Tubulin Binds Specifically to the Signal-transducing Proteins, Gαs and Gia1*

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Participation of cytoskeletal elements in regulation of hormonal response and responsiveness has been suggested by several laboratories. Addition of dimeric tubulin to rat cerebral cortex synaptic membranes causes stable inhibition of adenylyl cyclase, and the molecular basis for this effect appears to require a direct interaction between tubulin and G proteins. To test whether such tubulin-G protein interaction occurred, several purified G proteins were bound to nitrocellulose, and 125I-tubulin overlay studies were performed. 125I-Tubulin bound to the α subunits of Gs and Gi with high specificity and an apparent Kd of approximately 130 nM. Other G protein α subunits (αi23, αi23, αi33, and transducin) displayed a much lower affinity for tubulin, despite the much closer relationship of these proteins to αi than to αs. Association of βγ subunits with αi or αs did not alter the binding of tubulin to these G protein heterotrimers, and the binding of a hydrolysis-resistant GTP analog to the α subunits was similarly without effect. These results suggest that tubulin forms complexes with specific G proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades. These results also provide evidence of a functional distinction among the closely related α subtypes.

In a variety of transmembrane signaling systems, cell surface receptors are linked to effectors by GTP-binding (G) proteins which function as signal transducers (1). Several of these proteins have been described, and their classification and transducin) displayed a much lower affinity for tubulin, despite the much closer relationship of these proteins to αi than to αs. Association of βγ subunits with αi or αs did not alter the binding of tubulin to these G protein heterotrimers, and the binding of a hydrolysis-resistant GTP analog to the α subunits was similarly without effect. These results suggest that tubulin forms complexes with specific G proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades. These results also provide evidence of a functional distinction among the closely related α subtypes.

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(activated by transducin (Gαi). Although the family of signal-transducing G proteins had been considered to be a small one (including the above mentioned proteins plus Gs, a protein of unknown function which is abundant in mammalian brain), current evidence suggests that several subspecies of these proteins exist. Recent advances in molecular cloning of cDNAs for α (GTP-binding) subunits of G proteins have revealed four forms of αi (produced by alternative splicing of a single gene (2, 3) and three forms of αs (4, 5). Concurrent with the discovery of increasing numbers of G protein subtypes has been the assignment of new and varied physiological functions for these molecules. For Gs proteins, these roles include the gating of K+ channels as well as the inhibition of adenylyl cyclase. Despite the clear demonstration of molecular heterogeneity among the αi protein isoforms, no distinction with respect to physiological roles has been demonstrated.

Tubulin is also a GTP-binding protein, and significant functional similarity and amino acid sequence homology appear to exist between tubulin and the signal-transducing G proteins (6-8). Functional interaction between tubulin and synaptic membrane G proteins has also been demonstrated. In those studies, incubation of Gpp(NH)p-ligated dimeric tubulin with rat cerebral cortex synaptic membranes resulted in inhibition of adenylyl cyclase which persisted subsequent to washing of those membranes. This effect appeared to involve the transfer of guanine nucleotide from tubulin to Gi, and this process was thought to represent a unique feature of the regulation of neuronal adenylyl cyclase (9). These data suggested that tubulin molecules interacted physically with G proteins. Further suggestions of such interactions included observations that tubulin bound to a G protein affinity column and, as Gs, to a tubulin affinity column (10, 11). The current study was undertaken in an effort to elucidate the molecular details of tubulin-G protein interaction. In this study, the formation of tubulin-G protein macromolecular complexes is demonstrated. It appears that the ability to form these complexes reveals a clear distinction among the subtypes of αi.

