Tubulin Sequence Region β155-174 Is Involved in Binding Exchangeable Guanosine Triphosphate*

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Assembly-competent microtubule protein was directly photoaffinity labeled with [α-32P]guanosine triphosphate by UV irradiation. The labeled tubulin was digested with trypsin. The radioactive fragments were isolated and sequenced, revealing β-tubulin residues 155-174 to be the major labeled region. An antibody to a synthetic peptide comprising residues β154-165 inhibits GTP incorporation and tubulin polymerization.

Guanosine triphosphate is required for the assembly of microtubules (1). Two molecules of GTP are bound to the tubulin heterodimer (2, 3). One binds to an N site, where it is not exchangeable and is not involved in assembly in vitro (4). Nonexchangeable GTP labeled with 32P in cells in vivo remains unhydrolyzed after several cycles of assembly-disassembly in the presence of unlabelled GTP (5). At the other site, the exchangeable or E site, GTP is readily exchanged with added nucleotide and is hydrolyzed during incorporation into the microtubule (2, 4, 6).

However, tubulin can also polymerize in the presence of the nonhydrolyzable GTP analog guanyl-5′-y-l-imidodiphosphate (6, 7). The resulting microtubules are more resistant toward depolymerizing agents. Furthermore, GTP hydrolysis lags behind polymerization (8-10). Thus, the energy of hydrolysis is not required for polymerization. Possibly, GTP binding causes a tubulin conformation favorable for polymerization, whereas hydrolysis may be a consequence of cooperative interactions of the dimer with its neighbors and favor subsequent disassembly (11, 12).

Previously, the exchangeable site has been localized on the β-subunit with the photoaffinity analog 8-azido-GTP (13, 14) and by UV cross-linking with unmodified GTP (15, 16). Upon limited hydrolysis with chymotrypsin, the label is found on the amino-terminal fragment, comprising residues 1-281 (17, 18). Here we report that the β-tubulin sequence region 155-174 contributes to the binding site for exchangeable GTP.

EXPERIMENTAL PROCEDURES

RESULTS

Neither microtubule-associated proteins nor bound GTP was removed before covalent binding of [α-32P]GTP in order to minimize a possible denaturation of tubulin. This restricted the covalent incorporation of added [α-32P]GTP to 2% of the tubulin dimers. However, this reflects only the exchange of added radioactive GTP for already bound unlabeled nucleotide and not the total amount of nucleotide covalently incorporated into tubulin upon irradiation. The percentage of cross-linking is not known, but is probably much higher. To assess polymerizability of tubulin, one cycle of assembly-disassembly was carried out with the whole sample immediately before photoaffinity labeling, and with aliquots thereafter (Fig. 1). Irradiated tubulin polymerizes more rapidly than nonirradiated tubulin, but we observed the same final extent of assembly under all conditions tested, i.e. after 60 min of irradiation of tubulin without additional GTP, irradiation in the presence of a 20-fold excess of GTP over tubulin, as well as without irradiation.

Immediately after photoaffinity labeling, tubulin was separated from microtubule-associated proteins (MAPs)2 and unbound GTP by chromatography on DEAE-cellulose. The elution profile is shown in Fig. 2. While all of the MAPs and a small fraction of tubulin were found in the flow through, tubulin eluted at 0.4 M NaCl just behind the bulk of unbound GTP.

Gel electrophoresis and autoradiography of the labeled tubulin revealed that the label was bound exclusively to the β-subunit as demonstrated previously in more detail (15). Excess unlabelled GTP inhibited incorporation of the radioactive compound. Therefore, only fragments of the β-chain were considered as possible carriers of the label. Tubulin was then digested with trypsin, and the peptides were separated by gel filtration on Sephadex G-50 (Fig. 3). Two radioactive peaks were obtained at the very end of the absorption profile. Peak T1 contained 48% and peak T2 47% of the initial radioactivity. Further separation by HPLC anion exchange chromatography yielded one single radioactive peak originating from peak T2 (Fig. 4), while the radioactivity of peak T2 was split into four fractions (Fig. 4b). By sequence analysis of peak T1a, containing 44% of the radioactivity, we found residues β155-162 of the known amino acid sequence of porcine brain tubulin (26, 27). In peak T2a (16% of the total radioactivity) only α-peptide-305-308 could be identified, while peak T2c

