

Monoclonal antibodies localize the exchangeable GTP-binding site in β - and not α -tubulins

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A combination of several methods was used to localize the exchangeable GTP-binding site in the α -/ β -tubulin heterodimer: (i) direct photoaffinity labeling with [α - 32 P]GTP, (ii) specific labeling of α - and β -tubulin by tyrosylation and phosphorylation, respectively, and (iii) immunoprecipitation with specific monoclonal antibodies. Direct evidence was obtained that GTP binds exclusively to β - and not α -tubulins.

α -Tubulin	β -Tubulin	Direct photoaffinity labeling	Exchangeable GTP-binding site	GTP
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1. INTRODUCTION

The tubulin heterodimer consisting of α - and β -tubulin contains two GTP-binding sites [1]. At one site (E-site) the bound nucleotide is readily exchangeable with free GTP whereas no significant exchange occurs at the other, non-exchangeable GTP-binding site (N-site). While the N-site GTP appears to be an integral part of the tubulin dimer, hydrolysis of E-site GTP to GDP has been found to be a consequence, but not a prerequisite step for microtubule assembly [2,3]. Since nucleotide binding may be required to maintain a certain conformation of tubulin [3-5] it is of interest to localize the GTP-binding sites in the quaternary structure of the tubulin dimer. In all previous studies concerning the exchangeable GTP-binding site, GTP-analogues instead of GTP itself were used for the affinity labeling, and one result apparently contradicts the other: in one case using the photoreactive 8-N₃-GTP it was suggested that the bound nucleotide comigrates with the β -tubulin band in SDS-polyacrylamide gels [6,7]; in another case two different hydrolyzable GTP analogues modified in their ribose moiety were found to be almost equally associated with both the α - and β -subunit [8]. Therefore, we have used the direct photoaffinity labelling technique with non-derivatized GTP in

combination with a set of highly specific monoclonal antibodies directed to either α - or β -tubulin and here provide the direct evidence that only β - and not α -tubulin contains the exchangeable GTP-binding site.

2. MATERIALS AND METHODS

2.1. Preparation of tubulin and tubulin-tyrosine ligase

Tubulin was isolated through two cycles of assembly/disassembly [9] from a 125 000 \times g supernatant of bovine brain extract. Tubulin-tyrosine ligase was partially purified from the same source as in [10] with the modification that ADP-Agarose (P-L Biochemicals) was used for affinity chromatography.

2.2. Direct photoaffinity labeling

Direct photoaffinity labeling of tubulin by GTP was performed according to essentially the same procedures described for the labeling of myosin and actin by ATP [11,12]. Briefly, 30 μ g tubulin were preincubated at 0°C for 30 min with 0.2 mM [α - 32 P]GTP (New England Nuclear) in 50 μ l of a buffer containing 40 mM 2-(N-morpholino)ethanesulphonic acid (Mes), pH 6.4, 0.4 mM EGTA, 0.2 mM MgCl₂, and then irradiated by UV-light

(wavelength 254 nm) at a distance of 2–3 cm for 60 min unless otherwise indicated. For quantitation, the radioactivity of GTP covalently bound to tubulin was measured by the filter paper method [11].

2.3. Phosphorylation and tyrosylation of tubulin

For phosphorylation 105 μ g tubulin were incubated at 35°C for 20 min in the presence of 5 mM MgCl₂, 40 μ M [γ -³²P]ATP (Amersham) and 0.6 μ g of the catalytic subunit of the cAMP-dependent protein kinase (Sigma).

105 μ g detyrosylated tyrosine [13] were tyrosylated at 35°C for 15 min in a reaction mixture containing 25 mM Mes-KOH, pH 6.8, 150 mM KCl, 12.5 mM MgCl₂, 2.5 mM ATP, 1 mM DTT, 0.1 mM [³H]tyrosine (New England Nuclear) and an aliquot of partially purified tubulin-tyrosine ligase.

2.4. Gel electrophoresis and autoradiography

Tubulin subunits were separated on 7.5% SDS-polyacrylamide gels after carboxymethylation with iodoacetic acid [14]. ³²P was detected by autoradiography and ³H by fluorography [15] on Kodak X-ray films.

2.5. Immunological methods

For immunoblots the tubulin subunits were transferred from SDS-gels to nitrocellulose [16]. The nitrocellulose was then soaked in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% NaN₃ for 2 h to block all binding sites. Strips of this nitrocellulose were incubated for 3 h with 1:1000 dilutions of mouse monoclonal antibodies to either α - or β -tubulin (Amersham). After washing they were incubated overnight with a 1:300 dilution of ¹²⁵I-labelled goat anti-mouse antibody. Unbound goat antibody was removed by repeated washing. The radioactive peptide bands on the dried nitrocellulose paper were identified by autoradiography.