**EXPERIMENTAL PROCEDURES**

Membrane and Protein Preparation—Synaptic membranes were prepared from cerebral cortices of 21-day-old rats (12) and stored under liquid N2 until use. Gαs and Gαi were isolated from rat brain, through the DEAE-Tyopearl chromatography step, by the procedure of Goldsmith et al. (13), and fractions containing both of these G proteins species were employed in immunoprecipitation studies. Bovine brain Gαs and Gαi, as well as αi and αs, were obtained from Dr. E. Neer (Harvard). αi (45-kDa form (14)), αi23, and αi33 were expressed in Escherichia coli and were provided by Dr. A. G. Gilman and colleagues (University of Texas, Dallas). Purified Gαs and Gαi (and their respective αi subunits as well as αs) were provided by Drs. T. Katada and Y. Kaziro (Tokyo University). Bovine αs and Gs were obtained from Dr. H. Hamm (University of Illinois, Chicago). Human red blood cell Gs was obtained from Dr. J. Codina (Baylor). Ovalbumin (Sigma) was iodinated with IOODO-GEN (Pierce Chemical Co.) and used at a specific activity of 1800 Ci/mmol.

1 The abbreviations used are: Gαs: G protein from the rod outer segment (transducin); αi, the GTP-binding subunit of a given G protein; AAGTP, p-1,4-azidoanilido-p-1,3-GTP, EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid, 1.5 sodium; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonate; (Gpp(NH)p, guanylyl-5'-yl imidodiphosphate; GTP•S, guanosine 5'-O-(thiotriphosphate); SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.)
Tubulin was prepared by two cycles of polymerization from rat brain (15), and microtubule-associated proteins were removed by phosphocellulose chromatography (16). Tubulin was iodinated with IODO-GEN to a specific activity of 1800 Ci/mmol, and functional integrity of $^{125}$I-tubulin was verified by copolymerization with unlabeled freshly depolymerized (second cycle) tubulin (containing microtubule-associated proteins). Under those conditions, 91% of the iodotubulin repolymerized and 88.5% of the iodotubulin repolymerized. Thus, relative to the controls, the iodotubulin used in these experiments was 97% polymerization-competent. Purity of the $^{125}$I-tubulin was verified by silver staining and autoradiography. When 90 ng were run on 8.5% SDS-polyacrylamide gel electrophoresis, no impurities were detected by staining or autoradiography.

**Photoaffinity Labeling—Membranes** were incubated with $[^35]$I$P$NP$A$, 1,4-azidoanilido-$p'$-5'-GTP (AAGTP) or unlabeled AAGTP (0.12 μM) to a specific activity of 1800 Ci/mmol. Tubulin was iodinated with IODO-GEN to a specific activity of 1800 Ci/mmol and functional radiography.

**1,4-azidoanilido-$p'$-5'-GTP (AAGTP) or unlabeled AAGTP (0.12 μM)** were added to the reaction. The mixture was then UV-irradiated to a specific activity of 1800 Ci/mmol. Samples were loaded on a 1-mL sample desalting column (from Bio-Rad) which was equilibrated with Tris buffer. The unlabeled radioactive compound was removed by ultrafiltration, and the protein solution (90% of proteins were recovered) was used in the immunoprecipitation experiments.

**Immunoblotting and Tubulin Overlays—After electrophoresis, proteins were transferred to nitrocellulose (PH 7.5, 0.1-μm pore size; Scheicher and Schuell) by washing in blotting buffer (25 mM Tris, 192 mM glycine, pH 7.6) and transferred at 50 mA for 2 h at 4°C. Transfer efficiency was verified by silver staining of the dried gels. Nitrocellulose was incubated at room temperature for 2 h in 2% bovine serum albumin (BSA) blocking buffer containing 10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 0.02% Triton X-100. Nitrocellulose strips were then incubated with iodinated $^{125}$I-tubulin or $^{35}$I-ovalbumin (1.3 μg/ml) at a specific activity of 1800 Ci/mmol. After repeated washing of the nitrocellulose with blocking buffer, nitrocellulose strips were dried and autoradiography was performed using Kodak XAR film.

