α and βγ subunits of G proteins are thought to transduce signals from cell surface receptors to intracellular effector molecules. Gα and Gβγ have also been implicated in cell growth and differentiation, perhaps due to their association with cytoskeletal components. In this report Gβγ is shown to modulate the cytoskeleton by regulation of microtubule assembly. Specificity among βγ species exists, as β1γ2 stimulates microtubule assembly, and β1γ1 is without any effect. Furthermore, a mutant β1γ2, β1γ2(C68S), which does not undergo prenylation and subsequent carboxyl-terminal processing on the γ subunit, does not stimulate the formation of microtubules. β Immunoreactivity was detected exclusively in the microtubule fraction after assembly in the presence of β1γ2, suggesting a preferential association with microtubules rather than soluble tubulin. Crude microtubule fractions from ovine brain contain Gβγ, and electron microscopy reveals a specific association with microtubules. The decoration of microtubules by Gβγ appears to be strikingly similar to the periodic pattern observed for microtubule-associated proteins, suggesting a similar site of activation of microtubule assembly by both agents. It is suggested that reformation of the cytoskeleton represents an additional cellular process mediated by Gβγ.

G proteins play important roles in signal transduction by transferring signals from cell surface receptors to intracellular effectors. 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. Many studies have implicated the participation of the cytoskeleton in neurotransmitter signaling pathways (1–7). Although G proteins are likely to be membrane-bound when coupled to receptors, recent results from several laboratories suggest their association with several subcellular compartments. In Caenorhabditis elegans embryos, Gα1 is required for proper spindle orientation and transiently associates with the region of asters (the array of microtubules emanating from the centrosomes) just before and transiently associates with the region of asters (the array of microtubules from ovine brain contain Gα and Gβγ, and the synaptic membrane tubulin has been observed previously (2–11). Tubulin appears to activate G proteins directly, and complexes between tubulin and Gα have been isolated from plasma membranes. While some interaction between tubulin and Gβγ has been observed previously, the role of such interaction remains unclear. Modification of microtubule cytoskeleton by Gβγ might provide an explanation for the association of Gβγ with the mitotic spindle and its role in cell growth and differentiation. The present study was undertaken to explore the role of Gβγ in microtubule assembly and dynamics.

EXPERIMENTAL PROCEDURES

Purification of Proteins—PC-tubulin1 (tubulin free of microtubule-associated proteins) was purified from ovine brain by two cycles of assembly and disassembly (12) followed by phosphocellulose chromatography (13). The tubulin preparation made by two assembly-disassembly cycles (microtubule proteins) contains microtubule-associated proteins (MAPs). These MAPs were removed by phosphocellulose chromatography (13). β1γ2, purified from SF9 cell membranes (14) was kindly provided by Drs. T. Kosaza and A. G Gilman. The specificity of Gα1 and β1γ2(C68S), which does not undergo prenylation, and subsequent carboxyl-terminal processing on the γ subunit, was a generous gift from Drs. Peel, Nick Skiba, and Heidi E. Hamm of Northwestern University and Yee-Kin Ho of University of Illinois, Chicago. Mutant β1γ2(C68S) was generously provided by Drs. John Hepler and A. G Gilman.

Microtubule Assembly—Fresh ovine brains were obtained from a local abattoir and homogenized in PEM buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM MgCl2) containing 0.1 mM GTP, followed by centrifugation at 100,000 × g for 60 min at 4 °C. This initial pellet (P1), the crude membrane preparation, was resuspended in Hepes buffer (0.1 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride) and saved for immunoblotting. Tubulin from the initial supernatant (S1, cytosolic fraction) was added to assembly buffer (PEM, PH 6.9, 2 MM EGTA, 1 m M MgCl2) containing 0.1 mM GTP, for 60 min at 4 °C. This initial PC-tubulin in PEM buffer and 1 mM GTP was incubated with or without βγ (0.05 mg/ml) or βγ (0.1 mg/ml) at 0 °C for 10 min followed by assembly at 37 °C for 45 min to 1 h. Assembly was quantitated by centrifuging the polymer at 150,000 × g for 20 min at 37 °C, and pellets and supernatants were separated. Pellets were resuspended in cold PEM buffer and protein concentrations were determined both in pellets and supernatants. Protein was estimated by Coomassie Blue binding according to the method of Bradford (15) using bovine serum albumin (BSA) as a standard. All βγ combinations were exchanged in PEM buffer through a rapid spin

