RAPID COMMUNICATIONS

INTRACELLULAR LOCALIZATION OF THE HIGH MOLECULAR WEIGHT MICROTUBULE ACCESSORY PROTEIN BY INDIRECT IMMUNOFLUORESCENCE

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ABSTRACT

Microtubule accessory proteins were isolated from porcine brain microtubules by phosphocellulose chromatography, and the high molecular weight protein (HMW protein), purified from this microtubule-associated fraction by electrophoretic elution from SDS gels, was used to raise antisera in rabbits. In agarose double diffusion tests, the antiserum obtained forms precipitin lines with purified HMW protein but not with tau protein or tubulin. When rat glial cells (strain C6) are examined by indirect immunofluorescence, this serum specifically stains a colchicine-sensitive filamentous cytoplasmic network in interphase cells, a network indistinguishable from that seen when cells are treated with antitubulin serum. In dividing cells, specific staining of the mitotic spindle and the stem body is observed with the antiserum to HMW protein. These studies indicate that HMW protein, like tau protein, is associated with microtubules in intact cells.

KEY WORDS microtubules · microtubule accessory proteins · HMW protein · indirect immunofluorescence

Microtubules purified by successive cycles of polymerization and depolymerization contain, in addition to tubulin, a series of accessory proteins. When tubulin is separated from these accessory proteins by ion exchange chromatography, it will no longer assemble into microtubules under standard in vitro polymerizing conditions (buffered aqueous solutions, neutral pH, 1 mM Mg). Furthermore, it has been shown that two of these nontubulin proteins, the high molecular weight protein (HMW protein) (11, 14, 18) and tau protein (19), are necessary and sufficient for both the initiation and the elongation process of microtubule assembly in vitro (18, 22). They also cospurify with tubulin through successive cycles of polymerization in a constant stoichiometric ratio (2, 15).

We have studied the relationship between these microtubule accessory proteins and microtubules in vivo by examining their distribution by the indirect immunofluorescence technique. In a previous report (4), we showed that tau protein is associated with microtubules in intact cells. In the present study, we examine the relationship between HMW protein and microtubules and demonstrate that precipitating antisera prepared in rabbits to electrophoretically purified HMW protein give specific immunofluorescent staining of microtubules in rat glial cells. As a result of these studies, we conclude that HMW protein is associated with microtubules in glial cells.
MATERIALS AND METHODS

Preparation of Antisera

Porcine brain microtubules were purified by two cycles of polymerization and depolymerization using a modification of the method of Shelanski et al. (17) and Weingarten et al. (20), and tubulin was separated from accessory proteins by phosphocellulose chromatography (19). Tubulin and HMW protein were further purified by electrophoretic elution from polyacrylamide gels containing 0.1% SDS as previously described (4). Tau protein was purified by the method of Cleveland et al. (2) and further purified by elution from SDS-containing polyacrylamide gels.

Antisera to tubulin and HMW protein were prepared as follows: Female New Zealand White rabbits were injected subcutaneously with 250 μg of protein in complete Freund’s Adjuvant and a second time 20 days later. Another injection of 500 μg of protein in incomplete Freund’s Adjuvant was given 15 days after the second injection, and the rabbits were bled 2 wk after this last injection. Preimmune sera were obtained from the same animals before immunization.

Immunodiffusion Tests

Antisera were analyzed by Ouchterlony’s double diffusion test, with 1% agarose in phosphate-buffered saline (PBS) at room temperature. For absorption experiments, electrophoretically purified antigen at a concentration of 1 mg/ml was added to the antiserum well, and allowed to diffuse for 10 min before application of the antiserum.

Cells

For immunofluorescence, rat glial cells strain C6 (American Type Culture Collection, Rockville, Md.) were grown on 22-mm glass cover slips in alpha minimum essential medium supplemented with 10% fetal calf serum. In some experiments, culture medium was removed and replaced with the same medium containing Colcemid at 0.5 μg/ml for 60 min before fixation of the cells.

