

Purification and characterization of ensconsin, a novel microtubule stabilizing protein

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SUMMARY

In previous studies (Bulinski and Borisy (1979). *Proc. Nat. Acad. Sci.* 76, 293-297; Weatherbee et al. (1980). *Biochemistry* 19, 4116-4123) a microtubule-associated protein (MAP) of $M_r \sim 125,000$ was identified as a prominent MAP in HeLa cells. We set out to perform a biochemical characterization of this protein, and to determine its *in vitro* functions and *in vivo* distribution. We determined that, like the assembly-promoting MAPs, tau, MAP2 and MAP4, the 125 kDa MAP was both proteolytically sensitive and thermostable. An additional property of this MAP; namely, its unusually tight association with a calcium-insensitive population of MTs in the presence of taxol, was exploited in devising an efficient purification strategy. Because of the MAP's tenacious association with a stable population of MTs, and because it appeared to contribute to the stability of this population of MTs *in vitro*, we have named this protein ensconsin. We examined the binding of purified ensconsin to MTs; ensconsin exhibited binding that saturated its MT binding sites at an approximate molar ratio of 1:6 (ensconsin:tubulin). Unlike other MAPs characterized to date, ensconsin's binding to MTs was insensi-

tive to moderate salt concentrations (≤ 0.6 M). We further characterized ensconsin in immunoblotting experiments using mouse polyclonal anti-ensconsin antibodies and antibodies reactive with previously described MAPs, such as high molecular mass tau isoforms, dynamin, STOP, CLIP-170 and kinesin. These experiments demonstrated that ensconsin is distinct from other proteins of similar M_r that may be present in association with MTs. Immunofluorescence with anti-ensconsin antibodies demonstrated that ensconsin was detectable in association with most or all of the MTs of several lines of human epithelial, fibroblastic and muscle cells; its *in vivo* properties and distribution, especially in response to drug or other treatments of cells, were found to be different from those of MAP4, the predominant MAP found in these cell types. We conclude that ensconsin, a MAP found in a variety of human cells, is biochemically - and perhaps functionally - distinct from other MAPs present in non-neuronal cells.

Key words: 125 kDa MAP, HeLa cell, taxol, stable MTs

INTRODUCTION

Microtubules (MTs) serve many vital roles in non-neuronal cells, participating in organization of the cytoplasm, in cell motility, and in mitosis. In order to understand how MTs carry out these diverse functions, it is necessary to identify and characterize the functions of all of the components of cytoplasmic MTs. In addition to tubulin, several MT-associated proteins (MAPs) comprise cellular MTs; MAPs may serve as modulators of MT dynamics and/or as liaisons between MTs and other cellular machinery. The human cultured cell line, HeLa, has been widely used as a model system to study the self-assembly of non-neuronal MTs, and these experiments originally led to the biochemical identification of two prominent groups of HeLa MAPs, species of 125 kDa and 210 kDa (Bulinski and Borisy, 1979; Weatherbee et al., 1980). In subsequent studies, each was found to be associated with cellular MTs *in vivo* (Bulinski and Borisy, 1980a), and HeLa MAP mixtures con-

taining both the 125 kDa and 210 kDa MAPs were shown to stimulate *in vitro* polymerization of pure tubulin (Bulinski and Borisy, 1980b).

The 210 kDa HeLa MAP has been the topic of a wealth of further study (reviewed by Bulinski, 1994). Interspecific homologs were identified in mouse (Olmsted and Lyon, 1981), rat (Huber et al., 1985; Kotani et al., 1988) and cow (Murofushi et al., 1986), and the MAP was renamed MAP4, a better name given the slight variation in M_r values observed in different species and with different electrophoresis conditions (Parysek et al., 1984). MAP4 was shown to be expressed in most or all non-neuronal cell types in mammals (Bulinski and Borisy, 1980c; Parysek et al., 1984; Huber and Matus, 1990). Subsequent cDNA cloning revealed that, within an organism, MAP4 consists of a family of several proteins with structurally distinct MT-binding domains that bear homology to those of the assembly-promoting neuronal MAPs, MAP2 and tau (Aizawa et al., 1990; Chapin and Bulinski, 1991; West et al., 1991).

