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Analyzing the components of microtubules: Antibodies against chartins, associated proteins from cultured cells

(microtubule-associated protein/tubulin/affinity elution/immunofluorescence/immunoblotting)

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ABSTRACT **In previous work, we have identified cytoplasmic microtubule-associated proteins by isolating the microtubule organelles of several different cultured cells. Among those proteins are the chartins, a family of polypeptides with related sequence but of varying molecular weight and isoelectric point. Biochemical analyses of the distribution of the chartins in the cytoplasm, and among cells with different functions, suggest that they may regulate microtubule structure *in vivo*. We describe here the preparation and application of antibodies to chartins. These antibodies enable us to demonstrate that the chartins colocalize with assembled tubulin in the cytoplasm, as assessed by immunofluorescence, so that they fulfill a major criterion that has been applied to other putative microtubule components. The results also demonstrate that the τ proteins, which fractionate and copurify with chartins, are in fact clearly distinguishable from them. The implications of these results for evaluating microtubule composition are discussed.**

Microtubules occur in a variety of structures and apparently perform a variety of functions. This diversity displayed by a highly conserved structural element might be explained by subtle variations in the major microtubule component, tubulin, or by specificities among the minor components or other interacting elements. We have developed a fractionation procedure that enables isolation of microtubule organelles from cells and analysis of their molecular components without relying on *in vitro* reconstruction experiments (1). This analysis, applied to several different cell types in different contexts, supports the idea of a regulatory role for these minor components, the microtubule-associated proteins (2–4).

Among the proteins uncovered by this approach are a family of related polypeptides, which we here name chartins. In neuroblastoma cells, the major forms of the chartins have nominal molecular sizes of 69 kDa, 72 kDa, and 80 kDa, although in fact the family is subject to covalent modifications that affect not only their isoelectric point but produce variations in their apparent molecular size as well. The relationship among these proteins at the level of primary sequence has been demonstrated by peptide mapping; the 80-kDa form includes all the methionine-containing tryptic peptides present in the 69-kDa form, plus three more (4). This relationship becomes significant because the 69-kDa chartins can be found in preparations of interphase microtubules from all rodent cells, while the 80-kDa form is found in only a subset of those cells—those derived from neural tissue (2). Differences in the distribution and expression of these proteins could provide important insight into their function *in vivo*.

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As a first step toward that goal, we have prepared antibodies against the chartins. Our fractionation procedure, applied as it is to cultured cells, produces associated proteins in relatively small amounts, but antibody techniques recently developed by others have allowed us to circumvent that problem. In the present study, two such antibodies are characterized. They allow us to demonstrate the tissue distribution and intracellular localization of the chartins. In particular, they show that the chartins colocalize with assembled tubulin in the cytoplasm, as do other associated proteins identified by *in vitro* assembly (5–10). The antibodies also permit us to compare directly the results of different approaches toward identifying microtubule-associated proteins.

MATERIALS AND METHODS

Cell Culture. The origin and maintenance of the cultured cells lines—mouse neuroblastoma (NB2A), mouse 3T3, hamster fibroblast (NIL8), rat glioma (C6), and rat pheochromocytoma (PC12)—were as described (1–4).

Preparation of Antigens. Calf brain microtubule protein was isolated by two cycles of temperature-dependent assembly, in the absence of glycerol (11), and the microtubule-associated protein (MAP) fraction was purified by phosphocellulose chromatography and ammonium sulfate precipitation of the peak of the 0.35 M NaCl eluant (12). The pellet was redissolved to a final concentration of 15 mg/ml and fractionated by $NaDodSO_4$/PAGE (13), applying ≈4 mg per slab gel. Neuroblastoma protein was prepared as the calcium-mediated microtubule depolymerization extract (1, 4). Approximately 2 mg of protein was obtained in this fraction from 10^8 cells. The depolymerization extract was concentrated by acetone precipitation overnight at −20°C and fractionated by $NaDodSO_4$/PAGE as described above. The 69-kDa regions in both preparations were localized by Coomassie staining, cut out, and washed overnight in phosphate-buffered saline.

