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G Protein Binding and G Protein Activation by Nucleotide Transfer Involve Distinct Domains on Tubulin: Regulation of Signal Transduction by Cytoskeletal Elements[†]

Sukla Roychowdhury, Nan Wang,[‡] and Mark M. Rasenick[*]

Department of Physiology and Biophysics and the Committee on Neuroscience, University of Illinois, College of Medicine, Chicago, Illinois 60612-7342

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ABSTRACT: Cytoskeletal proteins have been demonstrated to interact with elements which mediate neuronal signal transduction. This laboratory has shown that tubulin forms complexes with certain G proteins and transfers GTP to them, and such nucleotide transfer correlates well with the inhibition of adenylyl cyclase. A previous study showed that tubulin–G protein complex formation occurred at regions of tubulin which were likely to be involved in binding to other tubulin dimers during the process of microtubule polymerization [Wang, N., & Rasenick, M. M. (1991) *Biochemistry 30*, 10957–10965]. This study represents an attempt to investigate the regions of tubulin which orchestrate the transfer of GTP from tubulin to G protein. It is demonstrated that two monoclonal antibodies, with determinants in similar regions of α (DM1a) or β (DM1b) tubulin, blocked (by 70–80%) the ability of tubulin dimers (with GppNHp bound) to promote a stable inhibition of adenylyl cyclase. Under the same conditions, a polyclonal antitubulin antibody caused only a slight reduction in adenylyl cyclase inhibition. None of the antibodies altered the inhibition of adenylyl cyclase induced by GppNHp in the absence of tubulin. Under conditions where tubulin–GppNHp inhibited synaptic membrane adenylyl cyclase, tubulin dimers with the photoaffinity GTP analog azidoanilido-GTP (^{32}P-AAGTP) bound transferred that nucleotide to $G_{\alpha i1}$ on the membrane. DM1a and DM1b blocked this nucleotide transfer. Similarly, tubulin–AAGTP transferred AAGTP to purified G proteins in solution and DM1a and DM1b blocked this process as well. Despite their ability to block the activation of G_{i1} by tubulin, neither antibody altered the ability of tubulin to bind to native $G_{i\alpha}$ affixed to nitrocellulose. These results suggest that multiple domains on tubulin are required for interactions with G protein, and the domains which are required for binding are distinct from those required for the transfer of nucleotide. Dimeric tubulin may activate specific synaptic membrane G proteins and, in doing so, might link elements of the cytoskeleton to the signal transduction process.

Guanine nucleotide-binding regulatory proteins (G proteins) mediate signal transduction across cell membranes for a variety of hormones or neurotransmitters. Modulation of G protein activation by various second messengers could represent an important way in which cells regulate hormone or neurotransmitter response or responsiveness. Generally, a G protein becomes activated in response to agonist occupancy of its cognate receptor. Nonetheless, several points of interaction exist among neurotransmitters. These interactions may involve phosphorylation of a receptor or G protein by a kinase activated through an alternate second messenger to that evoked by a given receptor–effector combination. Alternately, a protein, such as calmodulin, activated by the rise in Ca^{2+} elicited by a given neurotransmitter, could increase adenylyl cyclase directly (Tang et al., 1990; Choi et al., 1992) or in conjunction with a G protein (Harrison et al., 1989).

It has been suggested that, in neuronal systems, one locus of neurotransmitter interaction might be found in the association between cytoskeletal components and G proteins which modulate synaptic membrane adenylyl cyclase. Specifically, it has been observed that this enzyme is regulated by tubulin as a result of interactions between tubulin and $G_{s\alpha}$ or $G_{i\alpha1}$ proteins (Rasenick & Wang, 1988; Wang et al., 1990).

Brief incubation of tubulin–Gpp(NH)p[1] with synaptic membranes induced an inhibition of adenylyl cyclase which persisted after membrane washing (Rasenick & Wang, 1988). In these experiments, a hydrolysis-resistant GTP analog, bound to the exchangeable site on β tubulin, was required for the observed inhibition of adenylyl cyclase. Tubulin–Gpp(NH)p, however, had no effect on the partially purified adenylyl cyclase catalytic moiety, implying that tubulin does not substitute for G proteins. Further experiments with tubulin liganded with AAGTP (an hydrolysis-resistant photoaffinity GTP analog) suggested that a direct nucleotide transfer between tubulin and G_i represented the mechanism for inhibition of adenylyl cyclase by exogenous tubulin (Rasenick & Wang, 1988). Tubulin binds specifically (K_d = 130 nM) to $G_{\alpha s}$ and $G_{\alpha i1}$ or their heterotrimers but with a much lower affinity to all other

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[*] Address correspondence to this author 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: U20133@UICVM.Bitnet.