In contrast, following a few initial reports (Bulinski and Borisy, 1979, 1980a,b; Weatherbee et al., 1980), the 125 kDa HeLa MAP was not characterized further. There are several reasons for the 125 kDa MAP's ephemeral appearance in the scientific literature: first, in biochemical studies, the 125 kDa MAP exhibited aggregation properties that suggested that it was an intractable subject protein for biochemical studies. Also, aggregation of the 125 kDa MAP with itself or with tubulin suggested that this MAP might associate nonspecifically with MTs, rather than binding to saturable sites on the MT lattice (Bulinski and Borisy, 1980b). More importantly, because of the 125 kDa MAP's tendency to form aggregates, it could not be purified in standard MT purification protocols that involve high-speed centrifugation (e.g. the widely used taxol-method of Vallee and Collins, 1986). Yet another reason the 125 kDa MAP was not studied further stems from the lack of a high quality, abundant antibody to the MAP. We had difficulty in raising highly reactive rabbit antibodies against the 125 kDa MAP; in contrast to MAP4, which is a highly antigenic protein, the 125 kDa MAP proved to be less amenable to further research.

On the negative side, then, the unusual *in vitro* properties of the 125 kDa MAP impeded its further study. On the positive side, though, the atypical *in vitro* properties of this MAP suggested that the 125 kDa MAP might serve unusual functions *in vivo*. For example, the 125 kDa MAP's aggregation or binding to stable subsets of MTs might prove to be functionally significant *in vivo*. Therefore, to carry out further study of the 125 kDa MAP, we devised schemes to surmount each of the difficulties we had encountered in previous studies of this MAP. In the experiments described here, we have designed and utilized a purification strategy for the 125 kDa MAP, and we have characterized its *in vitro* properties. We have also renamed the 125 kDa MAP *ensconsin*, a name appropriate for a MAP with its MT-binding properties *in vitro* and *in vivo*. Finally, we have also demonstrated that, both in living cells treated with MT antagonistic drugs, and in lysed and extracted cells, the behavior of *ensconsin* differs from that of MAP4, the other prominent MAP present in the cell types we are studying. Our experimental results suggest that *ensconsin* plays a role in the stabilization or function of MTs *in vivo*, and that this role is most likely distinct from that of other known non-neuronal MAPs.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all solid chemicals were obtained from Sigma, Inc. (St Louis, MO), all liquid chemicals were purchased from Fisher, Inc. (Livingston, NJ), and all tissue culture supplies were obtained from GIBCO (Grand Island, NY). Taxol was a gift from the Drug Synthesis Branch of the National Cancer Institute, and nocodazole was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Purification and MT-binding assay of *ensconsin*

Twice-cycled MTs were purified from HeLa cells by an assembly-disassembly scheme in the absence of the MT stabilizing drug, taxol (Bulinski, 1986). Briefly, in each preparation, 10-20 g of packed HeLa cells was sonicated in buffer containing 0.1 M PIPES, pH 6.94, and 1 mM each of dithiothreitol, EGTA, MgCl₂ and GTP (PDEMG), in addition to a stock solution of proteolysis inhibitors (CLAP) that

contained 0.1 µg each of chymostatin, leupeptin, antipain and pepstatin. The HeLa cell lysate was centrifuged at 48,000 *g* for 30 minutes. Two assembly-disassembly steps were then carried out, including polymerization and sedimentation of MTs at 48,000 *g* at 37°C, followed by depolymerization and clarification of the depolymerized tubulin solution at 48,000 *g* at 0°C; each step was carried out for 30 minutes. A yield of ~5 mg of twice-cycled MTs was routinely obtained.

