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Direct Isolation of Neuronal Microtubule Skeletons

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The microtubule skeletons of cultured neuronal cells have been isolated in their original form utilizing taxol and rigorous extraction conditions. Continuous microtubules are visible throughout the cell body and into the processes. These preparations include the complex set of microtubule-associated proteins of 69,000 and 80,000 daltons previously identified in these cells.

Analysis of the structure and molecular composition of cytoplasmic microtubules has required strategies to overcome the lability and low abundance of these organelles. Recently, our laboratory has developed approaches that allow the analysis of these structures in situ in detergent-extracted cells (12). By using selective detergent extraction procedures and the well-characterized drug and ionic sensitivities of microtubules, it has been possible to identify microtubule-associated proteins in these preparations. This approach has been applied to a variety of cells in interphase and mitosis (3, 14). In this report, we describe our efforts to directly isolate the microtubule networks of interphase neuronal cells. These isolates preserve the microtubule networks in their in vivo distribution and are useful for both ultrastructural and biochemical analysis. These results suggest that the microtubules are continuous in the neurites of neuroblastomas. In addition, the isolates include the complex family of microtubule-associated proteins previously identified in this cell type.

Mouse neuroblastoma cells (NB-2A) (7, 10) and the clonal line of rat pheochromacytoma cells (5) were maintained as previously described (3). Microtubule networks were prepared by sequentially extracting cells in a microtubule stabilization buffer (0.1 M PIPES, 1 mM $MgSO_3$, 2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid], 2 M glycerol, 0.2% Nonidet P-40 [pH 6.9]) supplemented with 5 μg of taxol per ml and protease inhibitors, followed by the same buffer containing 100 μg of DNase I per ml at 25°C for 5 min and then with 1 mM PIPES (pH 6.9) (14). After the low-ionic-strength extraction, the isolated skeletons detach from the monolayer and are collected for analysis by sedimentation at 100 × *g* for 3 min.

Isolated microtubule networks from mouse neuroblastoma cells are viewed after critical point drying (Fig. 1A and B). The stabilized microtubules and some insoluble material remain attached to the nuclear envelope. If taxol is omitted from the extraction buffers or if microtubules are depolymerized by prior exposure to antimitotic drugs, intact microtubules are not preserved, and the only sedimenting structures are nuclear envelopes with some associated insoluble material (Fig. 1C).

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The taxol-stabilized microtubules in the isolated skeletons appear as uniform fibers 24 to 25 nm in diameter (9, 13). In the perinuclear regions of the cell, individual microtubules can be followed for distances of over 5 μm. Few lateral interactions are seen between microtubules, and some microtubule ends are clearly visible. The microtubules do not appear to arise from a single perinuclear focus but rather they appear to form a complex meshwork which surrounds the nucleus.

In virtually every cell examined, discrete groups of microtubules extend from the cell body. These groups are between 5 and 50 μm long, and their morphology makes it likely that they are from the neurites of the original cell (11). Individual microtubules can be traced for considerable distances (7 to 12 μm). At some point in each of these groups, the microtubules gather into a short dense bundle and then splay out again toward the distal end. Because of this bundling, it is impossible to trace individual microtubules from the cell body to the end of the process. However, these groups do contain similar numbers of microtubules on either side of the bundles. For example, by counting in regions where the individual microtubules are visible, the process in Fig. 2 contains ca. 31 (plus or minus 3) microtubules at both the proximal and distal ends. This is consistent with the notion that some microtubules are continuous through the entire neurite (1, 2, 4, 6).

The protein composition of isolated microtubule networks from rat pheochromacytoma cells and mouse neuroblastoma cells are examined directly on two-dimensional gels in Fig. 3 and 4, respectively. This is accomplished by examining the proteins of both the stabilized microtubule skeletons (Fig. 1A and B) and the control preparations lacking microtubules (Fig. 1C). The proteins that are present only in the stabilized preparations and absent in the controls lacking microtubules are the putative microtubule-associated proteins.

