tubulin, suggesting that bovine transducin $\beta\gamma$ does not interact with tubulin in a manner similar to bovine brain $G_{\beta\gamma}$. Several species of β and γ have been shown to exist (Simon et al., 1991), and they appear to show preferences for various forms of G as well as for specific receptors (Kleuss et al., 1992, 1993; Simon et al., 1991). Therefore it appears that tubulin dimer not only shows differential affinity for different G proteins, but it can distinguish among $\beta\gamma$ subunits derived from those G proteins.

Activation of GTPase in the Tubulin–$G_{\alpha i1}$ Complex. Since previous studies from our laboratory suggest that dimeric tubulin binds to α subunits of G protein at the sites where tubulin dimers bind to each other during microtubule formation (Wang & Rasenick, 1991), the possibility that tubulin–$G_{\alpha i1}$ complex formation displayed other characteristic behaviors of microtubule assembly was investigated. During microtubule assembly, association of tubulin dimers activates a GTPase, and GTP bound to the exchangeable binding site of tubulin is hydrolyzed to GDP, which then becomes nonexchangeable in the microtubule (Carlier et al., 1989; Margolis & Wilson, 1979). Thin layer chromatography (TLC) was used to determine the nucleotide bound to the protein. As shown in Figure 3, 72% of bound GTP was hydrolyzed to GDP in the

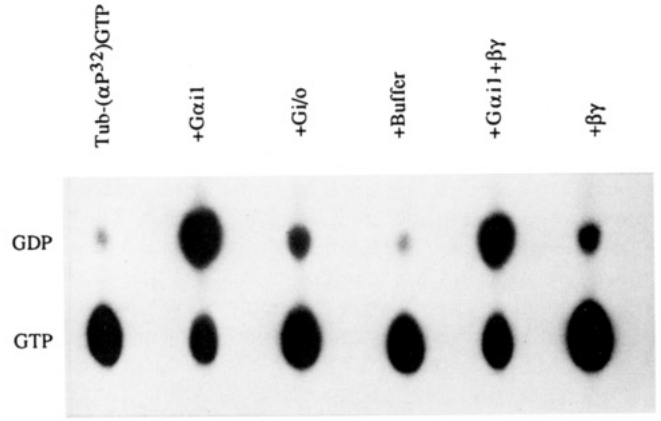

FIGURE 3: Tubulin–$G_{\alpha i1}$ interaction activates GTPase while $G\beta\gamma$ appears to inhibit GTP hydrolysis. Tubulin–[α-^{32}P]GTP (1.0 μM) was incubated with (lane 2) or without (lane 1) $G_{\alpha i1}$ (2.5 μM), $G_{i/o}$ (2 μM, lane 3), $G_{\alpha i1}$ + 0.8 μM $\beta\gamma$ (lane 5), 0.8 μM $\beta\gamma$ (lane 6), or Hepes buffer (lane 4) as indicated, for 20 min at 30 °C. The samples were then treated with 1% SDS and spotted onto a PEI cellulose plate. TLC was performed as described under Experimental Procedures. The autoradiogram of the plate is shown in the figure. One of two similar experiments are shown.

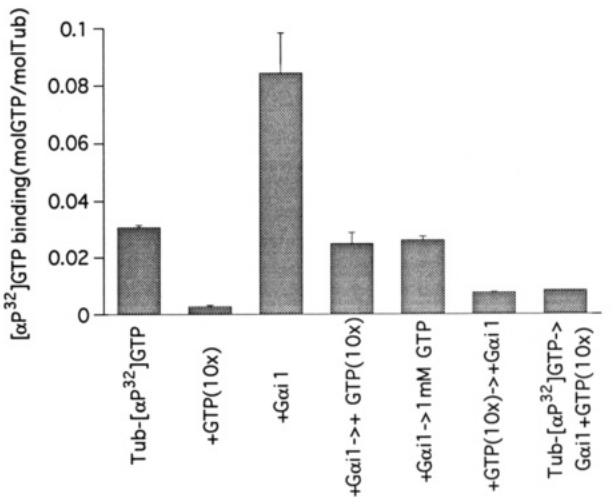

FIGURE 4: 30–50% of bound nucleotide is nonexchangeable in tubulin–$G_{\alpha i1}$ complex. Tubulin–[α-^{32}P]GTP (0.75 μM, 0.7 mol of GTP/mol of tubulin) was incubated with (lanes 3, 4, and 5) or without (lane 1) $G_{\alpha i1}$ (1.8 μM) or a 10-fold excess of free GTP (lanes 2 and 6) at 30 °C for 20 min, followed by a second incubation with a 10-fold excess of GTP (lane 4), 1 mM GTP (lane 5), or $G_{\alpha i1}$ (lane 6) as indicated in the figure. In lane 7, $G_{\alpha i1}$ was added to tubulin–[α-^{32}P]GTP after incubation with a 10-fold excess of free GTP. [α-^{32}P]-GTP binding was measured as in Figures 1 and 2. While the nucleotide in environment does displace some GTP from the tubulin–$G_{\alpha i1}$ complex (50–70%), significantly more is retained than could be accounted by exchangeable binding.