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‡ Portions of this paper (including "Experimental Procedures" and Figs. 2-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-2436, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

1 The abbreviations used are: MAPs, microtubule-associated proteins; HPLC, high performance liquid chromatography; MBS, 4-morpholineethanesulfonic acid; EGTA, bis[ethylenediamineiminocarboxyethyl]tetracetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TFA, trifluoroacetic acid.
GTP Binding Sequence in β-Tubulin

Fig. 1. Assembly competence of tubulin before and after UV irradiation for 60 min. Tubulin samples (1 mg/ml) were incubated at 37°C, and the change in absorbance at 330 nm was monitored. All samples were preincubated on ice for 30 min either in the presence (Δ, ▲) or absence (○, ●) of 0.2 mM GTP. Of each pair of samples, one was UV-irradiated (open symbols), while the other was kept on ice without irradiation (closed symbols).

Fig. 5. Inhibition of GTP incorporation into microtubule (MT) protein upon UV irradiation by preincubation with IgG. ○, anti-α154–165; ●, preimmune IgG from the same animal; △, anti-α389–412. Immunoglobulin fractions were adjusted to equal titers to native tubulin.

(8% of the radioactivity) contained residues 163–174 of β-tubulin. No peptides were identifiable in peaks T2b and T2d (5 and 18% of the radioactivity, respectively). In summary, these experiments indicate that sequence region 155–174 of β-tubulin forms part of the GTP-binding site.

The exact labeled residue was not identified. The radioactivity remained on the filter upon degradation while the phenylthiohydantoins were extracted and identified. The bond between GTP and peptide was apparently sensitive to the degrading chemicals.

To confirm these findings by an immunological procedure, we generated an antiserum to a synthetic peptide comprising β-tubulin residues 154–165. The IgG fraction of the serum was isolated on protein A-Sepharose and was found to bind to native tubulin (data not shown). The IgG fractions of the preimmune serum and of an antiserum generated to α-tubulin residues 399–412 were used as controls throughout. The antibodies were adjusted to equal titers to native tubulin and incubated in various concentrations with tubulin prior to photoaffinity labeling with GTP. Antibody to β154–165 inhibited GTP incorporation into β-tubulin at low concentrations (Fig. 5), whereas the control antibodies were inhibitory only at higher concentrations. Neither antibody incorporated GTP, illustrating the specificity of the photoaffinity labeling procedure.

Only the antibody to β154–165 and none of the control antibodies impaired tubulin polymerization (Fig. 6). This confirms the results obtained by sequencing photolabeled peptides.

DISCUSSION

From our studies of direct photoaffinity labeling of tubulin with [α-32P]GTP, we conclude that the β-tubulin sequence region 155–174 directly interacts with GTP and forms part of the exchangeable GTP-binding site. An antibody generated to a synthetic peptide corresponding to a part of this region specifically reduces incorporation of the photolabel and inhibits tubulin polymerization in vitro. This is in agreement with most of the previous less specific experimental evidence (13–17, 28). Only in chemical cross-linking studies with GTP analogs, carrying highly reactive groups in the ribose moiety, were α- and β-tubulin found to be labeled to an approximately equal extent (29).

Two caveats should be kept in mind when considering and comparing photoaffinity labeled sequences. 1) Usually several sequence regions of a polypeptide are involved in the binding of a nucleotide (30–32). It is, therefore, not surprising that a different labeling method quite correctly yields another contact region (33). 2) Not all amino acid side chains are activated by UV irradiation to form reactive radicals. Moreover, the radi-als may not be as short lived as anticipated, and as a consequence the final covalent bond may not necessarily mark the immediately contacting residue but rather a particularly reactive one nearby.

We could not identify the exact labeled residue within a sequence of six, but it is tempting to speculate that the bond between GTP and tubulin is formed with tyrosine β-159. Tyrosine, like other aryl compounds, can form long lived radicals upon UV irradiation (34). Moreover, in studies of Manser and Bayley (35) removal of E site nucleotide caused specific changes in the near-UV circular dichroism of tubulin. These were attributed to changes in protein conformation involving the interaction between tyrosine residues and the guanine moiety.

