A major 170 kDa protein associated with bovine adrenal medulla microtubules: a member of the centrosomin family?

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Abstract Microtubules isolated from bovine adrenal medulla cells contain a major 170 kDa protein (p170). p170 is heat-labile and is associated with microtubules in an ATP-insensitive manner. This protein was purified to near homogeneity using FPLC. A preparation containing purified p170 caused bundling of microtubules. By microsequencing of p170, two polypeptides were identified which appeared to be identical to a recently sequenced p167 centrosomin-related protein. Polyclonal affinity-purified anti-p170 antibody was found to immunostain microtubules and to recognize the 170 kDa polypeptide in culture cells. We suggest that p170 is a new member of a centrosomin family and is a new structural protein associated with microtubules in some cell types.

Key words: Microtubule; Adrenal medulla; Microsequencing; p170; HeLa; Centrosomin

1. Introduction

Microtubules (MTs) are dynamic cytoskeletal structures that are essential for proper cellular morphogenesis and for many types of cell motility including meiosis, mitosis, secretion and axonemal transport. MTs are made of polymerized tubulin and specific associated proteins, and a number of cellular proteins can co-operate with MTs as well. It has been shown in many experiments that behavior and functions of MTs depend on associated proteins rather than upon tubulin itself (reviewed in [1]). Proteins associated with MTs can be divided into several functional classes including both enzymes and structural proteins. The most functionally important are motor proteins – mechanochemical ATPases, which directly move cargoes along MTs. Structural MT-associated proteins (MAPs) usually serve as linkers between different tubulin subunits or between MTs and other cellular structures. MAPs can strongly modulate MT stability [2]. In addition, MAPs can either enhance the actions of motor proteins or inhibit them [3].

Many MAPs are species- and tissue-specific. MTs are easy to isolate from vertebrate brain and many MAPs in nervous cells have been described and studied. In non-nervous vertebrate cells MAPU (MAP4) protein was well established only as a structural MAP [4], and CLIP170 protein as a linker protein between MTs and membrane vesicles [5]. The properties of these proteins cannot completely elucidate the behavior of MTs [6]. Therefore one might expect that more MAPs exist in non-nervous cells that have not yet been described. Discovery of new MT proteins can lead to a better understanding of MT functioning in cells and to further clarification of cellular morphogenesis and motility.

In this work bovine adrenal medulla MT-associated proteins were studied. Adrenal medulla (AM) chromaffin cells are neural in origin, and contain many MTs, but they are similar to secretory epithelial cells in shape and function [7]. Thus, one could expect to find in AM cells some new MAPs that are rare in brain tissue. MTs isolated from AM cells contained a noticeable amount of 170 kDa protein (p170), which is rare in brain MTs. Using specific antibodies, this 170 kDa protein was found to be associated with MTs in the non-nervous flattened cultured cells. By microsequencing p170 was established to be closely related to p167 protein, a member of the centrosomin family [8]. So, the centrosomin family of proteins can be associated not only with the centrosome, but also with MTs, and could be involved in MT network regulation as well.

2. Materials and methods

All reagents used in this study were purchased from Sigma, Serva and Merck if not stated otherwise, and were of the best research grade. Taxol was kindly provided by Dr. Suffness (National Institutes of Health, USA). The composition of protease inhibitor cocktail (PIC) added to solutions was 1 μg/ml of pepstatin, 10 μg/ml of TAME, 1 μg/ml of leupeptin and 1 μg/ml of aprotinin.

2.1. Isolation, purification and functional testing of p170 from bovine adrenal medullae

Bovine brain MTs were obtained by the polymerization-depolymerization method described in [9]. Bovine brain MT tubulin was purified on a phosphocellulose column (Whatman).

Bovine adrenal glands were obtained directly from the slaughterhouse within several minutes after the electrocution of the animals and kept in ice before experiments for no more than 5 h. Adrenal medullae were homogenized in a 10× volume of buffer A (50 mM imidazole pH 6.7, 50 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) with 0.3 M sucrose). The homogenate was clarified by brief centrifugation; the supernatant was supplemented with PIC and centrifuged for 15 min at 27 000×*g*. Purified brain tubulin, 5 μM of taxol and 2.5 mM tripolyphosphate were mixed with supernatant, incubated at 37°C for 5 min, loaded to a cushion of the same buffer supplemented with 4 M glycerol and sedimented for 60 min at 120 000×*g* (22°C). In some experiments brain tubulin was omitted from the incubation mixture.