Immunizations. The acrylamide pieces containing 40–80 μg of the 69-kDa regions were mashed with a pestle, mixed with Freund's complete adjuvant, and injected subcutaneously into New Zealand White rabbits at multiple sites. Secondary injections were in incomplete adjuvant, began 1 month later, and thereafter occurred every 1–2 weeks.

Protein Blots. All procedures were essentially as described by Towbin *et al.* (14), except that the blocking and washing solutions contained 2% hemoglobin. The microtubule depolymerization extracts of untreated and drug-treated cells were prepared as described (1, 4). Bound antibody was detected by incubating the strips with 10^5 cpm of ^{125}I-labeled protein A and exposing the films to x-ray film with an intensifying screen at −70°C.

Affinity Elution. Ten to twenty strips (0.3 cm wide) of nitrocellulose bearing either brain or neuroblastoma protein

Abbreviation: MAP, microtubule-associated protein.

were used as substrate for adsorption. All procedures were as described by Olmsted (15). To prepare more concentrated antibody, the elution was performed with 0.2 M acetic acid (pH 2.8), and the eluant was immediately neutralized with 1 M ammonium carbonate. These samples were lyophilized in the presence of 100 μg of bovine serum albumin as carrier, redissolved in phosphate-buffered saline, and kept at 4°C.

Immunofluorescence. Detergent-extracted cytoskeletons of NIL8 or neuroblastoma cells were prepared for microscopy as described (1). The affinity-eluted and concentrated 4-81 serum was visualized with affinity-purified fluorescein-tagged goat anti-rabbit IgG. The mouse monoclonal antibody against tubulin was visualized with rhodamine-tagged sheep anti-mouse IgG. Both reagents were purchased from Cappel Laboratories (Cochranville, PA). Images were photographed on a Zeiss PhotoIII, using Kodak Tri-X film which was developed in HC-110 at dilution B.

RESULTS

Preparation of Antibodies. Several attempts to make monoclonal antibodies against the chartins were unsuccessful, regardless of the source of the protein, the injection protocol, and so forth. Antibodies against other lower abundance proteins in the same preparations of immunogen did arise, suggesting that the chartins may be less immunogenic. Only two of several attempts to raise polyclonal antibodies were successful, one using neuroblastoma protein and one using protein from bovine brain. Fig. 1 shows the regions of one-dimensional gels that were excised and injected from phosphocellulose-purified brain MAPs (lane A) and microtubule depolymerization extract from neuroblastoma cells (lane B). The appropriate region of the brain protein gel was determined by comparison with two-dimensional gels of the same preparations, from which spots have been isolated and shown to be related to chartins by peptide mapping (unpublished observations). The appropriate region of the neuroblastoma protein gel was determined by comparing it with a gel of the analogous extract from drug-treated cells, from which the chartins are missing (lane C). That the obvious complexity of these antigen preparations did not prevent the preparation of useful reagents is due to the power of two techniques, protein–antibody blotting and the affinity elution of antibodies from nitrocellulose strips worked out by Olmsted (15).

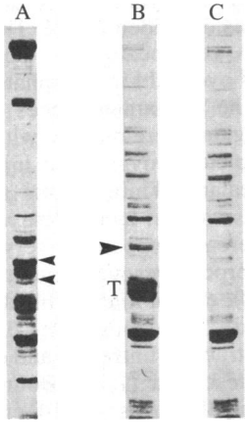

FIG. 1. Preparation of immunogens. Coomassie blue-stained gels of phosphocellulose-purified bovine brain MAPs (lane A), the microtubule depolymerization extract of untreated NB2A cells (lane B), and NB2A cells incubated with Nocodazole (lane C). Arrowheads indicate the regions of the gel around 69 kDa excised for injection. T indicates the position of the tubulin bands.

An Antibody Raised Against Brain Protein. One rabbit, injected with proteins of ≈69 kDa from the brain MAP preparations, produced a serum (3417) that stained at least five bands on immunoblots of neuroblastoma microtubule depolymerization extracts. The most prominent of those bands absolutely comigrated with the 80-kDa band previously identified as one of the chartins, and it was absent from the corresponding extract from cells previously treated with Nocodazole (Fig. 2, compare lanes A and B). No binding to a protein comigrating with the 69-kDa chartin was detected, despite the close homology between these proteins.

