PROTEINS ASSOCIATED WITH TUBULIN

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SUMMARY: The distribution of proteins on SDS-urea polyacrylamide (7.5%) disc gel electrophoresis is studied from rat brain tubulin purified by three different procedures, including ammonium sulfate precipitation followed by DEAE cellulose chromotography, three cycles of polymerization-depolymerization and colchicine-containing agarose affinity columns. Three tubulin-associated proteins other than the principal tubulin dimer are identified and characterized with respect to molecular weight, behavior on gel filtration chromatography and method of tubulin purification. One of these proteins (H_1) is released from the tubulin complex when colchicine is irreversibly bound to tubulin. These proteins may participate in processes related to microtubule assembly or function.

It is generally agreed that microtubules are polymerization products of the asymmetric, dimeric protein, tubulin. However, there are compelling reasons for believing that microtubules are formed by far more complex processes than the simple sequential association of tubulin dimers alone. For example, fractionation on Sephadex G-200 resolves purified tubulin into two discrete populations, present in the excluded (V_o) and included (V_i) column volumes, and the former is not only the only species which has the capacity to polymerize but it can also induce polymerization of the latter, included species (1-2). In addition, tubulin is known to polymerize in a polar, orderly and linear manner under appropriate conditions (3). By a series of different methods tubulin copurifies with a protein kinase activity (4-7) as well as with heterogeneous high-molecular weight materials which in part have been referred to as ATPase-containing dyneins (8,9). Also, morphological studies demonstrate that microtubules are coated with fibrillar structures (10) and are closely associated with different cellular organelles (11).

As a first step toward understanding more completely the biology of microtubule assembly and function, we have studied by polyacrylamide (7.5%) disc gel electrophoresis the proteins most consistently associated with different functional aggregates of tubulin.

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Abbreviation: DAC-Sepharose, deacetylcolchicine-Sepharose
SDS, sodium dodecyl sulfate

MATERIAL AND METHODS: Male rat brains were homogenized (4°) in one volume of 10 mM sodium phosphate buffer, pH 6.75, containing 5 mM $MgCl_2$ and 1 mM GTP (buffer A). This buffer was used throughout except when tubulin was purified by three cycles of polymerization-depolymerization, in which case the conditions were modified as described (12) in order to obtain optimal purification. The other methods of purification were ammonium sulfate precipitation followed by DEAE cellulose chromatography, as described by Weisemberg (13), and DAC-Sepharose affinity chromatography (7,14).

Fractionation of tubulin according to its state of aggregation was carried out (4°) on 30 x 2 cm Sephadex G-150 columns, equilibrated with buffer A. When indicated, the V_e tubulin fraction from Sephadex G-150 was further fractionated (4°) on a 30 x 2 cm Sephadex G-200 column equilibrated with buffer A.

Electrophoresis on SDS-urea polyacrylamide (7.5%) gels was carried out, on about 100 µg of protein, essentially as described by Eipper (15), except that β-mercaptoethanol was replaced by 3 mM dithiothreitol in the sampling mixture containing 8 M urea, 0.1% SDS, and $K_3Fe(CN_6)$ was not included in the gel. Since in preliminary experiments different boiling times of the sampling mixture (1, 2, 5, 20 min) did not modify the gel protein pattern, the boiling time was standardized at 5 min for the rest of the experiments. Some gels were also run in the absence of any SDS or urea, or according to the Laemli method (16). The volume of the sample varied between 300 µℓ and 100 µℓ depending on the thickness and length of the gels used. Markers for molecular weight determinations were: pyruvate kinase (10^5 daltons) α-casein (1.22×10^5 daltons), glyceraldehyde 3 phosphate dehydrogenase (2.7×10^4 daltons) cytochrome C (1.3×10^4 daltons). Electrophoresis (2.5 mamp per gel) was performed at 24° until the Bromophenol blue used as tracking dye was 1 cm from, or on the very bottom, of the gel. Protein was determined by the Lowry method (17) with appropriate corrections for the presence of GTP.