**Dot Blotting**—Purified G proteins (obtained from the indicated sources) were applied to nitrocellulose in the amounts indicated in the figure legends. Quantification of proteins bound after washing was performed by applying 100 ng of iodinated tubulin, ovalbumin, or α to nitrocellulose. These proteins (each at a concentration of 0.5 μg/ml) were in a 100 mM PIPES buffer containing 2 mM EGTA, 1 M NaCl, pH 7.4, and one of the following detergents: Lubrol PX, Triton X-100, SDS, CHAPS, sodium cholate (each 0.5%), or no detergent. Nitrocellulose was subjected to the washing procedure described above. Protein remaining on the nitrocellulose was quantified in an LKB Rackgamma counter. In each case, regardless of protein or detergent used, about 40% (30.8% average of two triplicate experiments for each protein) of the applied protein remained on the nitrocellulose after the washing steps.

**Immunoprecipitation—Labeled G proteins (0.6 μg) were incubated with 10 μg of purified tubulin at 23°C for 20 min followed by the addition of indicated amounts of anti-tubulin antisera (ICN 65095) and 0.15 M dithiothreitol, and boiled for 5 min. The supernatant was then incubated with 100 ng of iodinated tubulin, ovalbumin, or α to nitrocellulose. These proteins (each at a concentration of 0.5 μg/ml) were in a 100 mM PIPES buffer containing 2 mM EGTA, 1 M NaCl, pH 7.4, and one of the following detergents: Lubrol PX, Triton X-100, SDS, CHAPS, sodium cholate (each 0.5%), or no detergent. Nitrocellulose was subjected to the washing procedure described above. Protein remaining on the nitrocellulose was quantified in an LKB Rackgamma counter. In each case, regardless of protein or detergent used, about 40% (30.8% average of two triplicate experiments for each protein) of the applied protein remained on the nitrocellulose after the washing steps.

**RESULTS**

**Tubulin Binding Proteins in Synaptic Membranes—Initial attempts to demonstrate tubulin-G protein interaction involved SDS-polyacrylamide gel electrophoresis of rat cerebral cortex membrane proteins and transfer of those proteins to nitrocellulose. Subsequent incubation of that nitrocellulose with $^{125}$I-tubulin showed $^{125}$I-tubulin binding to eight proteins, four of which had electrophoretic mobilities corresponding to proteins labeled covalently by the photoaffinity GTP analog, AAGTP, and ADP-riboseylated by cholera or pertussis toxin (17). However, a number of subtypes of α exist; these proteins and α, are not separated well in SDS gel electrophoresis of synaptic membrane preparations. Furthermore, apparent co-migration in one dimension does not provide sufficient evidence to implicate G proteins as the targets of tubulin binding. Thus, it was necessary to examine the ability of tubulin to bind to purified G proteins.

**Tubulin Binding to Specific G Proteins—Tubulin bound preferentially to α (recombinant, 45-kDa form (14)) and α, but only slightly to α3, αβ, and α (Fig. 1). Recombinant αβ and αβ purified from porcine brain bound $^{125}$I-tubulin to a similar extent. Furthermore, both activated (GTP$\gamma$S-ligated) and inactive (GDP-ligated) forms of α, αβ, and α were tested. This did not alter the ability of $^{125}$I-tubulin to bind (or not bind) to these proteins. The binding of tubulin to Gα was specific, as either excess cold tubulin or denaturation of Gα by boiling abolished the binding of tubulin. Although tubulin did not bind to isolated βγ subunits on nitrocellulose, Gα (the αβ heterotrimer) bound tubulin with affinity similar to that of α, and heterotrimeric Gα, or G, were no more effective than their α subunits in the binding of tubulin (Fig. 2). Furthermore, whereas the α was used as a recombinant protein (expressed in E. coli), G, was purified from human erythrocytes (Fig. 2).