The same serum binds to several bands in other cell types, such as NIL8 and 3T3 cells, but those bands are present in the extracts from both drug-treated and untreated cells. No band corresponding to the 80-kDa chartin, either by apparent molecular size or fractionation properties, is found in these cells. This result is also consistent with previous biochemical data, suggesting that the 80-kDa chartin could not be detected in these cell types (2).

The antibodies in serum 3417 that recognize the 80-kDa chartin are distinct from those that recognize the several other proteins. That fact was demonstrated by fractionating the serum by affinity elution, using as substrate for the adsorption a number of nitrocellulose strips with bound neuroblastoma protein. The 80-kDa region alone was eluted, and neuroblastoma strips were reprobed. This preparation bound to only one band, of 80 kDa, present in extracts from control cells but not from drug-treated cells (Fig. 2, compare lanes C and D) and again absent from NIL8 cell extracts (lane 3). Curiously, no band is detected with the affinity-eluted antibody when phosphocellulose-purified brain MAPs are probed (lane F). In fact, the whole serum 3417 does recognize a complex series of proteins in these preparations (data not shown), but none of those activities copurifies with the anti-80-kDa activity. We attempted to use the purified anti-chartin activity of serum 3417 for immunofluorescence, but it produced images that were not clean and not usable.

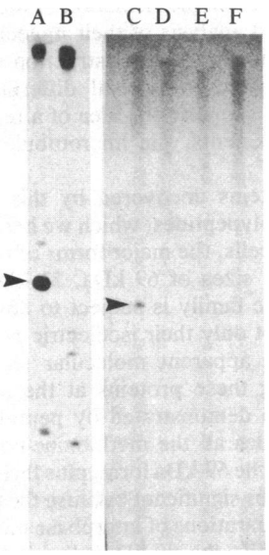

FIG. 2. Immunoblotting with serum 3417. Autoradiograms of blots probed with serum 3417 (lanes A and B) or antibodies affinity-eluted from the 80-kDa band (lanes C–F). The proteins on the nitrocellulose strips are from microtubule depolymerization extract, untreated NB2A cells (lanes A and C), the same extract from drug-treated NB2A cells (lanes B and D), microtubule depolymerization extract, untreated NIL8 cells (lane E), phosphocellulose-purified brain MAPs (lane F). Arrowheads indicate the position of the 80-kDa chartin band.

An Antibody Raised Against Neuroblastoma Protein. A rabbit injected with protein from the 69-kDa region of neuroblastoma microtubule depolymerization extracts produced a serum (4-81) that stained at least four major bands (Fig. 3, compare lanes A and B). The two smallest bands comigrate with tubulin and actin, and the two largest comigrate with the 69-kDa and 80-kDa chartins. In this example, and occasionally in others, the smaller chartin is resolved into two bands, probably corresponding to the 69-kDa and 72-kDa forms seen in two-dimensional gels (4). Those bands, and the presumptive tubulin band, are absent from extracts derived from Nocodazole-treated cells. Proteins of similar molecular size and fractionation are found in PC12 cells (Fig. 3, compare lanes C and D). In NIL8 cells (compare lanes E and F), a faint 69-kDa band, but not the 80-kDa band, is detected. In C6 cells (compare lanes G and H) a faint band at 80 kDa is detected, but not one at 69 kDa. The serum 4-81 also stains two bands in phosphocellulose-purified brain MAP preparations, again of ≈69 kDa and ≈80 kDa (lane I). This serum provides us with evidence that the 80-kDa chartin is present in brain.

We used the affinity elution procedure to fractionate the serum. In dealing with serum 4-81, we used phosphocellulose-purified MAP strips as the substrate for adsorption, because those proteins are much more readily available in significant quantities. After elution of several strips (see *Materials and Methods*) and reprobing of new strips, only two bands—of 69 kDa and 80 kDa—are recognized in extracts from control cells (Fig. 4, compare lanes A and B and lanes D and E). Both bands are present in the brain MAP preparations (compare lanes C and F). No other bands are detected. As the data in Fig. 4 show, antibodies that recognize both the lower and upper bands are adsorbed by each of them. These results are consistent with the known peptide homology between these two proteins, at least in neuroblastoma cells (4).

