PROTEINS FROM MORPHOLOGICALLY DIFFERENTIATED NEUROBLASTOMA CELLS PROMOTE TUBULIN POLYMERIZATION

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ABSTRACT

Clonal cells (N18) of the mouse neuroblastoma C-1300 can be induced to undergo a morphological differentiation characterized by the outgrowth of very long neurites (>150 μm) that contain many microtubules. Because the marked increase in the number and length of microtubules is apparently not due to an increase in the concentration of tubulin subunits, the possible role of additional macromolecules in the regulation of tubulin polymerization during neurite formation by N18 cells was examined. Using an in vitro system where the polymerization of low concentrations (<4 mg/ml) of purified brain tubulin requires microtubule-associated proteins (MAPs), high-speed supernates (250,000 g) from neuroblastoma and glioma cells were assayed for their ability to replace MAPs in the polymerization of brain tubulin. Only the supernates from “differentiated” N18 cells were polymerization competent. Electron microscope observations of these supernates failed to demonstrate the presence of nucleation structures (rings or disks). The active factor(s) sedimented at ~7S on sucrose gradient centrifugation and eluted from 4B Sepharose in the region of 170,000 mol wt proteins. Furthermore, the inactive supernates from other cells did not inhibit polymerization when tested in the presence of limiting MAPs. Thus, microtubule formation accompanying neurite outgrowth in neuroblastoma cells appears to be regulated by the presence of additional macromolecular factor(s) that may be functionally equivalent to the MAPs found with brain microtubules.

KEY WORDS microtubules · tubulin · neuroblastoma · neurites · differentiation

Nerve cell differentiation is characterized by the outgrowth of axons and dendrites, containing many long and well-organized microtubules. These microtubules are thought to function primarily as a cytoskeleton (24) and may provide an attachment site for contractile proteins related to neural function (20). Although there is a large increase in the number and length of microtubules during neuronal differentiation (15, 18), this process has been shown to be relatively independent of increased synthesis of tubulin subunits in a variety of nerve cell cultures (13, 15, 21, 22, 25, 28). These results suggested a possible role for additional macromolecules in the regulation of tubulin polymerization during nerve fiber outgrowth. Therefore, cell extracts of both morphologically differentiated and undifferentiated mouse neuroblastoma clone N18 and several other cell lines were compared for their ability to stimulate the in vitro polymerization of purified brain tubulin; the results of this study are presented here. An abstract communication of these results has appeared (10).
MATERIALS AND METHODS

Materials

Clonal cell lines of mouse neuroblastoma N18 and N103 have been described by Seeds et al. (22). The mouse glioma cell line, G26, was obtained from the National Cancer Institute and was originally described by Zimmerman and Arnold (29). All cells were grown in 150-mm plastic tissue culture dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.) containing Dulbecco's modification of Eagle's medium (DMEM) (Grand Island Biological Co., Grand Island, N. Y.) and fetal bovine serum (Rebeis Co., Inc., Phoenix, Ariz.). GTP (types II and VI) and trypsin were purchased from Sigma Chemical Co. (St. Louis Mo.). RNase and DNase were obtained from Worthington Biochemical Corp. (Freehold, N. J.) and Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, N. J.). All other reagents were of the purest analytical grade available.

Purification of Tubulin

Microtubules were isolated from lamb brain homogenates by two cycles of polymerization as described (14) and stored at -75°C until further use. Tubulin was isolated from the microtubule pellet by deacetylcolchicine acid (DAC) affinity chromatography as previously described (14). The purified tubulin was concentrated in an Amicon PM-10 ultrafiltration apparatus (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and de-salted by G-25 Sephadex column chromatography. The concentrated tubulin was centrifuged at 14,000 g to remove aggregates, and the resulting supernate was used immediately.

