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Heterogeneity of Tubulin Subunits

(sea urchin/*Strogylocentrotus*/pig brain/acrylamide gel electrophoresis)

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ABSTRACT Tubulin, the subunit protein of microtubules, is a dimer that sediments at 6 S and has a molecular weight of 110,000. Using high resolution polyacrylamide gel electrophoresis, we have demonstrated the presence of two peptide chains, of molecular weight 56,000 and 53,000, in tubulin purified from brain. Two peptide chains of similar molecular weight were identified in each of the A- and B-tubulins isolated from flagella of sea urchin sperm. In all cases, the protein concentrations in the bands were equal. Each of the subunits ran as a single band when eluted from the gel and electrophoresed again in the same type of gel. Chromatography of purified brain tubulin on DEAE-Sephadex columns gave only a single peak containing both subunits in equal amounts.

Cyanogen bromide peptides were prepared from each of the bands after elution from polyacrylamide gel. While certain of the peptides appear to be common to both subunits, substantial differences exist between them. The tubulin dimer is composed of two nonidentical subunits.

The microtubule subunit protein, tubulin, has been isolated from sperm flagella, cilia, mitotic apparatus, and other sources; in each case it has been found to be a dimer with a molecular weight of about 110,000 (1–4) that can be dissociated into monomers of about equal weight by treatment with 8 M urea, 6 M guanidine·HCl, or detergents. There are 2 mol of guanidine nucleotide bound per dimer (4, 5) and 1 mol of colchicine may be bound per dimer of tubulin, except in the case of sperm-tail outer doublets, where binding activity is apparently absent (1, 6, 7).

Recent studies on the outer doublet microtubules of flagella (8, 9) have demonstrated that each of the tubules of the doublet is composed of a distinct tubulin moiety. A-tubulin, derived from the A-tubule, and B-tubulin, derived from the B-tubule, differ in isoelectric point, amino acid composition, and tryptic-peptide maps and can be thermally fractionated (8). In material from tetrahymena cilia, two bands are resolved by polyacrylamide gel electrophoresis (2) that may correspond to A- and B-tubulins. However, brain tubulin (4), A-tubulin or B-tubulin (8), and the tubulin from the central pair of sperm-tail microtubules (6) all migrated as a single distinct band. All of these earlier studies used discontinuous gels containing 8 M urea, but no detergents.

In this study, we have used high resolution, sodium dodecyl sulfate (SDS)-containing gels to examine tubulin subunits. We have resolved two bands that we believe represent subunits of the tubulin dimer rather than two different species of tubulin.

MATERIALS AND METHODS

Preparation of tubulins

Tubulin was prepared from pig brain by the batch method of Weisenberg, Borisy, and Taylor (4). A- and B-tubulins isolated by thermal fractionation (8) of the outer doublet microtubules of the sperm tails of *Strogylocentrotus droebachiensis* were the generous gift of Dr. Ray Stephens. Tubulins from mouse brain and neuroblastoma were prepared by vinblastine precipitation (10).

Disc gel electrophoresis

Two gel systems were used. The first (SDS–urea) is a discontinuous 8 M urea–SDS system in which the discontinuous Tris–glycine system (11) was modified by the inclusion of 8 M urea and 0.1% SDS in the running gel, spacer gel, and sample. The running gel contained 7.5% acrylamide and was 7 cm long. The spacer was 2.5% acrylamide, with a volume of 0.25 ml. Samples run in SDS–urea were denatured, reduced, and alkylated at pH 8.8 as described by Renaud *et al.* (2), and then made 1% in SDS. The SDS–urea system was used for all comparative studies of tubulins from different sources. Separation in this system is on the basis of molecular weight over a range of 30,000–150,000 (see Fig. 1). Gels were run at 3 mA/gel until the tracking dye reached the end.