The 4-81 serum also enables us to resolve the relationship

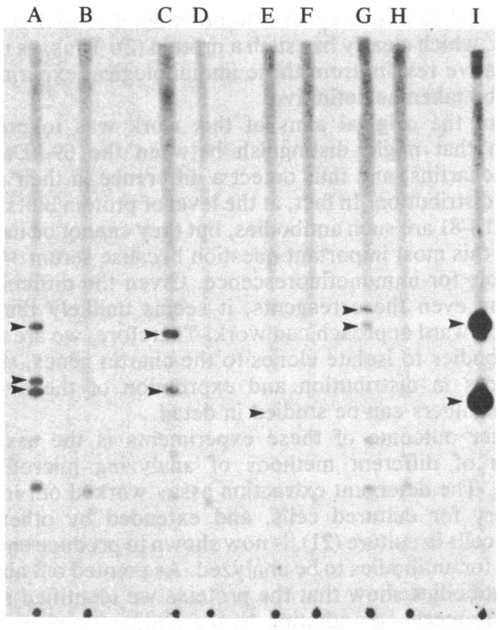

FIG. 3. Immunoblotting with serum 4-81. Autoradiograms of blots probed with serum 4-81. The proteins on the nitrocellulose strips are microtubule depolymerization extracts from untreated cells (lanes A, C, E, and G), drug-treated cells (lanes B, D, F, and H), or phosphocellulose-purified brain MAPs (lane I). Lanes: A and B, NB2A; C and D, PC12; E and F, NIL8; G and H, C6. Arrowheads point to the positions of the chartin bands detected by the antibody.

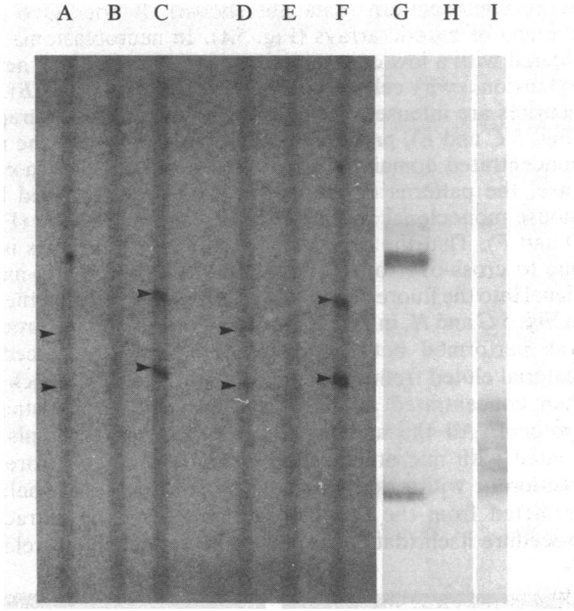

FIG. 4. Immunoblots with affinity-eluted 4-81 antibodies and with anti-τ antibodies. Autoradiograms of blots probed with antibodies eluted from the 69-kDa (lanes A–C) and 80-kDa (lanes D–F) bands of phosphocellulose-purified brain MAPs or with anti-τ antibody (lanes G–I). Proteins on the nitrocellulose strips are from microtubule depolymerization extracts from untreated NB2A cells (lanes A, D, and G), from drug-treated NB2A cells (lanes B, E, and H), or from phosphocellulose-purified brain MAPs (lanes C, F, and I). The 69-kDa and 80-kDa chartins are marked with arrowheads.

between the chartins and τ protein, with which they can be compared on the basis of copurification, presence in microtubules assembled *in vitro*, and approximate molecular size (2). Probing blots with the polyclonal serum used as the essential reagent for cloning the τ genes (17), discrete bands were labeled as described. In both brain MAP preparations (Fig. 4, lane I) and in neuroblastoma cell preparations (compare lanes G and H), the bands are clearly distinct from those recognized by 4-81. In particular, the bands labeled by the anti-τ antiserum do cofractionate with the chartins into the microtubule depolymerization extract of untreated cells only (compare lanes G and H), but they do not comigrate with the chartins. This fractionation of the τ polypeptides and their apparent size fit well with results reported by Kirschner and colleagues (18). Armed with hindsight, we have reexamined our analyses of neuroblastoma proteins, but we have been unable to detect τ proteins either as methionine-labeled, phosphate-labeled, or stained bands in our preparations. These results demonstrate that in the size fraction of brain MAPs, conventionally called the "tau" fraction, chartins, and perhaps other proteins as well, are present.