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

[1] Abbreviations: PC-tubulin, tubulin deprived of MAPs with phosphocellulose chromatography; tubulin–Gpp(NH)p, tubulin liganded with Gpp(NH)p; tubulin–AAGTP, tubulin liganded with AAGTP; 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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G proteins (Wang et al., 1990). It is suggested that, subsequent to the formation of a complex between tubulin and G protein, GTP is transferred from the former species to the later.

In order to understand tubulin–G protein interaction further, it is necessary to determine the articulating facets of these molecules. We have demonstrated that the regions of tubulin normally thought to bind other proteins [i.e., microtubule associated proteins (MAPs)] are not those involved in the association with G proteins. Rather, it appears that the sites on tubulin which bind G protein are similar or identical to those regions to which other tubulin dimers bind during the formation of microtubules (Wang & Rasenick, 1991).

Monoclonal antibodies with defined specificities have proven useful in the study of structural and functional domains of tubulin and the polymers it forms. These antibodies have been used to study regions of tubulin involved in microtubule assembly (Arevalo et al., 1990) as well as the sites where α and β tubulin interact in the formation of a dimer (de la Vina et al., 1988).

In this study, we attempted, initially, to localize the binding site on tubulin for the $G_{s\alpha}$ or $G_{i\alpha 1}$ subunit. To do this, we employed two well-defined monoclonal anti-tubulin antibodies, DM1a and DM1b (Blose et al., 1984). Surprisingly, we have found that these two antibodies could discriminate the nucleotide transfer process between tubulin and G_α from the physical interactions of these two proteins. Thus, a region near the COOH terminal of tubulin is operant in the transfer of nucleotide from that species to the α subunit of G_{i1} or G_s.

EXPERIMENTAL PROCEDURES

Tissue Preparations. Synaptic membrane-enriched fractions were prepared from 21-day-old male Sprague-Dawley rats as described (Rasenick & 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 supernatant of 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 100000*g*. The microtubule pellet was resuspended in the above buffer and depolymerized on ice. Nucleotides were removed from tubulin by charcoal treatment. A second polymerization step was performed with GppNHp, AAGTP, [^{32}P]AAGTP, or GTP (150 mM). This allowed a 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 contains microtubule-associated proteins (MAPs). These MAPs were removed by phosphocellulose chromatography with the eluting buffer of 100 mM Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgCl_2$. The resulting preparations (PC–tubulin) were greater than 97% tubulin as estimated by Commassie Blue staining. Tubulin preparations were stored under liquid nitrogen and used in less than 4 weeks. Prior to use tubulin–GppNHp or tubulin–AAGTP was desalted twice on a P6-DG column. After desalting, 0.4-0.6 mol of AAGTP or GppNHp were bound/mol of tubulin (Rasenick & Wang, 1988). The binding of nucleotide is entirely on the β subunit (Rasenick & Wang, 1988).

Purified PC–tubulin was iodinated as described by Wang et al. (1990). The iodinated tubulin (1800 Ci/mol, 1.04 mol iodine/mol tubulin) was 97% polymerization-competent relative to the control tubulin (non-iodinated). When compared with native tubulin on dot blots, the iodinated tubulin had a similar reactivity with DM1a, DM1b, and polyclonal anti-tubulin antibody.

Protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

Adenylyl Cyclase Assay. Synaptic membranes were thawed and resuspended at a protein concentration of ~2 mg/mL 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 or without indicated protein (tubulin or IgG) or nucleotide at 23 °C for 3 min or as noted. 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 assayed for adenylyl cyclase as described previously (Hatta et al., 1986). [^{3}H]cAMP formed was determined by the method of Salomon (1979).

Photoaffinity Labeling and Nucleotide Transfer. [^{32}P]AAGTP as well as AAGTP were synthesized by the method of Pfeuffer (1977). Synaptic membranes were washed and resuspended in 2 mM HEPES, pH 7.4, and 1 mM $MgCl_2$. Membrane suspensions (50 μg of protein in a final volume of 50 mL) were incubated with indicated concentrations of tubulin–[^{32}P]AAGTP for 3 min at 23 °C, and the reaction was terminated by dilution with 1 mL of the above buffer followed by centrifugation at 20000*g* for 10 min to remove unbound [^{32}P]AAGTP. Membranes were washed a second time and resuspended in the same buffer. The membrane suspensions were then UV irradiated for 5 min with a Spectroline UV lamp (254 nm, 9 W) on ice at a distance of 4 cm.

