IDENTIFICATION OF TUBULIN ASSOCIATED WITH RAT BRAIN MYELIN

Illana GOZES and Christiane RICHTER-LANDSBERG*
Department of Neurobiology, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

During development, axons in both the central and peripheral nervous systems become ensheathed with myelin [1]. Biochemical manifestations of myelination include rapid accumulation of the myelin membrane proteins [2,3]. These can be divided into three classes; the proteolipid proteins, the basic proteins and the Wolfgram proteins [3–6]. The Wolfgram proteins are characterized as the high molecular weight proteins of the myelin fraction [4]. The Wolfgram proteins can be resolved electrophoretically into several polypeptides [2,3,7–9], two of which, W1 and W2, have been characterized immunochemically [10].

The myelin membrane is principally derived from oligodendroglia plasma membrane [5,6]. A major protein component which is synthesized by glial and neuronal cells is tubulin [11–13], the subunit protein of microtubules [14,15]. Tubulin can be associated with membranes [16–18], and was shown to accumulate in the particulate fraction of brain with maturation [13], concomitantly with myelin formation [19]. Together, these observations suggest the possibility that tubulin may be a component of the myelin membrane.

By the criteria of SDS–polyacrylamide gel electrophoresis for molecular weight determination, peptide mapping and colchicine binding, we have now demonstrated that tubulin is a constituent of the high molecular weight portion of the myelin fraction.

* Present address: The Children's Hospital Medical Center, 300 Longwood Avenue, Boston, MA 02115, USA

2. Materials and methods

DEAE-cellulose (DE-52) was obtained from Whatman, [^{3}H]colchicine from New England Nuclear, vinblastine sulfate was a gift from Eli Lilly and *Staphylococcus aureus* V8 protease (36-900-1) was obtained from Miles Lab. [^{35}S]Methionine was obtained from the Radiochemical Centre, Amersham.

2.1. *Preparation of rat brain myelin*

Rat brain myelin was purified by differential centrifugation through sucrose by the method in [20] as modified [3]. This method yields highly purified myelin [3,20].

2.2. *Preparation of rat brain tubulin*

Rat brain tubulin was purified using DEAE-cellulose column chromatography as in [21], with slight modifications. Freshly obtained brains (2 g) from month 1 rats were homogenized using a Dounce homogenizer, in 4 ml 0.24 M sucrose/2.5 mM $MgCl_2$/50 mM Na PP_i (pH 7.0). The homogenate was centrifuged for 30 min at 30 000 × *g* and the supernatant was subjected to DEAE-column (0.5 × 4 cm). After extensive washing with 0.1 M NaCl/50 mM Na PP_i (pH 7.0)/2.5 mM $MgCl_2$, the tubulin peak was collected by elution with 0.26 M NaCl/50 mM Na PP_i (pH 7.0)/2.5 mM $MgCl_2$.

2.3. *SDS–gel electrophoresis*

Samples were mixed with sample buffer to yield final conc. 10% glycerol/5% 2-mercaptoethanol/1% SDS/0.0625 M Tris–HCl (pH 6.8)/0.001% bromophenol blue, and heated at 100°C for 5 min. Samples were subjected either to 1–40% gradient microgels as in [7], or 0.75 mm thick 10–20% gradient slab

gels using a discontinuous SDS-containing buffer system [22,23]. For quantitative evaluation, the photographic transparencies of the Coomassie brilliant blue stained gels were scanned at 560 nm in a Gilford 2400-S spectrophotometer.

2.4. *Colchicine binding assays*

The lyophilized myelin fraction was homogenized in 10 mM sodium phosphate (pH 7.4)/10 mM $MgCl_2$/0.5 mM GTP to final conc. 1 mg protein/ml. The colchicine binding activity was determined as for the particulate tubulin [13].