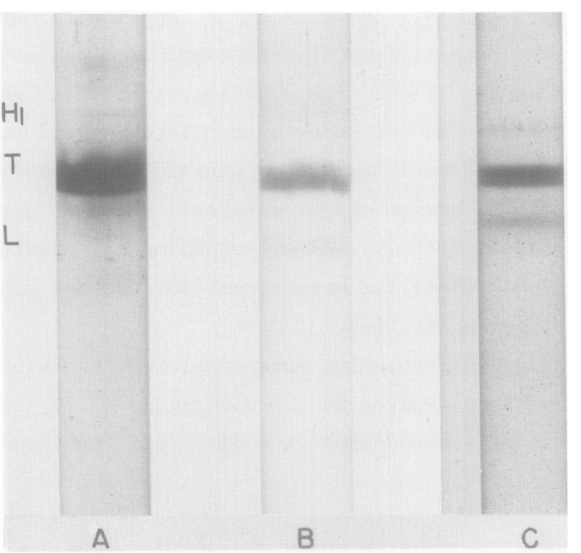

Fig. 1. SDS-urea polyacrylamide (7.5%) gel electrophoresis of unfractionated tubulin purified by three cycles of polymerization- depolymerization (A), ammonium sulfate DEAE cellulose chromatography (B), deacetylcolchicine-Sepharose affinity columns (C). Size of the gels is 7 x 1 cm. Volume of the sample, 300λ (100 µg of protein per gel). Electrophoresis was run at 2.5 mamp per gel (A,B) and 1 mamp per gel in C.

RESULTS: Tubulin purified either by ammonium sulfate precipitation followed by DEAE cellulose chromatography, or by DAC-Sepharose affinity chromatography, shows three principal bands on SDS-urea gels (Fig. 1). The central band represents tubulin (T), which is composed of two discrete bands (MW 53,000 and 55,000). A high-molecular weight (MW 100,000) band (H_1) and a low molecular weight (MW 32,000) band (L) are consistently observed.

Tubulin purified by three cycles of polymerization-depolymerization also shows the H_1 protein accompanying the major T band, but the L band is barely discernible or absent (Fig. 1). Furthermore, additional bands of molecular weight greater than that of H_1 appear consistently in this type of tubulin.

Regardless of the method used for tubulin purification, subsequent fractionation on Sephadex G-150 separates two major aggregate forms of the protein. The one which is present in the void, excluded volume (V_o tubulin) of the column contains (by electron microscopy) the proteins which usually coat microtubules (10). It is capable of polymerizing into microtubules and it can induce polymerization of the form which is present in the included protein peak (V_i), which alone is unable to undergo polymerization (1-2).

When the tubulin samples of the V_o and V_i fractions are examined on SDS-urea polyacrylamide (7.5%) gels, the major T band is always found as the predominant

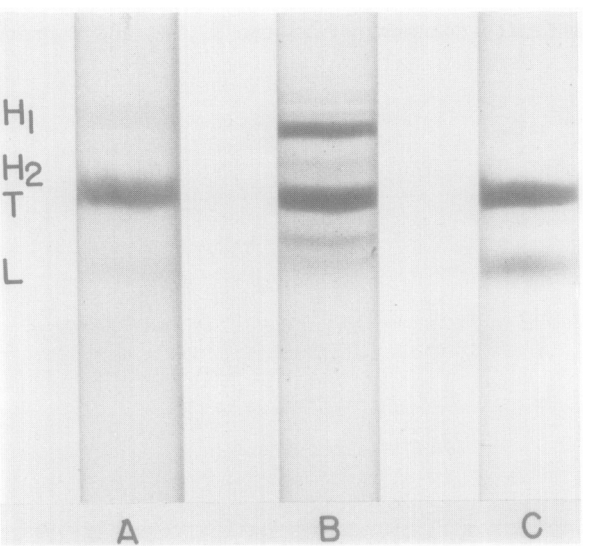

Fig. 2. SDS-urea polyacrylamide (7.5%) gel electrophoresis of tubulin purified by ammonium sulfate precipitation followed by DEAE cellulose chromatography (Weisemberg procedure). Unfractionated tubulin (A), excluded (B) and included (C) tubulin fractions from a 30 x 2 cm Sephadex G-150 column. Gel electrophoresis was done as described for gels A and B in Figure 1.