For nucleotide transfer studies between purified proteins, tubulin–[^{32}P]AAGTP (30 μg) was incubated with antibodies (1:2 ratio as determined by ELISA), control serum, or IgG at 30 °C for 20 min followed by another 20 min with the indicated G protein (the final ratio of tubulin–[^{32}P]AAGTP to G_α was 1:1). Membranes were UV irradiated on ice for 3 min.

Following UV irradiation (membranes or soluble proteins), reactions were quenched with ice-cold 2 mM HEPES, pH 7.4, 1 mM $MgCl_2$, and 4 mM DTT, followed by centrifugation at 20000*g* for 10 min. Membrane pellets were dissolved in 3% SDS Laemmli sample buffer with 50 mM DTT. Samples were heated for 4 min at 60 °C and electrophoresed in 10% SDS/polyacrylamide gels (unless otherwise noted) by the procedure of Laemmli (1970). After electrophoresis, gels were stained with Commassie Blue, dried, and radioautographed with Kodak XAR-5 film. AAGTP labeling was quantified by densitometric analysis of the autoradiogram (for synaptic membranes) or by radioactivity analysis on a Betagen blot analyzer (for purified G protiens).

G Proteins. G proteins were purified from porcine brain (Wang & Rasenick, 1991). Alternately, $G_{i1\alpha}$ was purified from *Escherichia coli* expressing the recombinant gene for that protein (supplied by Maurine Linder). *E. coli* were grown and harvested as described (Linder et al., 1990). Protein was purified by the method of Linder et al. (1990) except that DEAE-Sephacel was substututed for Mono Q and phenyl-Sepharose was substituted for heptylamine-Sepharose.

Immunoblotting and Tubulin Overlays. [^{125}I]Tubulin binding to $G_{i\alpha}$ immobilized on nitrocellulose was performed as described in Wang et al. (1990). Transfer to nitrocellulose and detection with anti-G protein antibody [antiserum 8730, provided by D. Manning-Philadelphia and used at a dilution of 1:500 (Carlson et al., 1989)] was performed as described in Wang et al. (1990). Gels used for immunoblotting were 10% polyacrylamide and 0.133% bisacrylamide and contained 6 M urea.

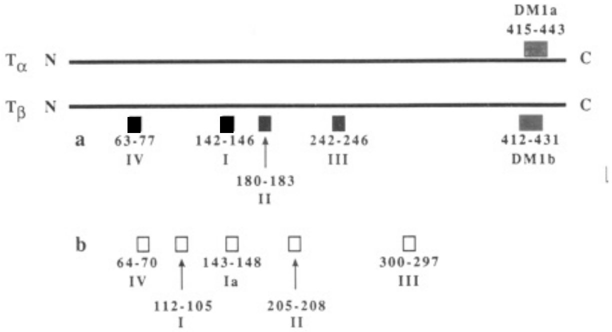

FIGURE 1: Proposed sequences of GTP/GDP-binding domains and antibody binding sites on tubulin: GTP-binding sequences proposed by (a) Linse and Mandelkow (1988) and (b) Sternlicht et al. (1987) from sequence homology with other nucleotide binding protein (Halliday, 1984) or limited proteolysis and pepide sequencing. The DMIa and DMIb binding sites on tubulin were suggested according to de la Vina et al. (1988) from limited proteolysis and peptide mapping.

Materials. [α-^{32}P]ATP (800 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]GTP were purchased from ICN (Irvine, CA). All nucleotides were from Boehringer Mannheim. *p*-Azidoaniline was synthesized by Dr. William Dunn (UIC). All other reagents used were of analytical grade. The monoclonal anti-tubulin antibodies, DM1a and DM1b, were from Amersham (Arlington Heights, IL). The polyclonal anti-tubulin antibody (code no. 65-095-1) was from ICN. Antibodies against $G_{i\alpha}$ were provided by D. Manning (Philadelphia), and an antibody against bovine rod transducin was provided by H. Hamm (UIC). 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 anti-tubulin were present (unless indicated otherwise).

RESULTS

Antibody and GTP-Binding Domains on Tubulin. Figure 1 illustrates consensus sequences for GTP binding on β tubulin as well as the putative domains for antibody binding. GTP-binding domains were proposed by Linse and Mandelkow (1988) or Sternlicht et al. (1987). Regions labeled I–IV are deduced from sequences proposed by Halliday (1984) to represent the GTP-binding domain. In the Sternlicht model, regions I and III gain their homology to consensus GTP-binding sequences to inversion of the amino acid sequence. Region Ia of the Sternlicht model corresponds to region I of the Mandelkow model. Regions proposed to represent the epitopes of DM1a and DM1b were determined by peptide mapping studies.