Immunoprecipitation was done with *Staphylococcus aureus* Cowan1 (SAC) cells (Calbiochem) [17] but the procedure used [18] was slightly modified: the tubulin samples labeled with [γ -³²P]-ATP, [α -³²P]GTP or [³H]tyrosine were boiled for 5 min in 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, 100 mM Tris-HCl, pH 6.8. After chilling on ice the samples were diluted 1:20 in 1% Triton X-100, 1% deoxycholate in Tris buffer so that the

final concentration of SDS was adjusted to 0.1% [18]. 2–4 μ g antibody was added to the samples (5.9 μ g tubulin/100 μ l) and the mixtures incubated at 4°C for 1 h. Then, 10 μ l washed SAC cells was added and the suspension gently shaken for 30 min at 4°C. The pellets were washed, boiled in SDS-sample buffer and, without carboxymethylation, subjected to SDS-polyacrylamide gel electrophoresis.

3. RESULTS AND DISCUSSION

Non-derivatized [α -³²P]GTP instead of GTP derivatives was used to localize the exchangeable GTP-binding site of tubulin by means of direct photoaffinity labeling for the following two reasons: (i) azido analogues of any nucleotide will bind not only to a binding site specific for the natural non-derivatized nucleotide but also, in a non-specific manner, to several other sites of the protein. (ii) GTP derivatives which contain the photo- or chemically reactive group at its ribose moiety instead of purine moiety may be less appropriate to use for the labeling of the GTP-binding site because it is the purine ring and not the ribose which determines the high specificity for the GTP binding to tubulin. Furthermore, to identify unequivocally the GTP-labeled peptide, we immunoprecipitated the labeled tubulin subunit by highly specific monoclonal antibodies, rather than simply relying on the apparent mobility of the labeled peptide(s) in SDS-polyacrylamide gels which may change due to the additional negative charge or conformation change upon GTP binding.

Fig. 1a shows the time course of the UV-induced covalent binding of GTP to tubulin. The incorporation of [α -³²P]GTP increased almost linearly at least up to 60 min. After 60 min, GTP was covalently linked to 7% of the tubulin dimers. The specificity of the GTP binding is demonstrated in fig. 1b: the addition of 10 mM non-radioactive GTP to the reaction mixture almost completely abolished the radioactivity incorporated into tubulin, while the slight labeling of several minor bands of higher M_r was not significantly affected, indicating that the binding to tubulin is highly specific.

Carboxymethylation with iodoacetic acid allows separation of α - and β -tubulin in SDS-polyacryl-