To purify *ensconsin* (which was formerly called 125 kDa MAP), twice-cycled HeLa MTs were first subjected to a third depolymerization and polymerization step; the third polymerization step was carried out in PDEMG containing 10 µM taxol. The taxol-stabilized MTs were homogenized at 0°C, in PDEMG containing 3 mM CaCl₂ (23 ml per gram of MT pellet), to depolymerize the calcium-labile taxol MTs (Vallee and Collins, 1986). Centrifugation of this calcium-containing MT solution revealed that only 40-55% of the MTs had depolymerized. Most of the tubulin and virtually all of the MAP4 were found in the supernatant. In contrast, *ensconsin* was greatly enriched in the pellet; that is, in the fraction containing MTs that had not been depolymerized by calcium. The final step in purification of *ensconsin* was to resuspend the pellet containing calcium-stable taxol MTs in PDEMG containing 0.75 M NaCl (typically 50 ml per gram of pellet), and boil this dilute solution for 20 minutes. The solution was then centrifuged at 48,000 *g* for 30 minutes, to sediment thermolabile tubulin, which was precipitated during the boiling step, along with most other contaminating proteins. *Ensconsin* was heat-stable under these conditions; it was found in the supernatant, which contained ≥75% pure *ensconsin* in all preparations. Pure *ensconsin* was desalted and concentrated with Centricon 30 units (Amicon, Danvers, MA).

Tubulin was purified from bovine brain and separated from brain MAPs by DEAE-Sephadex chromatography, according to Vallee (1986). Protein concentrations of most samples were determined by the bicinchoninic acid method (with directions and reagent from Sigma, Inc., St Louis, MO); bovine serum albumin was used as a standard. For very dilute protein solutions (e.g. *ensconsin* solutions were usually used at concentrations of less than 0.2 mg/ml), protein concentrations were determined by a slot blot assay (Vallee, 1986), using porcine γ-globulin as a standard.

In order to determine what structures were present at various stages during the purification and MT-binding assay of *ensconsin*, standard negative stain electron microscopy was used. Protein samples were applied to Formvar-coated 200 mesh grids, stained with 1% uranyl acetate, air dried, and observed and photographed the same day in a JEOL electron microscope.

Immunoblotting assays of *ensconsin*

Polyclonal mouse antisera were prepared using bands excised from Coomassie Blue-stained polyacrylamide gels of purified or partially purified *ensconsin* as immunogen. Methods of preparation and injection of antigen, and the immunization schedule, were exactly the same as those used to prepare rabbit antibodies to gel-purified 125 kDa MAP (Bulinski and Borisy, 1980a), except that the amount of immunogen used for mice (5-10 µg of *ensconsin* per immunization) was tenfold less than that used for rabbits, and the immunogen was injected intraperitoneally in mice. A small sample of blood was gathered from the tail vein for assay and, once a reasonable level of reactivity and specificity of the animal's serum had been obtained, a larger sample (typically ≥1 ml) was collected by terminal cardiac puncture. Serum was stored in aliquots at -80°C.

Immunoblots of purified *ensconsin* or HeLa MT protein enriched for *ensconsin* were also tested for cross-reactivity of *ensconsin* with antibodies prepared against other MAPs. Antibodies specifically reactive with kinesin, STOP and CLIP-170 were generously provided by Dr George Bloom (UT Dallas Southwestern Medical Center; Brady et al., 1990), Dr Fabienne Pirollet and Didier Job (INSERM, Grenoble, France; Pirollet et al., 1988), and Dr Thomas Kreis (Uni-

versity of Geneva; Rickard and Kreis, 1990), respectively. Antibody reactive with all forms of tau protein was purchased from Sigma, Inc. (St Louis, MO). These antibodies, as well as antibody against primate MAP4 (Chapin and Bulinski, 1991), were used according to published protocols or instructions provided with each antibody. A blot strip containing ensconsin at each stage of purification and a sample enriched for brain dynamin was also reacted with anti-dynamin antibody, by Dr Chris Burgess and Dr Richard Vallee (Worcester Foundation for Experimental Biology).

