IS TUBULIN A GLYCOPROTEIN?[1]

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Summary: [^{14}C]Glucosamine is incorporated in vivo in mouse brain into the major protein species present in purified tubulin preparations when analyzed both by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by isoelectric focusing. The radioactivity incorporated into tubulin can be recovered as a mixture of glucosamine and galactosamine.

Tubulin, the subunit protein of microtubules, has been extensively studied with respect to its protein backbone, but little attention has been paid to the possible association of sugars with the protein. Reports in the literature vary as to whether purified preparations of tubulin contain carbohydrate. Falxa and Gill (1) have reported the presence of 1% reducing sugar in tubulin preparations. However, the identity of the sugars involved was not established. These workers also did not establish whether the sugars were associated with a contaminant which co-purified with tubulin or whether tubulin itself was a glycoprotein. Margolis, Margolis and Shelanski (2), using tubulin purified by the batch methods of Weisenberg, et al. (3), found 1.3% carbohydrate consisting of glucosamine, galactosamine, galactose, mannose, fucose and sialic acid. 40% of the galactosamine was present in alkali-labile O-glycosidic linkages of N-acetylgalactosamine to serine and threonine residues. The remaining carbohydrate was attached to the protein in

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alkali-stable linkages. Again, the question of whether the carbohydrate in these preparations of tubulin was present in a contaminant was not considered. Eipper (4) devised a new purification scheme for tubulin and reported that tubulin purified in this way contained no amino sugars and no more than 0.2% neutral sugar.

The experiments reported below demonstrate that radioactive glucosamine is incorporated *in vivo* into the major protein species present in purified tubulin preparations when analyzed both by high resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis and by isoelectric focusing.

Methods

3-7 day old mice were injected intracerebrally with 10 μCi of D-[^{14}C]-glucosamine-1-hydrochloride (43 mCi/mM, New England Nuclear). The mice were sacrificed one hour after injection and tubulin was prepared from the high speed supernatant fraction by vinblastine precipitation as described previously (5).

Polyacrylamide gel electrophoresis was performed with discontinuous polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 8 M urea as described previously (6). Isoelectric focusing on polyacrylamide gels was accomplished by modifications of the techniques of Wrigley (7) as previously described (6). The isoelectric focusing gels contained 8 M urea, 1 mM dithiothreitol and 1% ampholytes (LKB) ranging in pH from 3-10. Initially, the protein was disolved in 8M urea and 1 mM dithiothreitol and was distributed uniformly throughout the gel.

Radioactive material released by limited acid hydrolysis was analyzed either by paper chromatography or by split stream column chromatography. The tubulin preparations were precipitated and washed with tricholoracetic acid and then hydrolyzed (4N HCl, 100°, 3 h for paper chromatography and 6 h for column chromatography). The descending paper chromatography system used butanol:pyridine:0.1 N HCl, 5:3:2 by volume. The lane containing the sample was removed from the dried chromatogram and cut into strips which were assayed for radioactivity by liquid scintillation counting. Standards were visualized by dipping in $AgNO_3$ and then NaOH.

For split stream chromatography, the long column of a JEOLCO 5 AH amino acid analyzer was used with an elution program that separated glucosamine and galactosamine from each other and from neighboring amino acids. A portion of the effluent from the column was monitored spectrophotometrically after mixing with ninhydrin and the other portion of the column effluent was collected in two-minute fractions and assayed for radioactivity by liquid scintillation counting. [^{14}C]valine was added to the sample to serve as an internal calibration standard for the difference in the time at which a radioactive compound was eluted from the column and the time at which it appeared in the spectrophotometer cell.

Results

The tubulin isolated by vinblastine precipitation was 90-95% pure

(Figure 1) when analyzed by sodium dodecyl sulfate-urea polyacrylamide gel electorphoresis. All of the colchicine-binding activity is removed from the high speed supernatant of brain homogenates by this procedure (8) suggesting that tubulin is quantitatively precipitated. Deliberately applying very large amounts of sample (2-3 mg) so as to be able to visualize contaminants, radioautography of stained gels demonstrated that the radioactivity was associated almost exclusively with tubulin and not contaminants (Figure 2).

Since gel electrophoresis in the presence of sodium dodecyl sulfate

Figure 1. Sodium dodecyl sulfate - urea polyacrylamide gel electrophoresis of tubulin prepared by precipitation with vinblastine. The same preparation is shown in Figure 2 using 10-15 times greater amount of protein.