complex. The degree of hydrolysis varied from 60% to 80% (n = 5). In the absence of $G_{\alpha i1}$ only 8–10% of bound GTP in tubulin was hydrolyzed to GDP. This result suggests that the tubulin-$G_{\alpha i1}$ interaction indeed activates a GTPase. Purified $G_{i/o}$ or $G_{\beta\gamma}$ did not induce any significant hydrolysis of the GTP bound to tubulin. In the absence of tubulin, $G_{\alpha i1}$ does not hydrolyze GTP in solution because, under the conditions of the assay, it does not bind free GTP.

Partial Nonexchangeability of Nucleotide Bound to the Tubulin–$G_{\alpha i1}$ Complex. Formation of a tubulin–$G_{\alpha i1}$ complex not only stabilized nucleotide binding and promoted GTP hydrolysis, but ~40% of the bound nucleotide in the complex was rendered nonexchangeable. [α-^{32}P]GTP bound to tubulin was displaced almost completely by a 10-fold excess of free GTP (Figure 4, lane 2) but only by 60% when tubulin was bound to $G_{\alpha i1}$ (compare Figure 4, lanes 2 and 4). The residual nucleotide stayed bound to the complex even in the presence of 1 mM GTP (compare Figure 4, lanes 2, 4, and 5). This result cannot be explained by the release of GTP from tubulin and subsequent binding of that nucleotide to $G_{i\alpha 1}$, because [α-^{32}P]GTP bound to tubulin can be chased almost completely by a 10-fold excess of free GTP under the same experimental condition. Nucleotide binding reached only 30% of the above level when tubulin–GTP was first exposed to a 10-fold excess of GTP before incubation with $G_{\alpha i1}$ (compare Figure 4 lane 6 with lanes 4 or 5). The GTP binding observed in lane 6 is likely due to [α-^{32}P]GTP in the medium binding to $G_{\alpha i1}$ and tubulin, since a similar low level of nucleotide binding is observed when $G_{\alpha i1}$, preabsorbed with a 10-fold excess of GTP, was added to tubulin–GTP (Figure 4, lane 7).

$G_{\alpha i1}$ Induces Similar Stabilization of GTPγS or GDP Binding by Tubulin. The nucleotide specificity required for tubulin–$G_{\alpha i1}$ induced stabilization of nucleotide binding was tested by comparing tubulin binding of the nonhydrolyzable GTP analog GTPγS with the binding of GDP. The ability of $G_{\alpha i1}$ to stabilize the nucleotide binding in the tubulin–$G_{\alpha i1}$ complex was comparable for both these nucleotdes (Figure 5). GTPγS binding to tubulin was very unstable, since about 96% of binding was lost when the sample was filtered through nitrocellulose. However, when tubulin–GTPγS was incubated in presence of $G_{\alpha i1}$, up to 90% of this binding was protected. Similarly, when tubulin–GDP was incubated in presence of $G_{\alpha i1}$ (1:2 molar ratio), an approximate 7-fold increase in nucleotide binding was observed. This result suggests that $G_{\alpha i1}$ induces a similar stabilizing effect on nucleotide binding, regardless of whether tubulin is in the GTP or GDP bound form.