Immunoblots of cultured cell extracts were prepared as described (Bulinski, 1986). First, cells were washed with phosphate-buffered saline, then they were solubilized by boiling in SDS sample buffer (Laemmli, 1970; 200 μ l buffer per 10 μ l of packed cell volume) containing CLAP protease inhibitors. In order that their protein concentration could be determined by the bicinchoninic acid method (Sigma, Inc., St Louis, MO), cell extract samples were first prepared in sample buffer lacking tracking dye or reducing agent. The composition of the sample buffer was adjusted after protein assay, and samples were electrophoresed on either 5% or 6.5% polyacrylamide gels (Laemmli, 1970). Electrophoretic transfer and analysis of western blots with antibodies against ensconsin, tubulin or MAP4 were performed as described previously for MAP4 (Bulinski, 1986). Mouse antisera against ensconsin were used at concentrations of 1/500 to 1/2500; preimmune serum from the same mouse was used as a control in each experiment.

Immunofluorescence of ensconsin in cultured cells

HeLa cells, MCF-7 cells (from Dr Jan Kitajewski, Columbia University), human myoblasts (strain H271, from Dr Stephen Hauschka, University of Washington), and human fibroblasts (HuT cells, obtained from American Type Culture Collection, Bethesda, MD, and strain 356 human foreskin fibroblasts obtained from Dr Robert DeMars, University of Wisconsin) were grown on acid-washed coverslips in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum. Myoblast differentiation was carried out when cells had grown to about half-confluency by shifting cultures to DMEM containing 2% fetal bovine serum that had been heat-inactivated by treatment at 55°C for 30 minutes. Coverslips were fixed by one of two methods: either they were rinsed in serum-free culture medium and then plunged directly into -20°C methanol for 5 minutes, or else they were first extracted for 2 minutes at 37°C in PDEMG containing 200 μ g/ml saponin, and then placed in -20°C methanol. Following fixation, coverslips were stored in Tris-buffered saline (10 mM Tris-HCl, pH 7.5) containing 0.05% sodium azide, until use. Immunofluorescence was performed as described previously for MAP4 (Bulinski, 1986), except that for anti-ensconsin sera (which were used at dilutions of 1/25 to 1/100) sheep anti-mouse biotinylated secondary antibody and Texas Red-labeled streptavidin (both used at a 1/100 dilution and purchased from Amersham, Arlington Heights, IL) were used to visualize ensconsin, while fluorescein-labeled secondary antibody was used to detect polyclonal anti-tubulin (Bulinski et al., 1988) or MAP4. Controls in which preimmune sera and sera preabsorbed with acrylamide gel pieces containing ensconsin were used as primary antibody showed dim fluorescence, with no obvious pattern of staining.

RESULTS

Purification of ensconsin

In order to perform further studies on ensconsin (formerly called 125 kDa HeLa MAP; Bulinski and Borisy, 1979, 1980a,b; Weatherbee et al., 1980), it was necessary to develop a strategy for its purification. In our protocol, we exploited the unusual solution properties of ensconsin: namely, its propensity to form sedimentable aggregates in solutions of HeLa MTs