Preparation of Cell Extracts

Mouse neuroblastoma clone N18 (without neurites, N18-), clone N103, and glioma G26 were maintained in logarithmic growth in DMEM containing 10% fetal calf serum, while neurite formation (N18+) was induced in N18- cells by culturing for 3 days in DMEM with 0.2% fetal calf serum (15, 22). The media were removed and the cells were washed twice with saline (0.138 M NaCl, 5.4 mM KCl, 1 mM Na2HPO4, 1 mM KH2PO4) plus 1 mM CaCl2, followed by a rinse with 0.1 M imidazole-Cl (pH = 6.8), 0.5 mM MgCl2, and 1 mM ethylene glycol-bis(β-aminoethyl ether)N,N',N'-tetraacetic acid (EGTA). The cells were removed from the plastic dishes by scraping and placed in a 10 × 75 mm tube at 4°C for sonication with a Heat System Sonifer microtip (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) for 40 s at a power setting of 2. Then glycerol was added to a final concentration of 1 M and the samples were centrifuged at 250,000 g for 90 min or as indicated, and the supernate (S-250) was retained.

Polymerization Assay

The polymerization of lamb brain tubulin in the presence of neuroblastoma and glioma extracts was assayed in a polymerization buffer (0.1 M imidazole-Cl [pH = 6.8], 0.5 mM MgCl2, 0.5 mM EGTA, 1 M glycerol, and 1 mM GTP) by the glass fiber filter assay as previously described (11, 12) or by the sedimentation (28,000 g for 20 min) assay of Olmsted et al. (19). These two methods have been shown to give essentially identical quantitation of tubulin polymerization (11).

Agarose Chromatography

Cell extract (S-250) from differentiated and undifferentiated neuroblastoma cells was fractionated on a Sephacryl 4B column (37 × 1 cm) equilibrated with 0.1 M imidazole-Cl (pH 6.8), 0.5 mM EGTA, 0.5 mM MgCl2. Fractions of 0.56 ml were collected at a flow rate of 9.0 ml/h and assayed for protein by the method of Lowry et al. (9), and for their ability to stimulate polymerization of affinity chromatographically purified tubulin as assayed by both the filtration and the turbidimetric methods (6). The Sepharose 4B column was calibrated by chromatography of 0.7 ml of a 2.5 mg/ml sample of ovalbumin, tubulin, or immunoglobulin G fraction and a 1 mg/ml solution of Blue Dextran 2,000.

Electron Microscope Observations

After incubation, aliquots were removed and fixed with 2% glutaraldehyde and negatively stained in 1% uranyl acetate on parlodian-coated grids and washed extensively with water. The specimens were observed in a Philips 300 electron microscope.

RESULTS

Lamb brain tubulin isolated by DAC-affinity chromatography is >98% tubulin and polymerizes only at high (5-8 mg/ml) tubulin concentrations (11, 14). Although microtubule formation does not occur at low (2-3 mg/ml) tubulin concentrations, the addition of brain microtubule-associated proteins (MAPs) can induce these solutions to polymerize (11). Thus, the induction of polymerization in dilute solutions of tubulin can serve as an assay for factors promoting microtubule formation.

Cloned cell lines of the mouse neuroblastoma C 1300, some of which can be induced to extend axonlike fibers, provide a homogeneous cell system for assessing the regulation of microtubule formation as related to neurite outgrowth. High-speed supernatants of neuroblastoma clone N18 cells of logarithmic (without neurites, N18-) and stationary (with neurites, N18+) growth
phases, as well as a glioma supernate were examined for their ability to promote the polymerization of purified lamb brain tubulin. As seen in Fig. 1, the extracts from the differentiated cells (N18*) markedly stimulate the polymerization of brain tubulin, while the N18- extract shows no effect and the mouse glioma G26 extract gives only a slight enhancement.

The possibility that this difference in polymerization was due to the presence of inhibitory factors (e.g., polyanions [2]) in the N18- cells was examined by using a limiting amount of brain MAPs in addition to the purified brain tubulin (Fig. 2, solid circles). If the inactive supernates contained inhibitory components, we would expect their addition to decrease the rate and extent of tubulin polymerization. Because there was no inhibition, we can conclude that the N18* supernate contains a stimulating factor(s).