DM1a and DM1b Antibodies Block Inhibition of Adenylyl Cyclase by Tubulin–GppNHp. Incubation of tubulin–Gpp(NH)p with synaptic membranes induced a ~50% inhibition of basal adenylyl cyclase which persisted subsequent to membrane washing (Rasenick & Wang, 1988; Figure 2). To further explore the G protein interaction domains of tubulin and the mechanism whereby nucleotide is transferred from tubulin to G protein, two monoclonal anti-tubulin antibodies, DM1a (anti-α tubulin) and DM1b (anti-β tubulin), were employed. Limited proteolysis and synthetic peptide mapping (de la Vina et al., 1988) have been used to suggest that the amino acid residues composing the epitopes are α(415–443) for DM1a and β(412–431) for DM1b (Figure 1). When preincubated in a 2:1 protein ratio with tubulin–Gpp(NH)p, both DM1a and DM1b were capable of blocking the inhibitory effect of tubulin–GppNHp upon adenylyl cyclase (Figure 2). When 1 μM tubulin–Gpp(NH)p was incubated with the membranes, maximum adenylyl cyclase inhibition was acheived. This was reduced 71% by DM1b and 78% by DM1a when those antibodies were preincubated with the tubulin–Gpp(NH)p. The polyclonal anti-tubulin antibody, used at the same concentration, was much less efficient and reduced the inhibition by only 20% (Figure 2). Tubulin–GppNHp inhibited adenylyl cyclase with an approximate IC50 between 3 and 4 × 10^{-8} M regardless of antibody used. The effect of these monoclonal anti-tubulin antibodies was specific for blocking tubulin-mediated inhibition of adenylyl cyclase. Gpp(NH)p, when incubated with synaptic membranes, could induce adenylyl cyclase inhibition with an efficacy and potency similar to that of tubulin–Gpp(NH)p (Rasenick & Wang, 1988). DM1a and DM1b, however, did not affect Gpp(NH)p-induced inhibition of adenylyl cyclase (Table I). Furthermore, they had no direct effect on adenylyl cyclase (Table I). A monoclonal antibody against bovine rod transducin which is capable of binding to other G proteins (Hamm et al., 1989) did not block the tubulin–GppNHp-induced inhibition of adenylyl cyclase when preincubated with tubulin.

DM1a and DM1b Block Transfer of Nucleotides from Tubulin to Synaptic Membrane G_i. While physical interaction between tubulin and G_i is required to effect the inhibition of adenylyl cyclase, the mechanism for this inhibition requires nucleotide transfer from tubulin to $G_{\alpha i}$ (Rasenick & Wang, 1988; Wang & Rasenick, 1991). Thus, the effect of the

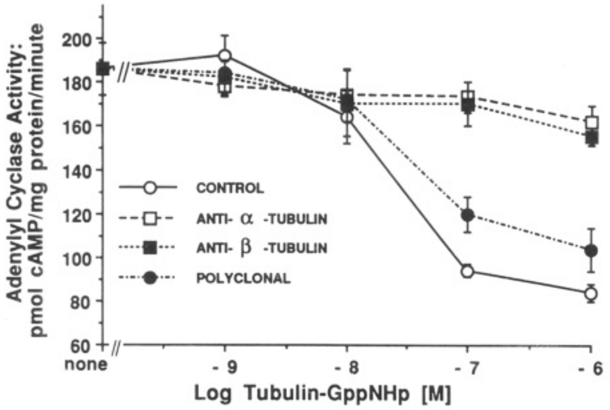

FIGURE 2: Effect of DMIa and DMIb on tubulin–GppNHp-mediated inhibition of adenylyl cyclase. Tubulin–GppNHp was incubated with the indicated antibodies in a 1:2 molar ratio at 23 °C for 30 min, followed by a 10-min incubation on ice. These mixtures were then incubated at the indicated concentrations with synaptic membrane (80 μg) and washed, and the adenylyl cyclase assay was performed as described under Experimental Procedures. One of three similar experiments (±SEM) is shown.

Table I: Effects of Antibodies on Persistent Inhibition of Adenylyl Cyclase in Synaptic Membrane Induced by GppNHp or Tubulin–GppNHp

preincubation antibodies	addition to preincubation		
	none	GppNHp	tubulin–GppNHp
IgG	107 ± 1[a]	50 ± 0.5	58 ± 0.5
DM1A	102 ± 0.5	54 ± 0.5	108 ± 1.0
DM1B	102 ± 0.5	56 ± 0.5	110 ± 1.0

[a] Picomoles of cAMP/(mg of protein·min) ± SEM. GppNHp or tubulin–GppNHp was preincubated with antibodies at a 1:2 molar ratio. The experiment was performed as described in the legend to Figure 2. Ovalbumin + GppNHp was used to control for tubulin–GppNHp in the preincubation with membrane. Data are from one of three similar experiments (triplicates ± SEM). Antibodies were preincubated with tubulin–GppNHp (10 μM), GppNHp (10 μM) plus ovalbumin (10 μM), or ovalbumin alone (none) as described in the legend to the Figure 1. These mixtures were incubated with synaptic membranes and washed, and adenylyl cyclase was assayed as described under Experimental Procedures.