As expected, the major difference in the protein profiles of the stabilized and control preparations is the presence of the large amount of tubulin in the controls. The tubulin in these preparations is heavily overexposed to emphasize the less-abundant-associated proteins and is only resolved as one large spot (Fig. 3A and 4A). In addition to tubulin, there are several other groups of proteins that are present only in cells that contain microtubules. These include the complex fam-

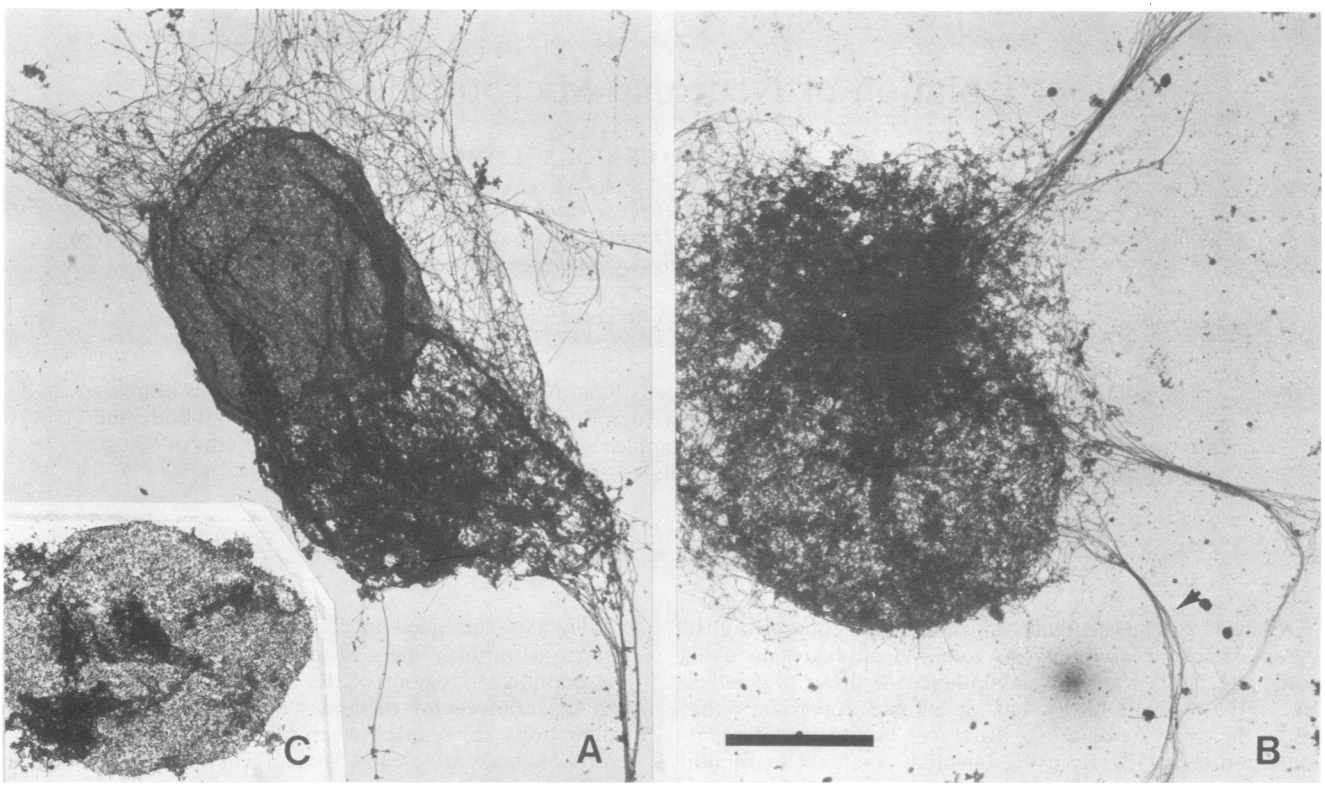

FIG. 1. Whole mount electron micrographs of isolated neuroblastoma microtubule skeletons. (A and B) Mouse neuroblastoma cells were detergent extracted in a microtubule stabilization buffer containing 5 μg of taxol per ml, and the microtubule skeletons adhering to the insoluble nuclear envelope were collected and processed for electron microscopy as described in the text. The process indicated with the arrow in (B) is examined at higher magnification in Fig. 2. (C) An identical preparation from a cell pretreated with 10 μg of Nocodazole per ml for 30 min before fractionation. Bar, 5 μm; magnification, ×2,850.

ilies of microtubule-associated proteins with molecular weights of about 69,000 (69K) and 80K previously identified as microtubule-associated proteins in these cells by different criteria (8). The 69K dalton (69Kd) and 80Kd proteins are closely related to each other and to the Tau proteins previously identified in brain microtubule preparations. Each protein exists as a set of structurally related polypeptides with different isoelectric points.