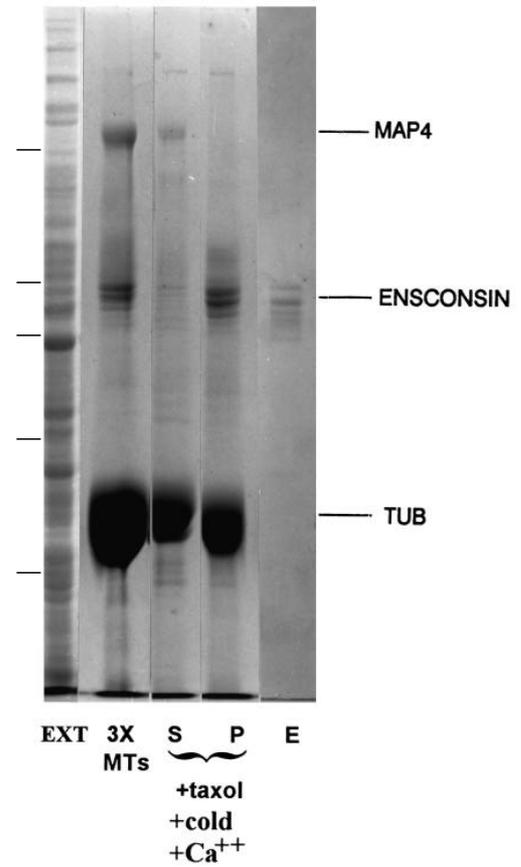


Fig. 1. Purification of ensconsin. Ensconsin was purified from HeLa extract (lane labeled EXT) by a standard, MT self-assembly/disassembly protocol in the absence of the MT stabilizing drug, taxol (see Materials and Methods for details). During the third polymerization step, taxol was added to the polymerization buffer; in the resultant MT pellet (lane labeled 3XMTs), both ensconsin (E) and MAP4 were enriched. The taxol-stabilized 3XMTs were homogenized at 0°C in PDEMG containing 3 mM CaCl₂, and centrifuged to yield a supernatant (labeled S) containing MTs depolymerized by cold and calcium, and a pellet (labeled P) containing MTs resistant to depolymerization. Ensconsin is the most prevalent non-tubulin protein visible in lane P. In contrast, MAP4 has obviously been depleted from the sample whose electropherogram is shown in lane P; it is the predominant MAP in the calcium, cold-labile MTs whose polypeptide composition is analyzed in lane S. Purification of ensconsin was completed by boiling the stable MT pellet in 0.75 M NaCl and centrifuging in order to sediment the heat-denatured proteins and separate them from the soluble, thermostable ensconsin (lane labeled E). Electrophoretic positions of MAP4, ensconsin and tubulin (TUB) are shown at right; molecular mass markers of molecular mass 200, 116, 94, 67 and 45 kDa are labeled at left.

(Bulinski and Borisy, 1980b). Fig. 1 shows an electropherogram of material obtained at each stage of the ensconsin purification scheme. Starting with an extract of HeLa cells (Fig. 1, lane labeled EXT), we carried out a standard reversible MT assembly scheme, in the absence of artificial stimulators of MT polymerization, such as taxol. After assembling and disassembling the HeLa MTs twice, we carried out a third polymerization step; in this step, however, taxol was added to the poly-

merization buffer. The composition of the MTs obtained at this stage; that is, after two cycles of polymerization and depolymerization without taxol, and one polymerization and sedimentation with taxol, is shown in Fig. 1 (lane labeled 3XMTs). Both MAP4 and ensconsin were enriched in thrice-cycled MTs; a massive tubulin band, as well as several minor species, can also be seen in the electropherogram. We homogenized the taxol-stabilized, thrice-cycled MTs in ice-cold buffer containing 3 mM CaCl₂, and centrifuged the solution in order to obtain a supernatant (Fig. 1, lane labeled S) containing MTs depolymerized by cold and calcium treatment, in addition to a pellet (Fig. 1, lane labeled P) containing MTs resistant to calcium-induced depolymerization. Ensconsin was the most prevalent protein other than tubulin, in the calcium-, cold-stable MTs (lane P), while MAP 4 had been depleted from this pellet fraction and was the predominant MAP in the calcium-, cold-labile MTs (lane S). Many previously characterized MAPs exhibit unusual thermostability, especially in the presence of moderate salt concentrations (Vallee, 1985); we found that ensconsin also exhibits this property. Accordingly, the final step in the purification of ensconsin consisted of boiling the pellet of calcium-stable MTs in the presence of 0.75 M NaCl and centrifuging to separate the precipitated, heat-denatured tubulin and other contaminant proteins from the soluble, heat-stable ensconsin (Fig. 1, lane labeled E).

Although one can see trace contaminants in the electropherogram of purified ensconsin, the purity obtained by our protocol was always $\geq 75\%$, as determined by densitometric scanning of stained electropherograms (data not shown); this represents greater than an 800-fold purification of ensconsin from cell homogenate. Note that in the preparation shown in Fig. 1, the CLAP cocktail of proteolysis inhibitors was not added to the cell extract until after sonication. Accordingly, we suspected that the four ensconsin bands that are visible in the electropherogram might represent proteolytic fragments of a single protein species. However, since all four bands copurify, exhibit identical solution behavior, and remain in approximately constant stoichiometry to one another (e.g. the second from the top is the most prominent), we have concluded that this heterogeneous group comprises ensconsin. Perhaps ensconsin, like MAP4 and the neuronal MAP, tau, consists of several electrophoretically distinct species. Nonetheless, we have taken the precaution of including CLAP in the cell homogenization buffer, in order to minimize ensconsin proteolysis.