Because the morphological differentiation of neuroblastoma cells is induced by lowering the concentration of fetal calf serum in the culture media, the difference in the cell extracts may reflect only the culture conditions and not the morphological transition of these cells. Therefore, N18 cells in media containing a low serum concentration were also cultured in plastic Petri dishes (untreated for cell culture) where the N18 cells do not attach and remain in suspension without

![Figure 1](image1.png)

**Figure 1** Polymerization of lamb brain tubulin in the presence of increasing concentrations of S-125 supernates from neuroblastoma and glioma cells. Tubulin purified by affinity chromatography was incubated for 16 min at 37°C at a final concentration of 2.1 mg/ml in a set of tubes containing increasing concentrations of the 125,000 g supernates from cells of differentiated neuroblastoma N18* (*), undifferentiated neuroblastoma N18- (○), and glioma G-26 (△), in a total volume of 0.2 ml of polymerization buffer. The extent of polymerization of each incubation mixture was assayed by the sedimentation assay as described in Materials and Methods, using appropriate control samples containing 1 × 10^{-4} M colchicine.

![Figure 2](image2.png)

**Figure 2** Kinetics of tubulin polymerization stimulated by limiting amounts of brain MAPs in the presence and absence of neuroblastoma and glioma supernates. Tubulin purified by affinity chromatography was incubated at 37°C at a final concentration of 3.0 mg/ml with 0.39 mg/ml of MAPs (0.3 M NaCl eluate of microtubule protein fractionated on diethylaminoethyl Sephadex [11, 16]) in a final volume of 1.0 ml of polymerization buffer. Aliquots of 75 μl were taken at times indicated and assayed for polymerization (●) by the glass fiber filter method. Other 1.0-ml samples of this mixture were incubated in separate tubes in the presence of 0.78 μg/ml of S-125 supernates from neuroblastoma N18* cells (●), N18- (○), and glioma G-26 (△), and assayed similarly. Parallel samples, containing 1 × 10^{-4} M colchicine incubated at 37°C and assayed under the same condition, served as blank values.

neurites. The 250,000 g supernates from these cells grown in low-serum suspension cultures were relatively inactive for inducing tubulin polymerization (Fig. 3). Also, Fig. 3 (solid square) shows that under these conditions the N18* supernates do not self-polymerize and that microtubule formation requires exogenous brain tubulin.

A study similar to that in Fig. 3 was carried out with another neuroblastoma clone, N103, that attaches to culture dishes but fails to extend neurites under a variety of growth conditions that often influence changes in cellular morphology.
Fig. 3 Neuroblastoma factor and its relationship to serum concentration and cell morphology. Tubulin purified by affinity chromatography was incubated for 20 min at 37°C at a final concentration of 2.05 mg/ml in 0.2 ml of polymerization buffer containing increasing amounts of concentrated S-250 from extracts of neuroblastoma N18 grown in 0.2% serum suspension culture, N18+ grown in 0.2% serum, and N18– grown in 10% serum. After incubation, the extent of polymerization was measured by the glass fiber filter assay as described in Materials and Methods. Polymerization of N18+ extracts in the absence of exogenous brain tubulin (11).

Fig. 4 Neuroblastoma factor and its relationship to neurite formation. Lamb brain tubulin purified by affinity chromatography was incubated at a final concentration of 1.6 mg/ml in 0.2 ml of polymerization buffer under the same conditions as those of Fig. 3, with increasing amounts of S-250 supernate from neuroblastoma N18+ (0.2% serum) (○), N103 (0.2% serum) (△), and N103 (10% serum) (■). After the incubation, the extent of polymerization was assayed by retention of microtubules on glass fiber filters as described.