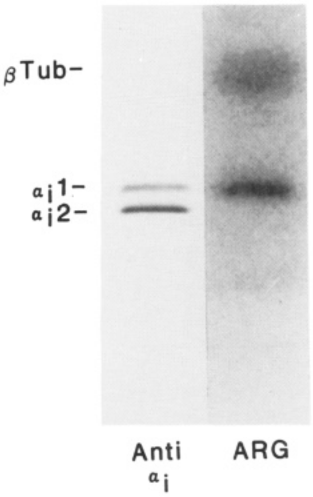

FIGURE 3: Transfer of AAGTP from tubulin to $G_{i\alpha}$ on synaptic membranes. Synaptic membrane (80 μg) was incubated with 15 μg of tubulin–AAGTP at 23 °C for 3 min. After UV irradiation and SDS gel electrophoresis, proteins were transferred to nitrocellulose membrane. Nitrocellulose was probed with anti-$G_{i\alpha}$ (common) (no. 8730 from D. Manning), and the immune complex was detected with alkaline phosphatase. The autoradiograph corresponding to the blot is on the right. One of two similar experiments is shown.

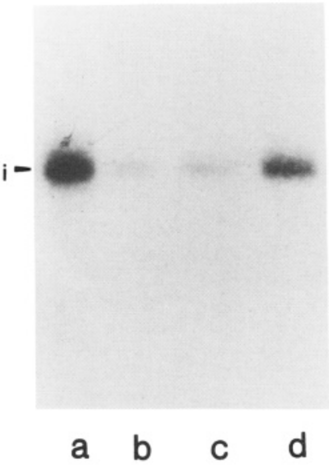

FIGURE 4: Effect of DM1a and DM1b on nucleotide exchange from tubulin to G_i in synaptic membrane. Synaptic membrane (80 μg of protein) was incubated with (a) 15 μg of [^{32}P]AAGTP–tubulin or tubulin preincubated with the following antibodies in a 1:2 molar ratio, (b) DM1a, (c) DM1b, and (d) polyclonal tubulin antibody, for 3 min at 23 °C. Membranes were then washed, UV-irradiated, subjected to SDS-PAGE, and autoradiographed as described under Experimental Procedures.

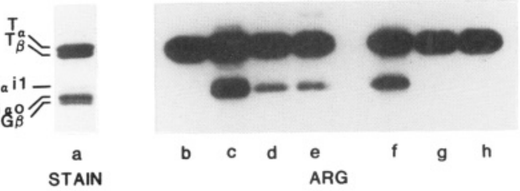

FIGURE 5: In vitro transfer of [^{32}P]AAGTP from tubulin to $G_{i\alpha}$ and the role of tubulin specific antibodies on the transfer process. [^{32}P]AAGTP–tubulin (5 μM) was incubated with or without DM1A, DM1B, or nonspecific IgG in 1:2 protein ratio at 30 °C for 20 min and cooled on ice. $G_{i/o}$ (5 μM) was then added (lanes a–f) and incubated at 30 °C for another 20 min. All lanes contain tubulin–AAGTP. Other additions are (a) $G_{i/o}$, (b) none, (c) $G_{i/o}$, (d) DMIA+$G_{i/o}$, (e) DMIB + $G_{i/o}$, (f) IgG + $G_{i/o}$, (f)DMIa, and (g) DMIb. Samples were then UV-irradiated as described under Experimental Procedures and subjected to SDS–PAGE and autoradiography. Lane a is Coomassie Blue staining, and lanes b–h are an autoradiogram of the samples.

