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*

1. INTRODUCTION

The tubulin heterodomer 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-N_3-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 × *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 μg tubulin were preincubated at 0°C for 30 min with 0.2 mM [α-^{32}P]GTP (New England Nuclear) in 50 μl of a buffer containing 40 mM 2-(*N*-morpholino)ethanesulphonic acid (Mes), pH 6.4, 0.4 mM EGTA, 0.2 mM $MgCl_2$, and then irradiated by UV-light

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(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 μg tubulin were incubated at 35°C for 20 min in the presence of 5 mM $MgCl_2$, 40 μM [γ-^{32}P]ATP (Amersham) and 0.6 μg of the catalytic subunit of the cAMP-dependent protein kinase (Sigma).

105 μg 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 $MgCl_2$, 2.5 mM ATP, 1 mM DTT, 0.1 mM [^{3}H]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]. ^{32}P was detected by autoradiography and ^{3}H 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% NaN_3 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 ^{125}I-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* Cowanl (SAC) cells (Calbiochem) [17] but the procedure used [18] was slightly modified: the tubulin samples labeled with [γ-^{32}P]-ATP, [α-^{32}P]GTP or [^{3}H]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 μg antibody was added to the samples (5.9 μg tubulin/100 μl) and the mixtures incubated at 4°C for 1 h. Then, 10 μl 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-^{32}P]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 bindding 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-^{32}P]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-

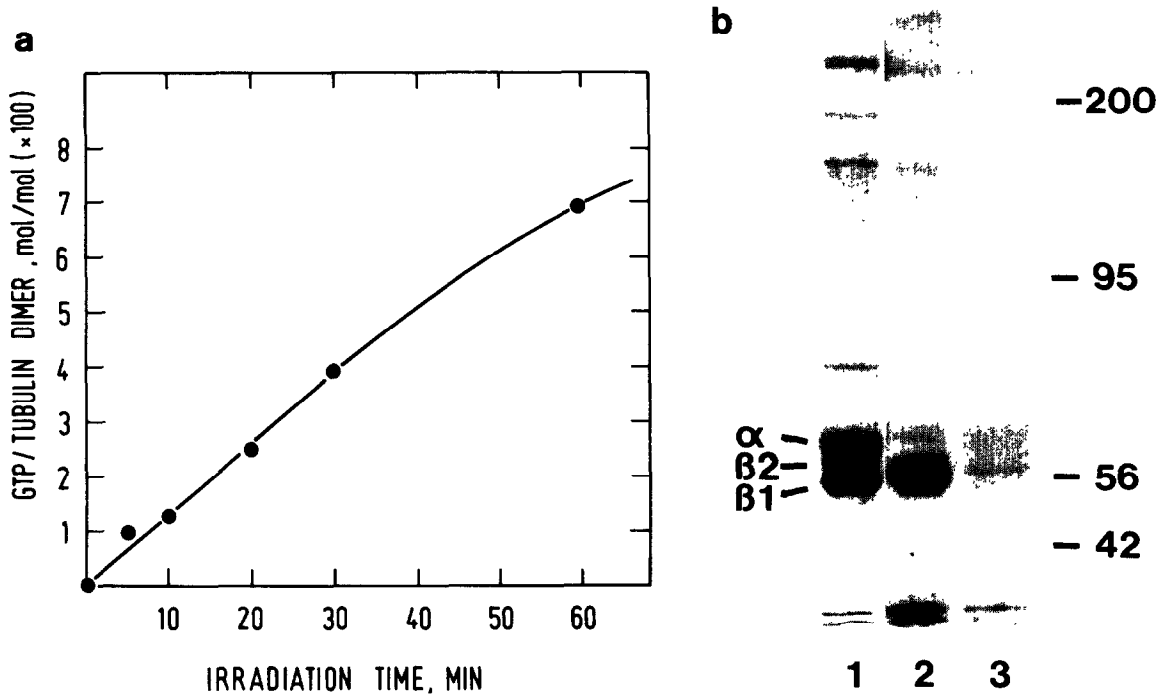

Fig.1. Covalent binding of GTP to the tubulin dimer by UV-irradiation. (a) Time course of the GTP-binding to the tubulin preparation. (b) SDS-polyacrylamide gel electrophoresis of [α-^{32}P]GTP-labeled carboxymethylated tubulin. (Lane 1) Coomassie blue stain. (Lanes 2 and 3) Autoradiographs of tubulin 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 towards 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