**Affinity and Stoichiometry of Tubulin-G Protein Interaction—The affinity of tubulin for α was determined by immobilizing α on nitrocellulose and performing overlay experiments with varying concentrations of $^{125}$I-tubulin. The binding was saturable and 86% specific at 0.5 nM of tubulin. Calculated Kd values for tubulin binding to α ranged from 110 to 140 nM, and immobilized α bound 0.3-0.6 mol of tubulin/mol of α (Fig. 3). Hill coefficients for tubulin binding to α were approximately 1 (0.976 ± 0.017; mean ± S.E.) for $^{125}$I-ovalbumin did not bind to the dotted G proteins, and boiled G protein failed to bind tubulin. Excess (400 ×) unlabeled tubulin completely eliminated $^{125}$I binding to G proteins.
A. purified G proteins were dotted on a nitrocellulose sheet in the amounts indicated and air-dried at room temperature. Following this, tubulin binding was assessed by overlay with [3H]-tubulin and autoradiography as described in Fig. 1. B, purified G protein subunits were dotted on a nitrocellulose sheet in the dilution series indicated (the protein amount of heterotrimeric G1 roughly doubled that of α and β) followed by overlay with [3H]-tubulin and autoradiography as described in Fig. 1.

**FIG. 3.** Saturation isotherm and Scatchard plot for tubulin binding to α1. Data were derived from dot blotting performed with a method similar to that described in Fig. 2 except that cold tubulin was added to adjust the final concentration of [3H]-tubulin to a specific activity of 278 Ci/mmol. Incubation volumes were 1 ml. Two pmol of α1 were applied to each spot. After autoradiography, triplicate nitrocellulose spots corresponding to the total binding and nonspecific binding (determined in the presence of 100-fold excess unlabeled tubulin) were cut out and counted in an LKB Rackgamma counter. The graph on the left shows the saturation isotherm for total (A), specific (■), and nonspecific (□) binding of [3H]-tubulin to the mean of three G protein spots. On the right is a Scatchard plot derived from these data. In the experiment depicted above, the Kd and Bmax were 134 nm and 0.664 pmol, respectively. A Hill plot of these data yields an nH of 0.995. One of three similar experiments is shown.

Each of the three determinations. It is possible that some of the tubulin binding sites on α1 were occluded when those regions of α1 bound to the nitrocellulose. Thus, this estimate of tubulin-α1 binding affinity could be low.

**Immunoprecipitation of Tubulin-G Protein Complexes**—Although tubulin bound specifically to α and α1, which were affixed to nitrocellulose, it was important to determine whether these proteins could form complexes in solution. Therefore, immunoprecipitation was performed with an anti-tubulin antibody which did not interfere with the tubulin-mediated inhibition of adenylyl cyclase or the binding of tubulin to G proteins on nitrocellulose. α1 formed complexes with tubulin and was immunoprecipitated only in the presence of tubulin (Fig. 4). Substitution of nonspecific antibody or elimination of tubulin from the incubation blocked α1 immunoprecipitation. Substitution of chicken uterus myosin and rabbit anti-myosin (both from Dr. P. de Lanerolle, University of Illinois, Chicago) for tubulin and anti-tubulin resulted in precipitation of myosin but not α1, indicating that the formation of a precipitable immune complex which has no specific interaction with α1, will not precipitate α1. AAGTP-labeled α1, although present in these preparations in amounts roughly equal to α1, was not immunoprecipitated even in the presence of tubulin, implying that no complexes were formed between tubulin and α1, a result consistent with the dot blotting experiments (see Fig. 2).

Since α1 immunoprecipitated with tubulin was prelabeled with [32P]AAGTP before incubation with tubulin, it was assumed that this was dissociated from βγ subunits. A similar experiment was performed in the presence of 100 μM GTPγS, a condition which should dissociate all α from βγ. In this experiment, tubulin and α1 were immunoprecipitated with anti-tubulin, but βγ subunits were not. To detect whether the αβγ heterotrimers of G formed complexes with tubulin, unlabeled G1 was incubated with tubulin and subjected to immunoprecipitation with anti-tubulin as described above. Immunoprecipitated β was detected by anti-β antibodies. Since tubulin did not bind to βγ subunits of G proteins and purified βγ subunits were not precipitated by anti-tubulin antibody (in the presence or absence of tubulin) those proteins detected by the specific anti-β antibody were a part of G1-tubulin complexes.