We used, in addition, a discontinuous SDS system (ref. 12, and personal communication) that also separates on the basis of molecular weight. Samples contained SDS and

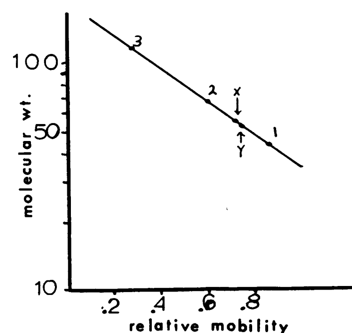

Fig. 1. Molecular weight calibration of the SDS–urea gel system. Standards are ovalbumin (*1*), albumin (*2*), and albumin dimer (*3*). The *X* subunit has an apparent molecular weight of 56,000 and *Y* has a molecular weight of 53,000.

Abbreviation: SDS, sodium dodecyl sulfate.

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mercaptoethanol, but were not alkylated. An acrylamide concentration of 7.5% was used to separate subunits, while 15% gels were used for peptide maps. Gels were 10 cm in length and were run at 1 mA/gel until the tracking dye reached the bottom.

For visual examination, gels were fixed in methanol–water–acetic acid 4.5:4.5:1 for 12 hr and stained either in 1% amido black or 0.1% coomassie blue. Destaining was by diffusion in 7% acetic acid.

Recovery of proteins from polyacrylamide gels

For all studies except peptide mapping, protein bands were cut out of fixed, stained gels, placed in the bottom of destaining tubes, and held in place by a glass rod. A small dialysis bag was tied around the bottom of the destaining tube. The band was then eluted electrophoretically using a 0.1% SDS–0.1 M sodium phosphate (pH 7) elution buffer at a current of 10 mA/gel slice for 1 hr. Recoveries ranged from 96–100%. The protein solutions in the dialysis bags were collected and concentrated by vacuum dialysis.

For subunit isolation, 12 gels were run under identical conditions in the second gel system (see above). One gel was fixed in 50% trichloroacetic acid in order to precipitate and visualize the bands. The other gels were then aligned with the fixed gel and cut into regions corresponding to the bands in the fixed gel. The slices were then eluted as described above and the purity of each band was checked by electrophoresis in the first system (see above).

CNBr peptide maps

Bands eluted from unfixed, discontinuous gels were reduced and alkylated in 8 M urea and then dialyzed for 24 hr against water to remove urea and SDS. The solution was made up to 70% in formic acid and a few crystals of CNBr were added. The solution was allowed to stand in the dark for 24 hr, then dried by evaporation with nitrogen. The specimen was resuspended and electrophoresed on 15% discontinuous SDS gels (system 2 above).

DEAE-Sephadex chromatography

A DEAE-Sephadex column (1×7 cm) equilibrated with 0.01 M MgCl–0.01 M phosphate (pH 6.5) was used. Elution was with 0.3 M KCl, followed by a gradient from 0.3 M to 0.8 M KCl.

Isoelectric focusing

Isoelectric focusing on polyacrylamide gels was performed by modifications of the techniques described by Wrigley (13). The gels contained 7.5% acrylamide, 1% ampholytes (LKB) ranging in pH from 3 to 10, 8 M urea, and 1 mM dithiothreitol. 0.5–1 mg of protein was dissolved in 8 M urea–1 mM dithiothreitol and was uniformly distributed throughout the gel. The gels were polymerized with ammonium persulfate. The final gel volume (1.5 ml) was contained in a 5-mm (inner diameter) glass tube (gel length, about 6 cm). The anodic and cathodic vessels were filled with 0.2% sulfuric acid and 0.4% ethanolamine, respectively. A maximum current of 1 mA/tube was maintained by gradually increasing the voltage up to 350 V. This voltage was maintained for at least an hour after the focusing pattern stabilized. Gels were removed from the tubes and protein zones were detected as white precipitation bands after immersion in 5% trichloroacetic acid. The gels were photographed by trans-illumination against a black background.