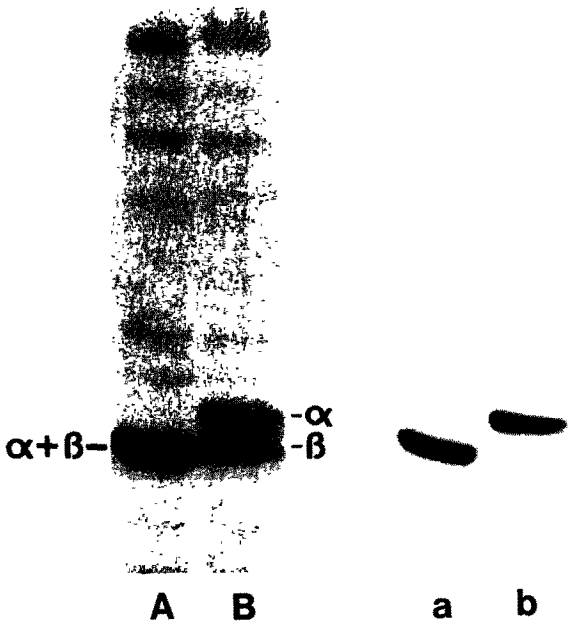

Fig.2. Identification of carboxymethylated α-tubulin in SDS-polyacrylamide gels by tyrosylation. Tubulin was tyrosylated by partially purified tubulin-tyrosine ligase with [^{3}H]tyrosine and separated on a 7.5% SDS-gel without (A,a) and after carboxymethylation (B,b). (A,B) Coomassie blue stain. (a,b)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

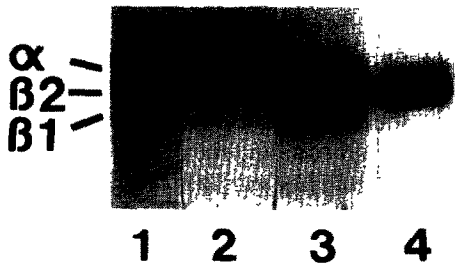

Fig.3. Specificity of monoclonal antibodies against α- and β-tubulins. Carboxymethylated tubulin was separated on a 7.5% SDS-gel and transferred to nitrocellulose. (Lane 1) Amido black stain; (lanes 2 and 3) autoradiographs of tubulin after treatment with monoclonal anti-α tubulin (lane 2) or monoclonal 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.

