Brain and Erythrocyte Microtubules from Chicken Contain Different \(\beta\)-Tubulin Polypeptides*

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\(\beta\)-Tubulin subunits isolated from chicken brain tissue and erythrocytes are distinguishable as unique biochemical species by electrophoretic and peptide mapping procedures. 1) The subunits of \(\beta\)-tubulin exhibit major differences in electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels that vary according to the pH and ionic strength of the gel. 2) The isoelectric points of urea-denatured \(\beta\) subunits from brain tissue and erythrocytes are pH 5.1 and 5.4, respectively, whereas those of both \(\alpha\) subunits are approximately pH 5.2. 3) Two-dimensional peptide maps prepared with \(\alpha\)-chymotrypsin or V8 protease show that \(\alpha\)-tubulin peptides are indistinguishable, whereas \(\beta\)-tubulin peptides are very different. Only one-third of the 15 major tyrosine-containing \(\beta\)-tubulin peptides prepared with \(\alpha\)-chymotrypsin are common to both \(\beta\)-tubulin species. The data indicate that the \(\beta\)-tubulin subunits of brain tissue and erythrocytes are biochemically distinct and may be different gene products. The presence of tubulin variants in brain tissue and erythrocytes may indicate special requirements for microtubule assembly and function in different cell types.

Chicken erythrocytes contain a circumferential band of microtubules that coils around the periphery of the cell close to the cell membrane. The band of microtubules is thought to generate and maintain the discoid shape that is characteristic of these and many other blood cell types. For discussions of the occurrence, properties, and function of microtubule bundles in blood cells see Behnke (1) and White (2). Recently we isolated microtubule protein from chicken erythrocytes by an in vitro assembly procedure, compared it to microtubule protein from chicken brain, and found major differences in protein composition and assembly properties. 1) Microtubule protein isolated from chicken brain by two cycles of in vitro assembly-disassembly contains 70% tubulin, 15% high molecular weight tubulin-associated proteins, and other trace components including (55,000–70,000 \(M_r\)) \(\gamma\) factors. Comparable material from chicken erythrocytes contains >95% tubulin with small amounts of \(\sigma\) polypeptides, hemoglobin, and trace amounts of spectrin-like polypeptides but no microtubule-associated proteins. 2) Assembly of erythrocyte microtubules in vitro at 37 \(^\circ\) C is distinguished from chicken brain material by low levels of nucleation (despite an abundance of ring oligomers at 5 \(^\circ\) C), longer lag times, slower assembly rates, and microtubules that are twice as long as brain microtubules assembled under identical conditions. The polypeptide composition and assembly characteristics of erythrocyte tubulin are described in a separate paper. These findings prompted us to examine whether there were chemical differences between the two tubulin species, since it would be informative to relate differences in tubulin composition and structure to differences in microtubule assembly and function. A number of tubulin variants have been identified by isoelectric focusing (3, 4), polyacrylamide gel electrophoresis (5–9) chromatography on hydroxylapatite (10), amino acid modifications (11, 12), intramolecular cross-linking (13), peptide mapping (14–16), amino acid sequencing (17), and in vitro assembly methods (18, 19), to name only a few. Yet the modifications that occur in some of these variants are subtle, and in many cases there has been the problem of isolating the tubulin in suitable quantity for in vitro studies. The attractive features of erythrocyte tubulin are that its cellular origin and function are known and that it can be purified in gram amounts.

In this paper we show that the \(\beta\)-tubulin subunits from brain tissue and erythrocytes have different electrophoretic mobilities and isoelectric points and that these differences may be due to alterations in primary structure as revealed by two-dimensional peptide mapping. In contrast, the \(\alpha\)-tubulin subunits could not be differentiated by these procedures. Based on these observations we consider the possibility that the \(\beta\) subunits in brain tissue and erythrocytes are different gene products and that they form tubulin variants that are biochemically distinct.

**MATERIALS AND METHODS**

**Isolation of Microtubule Protein**

Microtubule protein was isolated by two cycles of in vitro assembly and disassembly from chicken brain by the procedure of Dentler et al. (20). The assembly buffer was 0.1 M Pipes, \(pH 6.94\), containing 1 mM \(\text{MgCl}_2\), 2 mM EGTA, 1 mM GTP, and 4 mM glycerol. Microtubule protein was isolated from chicken blood erythrocytes in phosphate/glutamate buffer by a modification of the procedure described by Asnes and Wilson (21) which is summarized briefly as follows. Red cell pellets were resuspended in an equal volume of the assembly buffer (20 mM Na phosphate, 100 mM Na glutamate, \(pH 6.75\)) supplemented with 2 \(\times\) strength assembly factors (2 mM EGTA, \(\text{MgCl}_2\) and dithiothreitol, and 0.2 mM GTP), brought to 5 \(^\circ\) C and sonicated to disrupt the cells (Bronson sonifier, standard tip, full power output). Cell extract was prepared by centrifugation (30,000 \(\times\) g, 30 min).