2.5. *Protease digestion of tubulin*

Samples were digested by the method in [24]. After gel electrophoresis, stained tubulin bands were excised, soaked for 15 min in buffer consisting of 0.125 M Tris–HCl (pH 6.8)/0.1% SDS/1 mM EDTA. The gel slices were inserted into sample slots of 1.5 mm thick, 15–20% polyacrylamide slabs with a 4 cm high stacking gel. The slots were then filled with 10 μl buffer containing 20% glycerol and 0.001% bromophenol blue. Finally, 10 μl buffer was added containing 10% glycerol and 100 ng *Staphylococcus aureus* protease. Electrophoresis was carried out at 100 V for 90 min until the tracking dye reached the bottom of the stacking gel. Electrophoresis was then stopped for 30 min and thereafter resumed at 200 V until the tracking dye reached the bottom of the gel.

2.6. *Preparation of radioactively-labeled tubulin*

To get quick effective labeling of tubulin we used [^{35}S]methionine (900 Ci/mmol) and tissue culture cells. Neuroblastoma cells, N18TG-2, (a gift from Dr M. Nirenberg, NIH) were grown to confluency as in [25]. For a typical preparation we used 20 diam. 10 cm petri dishes and added 100 μCi [^{35}S]methionine to 4 dishes. After 24 h at 37°C the cells were homogenized with a Teflon–glass homogenizer and tubulin was prepared by DEAE-cellulose chromatography as in section 2.2.

3. Results

3.1. *Identification of tubulin in the myelin fraction by polyacrylamide gel electrophoresis*

The myelin fractions were analyzed by electrophoresis on microgels [7] for the presence of polypeptides with the same mobility as tubulin. When a DEAE-cellulose purified rat brain tubulin marker was added to a myelin fraction, enhancement of a pre-existing band was apparent, suggesting that tubulin is one of the constituents of the myelin (fig.1). A similar band was also found in beef brain myelin (unpublished data). When the rat brain myelin fractions were analyzed by SDS–polyacrylamide gel electrophoresis [23], the myelin fraction was resolved into about 8 major protein bands, one of which comigrated with a DEAE-cellulose purified rat brain tubulin marker (fig.2).

3.2. *Biological activity of tubulin in the myelin membrane*

Colchicine is known to bind specifically to tubulin and inhibit its polymerization into microtubules [14]. Moreover, the particulate fractions of mouse brain [16] and rat brain [13] were shown to have colchicine binding activity. By quantitative determination of the

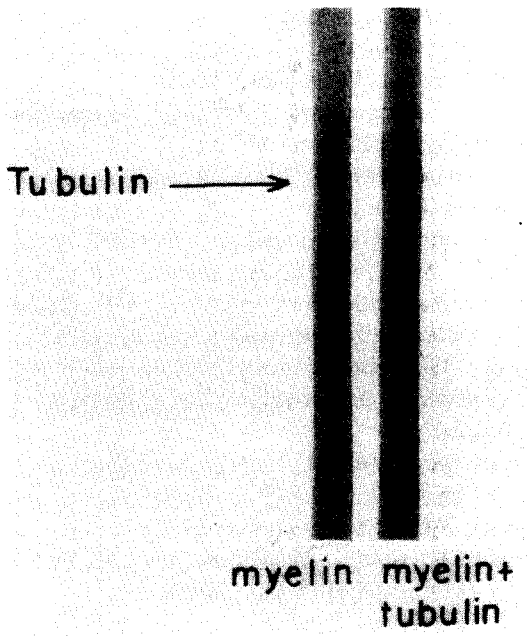

Fig.1. Analysis of rat brain myelin on microgels. Purified rat brain myelin was subjected to electrophoresis on microgels [7]. The position of the tubulin band was identified by mixing a myelin sample with rat brain tubulin which had been purified by DEAE-cellulose.