1 The abbreviations used are: PC-tubulin, tubulin free of microtubule-associated proteins, purified by phosphocellulose chromatography; MAP, microtubule-associated protein; BSA, bovine serum albumin; NGS, normal goat serum; PAGE, polyacrylamide gel electrophoresis; EMAP, a major microtubule-associated-protein of sea urchins and several other echinoderm membranes; βARK, β-adrenergic receptor kinase; PIPES, 1,4-piperazinediethanesulfonic acid.
incubation with anti-G antibodies were fixed by adding 0.5% glutaraldehyde in warm PEM buffer. For immunoelectron microscopy, microtubules were dissolved in 3% SDS Laemmli sample buffer with 50 mM dithiothreitol and subjected to SDS-polyacrylamide gel (10% acrylamide and 0.133% bisacrylamide) electrophoresis. For Western blotting, samples after electrophoresis, were transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad). The nitrocellulose membranes were incubated in 3% BSA in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) for 2 h at room temperature followed by an overnight incubation with anti-G\textsubscript{2} antibody. Detection of antibody binding was with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Pierce, Rockford IL). The membrane was washed with 0.02% Tween 20 in TBS, and the substrate solutions were added for color development.

Electrophoresis and Immunoblotting—Samples for electrophoresis were dissolved in 3% SDS Laemmli sample buffer with or without β\textsubscript{1}-2 (0.05 mg/ml) or β\textsubscript{1}-1 (0.1 mg/ml) at 0 °C for 10 min followed by assembly at 37 °C for 1 h. Samples were then divided into two aliquots. One aliquot was used for estimating polymer mass (see Fig. 3), and the other was processed for electron microscopy and viewed in a JEOL 100S electron microscope. Samples from the experiment described in Fig. 4 were used for electron microscopic analysis of microtubule assembly in the presence of β\textsubscript{1}-2(C68S). The bar represents 0.1 µm.

RESULTS AND DISCUSSION

Several species of G protein β and γ subunits have been shown to exist (16), and they appear to show preferences for various forms of G, as well as for specific receptors (17–20). β and γ subunits of G proteins function as a tightly bound complex, which is disrupted only by denaturation. Coexpression of both subunits are required to detect the activity of the complex and functional properties of the complex are dependent on contributions from both proteins (21). Although transducin βγ primarily consists of β1 and γ1 isotypes, βγ subunits purified from bovine brain are heterogeneous in composition.

β\textsubscript{1}γ\textsubscript{2} Promotes Microtubule Assembly—The effect of different combinations of βγ on microtubule assembly was tested. Tubulin purified free of microtubule-associated proteins was incubated at 37 °C in the presence of β\textsubscript{1}γ\textsubscript{2} or β\textsubscript{1}γ\textsubscript{1} (transducin βγ). Assembly was monitored by negative staining electron microscopy and measuring protein in polymers collected by centrifugation. Under these buffer conditions, tubulin does not assemble well unless glycerol (25–30% by volume) is present in the buffer. However, microtubule assembly was stimulated markedly, when β1γ2 was present at ~1:20 molar ratio with tubulin (Figs. 1, 2, and 3). In contrast, β1γ1 had no effect on microtubule assembly. Electron microscopic analysis indicated very few microtubules formed by tubulin alone or in the presence of γ1 alone (Fig. 1). In the presence of β1γ2, however, robust microtubule polymerization occurred. To quantify this response, eight random fields were examined by electron microscopy on a total of five grids from three separate experiments. The result is shown in Fig. 2. In a field of 5 µm in an average grid, 1–4 microtubules were found in the control samples (mean = 1.7) or samples incubated with β1γ1 (mean = 2.7), while 49–65 (mean = 54.7) isolated microtubules were detected in samples incubated with β1γ2. Protein estimation in the pellets also indicated a 71% increase in the presence of β1γ2 (Fig. 3A). No detectable change in pellet protein concentration (compared with controls) was observed in the presence of β1γ1. The maximal extent to which added β1γ2 if incorporated into the microtubule pellet could increase the pellet protein is calculated to be 13%. This suggests that the increase in pellet protein seen in Fig. 1 is indicative of β1γ2-induced enhancement of microtubule assembly. SDS-PAGE of the samples further confirmed...
the increase in tubulin concentration in the pellet formed in the presence of β1γ2 (Fig. 3B). This result clearly indicates that β1γ2 promotes microtubule assembly, perhaps by reducing the critical concentration of tubulin required for assembly. In the presence of β1γ2, PC-tubulin, at a concentration as low as 0.8 mg/ml, assembled readily into microtubules (15–20 microtubules/5-μm field in a grid).