Immunofluorescence

Cells grown on cover slips were rinsed twice in PBS, pH 7.0, and then fixed for 4 min in methanol, and 2 min in acetone, both at −20°C (16). Cells were then treated with one of the antisera at a dilution of 1:30 (in PBS), followed by fluorescein conjugated goat IgG to rabbit IgG (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) diluted 1:5 (4). Cells were examined with a Zeiss Photomicroscope II equipped with epifluorescence optics and photographed on Ilford FP4 film developed in Diafine. The antisera were absorbed with antigens as previously described (4).

RESULTS

The purity of tubulin and HMW protein, obtained by electrophoretic elution from SDS gels and used for the immunization of rabbits, is shown in Fig. 1. The HMW protein used as an antigen resolves into two bands in the acrylamide gel system of Sloboda et al. (18) and into four bands in very low percentage acrylamide gels. When tested by Ouchterlony double diffusion, the antiserum obtained to HMW protein forms a precipitin line with purified HMW protein and does not cross-react with either tau protein or tubulin (Fig. 2a). The precipitating activity of this antiserum to HMW protein could be abolished if the serum was absorbed with purified HMW protein (Fig. 2b) but not if absorbed with purified tubulin or tau protein (Fig. 2c and d). No precipitin lines were seen when the preimmune serum from the same animal was used. The antiserum to tubulin used in the present study was similar to those previously described (1, 4, 7).

To determine the in vivo relationship between the HMW protein, tubulin and the microtubule by indirect immunofluorescence, we treated rat glial cells with either anti-HMW serum or antitubulin serum. When glial cells were treated with anti-HMW serum, a filamentous network was clearly seen in the cytoplasm of all interphase cells (Fig. 3a), and the spindle and stem body were stained in mitotic cells (Fig. 3b and c). When cells were incubated with antiserum to tubulin, similar staining patterns were observed (Fig. 3d-f). This similarity in specific staining patterns indicates that HMW protein and tubulin share a common distribution in rat glial cells. When cells were incubated with Colcemid before treatment with either anti-HMW serum or antitubulin serum, only a very faint diffuse fluorescence staining was observed (Fig. 4). The same Colcemid treatment does not disrupt 10-nm filaments visualized by an antiserum to the 55,000 mol wt protein compo-
Analysis of antiserum to HMW protein by double diffusion tests in agarose. (a) Center well—25 μl of antiserum to HMW protein; outer wells—10 μl of tubulin (1 mg/ml) (T), tau protein (1 mg/ml) (TP), HMW protein (1 mg/ml) (HMW), and microtubule accessory protein fraction (2.5 mg/ml) (AP). (b–d) The antiserum well (bottom) was filled with HMW protein (b), tau protein (c), and tubulin (d) 10 min before application of 25 μl of antiserum to HMW protein. The antigen well (top) was filled with 10 μl of HMW protein (1 mg/ml). Neither preimmune sera from the same rabbits nor fluorescein-labeled goat antirabbit IgG alone stained interphase networks or mitotic spindles (not shown).

To ensure the specificity of the staining patterns observed, antiserum to HMW protein was absorbed with HMW protein before treatment of cells. As a result, fluorescent staining was greatly reduced (Fig. 5a), and neither spindles, stem bodies, nor filamentous networks were visible. If, however, tubulin or tau protein was used to absorb this serum, the staining of these structures remained undiminished (Fig. 5b and c). Similarly, virtually all staining was abolished if antitubulin serum was absorbed with tubulin but not when it was absorbed with HMW protein or tau protein.

DISCUSSION
The results obtained indicate that antiserum prepared against electrophoretically purified HMW protein gives the same staining patterns in interphase cells as antiserum to tubulin. Wiche and Cole (21) also detected a HMW protein in preparations of microtubules isolated from the same C6 glial cell line as used in these studies. The structures stained by HMW antiserum include the mitotic spindle, the stem body, and the cytoplasmic network of microtubules in interphase cells. The fact that the staining observed is Colcemid sensitive indicates that the structures visualized are indeed microtubules.