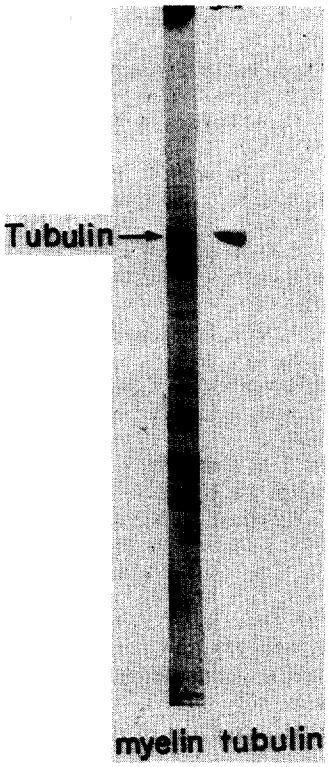

Fig.2. Identification of tubulin in rat brain myelin by SDS–polyacrylamide gel electrophoresis. Purified rat brain myelin was subjected to SDS–polyacrylamide gel electrophoresis [22,23]. The tubulin position was identified using a DEAE-cellulose purified tubulin marker.

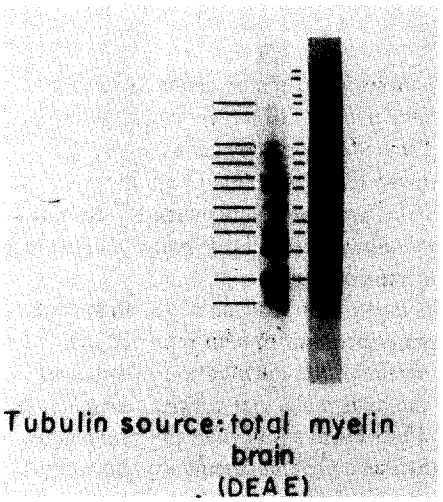

Fig.3. Peptide maps of tubulin bands excised from SDS–polyacrylamide gels. The tubulin bands were subjected to *Staphylococcus aureus* protease. The peptide analysis was performed on a 15–20% polyacrylamide gel containing 0.1% SDS [24].

colchicine binding activity we estimated the amount of tubulin at 1.5–4% of the total myelin protein. This value closely agrees with the result obtained by densitometric scanning of the SDS–polyacrylamide gel which was also ~4% (fig.2).

3.3. *Peptide mapping experiments*

The degree of relatedness between potential tubulin in the myelin fraction and tubulin prepared by DEAE-cellulose chromatography was analyzed further by peptide mapping experiments. After SDS–polyacrylamide gel electrophoresis of myelin and DEAE purified rat brain tubulin, the presumptive tubulin bands were excised, subjected to *Staphylococcus aureus* protease digestion and analyzed by SDS–polyacrylamide gel electrophoresis. As shown in fig.3, there is an overall similarity between the map of tubulin and of the corresponding polypeptides in the myelin fraction. Despite some differences, the peptide maps indicate that the two proteins are closely related.

3.4. *Is the association of tubulin with the myelin membrane due to contamination of cytoplasmic tubulin?*

To answer this question myelin was prepared from rat brains (1.4 g) in the presence of [^{35}S]methionine-labeled tubulin (10 000 cpm). We followed the amount of radioactivity associated with the myelin pellets throughout the purification procedure, which consists of 7 centrifugation steps. While the crude particulate fraction obtained after one centrifugation step still contained 16% of the counts the second centrifugation eliminated most of the radioactivity from the crude membrane fraction – only 1% of the original radioactivity added remained. After the third centrifugation step the crude myelin fraction was

devoid of radioactivity. This experiment indicates that tubulin is naturally associated with the myelin membrane and is not artifactually adsorbed during isolation.

4. Discussion

We have demonstrated the presence of tubulin among the heavy proteins of a highly purified myelin fraction. This extends observations in which tubulin was found to be associated with membranes [16–18]. Our experiments with [^{35}S]methionine-labeled tubulin suggest that tubulin is a natural myelin membrane component.

Differences in the composition of the high molecular weight components of myelin reported are probably the result of the purification procedures employed as the high molecular weight components can be washed out more easily than the proteolipid–protein and the basic proteins of the myelin fractions [8,10,26].

The minor differences observed between the peptide maps of the myelin tubulin and the DEAE-cellulose purified brain tubulin might be due to some variations in the primary structure and indicate the existence of a specific tubulin species in the myelin fraction. Tubulin, which is a major protein synthesized by glial cells [11] prior to myelin formation [19], could participate in the formation and maintenance of the myelin shape.

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