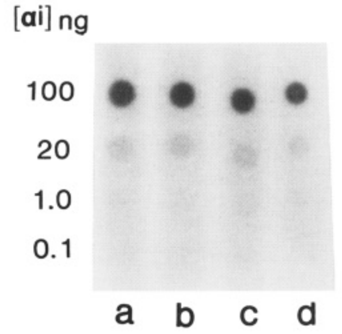

FIGURE 6: Effect of DM1A and DM1B on binding of tubulin to $G_{\alpha i}$. [^{125}I]Tubulin was preincubated in 1:3 protein ratio with (a) nonspecific antibody, (b) DM1A, (c) DM1B, and (d) polyclonal anti-tubulin antibody for 30 min at 23 °C and overlayed on $G_{\alpha i}$ applied, in the indicated concentrations, to a nitrocellulose membrane. One of two similar experiments is shown.

monoclonal antibodies on nucleotide transfer was examined. Brief incubation of tubulin–[^{32}P]AAGTP with synaptic membranes under conditions similar to those used to effect stable inhibition of adenylyl cyclase, followed by membrane washing and photolysis, resulted in photoaffinity labeling of synaptic membrane $G_{i\alpha 1}$ (Figure 3). Consistent with the effects upon adenylyl cyclase, nucleotide transfer from tubulin to $G_{\alpha i}$ was dramatically reduced by both DM1a and DM1b antibodies (~92% reduction in photoaffinity labeling of $G_{\alpha i}$ by DM1a and an ~86% reduction by DM1b). The polyclonal anti-tubulin antibody reduced AAGTP transfer by ~49% (Figure 4).

DM1a and DM1b Block Transfer of Nucleotides from Tubulin to $G_{i\alpha 1}$ in a Resolved System. In order to establish that the effects of anti-tubulin antibody were direct, the ability of tubulin–AAGTP to transfer AAGTP to G_i was examined in a resolved system. We found that when tubulin–[^{32}P]AAGTP was incubated at equimolar concentrations with purified brain G protein (as well as recombinant $G_{\alpha i}$), about 25% (25.6 ± 4.3%; $n = 7$) of the AAGTP was transferred to G_{ai}. Free AAGTP, added at the same concentration as tubulin–AAGTP, did not bind to $G_{\alpha i1}$ under these experimental conditions. This is consistent with the hypothesis that tubulin complexes with G_α and transfers AAGTP directly. This nucleotide transfer was inhibited by 57.6 ± 9.7% ($n = 7$) when tubulin was preincubated with DM1a and by 71.7 ± 8.9% ($n = 7$) when tubulin–AAGTP was preincuabted with DM1b. DM1a and DM1b did not alter the AAGTP-binding properties of tubulin or $G_{i\alpha}$ (Figure 5).

DM1a and DM1b Do Not Affect the Binding of Tubulin to $G_{i\alpha}$. The results above demonstrated that antibodies DM1a and DM1b interfere with inhibition of adenylyl cyclase induced by tubulin–Gpp(NH)p, as well as the nucleotide transfer between tubulin and $G_{i\alpha}$. These effects could be the consequence of DM1a and DM1b blocking the interaction between tubulin and $G_{\alpha i}$ or blocking only the nucleotide transfer process. Specific binding of tubulin to G proteins can be assesed by immobilizing G proteins on nitrocellulose and incubating the nitrocellulose with [^{125}I]tubulin (Wang et al., 1990). A similar approach was used in this study to examine the effect of DM1a and DM1b on binding of [^{125}I]tubulin to G_i. Despite a 50% increase in protein ratio of antibody to tubulin used, as compared with the adenylyl cyclase and photoaffinity labeling assay, DM1a, DM1b, and the polyclonal anti-tubulin antibody did not significantly affect tubulin binding to $G_{\alpha i}$ (Figure 6).

DISCUSSION

This study was initiated to further define the sites on tubulin which are responsible for interacting with G_{i1} and G_s.

Although the extreme C terminal of α or β tubulin contains the sites involved in the binding of microtubule-associated proteins, these regions are not required for tubulin to bind to G proteins (Wang & Rasenick, 1991). In order to assess the importance of other sites on α or β tubulin, two well-defined monoclonal antibodies were employed. Despite certain concerns over the precise location and composition of the epitope, α(415–443) and β(412–431) seem to comprise, respectively, the major part of the epitopes for DM1a and DM1b (de la Vina et al., 1988; Breitling & Little, 1986). These regions were close to carboxyl terminus of α and β tubulin, and have implicated as regulatory domains in microtubule polymerization as well as interactions between tubulin and other microtubule-associated proteins such as MAP2 and tau (Littauer et al., 1986; Arevalo et al., 1990).

DM1a and DM1b decreased the inhibition of adenylyl cyclase by tubulin–Gpp(NH)p when preincubated with tubulin–Gpp(NH)p in a 2:1 protein ratio (Table I and Figure 2). Higher ratios of DM1a and DM1b to tubulin were avoided due to nonspecific interactions at high final concentrations of antibody (Arevalo et al., 1990). Consistent with these results, the nucleotide transfer from tubulin to $G_{i\alpha1}$ was also blocked by DM1a and DM1b (Figure 4). The polyclonal anti-tubulin antibody could inhibit to some extent both the inhibition of adenylyl cyclase by tubulin–Gpp(NH)p and nucleotide transfer (Figures 2 and 4). These effects could be caused by certain subpopulations of anti-tubulin antibody which have epitopes simial or identical to those of DM1a or DM1b. It is also possible that the epitopes for the polyclonal anti-tubulin antibody are located in other regions on tubulin with which G proteins interact (vide infra).