protein (Fig. 2-3). This band represents about 70% and 80% of the total tubulin protein present in the V_o and V_i fractions, respectively. The H_1 band is restricted to the V_o fraction of tubulin, regardless of its method of purification (Fig. 1-2). Although the L band may be present in small amounts in the total V_o fraction of the Sephadex G-150 column, it is essentially absent in the earliest V_o fractions and its quantity increases notably along the fractions which approach the V_i volume of the column. In addition, when the whole V_o fraction is rechromatographed on Sephadex G-200, the L band is not present at all in the V_o of this column (data not shown). Thus, the presence of the L band in the V_o peak of Sephadex G-150 is almost certainly due to the incomplete separation and partial overlap of the V_o and V_i volumes of this column. In addition, a discrete band (H_2, MW about 60,000) is clearly discernible above the upper tubulin band (T) when tubulin is fractionated. This H_2 protein appears to parallel the H_1 distribution through all the fractions of the Sephadex G-150 column (Fig. 2-3) although its contribution to the V_o protein is considerably less than H_1. These patterns are basically independent of the method of tubulin purification (Fig. 1, 2, 3), of the concentration of SDS in the boiling mixture (in the range 0.1%-0.5%) and of the time of sample boiling before electrophoresis (1 to 20 min). Gels run in the absence of urea according to the method of Laemli (16), which is commonly employed in the gel electrophoretic study of tubulin (16), shows the same distribution of T, H_1 and L proteins through the V_o and V_i Sephadex G-150 fractions (Fig. 4). When the boiling step is omitted and SDS and urea are not included in the gels, none of the above bands enter the gels (data not shown).

DAC-Sepharose affinity columns retain the H_1, H_2 and T proteins present in the

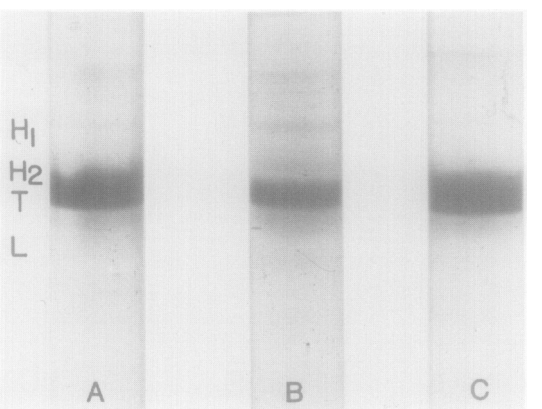

Fig. 3. SDS-urea polyacrylamide (7.5%) gel electrophoresis of tubulin purified by three cycles of polymerization depolymerization (Shelansky procedure). Unfractionated tubulin (A), excluded (B) and included (C) tubulin fractions from a 30 x 2 cm Sephadex G-150 column.

V_o samples of Sephadex G-150 columns chromatographed with crude rat brain homogenates (data not shown). Similarly, the affinity columns adsorb the T and L proteins from the V_i fractions of these Sephadex columns.

Affinity chromatography (4°) of Sephadex G-150 fractions (V_o and V_i) of tubulin previously purified by the Weisemberg procedure shows incomplete retention of the protein by DAC affinity columns under conditions which do not saturate the column. About 75% of the protein in the V_o sample, and 50% of that in the V_i sample, is retained by the affinity columns after extensive washing (25 bed volumes). This difference may be explained by the probable multi-point, more effective attachment of tubulin aggregates, compared to the dimer, to the affinity resin. In all cases