A MT pellet was resuspended in buffer A with 0.2 M NaCl and 20 μM of taxol (in some experiments 2 mM ATP was also added) and clarified twice by centrifugation for 40 min at 150 000×*g*. The resulting supernatant was used either for preparative electrophoresis or for

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protein chromatography using FPLC (LKB) using Sup6 and MonoQ columns.

For investigation of the MT bundling properties of p170, 20 μg/ml of taxol-stabilized bovine brain MTs polymerized from purified tubulin was mixed with various amounts of purified p170 and observed by video-enhanced microscopy. An Axiophot microscope (Zeiss) equipped with a 1.4 NA Plan-Neofluar lens and ICT condensor was used. Images were taken with a Hamamatsu 2400-01 Chalnicon video-camera and processed with an Argus-100 computer.

2.2. Antibodies

Supernatant from adrenal medulla MT washed with 0.2 M NaCl was subjected to preparative electrophoresis in the Laemmli buffer system as described in [10]. After electrophoresis gels were stained with 1% Coomassie R-250 dissolved in distilled water. The 170 kDa band was cut, homogenized and used for rabbit immunization. For purification of immune serum, the 170 kDa band was electroblotted to nitrocellulose Hybond-C (Amersham) strips. Strips were incubated overnight with immune serum immunoglobulins, washed, and the specific fraction was eluted with 0.5 ml of 0.2 M Tris-glycine buffer pH 2.7 immediately neutralized with 0.05 ml of 2 M Tris-base. The final concentration of antibody (named 170-Ab) was 0.1–0.15 mg/ml.

Monoclonal antibody (Ab) SN2-1F5, IgM class, was raised against a preparation of loach fish sperm centrosomes. Other primary antibodies used in this study were: monoclonal Ab to MAP1B protein heavy chain HM-1 (Sigma), polyclonal Ab to MAP2 and tau mixture (Sigma), and polyclonal Ab to CLIP170 protein kindly provided by Dr. T. Kreis.

2.3. Immunofluorescence staining of cultured cells and immunoblotting

HeLa human cervical carcinoma cells and PEK pig kidney embryo cells were plated in DMEM (Flow Laboratories) supplemented with 10% newborn bovine serum (Flow Laboratories) and 100 μg/ml of gentamicin and grown in 5% CO_2 and 95% air at 37°C. In some experiments 1 μg/ml of nocodazole (Calbiochem) or 10 μg/ml of taxol was added to cells overnight. For immunofluorescence staining, the cells were permeabilized with 0.5% Triton X-100 solution in buffer A supplemented with 4 M glycerol and fixed with 0.5% glutaraldehyde. Immunofluorescence staining was performed as described in [11]. Preparations were examined in a Photomicroscope-3 (Option).

Electrophoresis of cultured cell homogenates, animal tissue homogenates or protein preparations was done in a Laemmli buffer system as described in [10]; a 6–12% gradient PAGE was used. For Western blot analysis, the proteins were transferred to nitrocellulose Hybond-C (Amersham) by a semi-dry method in discontinuous buffers described in [12] and processed with Abs as described in [13]. Peroxidase was developed with 4-chlornaphthol/H_2O_2, alkaline phosphatase with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium system.

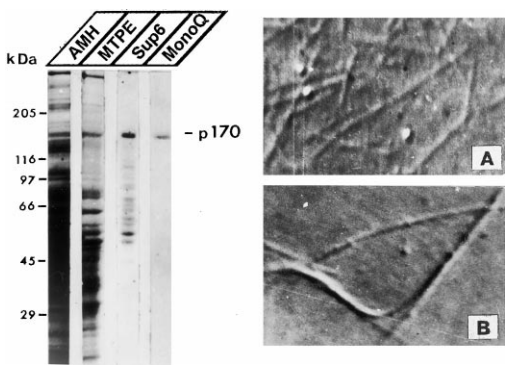

Fig. 1. Isolation and purification of p170 from bovine adrenal medullae and microtubule bundling caused by purified p170. Left panel: PAGE of proteins in 6–12% gradient gel. AMH, bovine adrenal medulla homogenate; MTE, proteins eluted from bovine adrenal medulla microtubules with 0.2 M NaCl; Sup6, protein fraction collected from the Sup6 column; MonoQ, protein fraction collected from the MonoQ column. 170 kDa protein band (p170) and molecular mass markers are indicated. Right panel: Video-enchanced microscopy of microtubule preparations. A: Taxol-stabilized microtubules polymerized from purified bovine brain tubulin. B: The same microtubules incubated with purified 170 kDa protein.

