A STRUCTURAL DIFFERENCE BETWEEN CYTOPLASMIC AND MEMBRANE-BOUND TUBULIN OF BRAIN

Jayasree NATH and Martin FLAVIN

Section on Organelle Biochemistry, Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institues of Health, Bethesda, MD 20014, USA

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1. Introduction

Tubulin tyrosine ligase catalyzes a unique post-translational modification of the α chain of tubulin, whereby, in an ATP-dependent reversible reaction, a tyrosine residue is added through peptide linkage to the α-carboxyl of C-terminal glutamate [1–4]. The enzyme has now been purified 300-fold and is active enough to ensure maximal tyrosylation of any tubulin preparation [5] and thus enables one to determine the fraction of tubulin susceptible to tyrosylation in a particular sample. It has been observed in our laboratory that every cytoplasmic tubulin preparation tested so far (>20), can be considered to be composed of three species: one which has pre-existing tyrosine; one which can accept further tyrosine in vitro; and one which appears not to be a substrate for ligase [5]. To investigate the function of this post-translational modification, we have begun to compare the state of tyrosylation of tubulin isolated from different sources.

In the present communication we wish to report that a membrane-bound fraction of brain tubulin, purified by vinblastine precipitation from a detergent extract, can be tyrosylated similarly to the brain cytoplasmic tubulin, but differs strikingly from the latter, by the complete absence of pre-existing tyrosine. In the course of this investigation we have also been able to obtain membrane tubulin which is 35% pure. This is the purest preparation of native membrane tubulin reported so far.

2. Experimental

We measured the amount of C-terminal tyrosine in tubulin samples by the difference in the amount of [^{14}C]tyrosine that could be added by ligase, before and after removal of pre-existing tyrosine with carboxypeptidase A (CPA). CPA removes pre-existing tyrosine without attacking the penultimate glutamate of the α-chain [4]. The ligase assay conditions were as in [3,4]. To determine the maximum capacity of various tubulin samples to accept tyrosine, conditions were similar to those for ligase assay, except that smaller amounts of tubulin were incubated in duplicate for 30 min with 2 large amounts of purified ligase, usually 0.5 and 1.0 unit/ml. The amount of [^{14}C]tyrosine fixed into trichloroacetic acid insoluble form was determined [3], and identical results indicated that tyrosylation was maximal.

Membrane-bound tubulin was isolated by a modification of the method in [6,7]. A crude 'synaptosomal' fraction which had been washed 4 times, was extracted for 30 min at 0°C with 0.5% Nonidet-P40 (NP40) in reassembly buffer, containing 100 mM K^+MES (pH 6.8) with 1 mM EGTA and 0.5 mM each $MgSO_4$ and GTP. For further purification (and stabilization) of membrane tubulin, the detergent-treated suspension was centrifuged 20 min at 100 000 × *g* and the clear supernatant was promptly made 2 mM in vinblastine and kept 60 min at 0°C. A visible precipitate was collected by centrifuging 60 min at 100 000 × *g* and the pellet dissolved in 0.5 ml reassembly buffer (with 0.1 mM GTP). All assays were done on the day of preparation.

Native tubulin was determined by DE-81 paper disc [^{3}H]colchicine binding assay [8,9]. Polyacrylamide gel electrophoresis was done in the SDS–urea system [10]. To identify protein bands labelled with

[^{14}C]tyrosine, Coomassie blue stained gels were vacuum-dried and exposed to Kodak single-coated blue-sensitive film. For quantitation of protein bands, gels were scanned at 550 nm. The chart was xeroxed and peak areas were cut out and weighed.

3. Results

A number of reports have shown that a substantial proportion of cellular tubulin in brain tissue can be present in a membrane-bound form [6,7,11,12]. A possible function of tyrosylation or detyrosylation, is to influence the partition of tubulin into subcellular compartments. The questions we ask here are: is brain-membrane tubulin, like soluble tubulin, a substrate for ligase and if so, does it differ in the extent to which it has pre-existing tyrosine, and/or, can accept additional tyrosine when incubated with excess ligase in vitro?