The chemical properties of this neuroblastoma factor(s) were examined. Table I shows that the polymerization-promoting factor(s) is heat labile, trypsin sensitive, and insensitive to RNase or DNase, suggesting that the factor is a protein.

Previous studies (15) have shown that the two different morphological types of N18 cells as well as N103 cells possess nearly identical amounts of tubulin; therefore, the stimulation of brain tubulin polymerization is not simply due to an increased concentration of tubulin subunits in the assay. However, the differentiated cells contain many more microtubules, and extracts from these cells may contain fragments of microtubules that could act as nucleation structures for polymerization. Although sonication in cold buffer should have depolymerized most microtubules and centrifugation of this sonicate at 250,000 g for 90 min should have removed any residual microtubule fragments, rings, or disks (1), these high-speed supernates were examined by electron microscopy for the presence of nucleation structures similar to those found in brain extracts (1, 3, 8) after microtubule depolymerization (Fig. 5A). Extensive examination of these neuroblastoma super-

TABLE I  
Stability of Neuroblastoma Factor(s)

| Treatment                          | µg MTs formed |
|-----------------------------------|--------------|
| None                              | 39.5         |
| 100°C for 5 min                   | 6.5          |
| Trypsin (10 µg/ml)                | 3.4          |
| Trypsin (10 µg/ml) + soybean trypsin inhibitor (100 µg/ml) | 28.5         |
| RNase (10 µg/ml)                  | 29.6         |
| DNase (10 µg/ml)                  | 32.5         |

A 0.1-ml aliquot (130 µg) of the N18+ S-250 supernate, treated with enzyme (37°C, 10 min) or heat and an untreated control sample were subsequently incubated for 20 min at 37°C with affinity chromatographically purified brain tubulin (2.3 mg/ml) in 0.2 ml of polymerization buffer. Tubulin polymerization was assayed by the glass fiber filter method, using appropriate colchicine-containing blanks. MTs, microtubules.
ates at high magnification failed to resolve any nucleation structures (Fig. 5B). However, the microtubules induced by the neuroblastoma factor(s) (Fig. 5C and D) appear similar to those formed by the addition of brain MAPs (not shown) and distinct from the double-walled tubules formed by polycationic molecules (3). The possibility of large (20-36S) nucleation structures was also examined by fractionation of the neuroblastoma supernates on 5-20% sucrose gradients.

FIGURE 5 Electron micrograph showing a typical view of an S-250 supernate from “differentiated” N18 cells (B) and, for comparison, a view of “rings” from depolymerized lamb brain microtubules at the same magnification (A). The microtubules induced to assemble by neuroblastoma factor and brain tubulin are shown in (C) and, in higher magnification, in (D) where the typical protofilament structure is readily observed. Bar, 1,000 Å.
The active fractions from the N18\(^{+}\) supernate were of relatively small size, with an approximate sedimentation coefficient of 7S (data not shown). The active supernate from the morphologically differentiated N18\(^{+}\) cells was resolved on Sepharose 4B column chromatography (Fig. 6). Although a very small amount of polymerization-promoting activity was found in the void fraction and in a high molecular weight region (fraction 30) of included molecules, the major peak of activity (fraction 38) eluted in the region of 170,000-dalton globular proteins and was clearly