DISCUSSION

The results presented here suggest that formation of a complex between tubulin and $G_{\alpha i1}$ changes the GTP-binding characteristics of both molecules. This interaction between tubulin and $G_{i\alpha}$ resembles that seen among tubulin dimers in the formation of microtubules. Previous studies from this laboratory have suggested that tubulin dimers in synaptic membrane may associate with α subunits of the specific G proteins and activate those G proteins by transferring GTP from the exchangeable GTP-binding site of tubulin to the α subunit of those G proteins (Roychowdhury et al., 1993; Wang & Rasenick, 1991; Wang et al., 1990). In reconstitution studies using tubulin–AAGTP, 25% of the AAGTP which had been bound to tubulin was transferred to $G_{\alpha i1}$ after tubulin–AAGTP was allowed to form complexes with $G_{\alpha i1}$ (Roychowdhury et al., 1993). Recently, in an attempt to determine the efficiency of the transfer process, it was observed that the transfer of AAGTP from tubulin to G protein varies with the G protein preparation. With some preparations of recombinant $G_{\alpha i1}$, AAGTP became equally distributed between $G_{\alpha i1}$ and tubulin at a 1:1 molar ratio of tubulin–AAGTP and G_{α} (S. Roychowdhury and M. M. Rasenick, unpublished observations).

In the present study, it has been shown that the interaction between tubulin and $G_{\alpha i1}$ is also associated with the stabilization of nucleotide binding. Filtration over nitrocellulose removes 60–80% of the GTP from tubulin. Addition of $G_{\alpha i1}$ protects this in a dose-dependent fashion. This increase in nucleotide binding in the presence of $G_{\alpha i1}$ is not due to release and rebinding of nucleotide since [α-^{32}P]GTP released from tubulin–[α-^{32}P]GTP after preincubation with a 10-fold excess of free GTP (which releases bound GTP completely) only

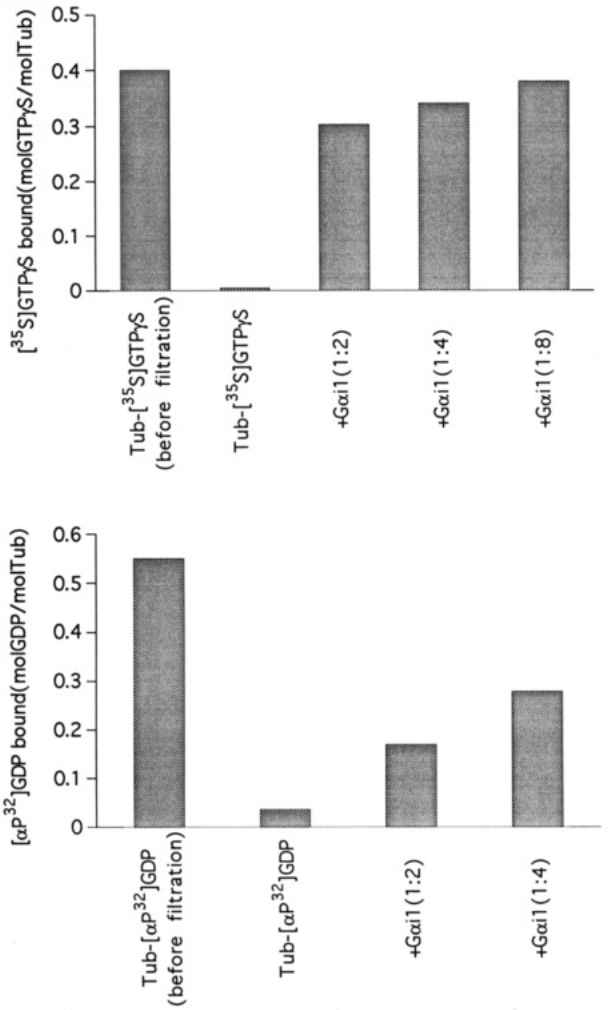

FIGURE 5: $G_{\alpha i1}$ induces similar stabilization of GTPγS or GDP binding in tubulin. (A, top) Tubulin–[^{35}S]GTPγS (1 μM) or (B, bottom) tubulin–[α-^{32}P]GDP (1 μM) was incubated with or without $G_{\alpha i1}$ at the indicated ratio at 30 °C for 20 min. The samples were then filtered through nitrocellulose, and nucleotide binding was determined as before. Lane 1 in both panels A and B indicates the amounts of [^{35}S]GTPγS or [α-^{32}P]GDP bound to tubulin before filtration.

binds $G_{\alpha i1}$ to 25% of the value observed for preformed tubulin–$G_{\alpha i}$ complex (compare lanes 4, 5, and 6 in Figure 4). Interaction of tubulin with heterotrimeric G protein did not induce a similar stabilization, rather it destabilized the nucleotide binding. Experiments using purified βγ showed that βγ itself can destabilize the GTP binding to tubulin. G_α expressed in *E. coli* binds $G_{\beta\gamma}$ rather poorly, while βγ does not disturb G_α binding to tubulin (in fact, heterotrimeric G_i binds to tubulin well) (Wang et al., 1990). It is possible that βγ has some effect on the process of nucleotide transfer through its interaction with tubulin. This raises the possibility that the functional interaction between tubulin and $G_{\alpha i1}$ in synaptic membrane is regulated by βγ subunits.