Due to the unstable nature of membrane tubulin in the NP40 extract [6], vinblastine precipitation was used to both purify and stabilize it [9,13,14]. We found that vinblastine did not interfere with our assay procedures and tyrosine acceptor capacity of purified brain cytoplasmic tubulin was not altered before and after precipitation with vinblastine. The purification of membrane tubulin from the washed membrane fraction was 50-fold in 70% yield, indicating that we did not isolate a minor sub-species of membrane tubulin. Both colchicine-binding assays and scans of Coomassie blue-stained electrophoretic gels (data not shown here) indicated that 30–35% of the protein was tubulin. This is the purest preparation of native membrane tubulin reported so far.

Figure 1 shows that membrane tubulin is a substrate for tubulin tyrosine ligase. This experiment was done with a freshly-prepared NP40 extract. When the extract was incubated with [^{14}C]tyrosine (with or without added ligase), radioactivity was fixed exclusively into tubulin α-chains. Identical amounts of tyrosine were fixed in reaction mixtures 2 and 3, showing that maximal tyrosylation was obtained. It is of interest to note that half as much was also fixed in mixture 1, without any added ligase. Thus the brain membrane fraction contains some firmly-bound ligase which is released and unmasked by NP40. The actual nmol tyrosine fixed/mg membrane protein

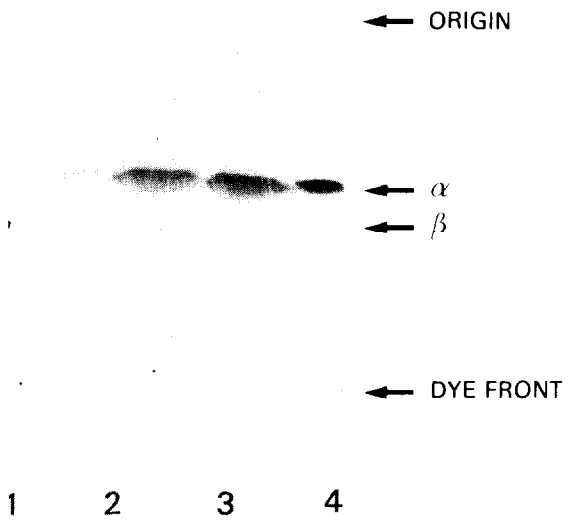

Fig.1. Radiogram showing fixation of [^{14}C]tyrosine into α chains of membrane tubulin. Aliquots from a freshly-prepared NP40 extract of washed membrane fraction were incubated with [^{14}C]tyrosine without (no. 1) or with 0.5 and 1.0 unit ligase/ml (no. 2 and 3, respectively). The incubation mixtures were applied to a polyacrylamide slab gel and electrophoresed as described in the text. Well no. 4 contained a reference sample of purified [^{14}C]tyrosylated-soluble tubulin. The slab gel was vacuum dried and exposed to X-ray film.

were 0.26 without and 0.50 with added ligase. Fixation in the original membrane fraction itself could not be determined as it caused nearly complete inhibition of tyrosylation of purified soluble tubulin by added ligase.

Table 1 compares the tyrosine acceptor capacities of soluble and membrane tubulin before and after CPA digestion. Soluble tubulin precipitated by vinblastine at 0°C, gave the same result as when purified by DEAE chromatography [5], the acceptor capacity increasing by 60–65% after CPA treatment. The membrane tubulin could accept a similar amount, but there was no further increase after CPA treatment. In separate experiments, two other membrane tubulin preparations gave the same results, 0.40–0.42 nmol/nmol tubulin, before and after CPA digestion, and 0.41–0.43 nmol/nmol tubulin. In both soluble and

Table 1
Tyrosine acceptor capacities of cytoplasmic and membrane tubulin from brain, before and after treatment with CPA

Sample	[^{14}C]Tyrosine fixed (nmol/nmol tubulin)	
	Before CPA	After CPA
1. Cytoplasmic tubulin in crude brain supernatant	0.22	0.36
2. Vinblastine precipitate from 1	0.24	0.38
3. Membrane tubulin precipitated with vinblastine as in text	0.42	0.43