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2. The abbreviations used are: PIPES, piperazine-\(N,N'\)-bis(2-ethanesulfonylic acid; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N,N'\),\(N'',N''\)-tetraacetic acid; SDS, sodium dodecyl sulfate.
P-Tubulin Variants

Fig. 1. Identification of α- and β-tubulin polypeptides. Samples of microtubule protein purified by two cycles of in vitro assembly-disassembly from chicken brain (B) and erythrocytes (E) were fractionated by SDS-gel electrophoresis at pH 8.3 and 9.1. Electrophoresis at pH 8.3 resolves the tubulin subunits of erythrocytes but not those of brain, while at pH 9.1 the subunits of both tissues are resolved. The erythrocyte polypeptides were identified as α- and β-tubulin by the immunoblotting method of Towbin et al. (33) using 125I-labeled rabbit antiserum to chicken brain tubulin. The autoradiogram labeled blot shows that both of the subunits fractionated at pH 9.1 are immunoreactive components.

 SDS-Polyacrylamide Gel Electrophoresis

Laemmli Gel, pH 8.3—Slab gels containing 12% acrylamide were prepared by the method of Laemmli (23) except that 0.4% dithiorthtartramide was used as the cross-linker (24). Acrylamide and diallyltartardiamide were obtained from Bio-Rad. SDS (99% as sodium dodecyl sulfate) was obtained from British Drug House. Gels were stained in Coomassie blue and destained as described by Fairbanks et al. (25).

Laemmli Gel, pH 9.1—Slab gels at pH 9.1 were prepared using the above procedures, only the resolving gel was prepared at pH 9.1 as employed by Cleveland et al. (26). SDS (99% as sodium dodecyl sulfate) was obtained from Bio-Rad.

0.1 M Phosphate Gel, pH 7.2—SDS tube gels containing 5% acrylamide were prepared by the method of Shapiro et al. (27) as described by Weber and Osborn (28). SDS (95%) and acrylamide were from Sigma.

Cleveland Gels—Partial digestion of α- and β-tubulin gel bands was performed as described by Cleveland et al. (29) using Staphylococcus

ng Protease

0 50 300 1000

Fig. 2. Two-dimensional gel of brain and erythrocyte tubulin based on the isoelectric focusing procedure of O’Farrell (30). Samples of phosphocellulose-purified, urea-denatured tubulin (purity >99%) were fractionated by isoelectric focusing in the first dimension followed by electrophoresis in SDS at pH 9.1 in the second dimension. The pH gradient in the focusing gel was determined by slicing control gels into twenty 0.5-cm segments, homogenizing the segments in 1-ml aliquots of distilled water and recording the pH. Erythrocyte and brain tubulin are shown in top and bottom, respectively. In both, β-tubulin is the faster migrating (lower) spot. The procedure was repeatable to within 0.05 pH unit.

Fig. 3. Analysis of tubulin peptides after partial digestion with V8 protease. Microtubule protein was fractionated by SDS-gel electrophoresis at pH 8.3, and gel bands made visible by brief staining in 1% aqueous Coomassie blue were cut out and electrophoresed on a second gel in the presence of varying amounts of Staphylococcus V8 protease which is indicated for pairs of erythrocyte and brain samples across the top of the figure. For each panel the peptides of a single α or β subunit from erythrocytes are compared with the combined peptides of both α and β subunits from brain. Brain tubulin peptides are compared with the erythrocyte α subunit in a and with the β subunit in b.
β-Tubulin Variants

V8 protease (Miles, Elkhart, IN). Details are presented in the legend to Fig. 3.

Carboxymidomethylation of Protein Samples—In some cases tubulin samples were carboxymidomethylated at pH 8.4 with iodoacetamide by the procedure of George et al. (3) prior to fractionation by SDS-gel electrophoresis.