RESULTS

A-tubulin, B-tubulin, and brain tubulin, prepared either by the methods of Weisenberg *et al.* (4) or vinblastine precipitation (10), each gave a doublet pattern in a discontinuous urea–SDS gel (Fig. 2). The two bands have apparent molecular weights of 56,000 (subunit X) and 53,000 (subunit Y) (Fig. 1). Identical results were obtained with brain tubulins in gels without urea. When the X and Y bands were independently eluted from the gel and rerun individually and as a mixture, each of the singlets gave one band, while the mixture gave a double band (Fig. 3). Eluted bands comigrated with the homologous bands in SDS–urea.

Protein concentrations in the bands were determined by densitometry on gels stained with coomassie blue and by elution of the individual bands and colorimetric assay of protein by the method of Lowry (14). The densitometric scans revealed protein ratios of 6:5, 5:5, and 5:4 in three independent experiments. Colorimetric determination of protein in four separate experiments showed the protein concentrations to be within 5% of each other in all cases.

Since these results are consistent either with equal concentrations of subunits in a dimer or with equal quantities of individual tubulin species, we attempted to further fractionate brain tubulin on a DEAE-Sephadex A-50 column. Protein prepared by a modification of the method of Weisenberg *et al.* (4), in which 0.4 M KCl rather than 0.5 KCl is used for the first batch elution from DEAE-Sephadex, was used. This protein was eluted as a single discrete peak with no apparent splitting into two bands. The material eluted had a sedimentation velocity of 6 S and was eluted in the void

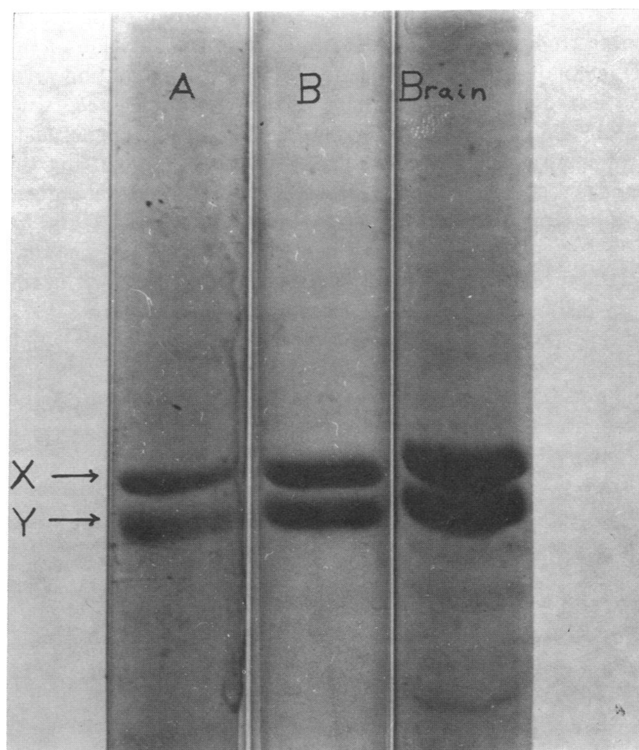

FIG. 2. Gel electrophoretic patterns of A-tubulin, B-tubulin, and brain tubulin on discontinuous SDS–urea gels, showing splitting into X and Y bands in each case.

