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CHANGES IN TUBULIN HETEROGENEITY DURING POSTNATAL DEVELOPMENT OF RAT BRAIN

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SUMMARY: Tubulin isolated from rat brain at various stages of postnatal development was subjected to isoelectric focusing on polyacrylamide gels. Multiple bands, indicative of the heterogeneity of the protein, were apparent at all developmental ages. When isoelectric focusing patterns of tubulin from brains of increasing developmental age were compared, changes in the distribution and relative intensities of the bands were observed. These changes were most pronounced between 8-12 days of age and were seen whether the tubulin was isolated by DEAE-cellulose chromatography or by successive cycles of assembly-disassembly. The isoelectric focusing pattern of tubulin isolated from the 22-day-old animal was indistinguishable from that of the protein obtained from 30-day-old rat brain. These developmental changes in tubulin heterogeneity may relate to changes in the assembly properties of the microtubule protein or may reflect age-dependent changes in the relative contributions of mitotic spindles, axons, dendrites, and glia to the total pool of tubulin in brain.

Heterogeneity of tubulin subunits has been demonstrated by isoelectric focusing techniques in several laboratories (1-5). The cause of the heterogeneity and its functional significance are not clear. Post-translational modifications, such as phosphorylation (6-9), tyrosylation (10-12), and glycosylation (13), could be important determinants of the assembly properties of tubulin. The reports by Fellous *et al.* (14-16) that tubulin formed during an early stage of rat brain development does not assemble into microtubules as efficiently as does tubulin from adult rat brain prompted us to look for developmental changes in tubulin subunits which might account for their observations. Our results show dramatic changes in the isoelectric focusing pattern of tubulin during the first two weeks of postnatal development of rat brain.

MATERIALS AND METHODS

All materials used for the preparation of tubulin and for the isoelectric focusing procedures were as described previously (5,17). Holtzman albino rats

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were used in all experiments. Tubulin was isolated from rat brain homogenates by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose column chromatography as described by Eipper (18) or by two cycles of assembly-disassembly by the method of Shelanski *et al.* (19). Purity of the preparations was assessed by SDS polyacrylamide gel electrophoresis carried out according to the method of Laemmli (20).

Isoelectric focusing of rat brain tubulin solubilized in 8 M urea was performed as described by O'Farrell (21). A linear pH gradient was established in a polyacrylamide gel support with ampholytes of pH range 4-6 and 5-7 (3:2 mixture). Gel tube dimensions were 6 x 120 mm. After polymerization, gels were pre-run for 15 min at 200 v, 30 min at 300 v, and 30 min at 400 v. Protein samples were then applied to the basic (top) end of the gel and electrophoresis continued for 16 hr at 300 v and finally for 1 hr at 800 v.

Fixing and staining of the gels were carried out according to the procedure of Righetti and Drysdale (22). Gels were fixed in 10% TCA for 6 hr, 5% TCA for 15 hr, and then in fresh 5% TCA for 6 hr. They were subsequently soaked overnight in 35% methanol-10% acetic acid-water (5:1:4), stained with Coomassie Brilliant Blue R250 in the same solvent, and destained by diffusion in 10% (v/v) acetic acid, 25% (v/v) methanol.

The pH gradient of the electrofocusing gels was determined from blank gels electrophoresed in parallel with sample gels. Slices (0.5 cm) of blank electrofocusing gels were suspended in 1 ml of 8 M urea 3 hr prior to measurement of the pH.

RESULTS AND DISCUSSION

In initial experiments, the isoelectric focusing patterns of tubulin isolated by DEAE-cellulose chromatography from the brains of 5-day-old and 30-day old rats were compared. The results of these experiments are shown in Fig. 1A. Numerous bands of varying intensity were resolved near pH 6.3. Nine discrete bands, indicated by the solid lines in Fig. 1A, are apparent in the electrophoretograms of neonatal and young adult tubulin. The distribution and relative intensities of the bands are markedly different in the gels of tubulin from the two ages, however. One band, indicated by the double triangles, is especially prominent in the electrophoretograms of tubulin from neonatal brain (Fig. 1A, gels a and b). Isoelectric focusing of the subunits of tubulin isolated by SDS polyacrylamide gel electrophoresis has shown this band to be associated with the β-subunit (data not shown). While this band is still present in the isoelectric focusing patterns of tubulin from the older animals (Fig. 1A, gels c and d), it is of no greater intensity than the several other more acidic β-bands, only one of which was