Because of the large difference in molecular weight between HMW protein and tau protein or tubulin, there seems little chance that the electrophoretically purified HMW protein used for immunization was contaminated with either of these proteins. In addition, Ouchterlony double diffusion tests showed no cross-reactivity between the antiserum to HMW protein and tau protein or tubulin. The absorption studies performed on antiserum to HMW protein are also consistent with the conclusion that HMW protein, tubulin, and tau protein are antigenically distinct proteins which are associated with microtubules in intact cells.

Several previous studies indicate that HMW protein is bound to in vitro polymerized microtubules (11, 14, 18). There are also reports from in vitro studies that the HMW protein can be visualized as projections on the outside of microtubules polymerized in the presence of HMW protein, whereas those polymerized in its absence are smooth and show no projections (5, 14). If HMW protein is bound to microtubules in vivo in an analogous fashion, then one would expect to see immunofluorescent staining patterns such as those observed in this study.

Although the results reported in this study show that HMW protein is somehow distributed along the microtubules in intact cells, they tell us nothing about its functions. HMW protein and tau protein are known to promote assembly of microtubules in vitro and to be necessary for both their initiation and elongation (18, 22), but whether or not they have a similar function in vivo is unknown, since many nonephysiological agents such as dimethyl sulfoxide (9), polycations (6), glycerol and high magnesium (13), or high magnesium alone (8) have been reported to substitute for this requirement in vitro. The demonstration that both HMW protein and tau protein (4) exist in close association with microtubules in vivo suggests that they...
are important in some way to the assembly, maintenance or function of microtubules in intact cells.

Whether microtubule accessory proteins exist in cells alone or are always found in association with microtubules or tubulin is not known. It has been postulated (12) that the tubulin in cells exists in two states, one of which is capable of being polymerized into microtubules and a second form which will not form microtubules. The way in
FIGURE 4 Immunofluorescent staining of rat glial cells with antisera to HMW protein after treatment of the cells with Colcemid. No cytoplasmic networks are seen, and only a diffuse background fluorescence remains (x 600). Bar, 10 μm.

which tubulin acquires the ability to form microtubules might be through binding of microtubule accessory proteins. It may well be that HMW protein and/or tau protein are synthesized in a rate limiting way, thereby exercising control over the assembly of tubulin in vivo.

It is noteworthy that we could not demonstrate any cross-reactivity between HMW protein and tau protein. Sloboda et al. (18) found that breakdown products of HMW protein still react with anti-HMW serum. However, they say nothing about its cross-reactivity with purified tau protein. In the present experiments, tau protein was first isolated by a series of column purification procedures and finally eluted from SDS containing polyacrylamide gels. This tau protein showed no cross-reaction with HMW protein in immunodiffusion tests, nor did it block immunofluorescent staining by the HMW antiserum, thus providing evidence that tau does not represent a breakdown product of HMW protein. The results obtained in this paper are in agreement with reports showing dissimilarity in peptide maps of purified tau protein and HMW protein (2, 3). In a previous report, we showed that tau protein detected by immunofluorescent staining is associated with microtubules in mouse fibroblasts (4). It is of particular interest to note here that when glial cells were treated with antisera to tau protein, little or no fluorescent staining was detected. We are currently examining these differences in staining patterns obtained with antisera to HMW protein and tau protein in various cell types.1

FIGURE 5 Immunofluorescent staining of rat glial cells after absorption of the antiserum to HMW protein with HMW protein (a), tau protein (b), and tubulin (c). Absorption with HMW protein has abolished the staining of the cytoplasmic network of microtubules (a), whereas after absorption with tau protein or tubulin the networks remain stained (b and c) (x 880). Bars, 10 μm.

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1 Connolly, J. A., V. I. Kalnins, D. W. Cleveland, and M. W. Kirschner. Manuscript in preparation.
We would like to thank Mark Cheung for technical assistance and Mike Wassmann for photography.

This work was supported by grant MT-3302 from the Medical Research Council of Canada to V. I. Kalnins, a grant from the Ontario Heart Foundation to V. I. Kalnins, United States Public Health Service grant GM 19667 to M. W. Kirschner, and American Cancer Society grant VC 213 to M. W. Kirschner.

J. A. Connolly is the recipient of a Studentship from the Medical Research Council of Canada.

Received for publication 9 November 1977, and in revised form 14 December 1977.

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