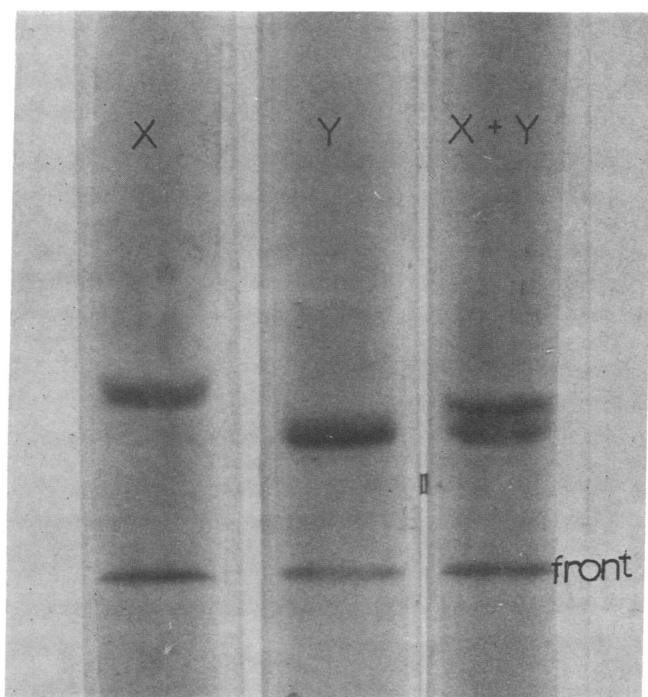

FIG. 3. Electrophoresis of the X and Y bands, and a mixture of X and Y, after preparation by elution from gels such as shown in Fig. 2.

volume from Sephadex G-100 column. Electrophoretic analysis of fractions taken from the forward edge, peak, and trailing edge of the peak (Fig. 4) all gave a doublet pattern, with the ratio of the protein in the bands about 1:1 in each case.

Cyanogen bromide peptides were prepared from bands eluted from unfixed gels. Electrophoretic examination of the X sample revealed a maximum of 5% contamination with Y. The Y sample was more heavily contaminated, with 15–20% X present. Due to the necessity of using unfixed proteins for this procedure, greater precision in cutting the bands was not possible. Nonetheless, marked differences can be seen between the peptide maps of X and Y (Fig. 5). The two samples also have a number of peptides that comigrate, but more detailed studies of the peptides are necessary before regions of homology can be established.

Isoelectric focusing of pig brain tubulin on polyacrylamide gels yielded two distinct bands, with isoelectric points of 5.5 and 5.7. However, when tubulin prepared by vinblastine precipitation from baby mouse brain (5-day-old) was examined in the same system, each of the major bands appeared to be split into two finer bands (Fig. 6).

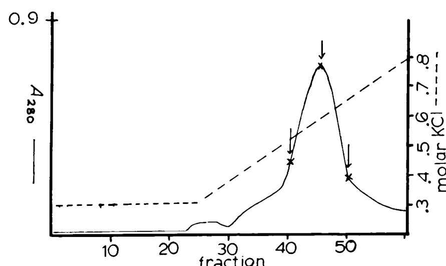

FIG. 4. Elution of tubulin from a DEAE-Sephadex column. *Arrows* indicate points at which samples were taken for electrophoretic analysis.

INTERPRETATION

The demonstration of two bands of similar molecular weight on electrophoresis of brain tubulin is open to several explanations. The two major possibilities are either that these represent two different species of tubulin, in analogy with the A- and B-tubulins of the sea urchin flagellum, or that these are nonidentical monomeric subunits that compose the tubulin dimer. Other possibilities include the artifactual generation of two bands from one by some mechanism such as the liberation of cyanide from urea during electrophoresis and the possibility that only one of the bands is tubulin, while the other is a nontubulin contaminant purified along with tubulin.

The possibility that these bands represent different tubulin species is rendered unlikely by the observation of similar bands in preparations of A- and B-tubulins. Since the A- and B-tubulins differ in both isoelectric point and tryptic peptides, it is also unlikely that these samples represent mixtures of one another in various proportions (8). The presence of double bands in tubulins from several clonal cell lines, including KB, L, HeLa, and neuroblastoma, also argues against the possibility that each of the bands has its origin in one or another of the major cell populations in the brain.