It is noteworthy that transfer of AAGTP from tubulin–AAGTP to G_i (on membranes) was blocked to a larger extent by the polyclonal antibody than was adenylyl cyclase inhibition by tubulin–GppNHp. This is not due to differences in AAGTP vs GppNHp, as nonradioactive (unilluminated) tubulin–AAGTP inhibits adenylyl cyclase with the same potency and efficacy as tubulin–GppNHp (Rasenick & Wang, 1988). Further, DM1a and DM1b effects on adenylyl cyclase were similar to those on transfer of AAGTP between tubulin and $G_{i1\alpha}$. It is likely, however, that G_{i1} is involved in several processes in addition to adenylyl cyclase inhibition (Simon et al., 1991). Thus, one might expect an inequity in the $G_{i\alpha}$ acting as recipients of AAGTP and those affecting adenylyl cyclase inhibition. This hypothesis is supported by the finding that tubulin–AAGTP showed maximal inhibition of adenylyl cyclase under conditions where only 15% of maximal $G_{i\alpha}$ labeling is acheived (Rasenick & Wang, 1988). It is also possible that various domains within the synaptic membrane might have selective populations of specific G proteins. The demonstrated interaction between G proteins and tubulin might subserve such microheterogeneity.

Despite the blockade by DM1a and DM1b of adenylyl cyclase inhibition by tubulin–Gpp(NH)p and of nucleotide transfer from tubulin to $G_{\alpha i}/G_{\alpha o}$, these monoclonal anti-tubulin antibodies had no effect on tubulin binding to $G_{\alpha i}$ (Figure 6). It could be argued that this discrepancy was caused by the different conditions used for adenylyl cyclase/nucleotide transfer assays vs dot blot hybridizations. This is unlikely because of the following: (1) Binding of iodinated tubulin to nitrocellulose-immobilized G_α is specific, saturable, and, presumably, resembles interactions between tubulin and G proteins under physiological conditions (Wang et al., 1990). (2) DM1a and DM1b can immunoprecipitate tubulin–G protein complexes; thus, the binding of tubulin to G proteins in solution is not disrupted by either antibody (Yan, 1992). (3) DM1a and DM1b did not significantly alter the EC_{50} of tubulin–Gpp(NH)p in inhibiting adenylyl cyclase (Figure 2), implying that the affinity for tubulin binding to $G_{\alpha i}$ is not changed. This is consistent with the results in Figure 6. Thus, it appears that while DM1a and DM1b interfere with the nucleotide transfer from tubulin to $G_{\alpha i}$, they do not alter tubulin binding to $G_{\alpha i}$.

It is suspected that, in order to acheive the transfer of AAGTP from tubulin to $G_{i\alpha1}$, as seen in Figures 3–5, tubulin and G proteins must form a physical complex. Such a complex would allow for the direct transfer of AAGTP between the proteins. It is noteworthy that the transfer of NAD has been demonstrated to occur among several mitochondrial hydrolases (Srivastava & Bernhard, 1986). Clearly, tubulin–AAGTP (or tubulin–GppNHp) presents GTP analogs to $G_{i\alpha1}$ in a unique fashion, since, under the conditions in Figure 5, free [^{32}P]AAGTP did not bind to the purified $G_{i\alpha}$ whereas tubulin–AAGTP transferred AAGTP to that G protein.

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 binding to other tubulin dimers in the formation of microtubules, is largely unknown. Studies with monoclonal or sequence-specific anti-tubulin antibodies have evoked the notion that, while parts of C-terminal domains of both α and β tubulin are exposed on the exterior of the microtubules, considerable regions of the N-terminal domains either are not exposed on this cytoskeletal filament or are masked by interacting proteins (Draber et al., 1989; Arevalo et al., 1990). The far C-terminal region of both subunits, though probably not directly involved in microtubule formation, is an important regulatory domain. It is this region to which MAPs and tau proteins bind and regulate microtubule formation. This region is not involved in G protein binding (Wang & Rasenick, 1991).