**DISCUSSION**

These data suggest that tubulin binds with high affinity to α1 and α1, but with much lower affinity to α2, α1, α1, and α1.
Given the very high (87-94%) amino acid sequence homology among the α, subtypes (18, 19) and the high (≥80%) homology among the pertussis toxin substrates (i.e., α, α, and α) (20) yet relatively low (<45%) homology between α and the others, this finding is surprising. In fact, the binding of tubulin represents the first demonstration of a functional similarity between α and one α subtype which is not shared among the three α subtypes.

Some functional as well as structural differences among the G subtypes have been demonstrated. G and G have been copurified from porcine brain, and only the GTPγS-activated α could inhibit membrane-bound adenyl cyclase (21). Furthermore, an affinity-purified polyclonal antibody against α did not recognize α, implying significant difference in the dominant immune determinants of α and α (22). Secondary structure analysis of the most diverse region of α subtypes, amino acid residues 80-130, revealed different predicted secondary structures among these three α subtypes (4). These structural differences may contribute to the binding specificity of tubulin to α subtypes. It is noteworthy that tubulin binding represents the first demonstration of a functional distinction between α and α.

The distribution of α subtypes among mammalian tissues presents an intriguing correlation with the results presented in this report. α is abundant in brain but less abundant in peripheral tissues (5, 23). The inhibitory effect of tubulin on adenyl cyclase, which appears to involve transfer of a hydrolysis-resistant GTP analog from tubulin to αa appears specific for neuronal tissue (9, 11). As the regulation of neuronal adenyl cyclase differs in several important aspects (e.g., calmodulin dependence and loss of receptor activation subsequent to cell disruption) from that of other tissues, it is possible that membrane-associated tubulin dimers participate in the intracellular regulation of neuronal adenyl cyclase. Such a process would participate in the modulation of neurotransmitter response (or responsiveness) and could provide a focus for a second messenger interaction (7, 9).

Tubulin itself is a GTP-binding protein with intrinsic GTPase activity and some structural homology with other GTP-binding proteins (8). Both tubulin and G proteins undergo GTP/GDP-dependent reversible complex formation, represented by microtubule formation in the case of tubulin and α, heterotrimer formation for the signal-transducing G proteins. Tubulin is also a substrate for both pertussis toxin- and cholera toxin-catalyzed ADP-ribosylation (24, 25).

Unlike the signal transducing G proteins, the primary biological role of tubulin is the formation of microtubules. During such an event, several "contact regions" on the tubulin dimer engage in a reversible association with other tubulin dimers. Given the various regions of homology between tubulin and G proteins, it is possible that α and α contain domains which are sufficiently "tubulin-like" that a reversible complex between these G proteins and tubulin is formed (7, 9). Since the binding of βγ subunits to α did not interfere with tubulin binding, it is likely that tubulin and βγ bind to α on separate domains.

While it is possible that a common sequence among G subunits is capable of being recognized by tubulin, certain conformational features need be preserved for this recognition to occur. This argument is supported by the observation that boiling of α1 abolished the ability of α1 to bind tubulin.

Recently, our laboratory suggested that there is a direct interaction and guanine nucleotide transfer between α and α, and that this process is involved in the regulation of neuronal adenyl cyclase (17). Functional complex formation between G proteins has also been proposed as a mechanism to explain coupling of the kinase and the receptor-effector coupling (26). The interaction and nucleotide exchange between G proteins and between G protein and tubulin may represent a form of intracellular regulation of neuronal adenyl cyclase. The significance of specific binding of tubulin to α and α, may be due to the likelihood that it is these proteins which stimulate and inhibit adenyl cyclase, and that it is enzyme which tubulin appears to modulate. Furthermore, this tubulin-G protein interaction represents a possible role for tubulin dimers which is independent of microtubule formation.

The precise relationship between tubulin and G proteins as well as the contribution of tubulin and/or other cytoskeletal components to the regulation of signal transduction is not yet known. Nonetheless, the possibility that an ordered presentation of members of the adenyl cyclase system and tubulin orchestrate cellular responses to external and internal signals remains intriguing.

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