Determination of Isoelectric Point

The isoelectric points of native tubulin dimers were determined by analytical electrofocusing in 0.5 mM thin layer agarose gels (LKB Instruction 1818A, LKB Produkt AB, Bromma, Sweden) using GelBond polyester support film (FMC Corporation, Rockland, ME) and a 3–10 pH gradient of ampholines. The isoelectric points of urea-denatured α- and β-tubulin subunits were determined by the two-dimensional electrophoretic procedure of O'Farrell (30). We used the modifications described by George et al. (3) for the isoelectric focusing of tubulin in tube gels (pH range 3–10).

Two-dimensional Peptide Mapping

Brain and erythrocyte tubulin samples were fractionated into α- and β-tubulin polypeptides by SDS-gel electrophoresis on Laemmli gels at pH 9.1. The bottom portion of each band was excised from the gel, iodinated by the chloramine-T method, and treated with α-chymotrypsin or V8 protease to produce peptides exactly as described by Elder et al. (31). The yield of counts as peptides from the gel slices was 80–90%. Two-dimensional peptide maps were prepared by electrofocusing and ascending chromatography as described by Elder et al. (32). For peptide mapping we used No. 5502 cellulose thin layer plates (E. Merck, Darmstadt, Germany).

Identification of Tubulin Polypeptides by Immunoblotting

Tubulin subunits were identified by examining the binding of tubulin antibody to tubulin subunits that were fractionated by SDS-gel electrophoresis and blotted by electrophoretic transfer onto nitrocellulose sheets by the method of Towbin et al. (33).

![Peptide maps of α- and β-tubulin made with α-chymotrypsin](image)

Peptides were prepared from iodinated gel slices of α- and β-tubulin subunits and two-dimensional maps were prepared by the method of Elder et al. (31, 32). Autoradiograms reveal tyrosine-containing peptides as the major spots. A large number of spots of uniform intensity are generated, since α-chymotrypsin cleaves proteins at tyrosine residues. Pairs of autoradiograms with sketches showing the best fit of the maps show complete maps for α- and β-tubulin from brain and erythrocytes. The positions of erythrocyte tubulin peptides (solid lines) and brain tubulin peptides (dotted lines) are indicated. The origins are visible in the lower left corners of the maps. Electrophoresis was performed in the horizontal direction in formic acid/acetatic acid with the cathode to the right. Ascending chromatography was performed in the vertical direction with butanol/pyridine/acetic acid.
Enzyme digestion with the following solutions: 1) 10 mM sodium phosphate, pH 7.5, with 0.15 M NaCl, 1 mM EGTA, 0.2% Triton X-100, and 1 mM NaN_3 (PTX) containing 4% Bovine serum albumin (30 min); 2) dilute antibody (1-2 µg/ml) in PTX4% BSA (overnight); 3) the following three rinses: PTX (4 times, 5 min each wash); 0.1 M glycine containing 2 M urea and 1% Triton X-100 (15 min); PTX (2 times, 5 min each wash); 4) PTX containing 125I-labeled protein A (6.7 x 10^5 cpm/ml for 1-2 h); 5) the rinsing steps described in 3 above. For autoradiography the nitrocellulose strips were dried on a slab gel drier, exposed to X-Omat AR x-ray film (Kodak) with an intensifier screen at -80°C for 30 min and developed in GBX developer (Kodak). Parallel strips were stained for protein immediately after electrophoretic blotting with Amido schwartz (34) as modified by Matus et al. (36).

**RESULTS**

**Differences in Electrophoretic Mobility on SDS-Polyacrylamide Gels**

Laemmli Gel, pH 8.3—On standard Laemmli slab gels the β subunit of erythrocyte tubulin migrated considerably faster than the corresponding polypeptide from chicken brain (Fig. 1). In contrast both α subunits appeared to have nearly identical electrophoretic mobilities. (On close examination each of the α and β bands could be resolved into two bands with the faster migrating bands representing 90% of the Coomassie staining material in both cases). The overall impression was that the tubulin subunits from erythrocytes were widely separated and clearly resolved whereas those from brain tissue were not resolved and ran essentially as a single band. We therefore examined the mobility of tubulin on two additional gel systems.

Laemmli Gels, pH 9.1—On this system the α and β subunits of both brain and erythrocyte tubulin are clearly resolved; however, the erythrocyte β-tubulin subunit still migrated somewhat faster than the β subunit from brain.

0.1 M Phosphate Gel, pH 7.2—On this gel the α and β subunits from both tissues migrated together as a single band (not shown).