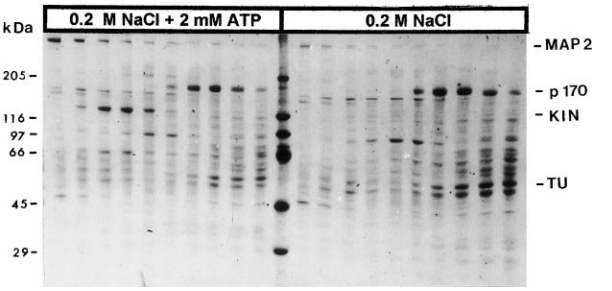

Fig. 2. Sup6 chromatography profiles of proteins eluted from adrenal medulla microtubules with either 0.2 M NaCl and 2 mM ATP or 0.2 M NaCl as indicated. PAGE of proteins in 6–12% gradient gel. KIN, kinesin heavy chain; MAP2, MAP2 protein; TU, tubulin. Molecular mass markers are indicated.

2.4. Microsequencing of p170

For microsequencing of p170, preparative electrophoresis of the final supernatant proteins was performed as described above, and a 170 kDa band was removed. A detailed description of protein microsequencing by mass spectrometry is given in [14].

3. Results

3.1. Purification of p170 from bovine adrenal medullae: p170 is an individual protein

In the adrenal medulla (AM) MT proteins eluted with 0.2 M NaCl a major 170 kDa band was seen (Fig. 1, MTPE lane). This 170 kDa band was not the most prominent one in total AM homogenate (Fig. 1, AMH lane) and in bovine brain MT preparation (data not shown). Many other MT-associated proteins were present in AM MT proteins, including MAP1A, MAP1B, MAP2 and tau proteins. All of them were recognized by commercially available specific Abs (data not shown). p170 was not recognized by any of these Abs. Thus, p170 was probably an MT-associated protein that was AM-specific or highly enriched in AM.

For further purification of p170, AM MT eluate was fractionated using FPLC Superose 6 sizing column (Figs. 1 and 2). The p170 was eluted after high molecular weight MAPs (280–350 kDa) and kinesin (360 kDa tetramer), and before tubulin (100 kDa dimer) (Fig. 2). Protein fractions enriched with p170 were collected and subjected to separation on FPLC with MonoQ ion-exchange column. Proteins were eluted from the column with a 0.05–0.3 M gradient of KCl. Thirty fractions were collected, and almost pure p170 appeared in fractions 22–24 (Fig. 1, lane MonoQ). No other polypeptides were found in the p170 preparation, which means that p170 was not tightly associated with other proteins.

3.2. p170 exhibits features of a structural MAP and not of a kinesin-like protein

p170 could belong either to structural MAPs or to motor proteins or to any other class of MT-interacting proteins (protein kinases, phosphatases, etc.). To determine if p170 is a kinesin-like protein one has to investigate whether MT binding of p170 is sensitive to ATP. 2 mM ATP added to the 0.2 M NaCl solution which was used for elution of p170 from MTs did not influence either the amount of eluted p170 or its

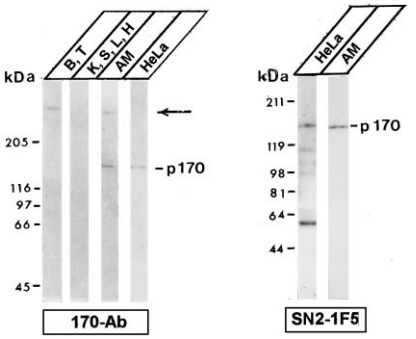

Fig. 3. Specificity of polyclonal Ab to p170 (170-Ab) and monoclonal Ab SN2-1F5. Western blot with either 170-Ab or SN2-1F5 as indicated at the bottom of the pictures. AM, preparation of MAPs from bovine adrenal medullae; B, T, rat brain or testis homogenate (both preparations showed identical reaction); K, S, L, H, rat kidney, spleen, liver, lung and heart homogenates (no reaction with 170-Ab). HeLa, HeLa culture cell homogenate. Molecular mass markers are indicated.

behavior on the Sup6 column (Fig. 2). In addition ATP did not influence MAP2 elution, but strongly increased the yield of kinesin in the eluate (Fig. 2). Thus, p170 is not a kinesin-like motor protein.