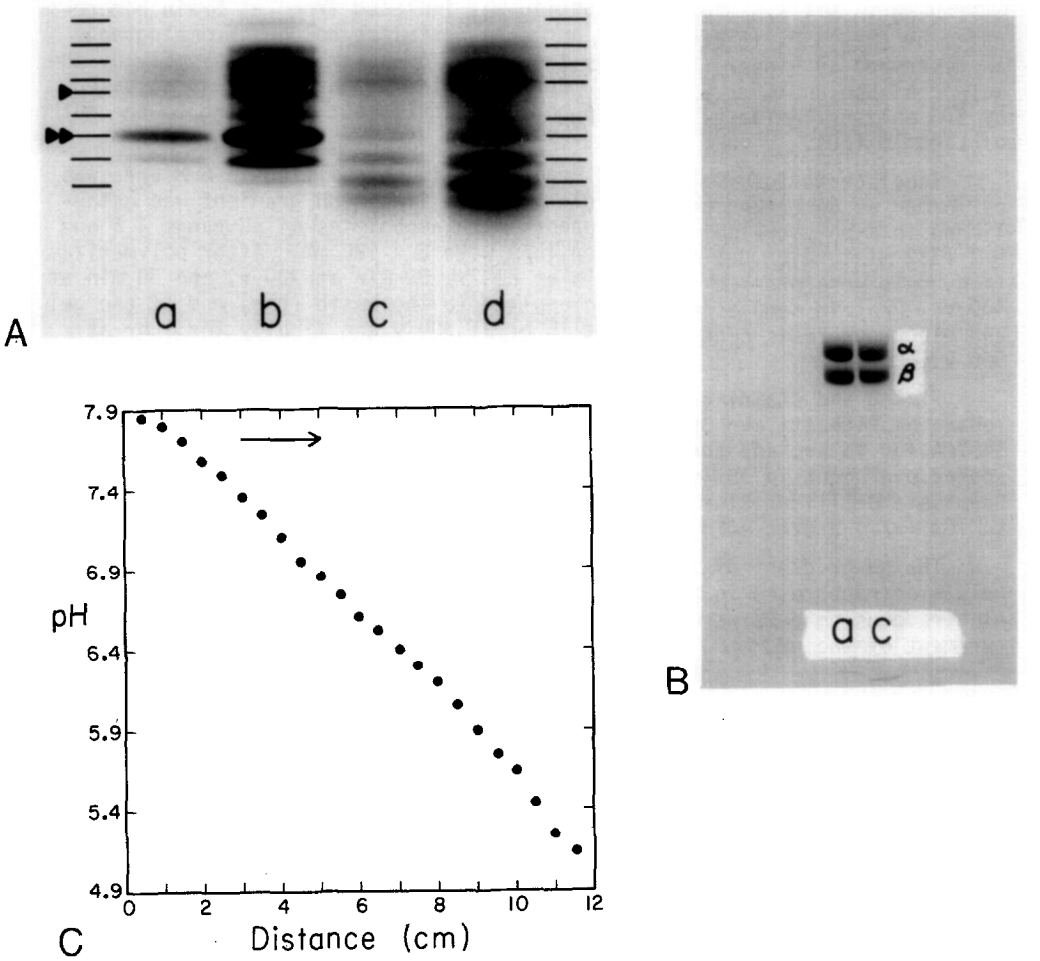

Fig. 1. (A) Isoelectric focusing patterns of tubulin isolated from the brains of 5-day-old rats (a,b) and 30-day-old rats (c,d). Tubulin was prepared by ammonium sulfate fractionation and DEAE-cellulose chromatography (18). Approximately 25 μg of protein were applied to gels a and c and 50 μg of protein to gels b and d. The acidic end of the gels is at the bottom. (B) SDS polyacrylamide gel electrophoresis of the tubulin isolated from the brains of 5-day old (a) and 30-day-old rats (c). Approximately 25 μg of protein were applied to each gel. The isoelectric focusing patterns of these tubulin preparations are shown in (A). (C) The pH gradient measured in cylindrical gels (6 x 120 mm) containing 1.2% ampholytes, pH range 4-6, and 0.8% ampholytes, pH 5.7. The arrow indicates the direction of migration. Tubulin subunits focus in the pH range from 6.2-6.4.

present in a significant amount in the electrophoretograms of neonatal tubulin. The other pronounced difference between the isoelectric focusing patterns of tubulin isolated from the brains at the two developmental ages is that the band designated by the single triangle in the electrophoretograms

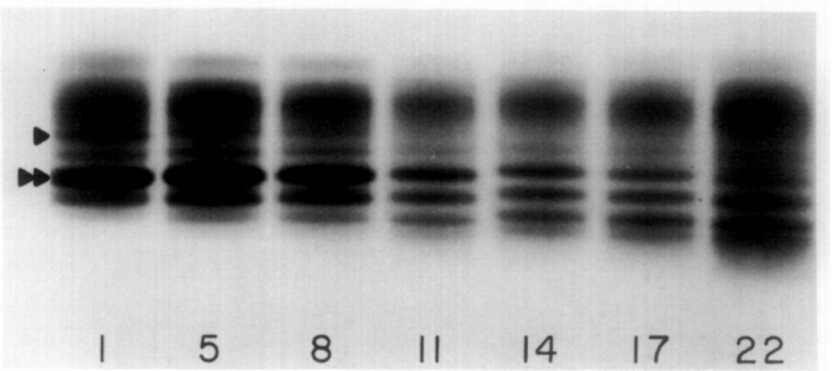

Fig. 2. Isoelectric focusing patterns of tubulin isolated from the brains of rats at 1, 5, 8, 11, 14, 17, and 22 days of age. Tubulin was isolated by ammonium sulfate fractionation and DEAE-cellulose chromatography (18). Approximately 50 μg of protein were applied to each gel.

of tubulin from the brain of the 5-day-old rat is not present in those of tubulin from the 30-day-old animal. We have not yet been able to assign this band to one or the other subunit of tubulin. However, autoradiograms of isoelectric gels of tyrosylated tubulin show the more basic bands above it to be associated with the α-subunit (data not shown). In addition, autoradiograms of isoelectric focusing gels of phosphorylated tubulin have shown the more acidic band below it to be associated with the β-subunit (5).