We isolated soluble (Triton-extractable) tubulin and membrane-associated tubulin (not extractable from ghosts after lysis with 0.5% Triton) from erythrocytes using Vallee’s taxol-supported microtubule assembly procedure (36) to compare the mobility of tubulin isolated from these two intracellular sites with that of tubulin in whole cell homogenates and with tubulin prepared by cycles of in vitro assembly. The pronounced separation of α and β subunits was observed on Laemmli slab gels for all four preparations. Thus, the in vitro assembled tubulin was representative of total erythrocyte tubulin pool and was not a special tubulin species that was selected for by the in vitro polymerization procedure.

**Isoelectric Point**

We examined the isoelectric points of urea-denatured α- and β-tubulin subunits from brain and erythrocytes on isoelectric focusing gels and established the identity of α and β subunits on two-dimensional gels (Fig. 2) prepared by the method of O’Farrell (30). The α-tubulin subunits had similar but not identical isoelectric points (pH 5.1 and 5.2 for brain and blood, respectively); however, this small difference may simply be due to experimental error). In contrast, the isoelectric points of the β-tubulin subunits were very different (pH 5.1 for brain β-tubulin and pH 5.4 for erythrocyte β-tubulin). Thus, β-tubulin is more acidic than α-tubulin in chicken brain but is more alkaline than α-tubulin in chicken erythrocytes. Some heterogeneity was observed for the erythrocyte β subunit and for the other subunits on these focusing gels. However, because tubulin is not very soluble in 1-2% ampholines even in urea (37), the significance of these trace variants is difficult to evaluate.

For native dimers the isoelectric points of both tubulin species were determined to be pH 5.8 (not shown).

**Antibody Cross-reactivity**

To confirm that the anomalous β-tubulin subunit from erythrocytes was really tubulin, we examined its ability to cross-react with a rabbit antibody which was determined previously by Cleveland et al. (26) to bind to both the α and β subunits of chick brain tubulin. As seen in Fig. 1, the antibody binds to both the α and β subunits of both brain and erythrocyte tubulin that have been separated by SDS-gel electrophoresis at pH 9.1.

**Analysis of Tubulin after Partial Proteolytic Digestion**

To confirm that the erythrocyte β subunit was tubulin by a direct biochemical test, we used the method of Cleveland et al. (29) for comparing the electrophoretic mobilities of partially digested polypeptides on SDS gels. In Fig. 3 we show
the peptides generated by exposure to increasing concentrations of Staphylococcus V8 protease. In Fig. 3a we compare the peptides of erythrocyte α subunit with the total tubulin (α and β bands) of brain. It is clear that there are several peptides with identical electrophoretic mobilities for each of the digests that were prepared. This confirms that a polypeptide very similar to erythrocyte tubulin is also present in the brain tubulin sample. In Fig. 3b we compare the peptides of erythrocyte β subunit with the peptides from both the α and β tubulin subunits from brain. In this case, the result is less distinct. Peptides with identical mobilities as well as unique peptides (designated □) can be identified. This result establishes a relationship of the β subunit from erythrocyte to brain tubulin but also suggests there may be differences in primary structure that distinguish the erythrocyte β subunit.

Two-dimensional Peptide Mapping

We compared two-dimensional maps of tyrosine-containing peptides of α- and β-tubulin subunits using the procedures described by Elder et al. (31, 32) for iodinating gel slices with chloramine-T and preparing peptide maps on cellulose thin layer plates. The results for digestion with α-chymotrypsin and V8 protease are shown in Figs. 4 and 5. For both proteases, the maps for α-tubulin appear nearly identical. The β-tubulin maps, however, showed marked differences for both proteases. For the α-chymotryptic maps only 5 of the approximately 15 major spots were similar; brain β-tubulin contained 7 major unique spots and erythrocyte β-tubulin contained 5 major unique spots. It is likely that these maps represent complete proteolytic digestion because the same patterns were observed for protein digested for different lengths of time and with different amounts of protease. Similar results were also obtained for a different protease, V8 protease from Staphylococcus (Fig. 5).

DISCUSSION

The β-Tubulin Subunits from Brain and Erythrocyte May Be Different Gene Products

We have shown that the β-tubulin polypeptides contained in brain and erythrocytes can be distinguished by differences in their electrophoretic mobilities, isoelectric points, and peptide maps. The peptide maps are the most compelling reason for considering these tubulin polypeptides as biochemically distinct species. This conclusion appears to be sound, since essentially the same maps were obtained when the procedure was repeated using different proteases and digestion times and different tubulin:protease ratios.