A characteristic feature of structural MAPs is the bundling of MTs in vitro [15]. The suspension of taxol-stabilized MTs polymerized from purified bovine brain tubulin was seen under microscopy as thin irregularly distributed filaments (Fig. 1A). After addition of 1 μg/ml of purified p170, prominent bundles of MTs resembling thick filaments with straggling ends appeared in the preparation (Fig. 1B). The bundling of MTs was not ATP-sensitive. Thus p170 exhibited the property of a structural MAP.

MAP2, MAP3, MAPU and tau MAPs are heat-stable [16–18]. As p170 is similar by molecular mass to MAP3 and MAPU proteins, its heat stability was tested. When a solution of partially purified p170 was incubated for 5 min at 100°C, it caused complete denaturation and precipitation of p170 (data not shown). Thus, p170 is not heat-stable and so it is not a member of the MAP2, tau and MAPU families. Purified p170 did not react with antibodies to CLIP170 protein (data not shown).

3.3. Microsequencing of p170: evidence of similarity of p170 with p167 centrosomin-related protein

The mass spectrometry method used for p170 microsequencing does not allow discrimination between Leu and Ile amino acid residues. Two p170 peptides, Val-Leu/Ile-Leu/Ile-Ala-Thr-Leu/Ile-Ser-Leu/Ile-Pro-Leu/Ile-Thr-Pro-Glu-Arg and Leu/Ile-Ala-Thr-Leu/Ile-Leu/Ile-Gly-Leu/Ile-Gln-Ala-Pro-Pro-Thr-Arg, were identified that appeared to be identical to amino acid residues 322–335 (VLLATLSI-PITPER) and 355–367 (LATLLGLQAPPTR) of the recently described human centrosomin-related p167 protein (GenBank accession number U58046) [8]. The same peptides were also found in the recent database submission of mouse p162 protein (GenBank accession number U14172), which is very closely related to p167 protein.

A third p170 peptide identified by microsequencing, Ala-Leu/Ile-Gly-Asn-Leu/Ile-Glu-Leu/Ile-Gly-Leu/Ile-Arg, appeared to be identical to amino acid residues 56–65 of MAP1B human protein [19] and did not correspond to p167 and p162 proteins. The similarity of the third peptide with MAP1B could be fortuitous, and one cannot exclude that the bovine adrenal medulla variant of p167 contains a peptide homologous or identical to the MAP1B fragment. Interestingly, the gross amino acid composition of the third peptide fits very well with the N-terminal part of p167 protein.

Monoclonal Ab HM-1 specific to MAP1B recognized one 300 kDa polypeptide in AM MTs and in bovine brain MTs and did not recognize the p170 band (data not shown). This indicates that the 170 kDa band did not contain the full-length MAP1B protein. It could, however, contain the N-terminal MAP1B proteolytic fragment. Purified p170 did not contain any light chains although the 120 kDa MAP1B N-terminal fragment does retain its association with light chains [20]. So, probably, the MAP1B fragment did not contaminate purified p170, at least after chromatographic steps of purification.

3.4. p170 is associated with microtubules in culture cells

Polyclonal Ab to p170, 170-Ab, recognized 170 and 300 kDa bands in bovine adrenal medulla homogenates (Fig. 3). When rat tissues were tested with 170-Ab, no cross-reactivity was observed in liver, kidney, spleen, lung and heart, and the 300 kDa band was detected in brain and in testis (Fig. 3). The 300 kDa band could represent either MAP1B protein or a putative high molecular mass protein homologous to p167, as was speculated in [8]. Monoclonal Ab HM-1 to MAP1B recognized the 300 kDa band in brain and in testis as well (data not shown).

In homogenates of HeLa cells 170-Ab recognized one 170 kDa polypeptide (Fig. 3). HM-1 Ab did not react with any polypeptides (data not shown). In subconfluent flattened HeLa cells, 170-Ab uniformly immunostained the cytoplasmic fibrillar network (possibly MTs) and nuclei in a punctate pattern (Fig. 4A). After treatment of HeLa cells with nocodazole no cytoplasmic staining was detected (Fig. 4B), and after treatment with taxol bundles of cytoplasmic MTs were brightly stained with 170-Ab (Fig. 4C). Very similar results were obtained using 170-Ab with PEK cells (data not shown).