Our observation that ensconsin remained associated with insoluble material in taxol MT preparations homogenized in the presence of calcium suggested that ensconsin might be capable of forming aggregates, either with itself or with tubulin. Alternatively, ensconsin in these preparations might be bound to a population of MTs that was resistant to depolymerization by calcium and cold. To distinguish among these possibilities, we used negative stain electron microscopy to examine material from each of the final steps in the purification of ensconsin. Fig. 2A shows electropherograms of these fractions, while Fig. 2B shows electron micrographs corresponding to the two relevant samples. Long, unremarkable MTs were the most prevalent structures seen in preparations of thrice-cycled MTs, either before (micrograph a') or after (micrograph c') homogenization with PDEMG buffer containing 3 mM CaCl₂ and sedimentation to enrich for the material resistant to calcium depolymerization. Although these MT

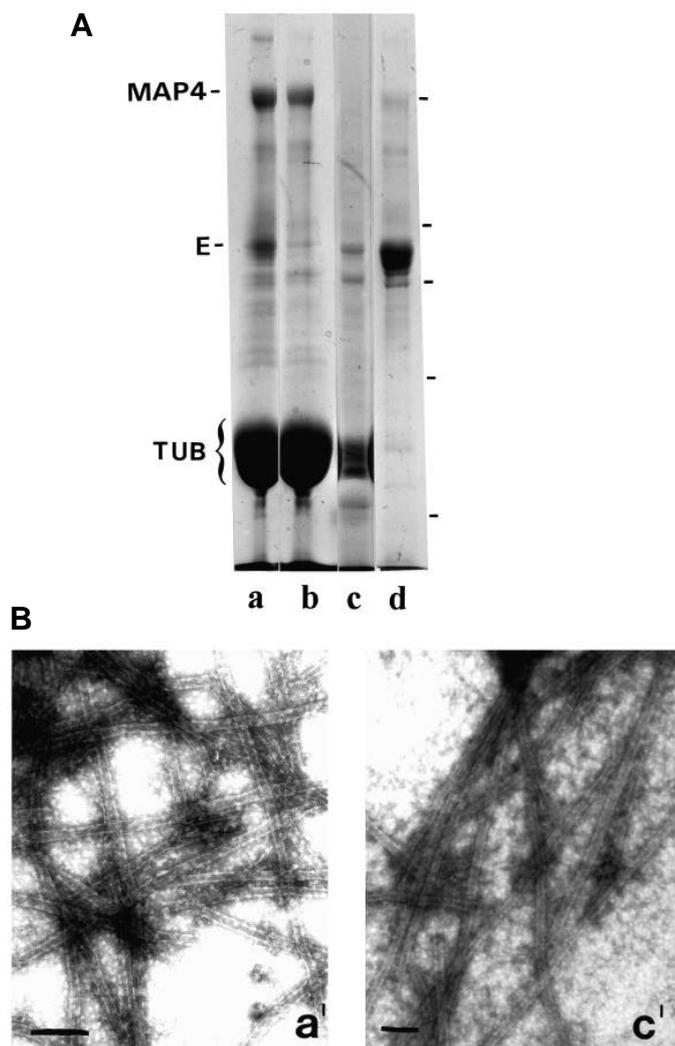


Fig. 2. Preferential association of ensconsin with calcium- and cold-stable MTs *in vitro*. Electropherograms and corresponding electron micrographs of samples obtained during ensconsin purification. (A) Electropherograms shown are: lane a, 100 μ g 3XMTs, as described in Fig. 1; lane b, 90 μ g calcium- and cold-labile fraction of 3XMTs; lane c, 38 μ g calcium- and cold-stable fraction of 3XMTs; lane d, 30 μ g purified ensconsin. The labels MAP4, E and TUB demarcate the electrophoretic positions of MAP4, ensconsin and tubulin; positions of molecular mass markers of 200, 116, 94, 67 and 45 kDa are shown as horizontal bars at right. (B) Micrographs of negatively stained MT samples corresponding to selected electropherograms above: (a') 3XMT sample shown in lane a, above; (c') sample of calcium- and cold-labile fraction of 3XMTs shown in lane c, above. Bar, 100 nm.