The epitopes for DM1a and DM1b are located near the C-terminal. These epitopes neighbor the MAP2 binding site on tubulin. It has long been known that MAP2 regulates the microtubule assembly in vitro, suggesting that binding of MAP2 to C-terminal domains of tubulin somehow affects the interactions among tubulin dimers in formation of microtubules. Analysis of MAP binding to microtubules in which the ratio MAP/tubulin is varied shows a polymorphism of tubulin–MAP oligomers (Carlier et al., 1984). Similarly, there is evidence indicating that binding of antibody to these C-terminal regions could also affect the morphology of polymers assembled from tubulins (Arevalo et al., 1990). Therefore, binding of DM1a and DM1b to the C-terminal region of α and β tubulin might induce certain conformational changes in other parts of the molecule, possibly the N-terminal domains including the GTP-binding site on β tubulin. Finally, it appears that polymerization of microtubules alters somewhat the cleavage of the C terminal by subtilisin. This suggests that, even though the C terminal may be accessible in the microtubule, its structure is modified somewhat (Lobert & Correia, 1992).

Evidence from this laboratory indicates that tubulin-G_α interactions resemble the interactions among tubulin dimers in formation of microtubules. 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). Since tubulin–tubulin interactions in microtubules may involve both C- and N-structural domains, G_α may bind to tubulin by interacting with the later regions.

This is also consistent with the hypothesis that tubulin transfers nucleotide directly to $G_{i\alpha1}$ from the exchangeable GTP-binding site, which is in the N-structural domain of β tubulin. Thus, it is possible that binding of DM1a and DM1b to tubulin dimers alters the conformational status of the N-structural domains of these molecules and, subsequently, interferes with the nucleotide transfer from tubulin to G_α. These changes are likely subtle, since neither the physical interaction between tubulin and $G_{\alpha i}$ or the ability of β tubulin to bind GTP is affected significantly by DMIa or DMIb.

It is noteworthy that the exchangeable nucleotide-binding site is on β tubulin (Geahlen & Haley, 1979), while DM1a, which binds to C-terminal domain of α tubulin, interferes with the nucleotide transfer between tubulin and $G_{\alpha i}$. This observation is consistent with a structural model for tubulin heterodimers in which the N- and C-structural domains of each subunit are in contact with each other (Sackett & Wolff, 1986; Serrano & Avila, 1985; Linse & Mandelkow, 1989).

It is reasonable to postulate that antibody molecules bound to one site of a molecule will cause sufficient distortion that other regions on the molecule are affected. The DM1b-mediated block of MAP2 binding (Arevalo et al., 1990) and the anti-N-terminal G protein antibody disruption of receptor–G protein coupling (Hamm et al., 1989) may serve as examples of this phenomenon.

This laboratory has proposed that tubulin may regulate neuronal membrane adenylyl cyclase by interacting with and transferring nucleotide to $G_{\alpha s}$ or $G_{\alpha i}$ (Yan & Rasenick, 1990; Rasenick & Wang, 1988). We have provided a rationale for such regulation as a locus for interaction among various second messenger systems. Recent evidence from this lab (Yan, 1992) suggests that tubulin–GppNHp can bypass a tightly coupled receptor to activate $G_{s\alpha}$, and we have hypothesized that tubulin dimers associated with the synaptic membrane might be released from constraint by MAP2 subsequent to a calcium signal (Rasenick & Wang, 1988; Wang & Rasenick, 1991). Although our investigations have centered on adenylyl cyclase, it is possible that other G protein mediated processes could be regulated in a like manner.

Recent evidence indicates that G proteins may form polymers among the same or different subspecies (Nakamura & Rodbell, 1990; Vaillancourt et al., 1990; Hingorani et al., 1989; Hatta et al., 1986). Nucleotide transfer between G_s and G_i has also been observed (Hatta et al., 1986; Rasenick & Childers, 1989) to accompany the regulation of adenylyl cyclase, and microtubule-disrupting drugs have also been shown to modify synaptic membrane adenylyl cyclase (Rasenick et al., 1981). Structural (Mandelkow et al., 1985; Sternlicht et al., 1989) and biochemical studies [reviewed in Rasenick et al. (1989)] suggest that tubulin shares certain features with many GTP-binding proteins. Therefore, tubulin–G_α (or G_α–G_α) complex formation and nucleotide transfer could represent a common feature among the members of this superfamily, as well as a potentially important step in the regulation of G protein mediated signal transduction.

In summary, we have demonstrated that anti-tubulin monoclonal antibodies DM1a and DM1b interfere with the inhibition of adenylyl cyclase by tubulin–Gpp(NH)p in rat synaptic membranes. We suggest that DM1a and DM1b exert their effect by inhibiting nucleotide transfer from tubulin to $G_{\alpha i}$. DM1a and DM1b could be potentially useful tools in further studies on tubulin–$G\alpha$ interactions.

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