![Figure 6 Molecular sieve chromatography of the neuroblastoma factor. A 0.7-ml aliquot (11.8 mg protein/ml) of S-250 from N18\(^{+}\) cells (upper panel) or N18\(^{-}\) cells (lower panel) was chromatographed on a 37 × 1 cm column of Sepharose 4B and fractions of 0.56 ml were collected. The brain tubulin polymerization enhancement activity of the fractions (○) was assayed by incubation for 20 min at 37°C of an 80-μl aliquot from each fraction with purified tubulin (1.6 mg/ml) in a total volume of 0.16 ml of polymerization buffer and retention of microtubules formed on glass fiber filters under conditions described in Materials and Methods. The protein concentration of the fractions is also shown (○). The arrows indicate the elution volume of molecular weight standard proteins: immunoglobulin G (165,000), tubulin (110,000), and ovalbumin (45,000 daltons).]
distinct from tubulin, which eluted in fraction 41. Supernates from the N18- cells were also characterized on this column and gave a similar profile for the elution of protein but, as expected, were without polymerization-promoting activity. SDS-polyacrylamide gel electrophoresis of fractions from N18+ and N18- showed (Fig. 7) that fraction 30 from both samples displayed several bands that co-migrated with the high molecular weight MAPs of brain, although these fractions produced little or no polymerization, respectively. The protein staining of polyacrylamide gels containing fraction 38 from N18+ and N18- was similar; however, several bands including a major component (*) displayed a slightly slower migration in the active N18+ fraction compared to the inactive N18-. The possibility that this difference in migration may represent a modified protein is currently being examined, as well as the purification and identification of the active component(s) in the N18+ extracts.

DISCUSSION

The studies presented in this report have shown that, concomitant with neurite outgrowth, neuroblastoma cells acquire a protein factor that promotes the in vitro polymerization of tubulin. This protein either is absent or is present in greatly reduced amounts in the "undifferentiated" or neurite-minus neuroblastoma cells, in contrast to tubulin which is present in an equal amount (15). These results are similar to those of recent studies (4, 5) with rat brain homogenates, where extracts from 30-day-old animals showed markedly greater rates and extents of tubulin polymerization than did extracts from 9-day-old rats, although the younger animals possessed higher concentrations of tubulin. Additional studies have shown that these brain extracts from young animals appear to be lacking an initiation protein, tau (26), or similar component, since the addition of high concentrations of tau factor stimulates the rate of tubulin polymerization in extracts from young animals, while it exerts no effect on tubulin polymerization in extracts of old rats (4). Thus, during this time of active neurite outgrowth and synaptogenesis (day 9-30), the developing rat brain tissue acquires a factor that promotes in vitro polymerization of brain tubulin. These studies and the results presented in this report suggest that neuronal differentiation is accompanied by the formation of protein factors that promote microtubule formation.

Obviously, the neuroblastoma factor participates in initiation of microtubule formation in vitro (Fig. 1) and possibly in elongation events (Fig. 2), since the extent of microtubule formation in the presence of MAPs is increased by the N18+ factor. However, it remains to be demonstrated that these neuroblastoma or brain factors are required for initiation or elongation events in situ, and are not merely stabilizers for microtubule networks in the differentiated cells.

Several protein factors that have been isolated by different methods enhance or are required for
tubulin polymerization in vitro and are proposed to regulate microtubule assembly in situ (7, 16, 23, 26). One is the heat-stable, cationic protein of 64,000 daltons called tau (26). Others are the high molecular weight proteins (HMWs) or MAPs, a pair of closely spaced bands (300-350,000 mol wt) after electrophoresis on SDS polyacrylamide gels, representing 5-20% of the microtubule proteins in some preparations (16, 23). At present, our studies (Table I and Fig. 6) suggest that the active component in extracts from neuroblastoma cells (heat labile, ~170,000 mol wt) is distinct from both of these factors as well as tubulin; current studies on the purification of this factor should permit a definitive answer to their relationship.

Although neuroblastoma tubulin has been reported to undergo self-assembly (17), Fig. 3 shows that under our assembly conditions neuroblastoma extracts did not display microtubule self-assembly and agree with the earlier observation of Wiche and Cole (27); however, the relatively low tubulin concentration (~0.1-0.2 mg/ml) in the assay may be below the critical concentration for polymerization. Self-assembly of these N18 extracts at very high protein concentrations or of purified neuroblastoma tubulin has not been examined.

The demonstration that a protein which promotes microtubule formation is present in a specific morphological state of a clonal nerve cell culture, and the reversibility of this morphological transition (22) should permit a variety of studies related to the in situ functions of this protein that may not be possible in a heterogeneous tissue such as brain.

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