Membrane tubulin was purified as in the text. Vinblastine precipitated 25% of the protein and 85% of the colchicine-binding activity from crude brain supernatant. The amount of tyrosine fixed was shown to be maximal by obtaining the same result with 0.5 and 1.0 unit ligase/ml. When CPA was added after ligase incubations 95% of the radioactivity was released from samples 2 and 3

membrane tubulin preparations, CPA released >95% of the [^{14}C]tyrosine added in vitro. Since this data indicated, as suggested [15], that membrane-tubulin contained no pre-existing C-terminal tyrosine, we did a control experiment to determine whether tyrosine might have been enzymatically removed during membrane extraction and subsequent procedures. The washed membrane preparation did not liberate any free tyrosine from added [^{14}C]tyrosylated soluble tubulin at 0°C, the temperature at which these membrane tubulin preparations were made. Control experiment was extended through precipitation of membrane tubulin by vinblastine and the result was the same as stated above.

Table 2 summarizes the results in terms of the proportions of native tubulin which have pre-existing tyrosine, can accept additional tyrosine, or are not a ligase substrate. As determined by [^{3}H]colchicine binding, the native tubulin content was 72% in the vinblastine precipitate from brain soluble fraction and 36% in the brain membrane fraction. The purity of tubulin in the vinblastine precipitates is less when its concentration is lower in the starting extract.

It would be worthwhile to mention here that a distinct detyrosylating enzyme has been discovered [16], which in our hands is not active in the brain membrane fraction. However, after NP40 extraction, or freezing and thawing, a weak detyrosylating activity could be unmasked (M. F., unpublished results). Thus, the brain membrane fraction contains firmly bound ligase and also the detyrosylating enzyme, both of which could be released upon detergent extraction.

Table 2
Proportions of 3 tubulin species in brain cytoplasmic and membrane preparations

Rat brain tubulin preparation	% Native tubulin (determined by colchicine binding)		
	Pre-existing tyrosine	Can accept additional tyrosine	Not a ligase substrate
Soluble	14	24	62
Membrane-bound	0	42	58

To isolate soluble tubulin, brain tissue was homogenized in reassembly buffer (100 mM K^+MES, (pH 6.8), 1 mM EGTA, 0.5 mM $MgSO_4$, 0.5 mM GTP). Supernatants from high-speed centrifugation were made 2 mM in vinblastine, and the precipitates which formed after 1 h at 0°C were collected and dissolved in reassembly buffer. Membrane-bound tubulin was precipitated with vinblastine from the supernatant after detergent extraction, obtained as described in the text. All procedures were done at 0°C. The % tubulin that can accept additional tyrosine was determined by incubating tubulin and [^{14}C]tyrosine with a large excess of ligase (0.5 and 1.0 unit/ml). Pre-existing tyrosine was determined as the increase in the amount that could be fixed when tubulin was first incubated with CPA, then with ligase + β-phenylpropionate (to inhibit CPA). Non-substrate tubulin is the moiety that could not accept [^{14}C]tyrosine even after CPA digestion

4. Discussion

The exact nature and function of the membrane-bound fraction of cellular tubulin is not known. Colchicine effects on membrane microviscosity changes [17] and on membrane-related cell functions (like lateral mobility of membrane receptors, secretion, exocytosis and organelle motility) which have been attributed to disassembly of cytoskeletal microtubules, could instead point to possible functions of membrane-bound tubulin.

The complete absence of C-terminal tyrosine from the α-chains of membrane-bound tubulin as isolated from brain, is the first structural difference that has been detected between it and the cytoplasmic tubulin. The most obvious interpretation of this difference is that detyrosylation is a step in the fixation of tubulin into membranes. However, one should recall that the brain-membrane fraction contains firmly bound ligase and also the detyrosylating enzyme, both of which could be unmasked upon NP40 extraction. Thus, as an example of a totally different and more fanciful interpretation, the absence of tyrosine could simply reflect a steady state of membrane tubulin constantly undergoing tyrosylation and detyrosylation in situ, possibly in relation to some function of tubulin in the membrane. Thus, if tubulin were a transmembrane protein with receptor sugars at the external N-terminus [18,19], reversible tyrosylation might mediate message transmission on the inside.

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