Previous results from this laboratory have suggested that the tubulin–G protein interaction sites may involve microtubule polymerization domains. The ability of tubulin to bind to G_α is decreased upon tubulin polymer formation, and binding of G_α to tubulin decreases tubulin polymerization (Wang & Rasenick, 1991). A significant functional similarity and amino acid sequence homology appear to exist between tubulin and signal-transducing G proteins (Linse & Mandelkow, 1988; Sternlicht et al., 1987; Yan & Rasenick, 1990). Tubulin is also a GTP-binding protein. It has two GTP (guanine nucleotide)-binding sites per dimer, one nonexchangeable at the α subunit, and the other exchangeable at the β subunit. Tubulin dimers can undergo polymerization and depolymerization in a Mg^{2+}- and temperature-dependent manner. Unlike the "conventional" G proteins, where GTP appears to facilitate the separation of the heterodimer, the presence of GTP enhances the polymerization process, and hydrolysis of GTP to GDP by what appears to be a tubulin intrinsic GTPase occurs subsequent to microtubule polymerization. The bound GDP becomes nonexchangeable in the polymer; however, the precise relationship between polymerization and GTP hydrolysis is not yet clear (Carlier & Pantaloni, 1981; Weisenberg et al., 1968). As with the "conventional" G proteins, tubulin is also a substrate for pertussis and cholera toxin catalyzed ADP-ribosylation (Amir-Zaltsman et al., 1982; Lim et al., 1985). Such similarities evoke the possibility that tubulin, which is associated, perhaps intrinsically, with synaptic membranes (Stephens, 1986; Zisapel et al., 1980), may interact with other synaptic membrane G proteins, including G_s and G_i. This might create an interdependence between synaptic shape and response to neurotransmitters. Recently, it has been demonstrated that, in rat cerebral cortex, tubulin and $G_{\alpha s}$ or $G_{\alpha i1}$ exist in preformed complexes (Cohen et al., 1993). Perhaps this interaction is related to the absence of microtubules associated with synaptic membrane despite the abundance of tubulin there. Sustained neural transmission has been reported to modify synaptic form in Aplysia (Schacher et al., 1993) as well as the formation of dendritic spines in the hippocampus (Lisman & Harris, 1993).

Indeed, tubulin–G protein interaction displayed several characteristic behaviors of microtubule assembly, i.e., activation of GTPase and partial nonexchangeability of nucleotide bound to the complex. The ability of $G_{\alpha i1}$ to interact with tubulin in a fashion similar to the formation of tubulin polymer indicates a possibility that tubulin and $G_{\alpha i1}$ in synaptic membrane can exist as polymer-like structure as suggested by Nakamura and Rodbell (1990) and Lisman and Harris (1993). Absent this, previous data from these labs have suggested that at least two G_α subunits form dimers and can transfer GTP among themselves (Hatta et al., 1986). Perhaps "tubulin-like" regions of G_α subunits participate in these processes.

Although the primary structure of α and β tubulin was determined a decade ago (Valenzuela et al., 1981), the three-dimensional structure of the molecule, as well as the functional domains involved in the binding to other tubulin dimers in the formation of microtubules, is largely unknown. The initial efforts from our laboratory to characterize the tubulin–$G_{\alpha i1}$ interacting domain suggest that the carboxy-terminal domain of tubulin dimer is not crucial for its interaction with $G_{\alpha i1}$ (Wang & Rasenick, 1991).

Transfer of GTP from tubulin to G_α represents the mechanism by which G_α becomes activated by tubulin. The idea of a transfer of nucleotide between proteins is not new and has been studied extensively in a system of mitrocondrial hydrolases, which appear to transfer NAD without ever releasing it to the medium (Srivastava & Bernhard, 1986). Perhaps the nucleotide transfer from tubulin to $G_{\alpha i1}$ allows the both proteins in the complex to attain a conformation where the nucleotide-binding sites are more protected and the bound GTP is retained, sustaining the activation of $G_{i\alpha}$ or the tubulin–G_α complex. Further, tubulin appears to activate G_α via transfer of GTP under conditions where that G_α does not bind GTP available in the medium (Roychowdhury et al., 1993). It is suggested that these new phenomena interact to allow for the interface between the cytoskeleton and G-protein-mediated signal transduction.

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