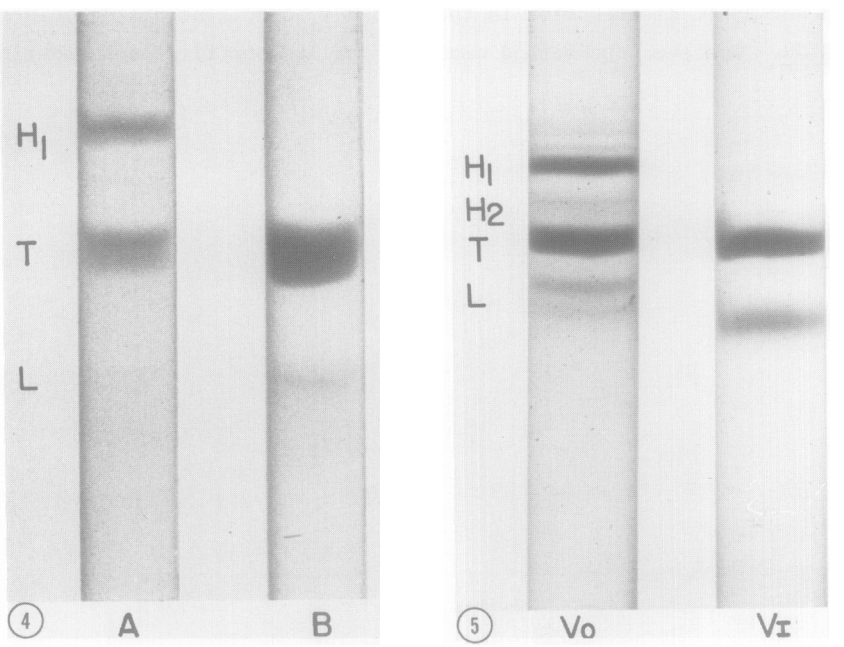

Fig. 4. SDS polyacrylamide (7.5%) gel electrophoresis run, in the absence of urea, according to the Laemli procedure (16). Size of the gels is 7 x 0.8 cm, while the other conditions are as described for gels A and B in Figure 1.

Fig. 5. SDS-urea polyacrylamide (7.5%) gel electrophoresis of the excluded (V_o) and included (V_i) Sephadex G-150 fractions of tubulin previously purified by the Weisemberg procedure and trapped by DAC-Sepharose columns. Tubulin purified by the Weisemberg procedure was chromatographed on a 30 x 2 cm Sephadex G-150 column and the V_o and V_i fractions eluting from this column were immediately applied on separate 2 ml (bed volume) DAC-Sepharose columns. After sampling, the affinity columns were washed with 50 ml buffer A and the total adsorbed protein was eluted with 7 M guanidine·HCl, pH 7.2. The guanidine was removed from the eluates by extensive dialysis and the samples were lyophylized before gel electrophoresis. The gel electrophoresis conditions were identical to those described for gels A and B in Figure 1.

the affinity columns retain proportional quantities of the H_1, H_2 and T proteins when the V_o samples are chromatographed, or of the L and T proteins when the V_i samples are examined (Fig. 5).

Affinity chromatography of the V_o protein on DAC-Sepharose columns shows that the H_1 protein is present predominantly in the breakthrough, non-adsorbed fractions and in the samples eluted with 0.1 M NaCl (Fig. 6). This protein is greatly decreased in the most tightly bound tubulin fraction, which is subsequently eluted with 7 M guanidine - HCl, pH 7.2 (Fig. 6). In contrast, the H_2 and L proteins are distributed evenly through these three fractions.

Centrifugation of the initial brain homogenates at 150,000 x g for 2.5 hours, which causes a considerable decrease in the V_o protein, or omission of GTP in the Sephadex G-150 column, procedures known to decrease the capacity of tubulin to polymerize (18-19), affect neither the proportion of H_1, H_2 and L proteins associated with tubulin nor their distribution in the V_o and V_i fractions, as described above.

DISCUSSION. Whatever the method employed for its purification, tubulin can be

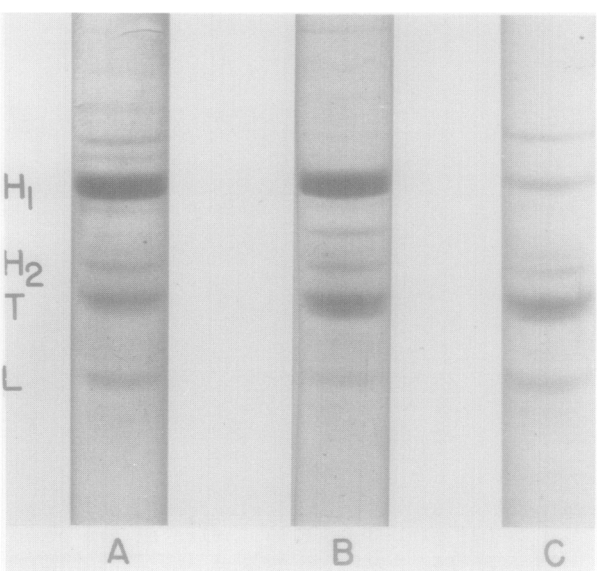

Fig. 6. Distribution of the H_1, H_2 and T proteins in different fractions of tubulin eluted from DAC-Sepharose columns. The whole V_o fraction of a Sepahdex G-150 column, on which tubulin purified by ammonium sulfate precipitation-DEAE cellulose had been chromatographed (4°), was sampled on a 2 ml (bed volume) DAC-Sepharose affinity column (4°). The washing volume (50 ml of buffer A) of the column was collected (A), and the column was eluted with 10 ml of 0.1 M NaCl (B) followed by 10 ml of guanidine·HCl, pH 7.2 (C). SDS-urea polyacrylamide (7.5%) gel electrophoresis of the three fractions (A, B and C) was run and the distribution of H_1, H_2 and T proteins studied. Size of the gels, 7 x 0.8 cm. Volume of the sample applied, 200λ (100 µg of protein per gel); 2.5 mamp per gel.