Prenylation of β1γ2 Is Required for Promotion of Microtubule Assembly—The finding that β1γ2 stimulated microtubule assembly, while β1γ1 was ineffective, suggests that the γ subunit is the determining factor for the observed difference. One major difference between γ1 and γ2 is the modification at the carboxyl terminus of γ; while γ1 is farnesylated, γ2 is geranylgeranylated (22–25). Furthermore, the amino acid sequences of γ1 and γ2 are substantially different. Thus, differential effect of β1γ1 and β1γ2 in microtubule assembly might be attributed to the γ subunit and the type of isoprenoid moiety attached to it.

Although prenylation and/or further carboxyl-terminal processing of Gγ is not required for assembly of βγ complexes, such modifications are indispensable for the function of βγ. High affinity interactions of βγ with either G protein α subunits or effector molecules require that the γ subunit be prenylated (21, 26, 27). To examine the role of the γ subunit carboxyl-terminal processing, a mutant β1γ2, β1γ2(C68S), which cannot undergo prenylation and further carboxyl-terminal processing was used. This mutant βγ was inactive in all functional assays (21).

As shown in Figs. 1 and 2, β1γ2(C68S) did not stimulate microtubule assembly. Analysis of microtubules from electron microscopic data (Fig. 2) indicated no significant difference from control (3 ± 1.0 versus 1.66 ± 0.49 per 5-μm field in an average grid). However, larger tubulin aggregates were seen frequently in samples assembled from β1γ2(C68S) (Fig. 1). β1γ2(C68S) did not alter total polymer mass over controls as determined by protein estimation in the pellet (data not shown).

β1γ2 Shows Specific Binding to Microtubules—SDS-PAGE and immunoblot analysis of pellet and supernatant fractions indicate β immunoreactivity in microtubule pellets after assembly in the presence of β1γ2 (Fig. 4), suggesting a preferential association with microtubules rather than soluble tubulin.

However, the microtubule and tubulin aggregates formed in the presence of β1γ1 or β1γ2(C68S) did not bind these βγ species, as all β immunoreactivity remained in the supernatant (Fig. 4). To rule out the possibility that the observed association of β1γ2 to microtubules is not due to aggregation and subse-
quent precipitation of the less soluble geranylgeranylated β1γ2, β1γ2 and β1γ2(C68S) were subjected to similar incubation and centrifugation conditions as in Fig. 4, except that tubulin was excluded from the samples. Greater than 90% of the β1γ2 or β1γ2(C68S) remained in the supernatant fractions, confirming that the precipitation of β1γ2 in the microtubule pellet is due to its specific interaction with microtubules.

β1γ1 and β1γ2(C68S) Do Not Inhibit Microtubule Assembly and Fail to Bind to Microtubules—Even though significant microtubule formation occurred at subcritical tubulin concentrations in the presence of β1γ2, it was necessary to determine if β1γ1 or β1γ2(C68S) inhibited microtubule assembly (as opposed to failing to promote assembly). To address this issue, microtubule assembly was induced by adding 30% glycerol to the incubation buffer, which reduces the critical concentration for assembly of purified tubulin (12, 28). Utracentrifugation studies and electron microscopic analysis demonstrated normal microtubule formation. Addition of β1γ2(C68S) or β1γ1 did not alter the level of assembly as detected by protein assays of the pellets as well as by electron microscopic observation. Since the degree of microtubule assembly was similar with all βγ species in the presence of glycerol, association of Gβγ with microtubules was tested under this condition. Consistent with the data in Fig. 4, only β1γ2 was associated with microtubules. The other two βγ species (β1γ1 and β1γ2(C68S)) remained exclusively in the supernatant fractions (data not shown).