It is possible, although unlikely, that the different β-tubulin maps are due to extensive post-translational modifications that selectively affect the β-tubulin subunit. However, the covalent modifications that are known to occur such as phosphorylation of the β subunit (11) and tyrosination of the α subunit (12) each involve only one amino acid residue and are probably insufficient to account for the unique mapping positions of so many peptides. (Of 15 major peptides produced in brain β-tubulin by α-chymotrypsin, only 5 were common to both β-tubulin subunits.) The fact that the α subunits have identical peptide maps further suggests that post-translational modification is not the principal cause for these differences.

It is also unlikely that modification occurs through terminal cleavage of the polypeptides. Since the chain weights of both β subunits are similar, one would expect to see only minor changes in the map by this mechanism, such as the deletion of one or two peptides from one of the maps, and this is clearly not the case.

These preliminary results suggest it is possible that the β-tubulins may be the products of different genes. This hypothesis could be tested by determining the nucleotide sequence of β-tubulin mRNA obtained from immature chicken erythrocytes. The corresponding amino acid sequence of the erythrocyte polypeptide could then be compared with the sequence that has already been determined for the β-tubulin subunit from chicken embryo brain by Valenzuela et al. (38). If differences in primary structure were confirmed, it would indicate that chicken brain tissue and erythrocytes possess different tubulin variants containing unique β-tubulin subunits.

The Occurrence of Different Tubulin Polypeptides

From tubulin gene cloning and hybridization studies, it is known that the number of β-tubulin genes in the eukaryotic genome ranges from possibly as few as 1 in the case of yeast to approximately 20 in sea urchin (39). In both chicken (39) and Drosophila (40, 41) 4 α- and 4 β-tubulin genes have been identified. In humans there may also be as many as 10 β-tubulin genes (39), but some of these appear to be nonfunctional pseudogenes (42, 43). At the present time the functions and tissue and cell specificity of the various tubulin polypeptides are incompletely understood. Kalfayan and Wensink (44) have determined that each of the four α-tubulin genes in Drosophila is transcribed, suggesting that the developmental sequence of tubulin gene expression may be complex. However, from a combination of genetic and biochemical analysis of mutants in suitable organisms, it may be possible to determine the function and significance of the different tubulin species.

In Aspergillus, β-tubulin mutants have been selected that affect nuclear migration and sensitivity to the drug benomyl (45-47). From preliminary studies in Drosophila it appears that there may be differential expression of β-tubulin genes for meiosis (48, 49) and for embryonic development (50) which are distinct from the β-tubulin gene that is expressed in adult somatic tissues. However, to understand the functions of these different tubulin gene products, it will be necessary to isolate and purify the tubulin variants and examine their properties in vitro. In these respects the erythrocyte tubulin dimer is an attractive protein for further investigation, because its origins and functions are well defined and because it can readily be obtained in large quantities for biochemical study.

Since brain β-tubulin has been shown to contain a number of variants, it is possible that the erythrocyte β-tubulin variant corresponds to one of the brain β subunits and is therefore not unique to erythrocytes. Clearly, our observations do not rule out this possibility. However, by isoelectric focusing we did not observe a pH 5.4 β variant in brain microtubules nor did we see traces of the erythrocyte β peptides by two-dimensional peptide mapping of brain β tubulin. Therefore, it seems likely that if the erythrocyte variant occurs in brain tissue it probably is present in only small amounts.

Microtubule Assembly and Function in Erythrocytes

The erythrocyte microtubule band is a unique structure, consisting of long microtubules in a compact coil closely apposed to the cell membrane. Preliminary studies show that the polymerization of erythrocyte microtubule protein in vitro is characterized by reduced levels of nucleation, resulting in long, stable microtubule polymers (51). These properties are distinct from neuronal microtubules and closely resemble the properties of microtubules in intact erythrocytes, but so far it has not been determined if the differences in neuronal and

3 D. Botstein, unpublished observations.
erythrocyte microtubules are due to the tubulin dimers or are due to other factors such as microtubule-associated proteins. Questions of even greater complexity, such as whether these tubulin variants make copolymers or form biochemically distinct microtubules during polymerization, have not yet been investigated. From further in vitro studies it may be possible to determine how the unique tubulin dimers are related to microtubule structure, assembly, and function in erythrocytes, and thereby gain insight regarding the function of tubulin variants in other cell types.

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