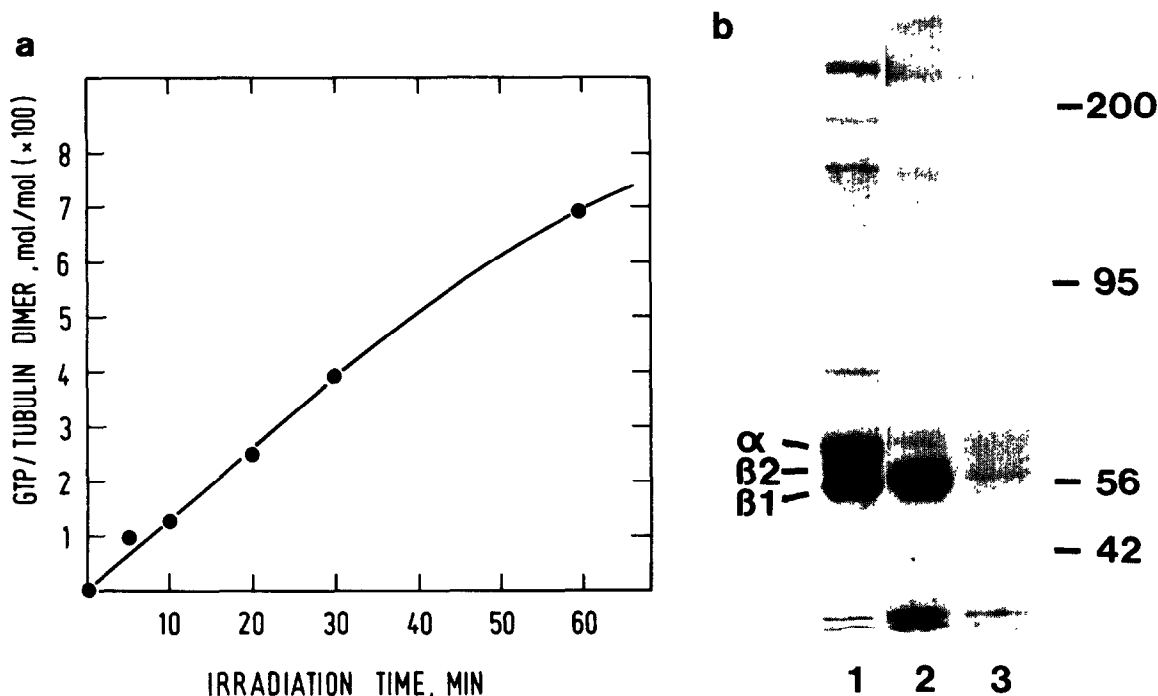


Fig.1. Covalent binding of GTP to the tubulin dimer by UV-irradiation. (a) Time course of the GTP-binding to the tubulin preparation. (b) SDS-polyacrylamide gel electrophoresis of [α - 32 P]GTP-labeled carboxymethylated tubulin. (Lane 1) Coomassie blue stain. (Lanes 2 and 3) Autoradiographs of tubulin samples irradiated in the presence of 0.1 mM [α - 32 P]GTP (lane 2) or in the presence of 0.1 mM [α - 32 P]GTP + 10 mM GTP (lane 3).

amide gels [19]. When chordate brain is used as the source of tubulin two β -tubulin components, the major β_1 -tubulin and a minor β_2 -tubulin, can be clearly distinguished from the more slowly migrating α -tubulin (fig.1b, lane 1) [20,21]. The specific labeling obtained by UV-irradiation of tubulin in the presence of [α - 32 P]GTP is distributed into two bands (fig.1b, lane 2). However, none of the obtained signals correlates exactly with any of the 3 peptide bands visible in the Coomassie blue stain: the predominant signal originates from just below the β_2 -band, the minor signal is located between β_2 - and α -tubulin. Although this labeling pattern might suggest that GTP is bound to the two β -subunits thereby causing a shift of their positions in the SDS-gel, several other possibilities could not be excluded. To eliminate at least the possibility of incomplete carboxymethylation we made use of the specificity of tyrosylation for the C-terminal end of α -tubulin [22,23]: the shift of all of the [3 H]tyrosine label after carboxymethylation to-

wards the position of alkylated α -tubulin clearly shows that no α -tubulin either remains in its original position or is present in the positions of alkylated β -tubulins (fig.2).

To obtain more direct evidence for the location of the exchangeable GTP-binding site we used a set of highly specific monoclonal antibodies directed to either α - or β -tubulin. As shown in fig.3, there is no cross-reactivity of the monoclonal anti- α tubulin with the two β -tubulins (lane 2) and the monoclonal anti- β tubulin recognizes only β_1 - and β_2 -tubulins (lane 3). When the pattern of [α - 32 P]-GTP-labeled tubulin (lane 4) is compared with those of the immunoblots in lanes 2 and 3 this again indicates that GTP is bound exclusively to the β -subunits although the bands do not exactly match with each other.

The subunit which contains the exchangeable GTP-binding site was directly identified by immunoprecipitating the labeled peptide(s) with monoclonal antibodies. As shown in fig.4, the

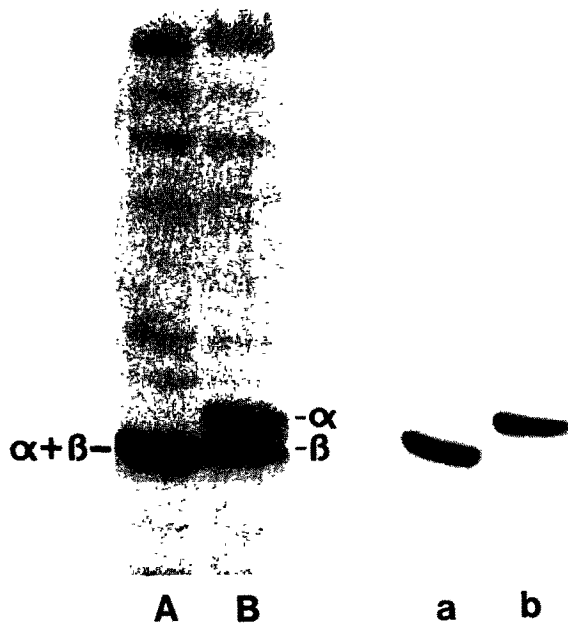


Fig.2. Identification of carboxymethylated α -tubulin in SDS-polyacrylamide gels by tyrosylation. Tubulin was tyrosylated by partially purified tubulin-tyrosine ligase with [3 H]tyrosine and separated on a 7.5% SDS-gel without (A,a) and after carboxymethylation (B,b). (A,B) Coomassie blue stain. (a,b) Fluorography of A and B, respectively.