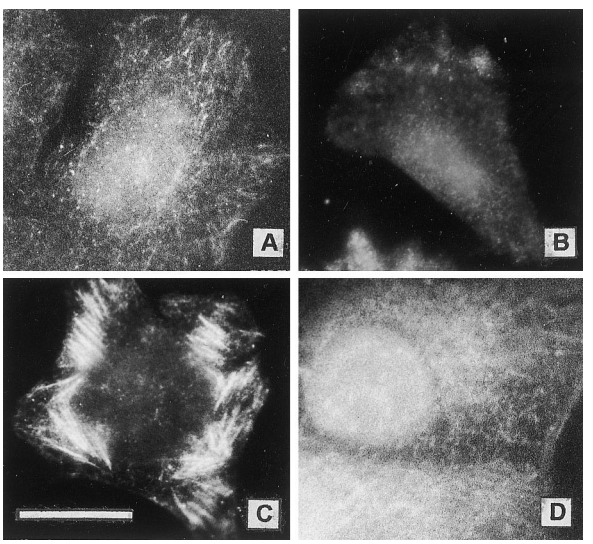

Fig. 4. Immunofluorescence staining of HeLa cells with polyclonal Ab to p170 (A–C) or with monoconal Ab SN2-1F5 (D). A, D: Control cells. B: Cells treated with nocodazole. C: Cells treated with taxol. Bar = 10 μm.

Thus, the cross-reaction of polyclonal 170-Ab with MAP1B protein could not be excluded, and therefore the data needed to be confirmed using another antibody. For that reason a monoclonal Ab was also used for p170 identification and immunolocalization. Monoclonal Ab SN2-1F5 was initially raised against a centrosome preparation isolated from loach fish sperm. This Ab strongly reacted with the 170 kDa band in adrenal medulla homogenate and did not react with the 300 kDa band (Fig. 3). SN2-1F5 Ab recognized major 170 and 50 kDa and several minor near 100 kDa bands in HeLa cell homogenate (Fig. 3). This Ab immunostained in HeLa and in PEK cells MTs, centrosomes and nuclei in a speckle-like pattern (Fig. 4D), a pattern very similar to 170-Ab staining.

4. Discussion

In this work a major 170 kDa protein, named p170, was identified in MTs of bovine adrenal medulla cells. From chromatography data and functional testing it became evident that p170 is an individual protein, is not a kinesin-like protein and could possibly be a structural MAP that causes bundling of MTs in vitro. Investigation with 170-Ab and Ab SN2-1F5 established that MT-associated p170 is restricted to particular cell types (adrenal medullae) and/or specific cell conditions (flattened transformed culture cells). It can be speculated that p170 belongs to that group of proteins that are regulated by cell interaction with substrate or expressed after cell transformation.

What is the molecular nature of p170? It is not thermostable like MAPU (190–220 kDa) and MAP3 (190 kDa) proteins and does not react with Ab to CLIP170 protein. A number of observations confirm the kinship of p170 and p167 centrosomin-related protein described in [8]: (1) two common peptides (14 and 13 amino acid residues long) were revealed by microsequencing; (2) the similar molecular mass of both proteins; (3) p170 is recognized by monoclonal antibody SN2-1F5 that was initially raised against centrosomes that contain centrosomin; (4) p170 is localized in both nuclei and cytoplasm like p167; (5) p170 is expressed in transformed cells, and p167 tightly interacts with tyrosine kinase $pp60^{src}$ (oncoprotein) substrates [8]. At least four members of the centrosomin family have been identified yet: centrosomins A and B [21,22], p167 [8] and p162. Centrosomin A (35 kDa protein) has been shown to be localized in mammalian centrosomes [21,22], and centrosomin B in nuclei [22]; p167 also is not restricted to centrosomes [8]. Some centrosomal components artificially over-expressed in cells can interact with MTs [23], so p167 could be a naturally over-expressed centrosomal protein associated with MTs. p170 can be either identical to p167 or be the fifth individual member of the centrosomin family having strong homology to p167.

Three possible explanations could be proposed for the cross-reaction of 170-Ab with both p170 and 300 kDa protein: (1) a fragment of MAP1B contaminated the 170 kDa band in preparative PAGE; (2) a 300 kDa homologous to p170 protein is expressed in brain, adrenal medullae and testis; (3) p170 has some homology to MAP1B that can lead to cross-reaction of Abs. Our further investigations of p170 will include its molecular cloning from adrenal medullae, and we hope to find the true explanation. It would also be interesting to study the function of p170 and its regulation in cells.

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