A more detailed developmental profile is presented in Fig. 2 in which the isoelectric focusing patterns of tubulin isolated from the brains of 1-, 5-, 8-, 11-, 14-, 17-, and 22-day-old rats are compared. Again the prominent band associated with the β-subunit is seen in the electrophoretograms of tubulin isolated from the brains of newborn animals. Its intensity decreases with developmental age in association with the appearance of the more acidic β-bands in the gels. The gradual diminution and ultimate disappearance of the band designated by the triangle in the electrophoretograms of tubulin from the newborn animal is also seen. Both of these changes appear to be complete by the time the animal is 10-12 days of age. It is of interest that it is after this period that the elaboration of neuronal processes is most intense in brain (23). Maximal brain growth is attained by about 21 days of age. In this work

assigned to the enriched methionine-29 C^{ε} carbon of native RNAase. Its chemical shift is 1.4-1.6 ppm upfield of the C^{ε} carbon in free methionine and in the peptide GlyGlyMetGlyGly which occur between 15.2-15.4 ppm from TMS ((7) and F. W. Benz, unpublished observations).

To test the feasibility of using ^{13}C NMR to study the behavior of individual methionine residues in RNAase during an equilibrium denaturation experiment, we treated the sample of [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase with guanidine deuterochloride. Our eventual goal is to study this denaturation process with labelled RNAase rather than the sulfonium derivative, however, we chose the latter protein at present because of the considerably larger amounts in hand. Figure 3 illustrates the behavior of the C^{ε} sulfonium carbon at three concentrations of guanidine. Previous ^{1}H NMR studies on the unfolding of RNAase have shown that the major unfolding transition occurred between 2.0-3.3 M guanidine (3). Thus at 1.9 M guanidine, a single resonance for [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase in the native state is observed at 26.9 ppm while at 3.1 M a single resonance at 26.3 ppm is seen for the same carbon in the denatured state. In 2.5 M guanidine both states are present and observed to be in slow exchange on the NMR time scale with an exchange rate of less than 92 sec^{-1}. It is interesting to note that the chemical shift of this carbon in the denatured state (3.1 M GuDCl) is identical to that reported by Jones et al. (7) for denatured myoglobin and free S-methylmethionine. In the native state, the RNAase sulfonium signal is even further downfield. In contrast, the signal from [Met^{29}-$^{13}C^{\varepsilon}$] RNAase is upfield of the free amino acid shift. This suggests that the C^{ε} carbon of Met^{29} does not occupy a similar position above Phe^{46} in both the sulfonium derivative and the native enzyme. This is not surprising since, in the sulfonium form, the sulfur carries a positive charge and should move away from Phe^{46} toward the solvent whereas uncharged Met^{29} would do the opposite. Despite the fact that the sulfonium C^{ε} shift suggests that it is more exposed to the solvent in the folded state than is the [Met^{29}-$^{13}C^{\varepsilon}$] carbon in native RNAase, it still is able to monitor the

amide electrophoresis and found less of an 82,000 molecular weight microtubule associated protein present in tubulin preparations from the brains of 5-day-old animals than in preparations from 30-day-old animals. Seeds and Maccioni (31) have shown that neurite outgrowth in neuroblastoma cells, which is associated with a marked increase in number and length of microtubules, appears to be facilitated by the presence of additional macromolecular factor(s) that may be functionally equivalent to the microtubule associated proteins found with brain microtubules (32).

Our results suggest that post-translational modifications of tubulin may also be important determinants of microtubule assembly. The changes we observe are most pronounced at the time when neuronal differentiation has been reported to begin in rat brain and appear to be complete by the time maturation occurs at the end of three weeks of postnatal life. Since tubulin isolated in our preparation is derived from several sources, mitotic spindles, axons, dendrites, and glia (24), our results could also reflect changes in the relative proportions of these sources of tubulin as the brain matures. Recently Rodriquez and Borisy (33) reported that the fraction of tubulin molecules containing hydrolyzable tyrosine changes with the stage of development of chick brain and suggest that tyrosylation may be important in modulating the function of microtubules *in vivo*. On the other hand, Raybin and Flavin (12) had previously reported no differences between the assembly properties of tyrosylated and non-tyrosylated tubulin. Experiments are currently in progress in our laboratory to ascertain the post-translational modifications responsible for the various bands observed on isoelectric focusing gels and to elucidate their functional roles in brain.

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