Immunofluorescence with Anti-Chartin. The problems of making an antibody to a low-abundance protein that behaves as if it were poorly immunogenic are compounded by the special demands of immunofluorescence. We were trying to determine whether the 4-81 serum would stain microtubules in cells. Estimating from the biochemical analyses, the chartins probably represent ≈1% of the tubulin in the assembled pool of microtubules. The expected low-intensity signal could be satisfactorily amplified by the photographic procedures described in *Materials and Methods*, and the whole serum could be seen to stain microtubule-like patterns. To be certain that staining was not due to antibodies against tubulin, we used affinity elution, modified to give a more concentrated antibody. This antibody stained interphase NIL cells very poorly, although that staining does suggest a

microtubule pattern (data not shown). It does give clear staining of mitotic arrays (Fig. 5A). In neuroblastoma cells treated with a low concentration of serum to induce neurite extension, every cell is stained by the antibody (Fig. 5B). The neurites are intensely stained and are readily photographed (Fig. 5 C and E), probably because they represent the most concentrated domain of microtubules in the cells. In every case, the patterns correlate well with that produced by a mouse monoclonal antibody directed against tubulin (Fig. 5 D and F). That the correspondence of these patterns is not due to cross-over of the rhodamine-illuminated anti-mouse signal into the fluorescein channel is demonstrated by the pair in Fig. 5 G and H, in which the same double-staining protocol was performed but eluted serum 4-81 was replaced by material eluted from a blank region of the strips, which was then concentrated and used in the same double-staining protocol. All the staining disappears when the cells are treated with microtubule depolymerizing drugs before extraction or with calcium ions after extraction, as would be predicted from the blotting data and from the extraction procedure itself (data not shown). The excellent correlation between the anti-chartin and anti-tubulin patterns also holds for the finer, and probably individual, microtubule elements in the cell body, but these do not photograph well in these cells, in part because they are not coplanar. The results suggest that the chartins colocalize with assembled tubulin in these cells and fit with previous findings, suggesting that the chartins and microtubules are in close proximity in the cytoplasm.