samples were strikingly different in their content of ensconsin and MAP4 (compare electropherograms in lanes a and c, in Fig. 2A), the appearance of the samples in negative stained preparations was indistinguishable (e.g. typical fields shown in Fig. 2B, a' and c'). In each sample, both single, well separated MTs and bundles of two to four MTs were occasionally observed; however, MT bundles were never the predominant structure in any sample or field of view. We also examined negative stained samples of purified ensconsin (lane d, Fig

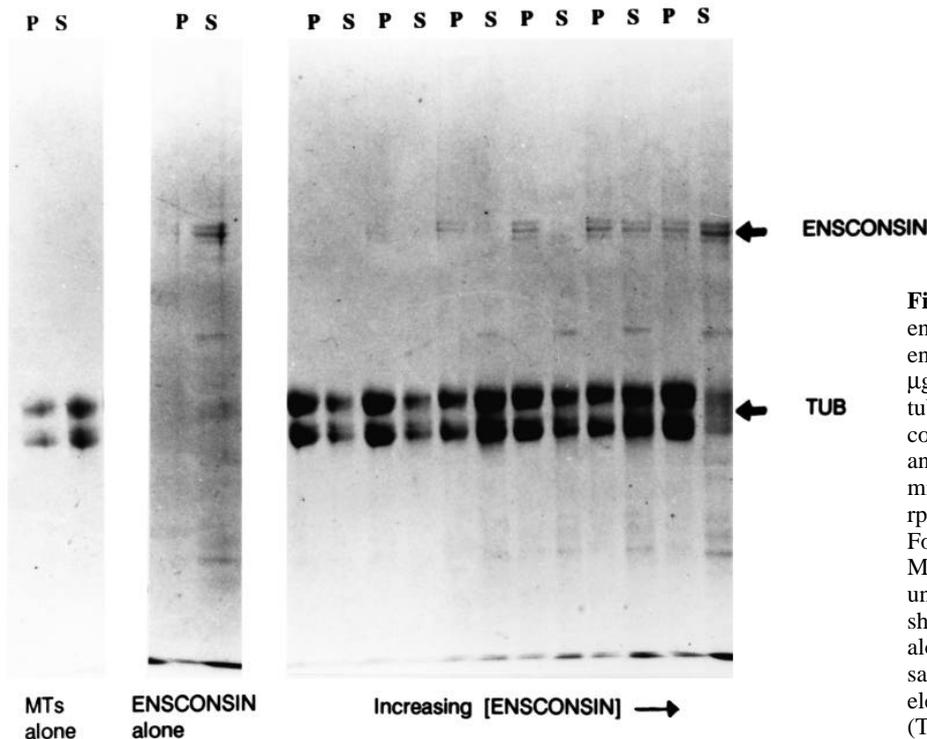


Fig. 3. Electropherogram of MT-binding assay of ensconsin and MTs. Increasing amounts of ensconsin, from left to right (8, 16, 22, 32, 68 $\mu\text{g/ml}$), were added to a constant concentration of tubulin (0.22 mg/ml), polymerized in PDEMG containing a final concentration of 0.6 M NaCl and 10 μM taxol. After incubation at 37°C for 25 minutes, the mixtures were centrifuged at 70,000 rpm in a TLA 100.3 rotor at 37°C for 7 minutes. For each mixture, the electrophoretic pattern of MT-bound material in the pellet (lanes P) and unbound material in the supernatant (lanes S) is shown. Ensconsin alone and pure tubulin MTs alone were assayed as controls, following the same procedure as for the mixtures, above. The electrophoretic positions of ensconsin and tubulin (TUB) are indicated at right.