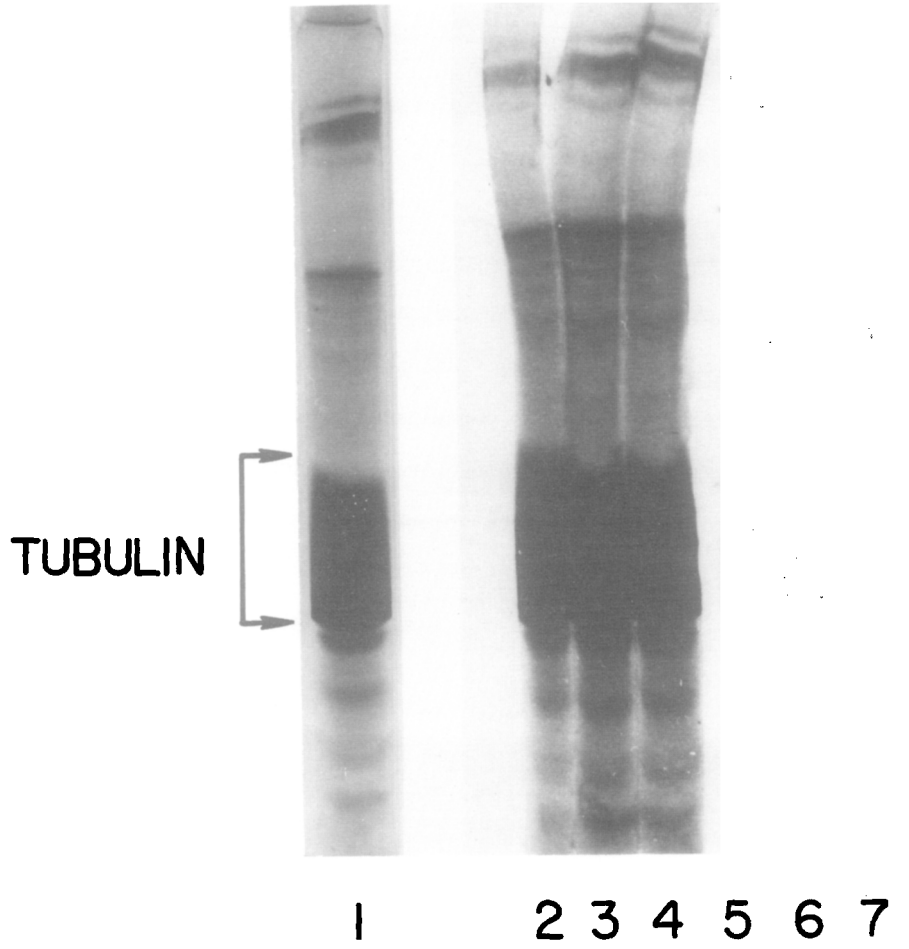

Figure 2. Radioautography of [^{14}C]glucoasmine labeled tubulin after sodium dodecyl sulfate - urea polyacrylamide gel electrophoresis. (1) shows the stained gel that was deliberately overloaded to reveal contaminants. The position of tubulin is indicated. (2-4) are three stained longitudinal slices from (1) after drying. (5-7) is the corresponding radioautogram.

separates proteins principally by molecular weight (9), it remained possible that the glucosamine has been incorporated into a protein contaminant with the same molecular weight as tubulin. To investigate this possibility further, tubulin labeled with [^{14}C]glucosamine was analyzed by isoelectric focusing on polyacrylamide gels. As previously reported (6) this technique resolved brain tubulin prepared from 3-7 day old mice into two major bands, each of which could be resolved further into a doublet

(4 bands total) and some minor bands. Radioautograms of the isoelectric focusing gels also demonstrated the association of the radioactive glucosamine with all of the tubulin subunit species resolved by this technique (Figure 3).

The nature of the radioactive moiety present in tubulin preparations after the intracerebral injection of radioactive glucosamine was investigated. After limited acid hydrolysis and paper chromatography, radioactivity was detected at positions corresponding to [^{14}C]glucosamine and [^{14}C]galactosamine (Figure 4). Radioactive glucosamine and galactosamine were also recovered by split stream column chromatography (Figure 5).