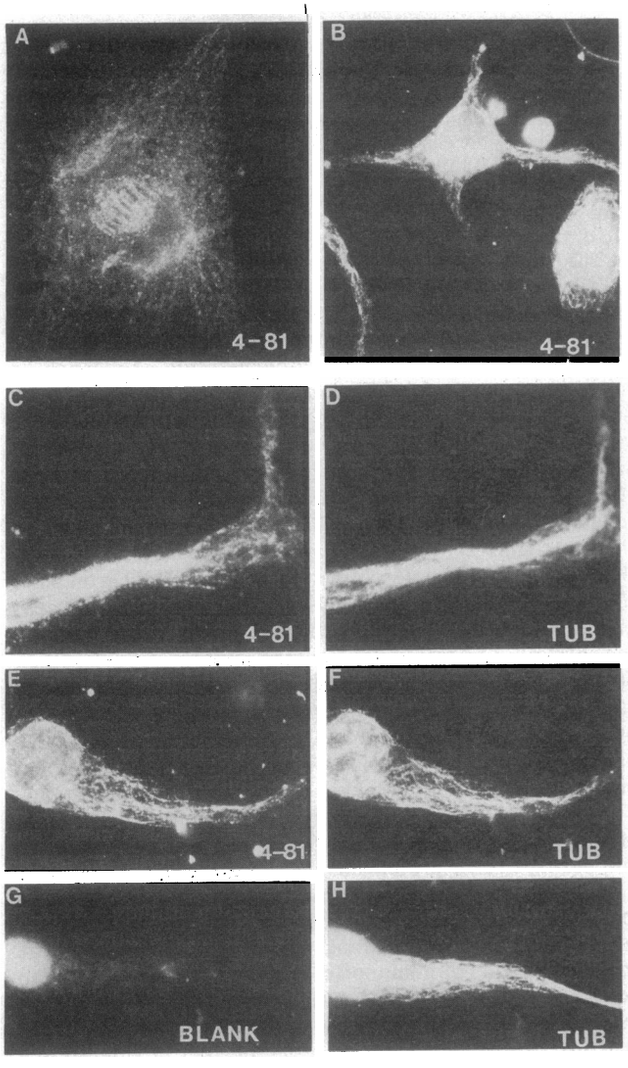

FIG. 5. Immunofluorescent staining with affinity-eluted anti-chartins. Antibodies against the 69-kDa chartin are purified as described in Fig. 4, and reacted with NIL8 cells (A) and NB2A cells (B, C, and E). In double-staining experiments, the same preparations are reacted with both mouse anti-tubulin (TUB) (D, F, and H), the affinity-eluted antibodies (C and E), or material eluted from a blank region of the gel (G). The images in E and G and in F and H were photographed and developed under identical conditions.

DISCUSSION

The results described above use antibodies that specifically recognize the chartins to study their intracellular localization and their occurrence in several cell types. The immunofluorescent experiment shows that the chartins colocalize with assembled tubulin in the cytoplasm. That result has been obtained for other putative MAPs identified by quite different criteria—assembly properties *in vitro*—and demonstrates that colocalization is another property shared by at least some of the microtubule components identified in these two different ways. In fact, any other result would have been surprising, given the assay used to identify chartins and their ability to stay with isolated microtubules after multiple centrifugations *in vitro* (3, 19). Apparently, however, not all proteins associated with microtubules *in vitro* are associated with them *in vivo*. Asai and colleagues have demonstrated this disparity for MAP-1, and others called into question the rigor of the *in vitro* criterion (20).

The antibodies were also used to further study the tissue distribution of the chartins, but with rather limited success. It is true that the immunological data are all consistent with the biochemical data—the 80-kDa chartin is found only in neural cells, while the 69-kDa protein is found in all rodent cells. But the negatives in this case are even less compelling than usual. For example, affinity-eluted antibodies from serum 3417 stain an 80-kDa band in neuroblastoma cells but not in NIL8 cells, nor do they stain bands in brain MAPs, against which the serum was raised. In addition, affinity-eluted antibodies from serum 4-81 do not stain an 80-kDa band in NIL8 cells; they also do not stain a 69-kDa band in C6 cells, which clearly has such a protein (2). Thus, as usual, the negative results from these immunological experiments cannot be taken as definitive.

One of the original aims of this work was to prepare reagents that might distinguish between the 69-kDa and 80-kDa chartins, and thus detect a difference in their intracellular distribution. In fact, at the level of protein blots, sera 3417 and 4-81 are such antibodies, but they cannot be used to address this most important question because serum 3417 is not usable for immunofluorescence. Given the difficulty in preparing even these reagents, it seems unlikely that the straightforward approach can work. Therefore, we are using the antibodies to isolate clones to the chartin genes, so the differences in distribution and expression of the distinct family members can be studied in detail.

Another outcome of these experiments is the assay it provides of different methods of analyzing microtubule function. The detergent extraction assay worked out in this laboratory for cultured cells, and extended by others to primary cells in culture (21), is now shown to produce enough material for antibodies to be analyzed. As pointed out above, those antibodies show that the proteins we identified share another property—in addition to the ability to coassemble (1)—with standard MAPs; that is, colocalization in the cytoplasm. They also show that the 80-kDa chartin is present in brain, although such a protein has not previously been identified. That omission is likely due to the fact that those preparations have largely been assessed with Coomassie staining, so that many minor bands are ignored (see ref. 16 for a silver-stained gel of such a preparation). Conversely, by

using anti-τ antibodies, we show that the protein originally identified from brain as an assembling species *in vitro* is also associated with assembled microtubules by the detergent extraction assay and that it cofractionates with chartins, although they are clearly different species. We missed the τ proteins in our analyses, probably because they are present in relatively low abundance compared to the chartins. Similarly, the composition of the "tau fraction" is likely to become more complex as more sensitive techniques for detecting minor proteins become available. All together, these results suggest that the results of different methods are likely to converge, at least to some extent.

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