In the neuroblastoma preparations, there are about six proteins of 69Kd with isoelectric points of 6.5 to 7.5 that fit the criteria for microtubule-associated proteins. There are also several major proteins of 80Kd that are present if and

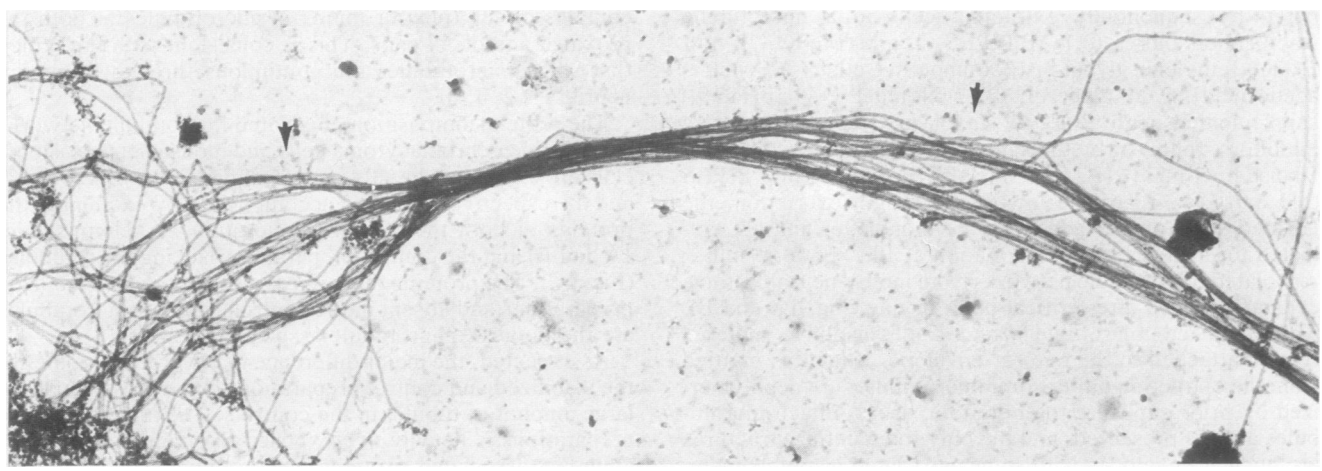

FIG. 2. Microtubule bundles in the processes of extracted neuroblastoma cells. A process of the cell in Fig. 1B is observed at higher magnification. There are ca. 31 (plus or minus 3) microtubules at the regions indicated by the arrows proximal and distal to the region of bundling. Magnification, ×14,200.

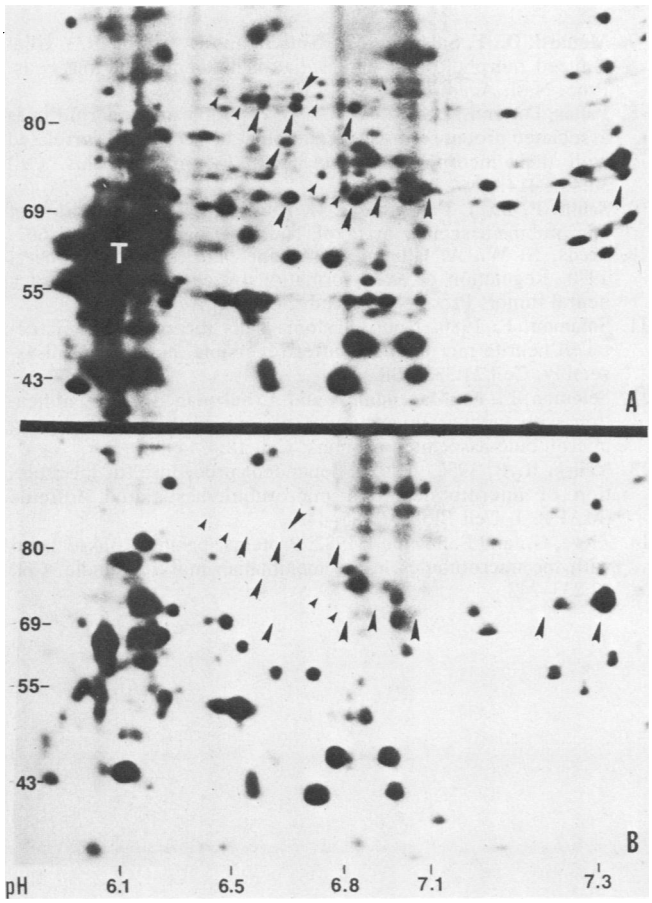

FIG. 3. Polypeptide analysis of isolated microtubule skeletons from neuroblastoma cells. Neuroblastoma cells were metabolically labeled for 16 h in medium containing 10% the normal methionine level and 10 μCi of [^{35}S]methionine per ml. At 30 min before extraction, half the cells were exposed to 10 μg of Nocodazole per ml. Equal numbers of control and experimental cells were then extracted to produce microtubule skeletons as described in the text. (A) Profile of normal experimental cells containing stabilized microtubule skeletons. (B) Control cells lacking intact microtubules because of exposure to Nocodazole before lysis. By our criteria, microtubule-associated proteins are those proteins present in the isolates in (A) and lacking in (B). The location of the major proteins that fit these criteria for microtubule-associated proteins are indicated with arrowheads in both the experimental and control preparations. Some minor spots are not marked for the sake of clarity. The tubulin (T) subunits are indicated.