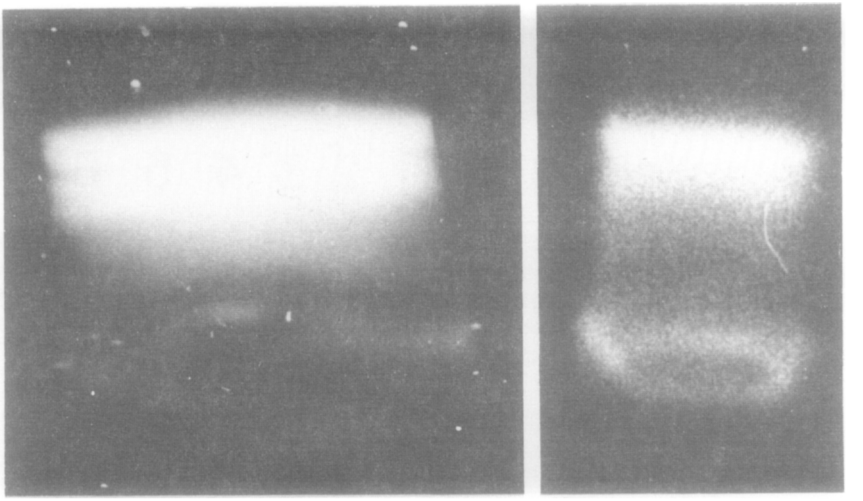

Figure 3. Separation of [^{14}C]glucosamine labeled tubulin subunits by isoelectric focusing. Tubulin was dissolved in 8 M urea in the presence of 1 mM dithiothreitol. The 7% polyacrylamide gels contained 1% ampholynes with a nominal pH range of 3-10, 8 M urea and 1 mM dithiothreitol. At the start of focusing, the protein was distributed uniformily throughout the gel. After focusing was completed, the gels were soaked in 5% trichloroacetic acid and the precipitated bands were photographed by transillumination against a black background, as shown on the left. Two major bands, each of which was itself a doublet, were present although difficult to discern in the photograph. Alkaline pH is towards the top. The gel was then sliced longitudinally and the slice was allowed to dry on paper. An autoradiogram was prepared and is shown on the right as a direct positive print.

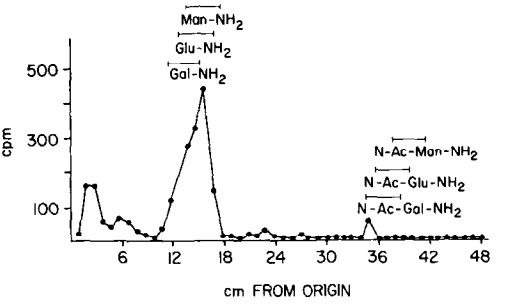

Figure 4. Paper chromatography of a limited acid hydrolysate of [^{14}C]glucosamine labeled tubulin. Tubulin was isolated from mice which had been injected intracerebrally with 10 microcuries of [^{14}C]glucosamine and killed one hour later. The protein was precipitated with vinblastine and washed with 5% trichloroacetic acid and then hydrolyzed (4N HCl, 100°, 3 h). The hydrolysate was examined by descending paper chromatopgrahy. The lane containing the sample was cut into 1 cm strips and counted. Parallel lanes contained the indicated standards.

Discussion

Tubulin purified either by conventional means (1,2) or by vinblastine precipitation contains carbohydrate. The above results show that the carbohydrate moeity present in purified tubulin preparations cannot be separated from tubulin itself either on the basis of molecular weight or net charge. It thus seems quite certain that brain tubulins are glycoproteins. This does not necessarily mean that every tubulin molecule contains carbohydrate, however. Tubulin may exist in glycosylated and non-glycoslated forms. The method used to purify tubulin in this study is quantitative and would result in the recovery of both glycosylated and non-glycosylated tubulin. The failure of Eipper (4) to detect amino sugars in tubulin prepared by her method may be because her method does not result in quantitative recovery of tubulin and may select for non-glycosylated tubulin.

The role of the carbohydrate moiety in tubulin is uncertain, but glycosylation may regulate a variety of biological processes in which microtubules and/or tubulin are involved. The ability of the protein to polymerize into microtubules, a process which is known to depend on calcium, GTP, temperature

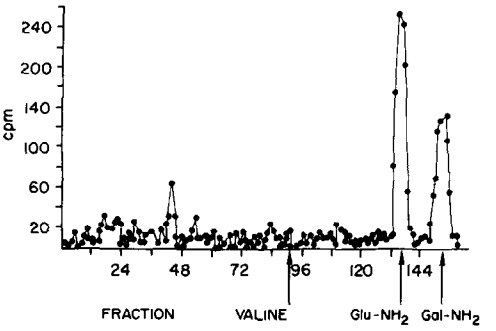

Figure 5. Split stream column chromatography of a limited acid hydrolysate of [^{14}C]glucosamine labeled tubulin. Tubulin was prepared as in Figure 3 except that hydrolysis was for 6 h. The hydrolysate was chromatographed on the long column of a JEOLCO 5 AH amino acid analyzer. Part of the effluent from the column was monitored spectrophotometrically after reaction with ninhydrin and the other part of the column effluent was collected in two minutes fractions and counted. [^{14}C]valine was added as an internal standard and used to determine the fractions which would contain glucosamine and galactosamine.

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and a number of cytoplasmic factors (10-12), could also be influenced by whether or not the protein is glycosylated. In their suspected role as one of the mediators of intracellular transport, microtubules must have sites at which membrane bound vesicles form contacts (13). These sites might depend on the distribution of carbohydrate in microtubules, if the polymerized form of the protein contains carbohydrate. In brain, tubulin has been shown to be a component of other subcellular organelles besides microtubules, in particular, synaptic plasma membranes (5,14-16) and the postsynaptic density (16). Tubulin associated with these synaptic organelles is strongly positive with the periodic acid Schiff reagent whereas soluble tubulin does not stain with this reagent, presumably because of the low content of carbohydrate. The association of tubulin with these subcellular organelles may depend on or otherwise involve a change in the extent of glycosylation of tubulin.

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