resolved into two major fractions by gel filtration chromatography. The material present in the excluded volume (V_o), which consists primarily of material with a sedimentation coefficient of 36S, is known to be constituted of rings and spirals which have the capacity to polymerize under appropriate conditions into microtubules (20). Furthermore, this fraction can induce the polymerization of the material present in the V_i fraction of the Sephadex column (1, 21). The latter protein, which has a sedimentation coefficient of 6S and a granular appearance by electron microscopy, is unable to polymerize by itself (1, 21) although it can be incorporated directly into microtubules and it can contribute to their elongation (21).

Treatment of the 36S material with high salt gives a major fraction of 6S protein which is able to exchange freely with the 6S protein present initially in the V_i fraction (21). Homogeneous 6S material, the tubulin dimer, can therefore be considered to constitute the major part of both the 36S and 6S tubulin fractions. Its integration into one or the other of these molecular species may be dependent on one or more factors, possibly proteins, which may be able to influence the equilibrium 36S $\rightleftarrows$ 6S in one or the other direction. This equilibrium has been demonstrated to constitute the first step in the polymerization of the 6S, tubulin dimer into microtubules (21). Therefore, the general equilibrium can be formulated as follows:

initiated microtubules $\rightleftarrows$ 6S (tubulin dimer) $\rightleftarrows$ 36S (coiled protofilaments) $\rightleftarrows$ microtubules

Although polymerization of tubulin into microtubules has been achieved *in vitro* (19), the molecular mechanisms and the precise locus of action of various factors (13, 19, 22) which are known to affect the equilibrium remain unknown.

The present studies describe the existence of three well defined proteins, H_1, H_2 and L which accompany tubulin when it is purified by grossly different methods. Furthermore, the distribution of H_1 and L proteins through the 6S and 36S fractions is clearly asymmetric, since the H_1 protein is restricted to the 36S fraction while the L component is present exclusively in the 6S fraction. This distribution may have some relevance to the different functional properties of the 36S and 6S fractions For example, the exclusive presence of the H_1 protein in the tubulin fraction which is able to polymerize by itself or to induce polymerization may contribute to these properties. Recently Weingarten *et al.* (21) have reported that the transformation of 6S to 36S, and therefore the formation of microtubules, depends on the presence of basic protein(s) associated with tubulin. Protein H_1, because of its peculiar association with the 36S fraction of tubulin, may be a good candidate for such a role, either in concert with other protein factors or by itself.

The involvement of a given protein in the 6S→36S transition does not exclude an additional, direct involvement in the subsequent process of microtubule assembly

from 36S protofilaments. For example, such an active role may be imagined if, after forming part of the coiled protofilaments (36S), the tertiary structure of the protein factor is modified, under conditions required for the 36S microtubule transition (13), in a way that causes a conformational change of the coiled protofilaments which facilitates their integration into a mature microtubule. Alternatively, the 36S structure may contain a protein whose function is expressed only in subsequent polymerization reactions.

It is well known that colchicine drugs cause microtubule depolymerization. We have observed that in colchicine-containing affinity columns the tighter the tubulin-colchicine complex, the greater is the exclusion of the H_1 protein from tubulin. If a role of H_1 in the polymerization of the tubulin dimer into microtubules can be demonstrated directly, it may be possible to test the hypothesis that the antimicrotubular activity of colchicine resides in its ability to exclude H_1 from ordered tubulin aggregates.

Preparations of tubulin purified by three cycles of polymerization-depolymerization contain less H_1 protein than tubulin purified by the Weisemberg procedure. Successive cycles of polymerization-depolymerization cause a diminution in the total amount of tubulin recovered, which may be due to exposure to depolymerizing conditions (cold, calcium treatment) which can lead to the loss of regulatory factors.

The exclusive presence of the L protein in tubulin fractions which are unable to form protofilaments (36S), and its scarcity in tubulin purified by cycles of polymerization-depolymerization, may also be causatively related phenomena. A possible role of the L protein in the elongation of microtubules by the addition 6S subunits (21) must also be considered.

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