only if the microtubules are present. In addition, there are a number of minor proteins with higher apparent molecular weights and more acidic pI's that seem to radiate from several of these major spots. Most of these are marked with arrowheads but a few have not been marked to reduce confusion (Fig. 3). Almost all these proteins are phosphorylated, and the minor families of proteins radiating from the major spots are more obvious when preparations are labeled with [^{32}P]phosphate. Differences in phosphorylation account for many of the variants (8). In the pheochromacytoma preparations, the families of 69Kd and 80Kd microtubule-associated proteins are less complex. There are a number of 69Kd proteins but only one major 80Kd protein (Fig. 4).

Control experiments demonstrate that there is no artifactual association of tubulin or any other proteins with the isolated microtubule networks during the isolation procedure. However, this analysis may miss some classes of microtubule-associated proteins. In particular, it will not identify proteins removed from the microtubules by either the detergent extraction or the low ionic strength. As previously demonstrated with the taxol-stabilized isolated spindles, the 220Kd microtubule-associated protein in these cells is lost from the stabilized microtubules by the low-ionic-strength extraction procedure (14).

We are presently preparing specific antibodies directed against the microtubule-associated proteins identified in the neural cells. The availability of specific antibodies will allow the localization of these proteins at both the light and electron microscopic level. The isolated microtubule networks will be useful preparations for the immunoelectron microscopic localization of the microtubule-associated proteins. The ultimate goal of this work is to understand how the microtubule-associated proteins contribute to the unique geometry and function of the microtubules in the neurites of the neural cells.

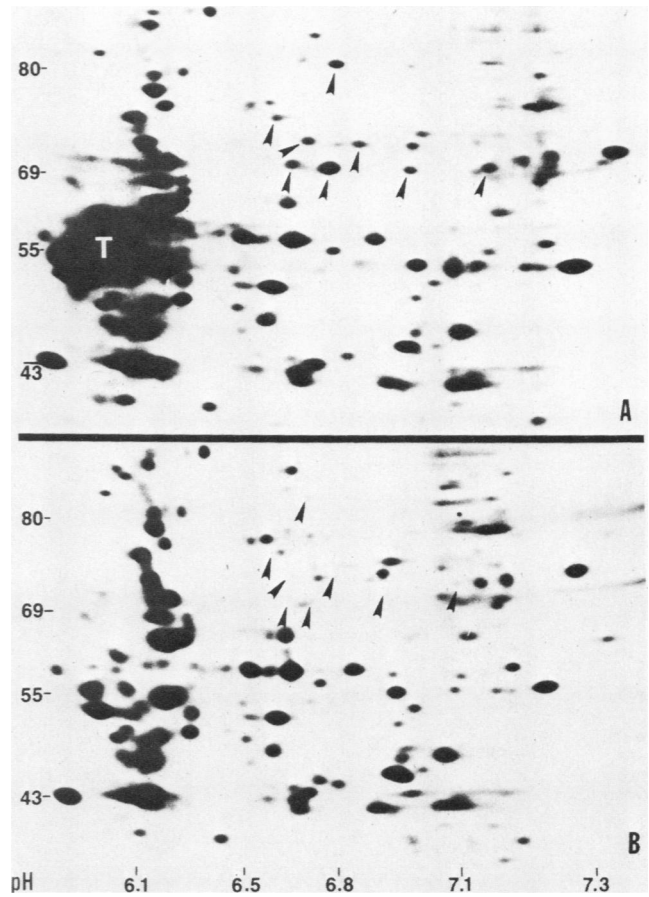

FIG. 4. Polypeptide analysis of isolated microtubule skeletons from rat pheochromacytoma cells. PC 12 cells were metabolically labeled with [^{35}S]methionine and extracted as described in Fig. 3. Equal numbers of skeletons from experimental and control cells were then analyzed by two-dimensional gel electrophoresis. (A) Protein composition of preparations containing intact microtubule skeletons. (B) Composition of controls lacking microtubules. The tubulin (T) subunits are marked. The locations of the families of proteins of 69K and 80K molecular weight that fit our criteria as microtubule-associated proteins are marked with arrowheads.

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