The results in Figs. 1–4 suggest that the functional interactions of βγ subunit of G proteins with tubulin/microtubule systems require a similar structural specificity of βγ to those which determine Gγγ interactions with Gα or effector molecules such as phospholipases or ion channels. In this context, tubulin/microtubules may represent a new class of effector system for βγ subunits of G proteins. As a corollary to this, βγ subunits might represent a new class of microtubule-associated protein. It is noteworthy that the major microtubule-associated protein (MAP) of sea urchins and several other echinoderms is a 77-kDa protein (EMAP) with no sequence homology with any other identified MAPs. However, EMAP exhibits a significant homology with β subunits of heterotrimeric G proteins (29).

Gβγ Binds to Microtubules at Regular Intervals, when Used to Promote Assembly—The suggestion that Gβγ might be behaving as a MAP for the promotion of microtubule polymerization elicited the question whether the binding of Gβγ to microtubules resembled that seen with MAPs. A polyclonal antibody directed against the β γ subunit was used to determine the nature of association of ββγ with microtubules assembled in the presence of β1γ2. βγ subunits were detected upon short projections which extended laterally from microtubules at regular intervals (Fig. 5). In addition, the β subunit was bound to some of the oligomeric structures. Similar structures, consisting of high molecular weight MAPs and tubulin, are frequently found in microtubule preparations and are suggested to be intermediate structures for microtubule formation (30). The decoration of microtubules by Gβγ appears to be strikingly similar to that observed by MAPs (31), suggesting perhaps, a similar site of activation of microtubule assembly by both agents.

Crude Microtubule Fractions Contain Gβγ—To assess the binding of βγ to microtubules in situ, βγ immunoreactivity was tested in crude microtubule fractions prepared from ovine brain homogenate. As shown in Fig. 6A, βγ was detected clearly in the crude microtubule preparation. In addition, βγ was detected in the unpolymerized cytosolic fraction (S2), although in lesser amount. Since particulate structures were removed prior to microtubule polymerization, the βγ associated with microtubules was present in the cytosol. The bulk of this βγ was able to associate with the crude microtubule fraction during the polymerization step (Fig. 6A).

Gβγ immunoreactivity in the S2 fraction may represent βγ species that do not bind to microtubules. Alternately, one or several MAP(s) may compete with Gβγ for microtubule binding, releasing some β1γ2 into the S2 fraction. This is supported by the observed association of βγ with the crude microtubule pellet. As shown in Fig. 6B, the β subunit is detected laterally on microtubule walls as a short projection. Note that the periodic association of Gβγ was seen when Gβγ-induced microtubule polymerization (Fig. 5) was not observed. Further, βγ was not detected in the two cycle purified microtubule fraction (microtubule protein), suggesting the loss of association of βγ with microtubules during the purification cycle.

In addition to regulating effector molecules, βγ subunits may play a role in macromolecular assembly (32). They enhance the association of a subunits with receptors. βγ binding to the pleckstrin homology domains of β-adrenergic receptor kinase (βARK), permits the translocation of βARK from cytosol to membrane and facilitates its association with target receptors (33, 34). The observed stimulation of microtubule assembly by β1γ2 further supports its role in the macromolecular organization of the cell. Although the assembly properties of βγ subunits are partly attributable to the WD-40 repeating units (a 40–43-amino acid tandem repeat usually punctuated with tryptophan-aspartate) present in the β subunit of the βγ complex (32), prenylation of γ subunits may play a crucial role in mediating the association between βγ and other proteins (35, 36). Furthermore, it appears from this study that prenylation...
of the γ subunit is critical for the interaction of βγ with tubulin for stimulation of microtubule assembly.

In mammalian cells, Gβ subunits have been associated with microtubules in mitotic spindles and with the plasma membrane (9). Spindle morphogenesis is associated with changes in the level of microtubule polymers and microtubule dynamics, and these two processes are tightly coupled (37). Thus, the association of Gβγ with microtubules in mitotic cells might represent a vehicle for coordination of microtubule assembly and dynamics, precise spatial and temporal control of the process is unknown. The data presented here point toward Gβγ as a physiological regulator for microtubule assembly and dynamics. These data further suggest a role for membrane-associated βγ, perhaps as a focal point for microtubule-membrane interaction. Exocellular signals to G protein-coupled receptors could modify a variety of microtubule-dependent events, using G proteins themselves as a second messenger. Regulation of microtubule assembly by Gβγ may provide an alternative pathway by which G proteins function in cellular signaling.

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