The differences in peptide maps between X and Y rule out

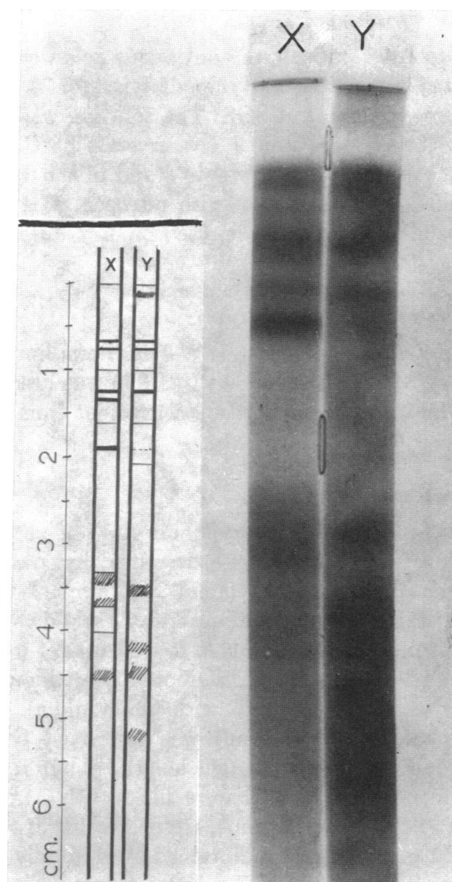

FIG. 5. Disc gel patterns of CNBr peptides prepared from X and Y bands eluted from SDS gels (system 2). *Inset*: graphic interpretation of photographic image.

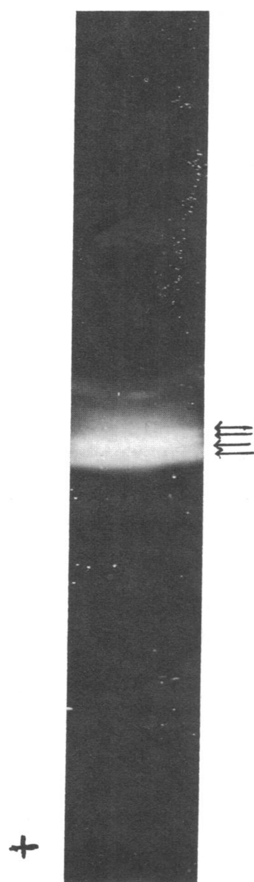

FIG. 6. Isoelectric focusing of tubulin prepared from 5-day-old mouse brain by vinblastine precipitation. Note splitting of each of the major bands.

the possibility that one of these species is derived from the other by some artifactual means, such as removal of a single peptide. This possibility is further argued against by the preservation of the single band when eluted samples are rerun in the same or the alternate system. It is also unlikely that one of the bands is a contaminant, since both A- and B-tubulins are derived from what are apparently pure preparations of microtubules. The similarity of a portion of the bands in the CNBr maps also makes this unlikely.

Thus, it would appear that the best interpretation of these observations is that the X and Y bands represent nonidentical subunits of the tubulin dimer and that subunits of molecular weights identical to those of X and Y exist in all tubulin species. This view is supported by the equality of protein concentration in each of the bands and by the fact that neither further resolution of the bands nor changes in their concentration ratio was achieved by chromatography on DEAE-Sephadex. These findings do not conclusively rule out the possibility of XX or YY dimers that were not resolved on the DEAE-Sephadex column and are present in equal concentrations.

The CNBr peptide maps support the nonidentity of the subunits. No attempt has been made to resolve the maximum number of peptides obtainable in each case; the maps are presented solely to establish differences between the subunits. It is of interest that the native tubulin dimer has been reported to have 1 mol of exchangeable GTP bound per 110,000 daltons and 1 mol of nonexchangeable GTP per 110,000 daltons (4). These observations are consistent with 1 mol of GTP bound per monomer and with nonidentity of the monomers. Furthermore, only 1 mol of colchicine is bound per 110,000 daltons (1), raising the possibility that only one of the monomers possesses colchicine-binding activity. It will be necessary to find gentle conditions to separate the monomers before the ligand binding can be correlated with the subunits.

We feel that the results presented here strongly support the hypothesis that the X and Y bands are nonidentical monomers that form the tubulin dimer and that a similar subunit structure is present in various tubulin species. Our findings do not argue against the presence of more than one species of tubulin, each with X and Y subunits, in brain. Indeed, the presence of four bands in the isoelectric focusing pattern of immature mouse tubulin raises this possibility.

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