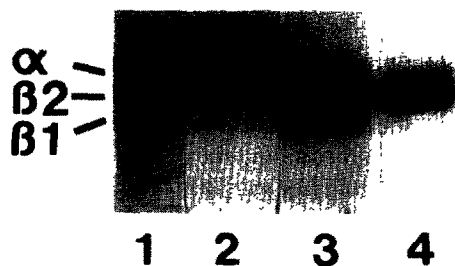


Fig.3. Specificity of monoclonal antibodies against α - and β -tubulins. Carboxymethylated tubulin was separated on a 7.5% SDS-gel and transferred to nitrocellulose. (Lane 1) Amido black stain; (lanes 2 and 3) autoradiographs of tubulin after treatment with monoclonal anti- α tubulin (lane 2) or monoclonal anti- β tubulin (lane 3) and [125 I]-labelled goat anti-mouse antibodies; (lane 4) autoradiograph of tubulin photoaffinity-labeled with [α - 32 P]GTP.

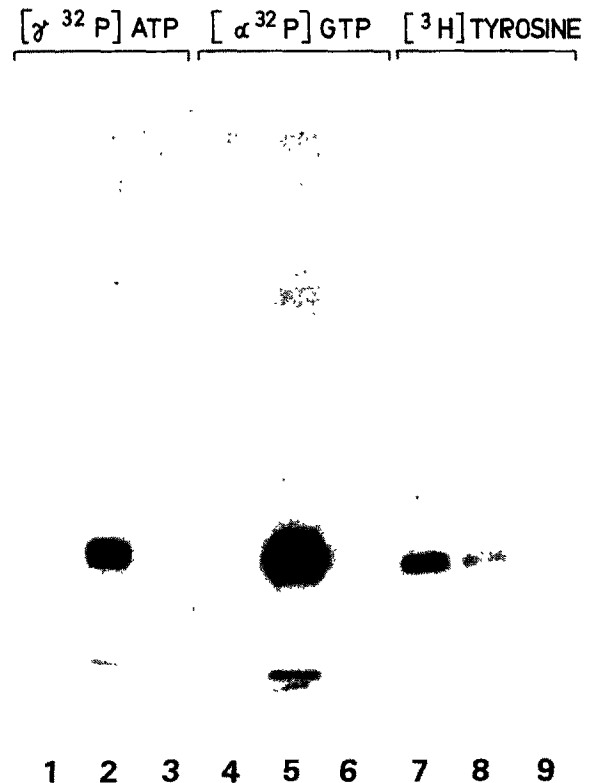


Fig.4. Immunoprecipitation of phosphorylated, [α - 32 P]-GTP-labeled and tyrosylated tubulin by monoclonal antibodies against α - and β -tubulins. [γ - 32 P]ATP-labeled (lanes 1-3), [α - 32 P]GTP-labeled (lanes 4-6) and [3 H]-tyrosylated tubulin (lanes 7-9) was precipitated in the presence of monoclonal anti- α tubulin (lanes 1,4,7), monoclonal anti- β tubulin (lanes 2,5,8) or only buffer for control (lanes 3,6,9).

[α - 32 P]GTP label was precipitated only by monoclonal anti- β tubulin and not by monoclonal anti- α tubulin (lanes 4-6). As an additional control, phosphorylation with [γ - 32 P]ATP by the catalytic subunit of the cAMP-dependent protein kinase [24,25] as well as tyrosylation with [3 H]tyrosine by the tubulin-tyrosine ligase [23] were used as specific markers for β - and α -tubulins, respectively. The [3 H]tyrosine label was precipitated mainly by monoclonal anti- α tubulin (lane 7). Only a small amount, however, was precipitated by anti- β -tubulin (lane 8). This may result from the incomplete dissociation of the tubulin dimers since cross-reactivity of the antibodies could be excluded (fig.3) and tyrosylation was shown to be specific

for α -tubulin (fig.2). A similar situation was observed in the case of phosphorylation (fig.4, lanes 1-3) where the ^{32}P label was predominantly precipitated by anti- β tubulin and only insignificantly by anti- α -tubulin. Both phosphorylation and tyrosylation, but not GTP binding, were performed at 35°C in the presence of Mg^{2+} -ATP which concomitantly induced microtubule assembly. This most likely prevented the complete dissociation of the tubulin dimer into its subunits.

These immunological data now directly show that the exchangeable GTP-binding site is indeed located exclusively on the β -subunit of tubulin. Based on a more indirect approach, a similar conclusion was reached in [7,26] by using GTP derivatives for the affinity labeling and determining the binding site by the apparent mobility of the labeled peptide in SDS-polyacrylamide gels. A preliminary report appeared recently which claimed that the non-exchangeable GTP-binding site is located on α -tubulin [27]. If this can be confirmed more directly, this and our present data together would clearly establish that the exchangeable GTP-binding site resides in β -tubulin, whereas the non-exchangeable GTP-binding site is located on α -tubulin.

Proteolytic digestion of the GTP-bound subunit, isolation of the GTP-bound fragment(s), and analysis of the amino acid composition and sequence we now underway and will make it possible to map the exchangeable GTP-binding site in the known amino acid sequence of brain β -tubulin [28].

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