Predictions of secondary structure by the methods of Chou and Fasman (36) and Garnier et al. (37) indicate that this tyrosine is the last residue of a short helix and the first of a reverse turn or loop. One should expect to see the essential residues of the binding site conserved in all species. However,

Fig. 6. Inhibition of tubulin polymerization in vitro by IgG from anti-β154–165. a, control polymerization without antibody. Preincubation with IgG from preimmune serum and with IgG from anti-α389–412 at a molar ratio of tubulin: IgG of 1:0.5 yielded identical results and is therefore not indicated separately. b, preincubation with IgG from anti-β154–165, molar ratio 1:0.25, c, same as b, molar ratio 1:0.5.
of the sequences available to date (apart from pig brain tubulin, they were all established on the nucleotide level), two appear to have the tyrosine exchanged for Phe (human 59) (38) and Leu (yeast) (39), respectively. On the basis of conservation, the important invariant residues seem to be Glu-157, Pro-160, Asp-161, and Arg-162. They may be in close proximity in the predicted helix-turn conformation.

Interestingly, there is another invariant region, HSLGGGTGSG at residues 137-146, ending only 13 residues upstream of Tyr-159. On the basis of sequence similarities with the Rossmann fold of dinucleotide-binding proteins (40), we have predicted it to be a triphosphate binding loop (27). Recent data, however, indicate that the function of the typical glycine loop resides rather in its high flexibility than in direct interaction with the phosphate groups, at least in two well-examined cases (30, 31), thereby controlling the access of the nucleotide to its binding site.

Kim et al. (33) have used 8-azido-GTP as a photoaffinity label to elucidate the sequence regions in β-tubulin involved in binding the exchangeable GTP and have found peptide-63-77 to be labeled by the photogenerated nitrene at position 8 of the purine base. From the combined results of the two investigations, a more complete image of the binding site emerges: residues in regions β-63-77 are binding to the purine moiety, whereas side chains in the region 155-174 may contact the ribose and a flexible loop in 137-146 may regulate the access to the binding site.

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GTP Binding Sequence in β-Tubulin

EXPERIMENTAL PROCEDURES

Tubulin was isolated from guinea brain by the cycles of assembly/disassembly (I) and stored at 70°C. Only before use the pellets were resuspended in reswelling buffer (100 mM NaCl, 1 mM MgCl2, 10 mM imidazole, 0.1 mM PMSF, and 10% glycerol). The assembly was carried through another cycle of assembly/disassembly. The pellet obtained was reassembled to 1 mg/ml and centrifuged for 20 min. The assembly competence of tubulin was tested by incubating, microtubule protein (1 mg/ml) at 37°C in reassembly buffer containing 8 mM glycerol and 0.1 mM ATP. The absorbance at 350 nm was measured at various time points.

Separation of the tubulin to fractions: The tubulin was separated by Sephadex G-50. The tubulin was mixed with 200 ml of 1% SDS (2M), New England Nuclear) and precipitated with 1 ml of ice for 30 min. The mixture was then poured on a plastic plate placed on ice and centrifuged at 11000 g at a distance of 3 cm for 45 min. The sample was concentrated by filtrating through the Cenosphere (3 ml) in 2.5 ml of the supernatant. The tubulin containing fractions, identified by SDS-polyacrylamide gel electrophoresis, were pooled, digested, and concentrated by lyophilization.

SUPPLEMENTARY MATERIAL TO TUBULIN SEQUENCE REGION 015-171

FIGURE 1. Separation of αI and αII tubulin on SDS-PAGE gel electrophoresis. The tubulin was separated in 5% gels of each fraction (25%)

FIGURE 2. Separation of αI and αII tubulin on Sephadex G-50. The tubulin was separated in 5% gels of each fraction (25%).

FIGURE 3. Separation of αI and αII tubulin on Sephadex G-50. The tubulin was separated in 5% gels of each fraction (25%).

FIGURE 4. PAGE analysis of the tubulin. The tubulin was analyzed by SDS-PAGE gel electrophoresis in 5% gels and stained with Coomassie blue. The tubulin was separated in 5% gels of each fraction (25%).

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