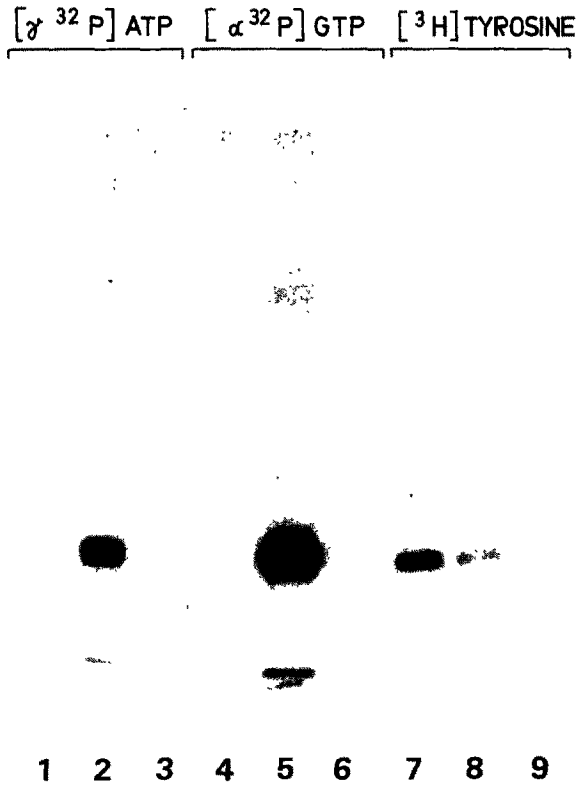

Fig.4. Immunoprecipitation of phosphorylated, [α-^{32}P]-GTP-labeled and tyrosylated tubulin by monoclonal antibodies against α- and β-tubulins. [γ-^{32}P]ATP-labeled (lanes 1–3), [α-^{32}P]GTP-labeled (lanes 4–6) and [^{3}H]-tyrosylated tubulin (lanes 7–9) was precipitated in the presence of monoclonal 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 precipitaed 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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REFERENCES

[1] Weisenberg, R.C., Borisy, G.G. and Taylor, E.W. (1968) Biochemistry 7, 4466–4479.
[2] Weisenberg, R.C., Deery, W.J. and Dickinson, P.J. (1976) Biochemistry 15, 4248–4254.
[3] Penningroth, S.M. and Kirschner, M.W. (1977) J. Mol. Biol. 115, 643–673.
[4] Spiegelman, B.M., Penningroth, S.M. and Kirschner, M.W. (1977) Cell 12, 587–600.
[5] Weisenberg, R.C. (1980) J. Mol. Biol. 139, 660–677.
[6] Geahlen, R.L. and Haley, B.E. (1977) Proc. Natl. Acad. Sci. USA 74, 4375–4377.
[7] Geahlen, R.L. and Haley, B.E. (1979) J. Biol. Chem. 254, 11982–11987.
[8] Maccioni, R.B. and Seeds, N.W. (1983) Biochemistry 22, 1572–1579.
[9] Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[10] Murofushi, H. (1980) J. Biochem. 87, 979–984.
[11] Maruta, H. and Korn, E.E. (1981) J. Biol. Chem. 256, 499–502.
[12] Maruta, H., Knoerzer, W., Hinssen, H. and Isenberg, G. (1984) Nature, in press.
[13] Martensen, T.M. (1982) Methods Cell Biol. 24, 265–269.
[14] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[15] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Kessler, S.W. (1975) J. Immunol. 115, 1617–1624.
[18] Mose-Larsen, P., Bravo, R., Fey, S.J., Small, J.V. and Celis, J.E. (1982) Cell 31, 681–692.
[19] Eipper, B.A. (1974) J. Biol. Chem. 249, 1407–1416.
[20] Little, M. (1979) FEBS Lett. 108, 283–286.
[21] Ludueña, R.F., Roach, M.C., Troka, P.P., Little, M., Palanivelu, P., Binkley, P. and Prasad, V. (1982) Biochemistry 21, 4787–4794.
[22] Raybin, D. and Flavin, M. (1975) Biochem. Biophys. Res. Commun. 65, 1088–1095.
[23] Raybin, D. and Flavin, M. (1977) Biochemistry 16, 2189–2194.
[24] Goodman, D.B.P., Rasmussen, H., DiBella, F. and Guthrow, C.E. jr. (1970) Proc. Natl. Acad. Sci. USA 67, 652–659.
[25] Eipper, B.A. (1972) Proc. Natl. Acad. Sci. USA 69, 2283–2287.
[26] Nath, J.P., Eagle, G.R. and Himes, R.H. (1983) J. Cell Biol. 97, 211a abstr.
[27] Steiner, M. (1984) Fed. Proc. 47, 2015 abstr.
[28] Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, W. and Ponstingl, H. (1981) Proc. Natl. Acad. Sci. USA 78, 4156–4160.