2A); no recognizable structures were observed by electron microscopy (not shown). Although electron microscopy provides only a qualitative assessment of the structures present in a sample, our data suggest that, during purification, ensconsin is enriched in association with a stable subset of MTs, rather than in association with other types of non-MT aggregates.

To obtain a more quantitative determination of ensconsin's association with stable MTs, we compared preparations of thrice-cycled HeLa MTs purified by the taxol method of Vallee and Collins (1986), which do not contain ensconsin, with thrice-cycled MTs prepared according to our ensconsin purification protocol. We determined that in the former, 60-75% of the MTs could be depolymerized by homogenization in the presence of calcium, while in the preparations in which ensconsin was present, only 40-55% of the MTs were calcium-labile ($n=8$).

These results suggest that ensconsin not only binds to a stable subset of MTs *in vitro*, but it also appears to contribute to the resistance of these MTs to depolymerization by cold and calcium treatment. In order to gain more definitive information about ensconsin's MT-binding properties, we performed *in vitro* experiments with purified ensconsin and MTs.

MT-binding properties of ensconsin

In order to study binding of ensconsin to MTs, independent of any stimulation of MT polymerization, binding assays were performed using taxol-stabilized MTs. This is a potentially critical feature of our experimental design; since a small amount (2-5%) of contaminating MAP4 was present in most ensconsin preparations (e.g. see Fig. 2A, lane d), and MAP4 is known to be a potent stimulator of MT polymerization (Murofushi et al., 1986), attempts to measure the polymerization stimulatory activity in these ensconsin preparations could

yield misleading results. The results of a typical MT-binding experiment performed with purified ensconsin are shown in Fig. 3. As expected, the quantity of tubulin sedimented with increasing ensconsin concentrations was roughly constant. In Fig. 3, lanes from left to right show the composition of fractions in which a constant concentration of taxol-polymerized tubulin was mixed with increasing ensconsin concentrations and centrifuged. For each mixture, both the pellet (containing protein bound to MTs) and the supernatant (containing protein not bound to MTs) are shown. Purified ensconsin exhibited saturable binding to taxol-stabilized MTs in these assays. Surprisingly, inclusion of moderate concentrations of salt (≤ 0.6 M NaCl) in the MT:ensconsin mixtures did not significantly affect MT binding; with or without salt, the presence of MTs with apparently normal morphology was observed by electron microscopy. In Fig. 4, data from four different MT-binding assays performed with purified ensconsin, in the absence or presence of salt, have been displayed graphically. MT-binding increased in an approximately linear manner, reaching a saturating level that corresponded to a molar ratio (ensconsin:tubulin dimer) of approximately 1:6. These MT-binding assays revealed that purified, heat-treated ensconsin differs from other MAPs (e.g. tau, MAP2 and MAP4) in its capacity to bind to MTs in the presence of moderate concentrations of salt. In contrast, ensconsin is similar to these other MAPs in the molar ratio at which it saturates its binding sites on MTs (see Discussion).

Immunological comparison of ensconsin with other MAPs

A number of other proteins reported to associate with MTs possess electrophoretic mobilities similar to that of ensconsin (~ 110 kDa). We tested the cross-reactivity of ensconsin with antibodies to a number of other MAPs, in order to determine

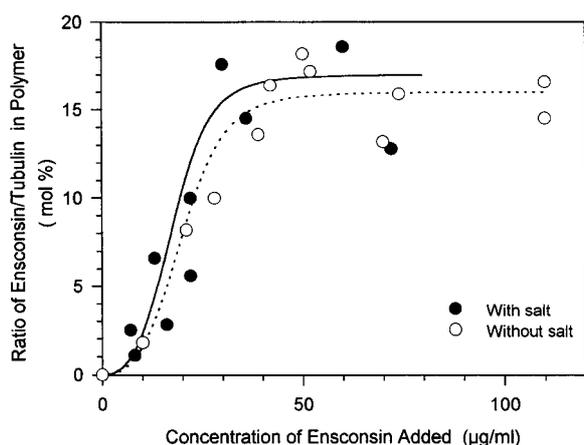


Fig. 4. Quantification of MT-binding assay of ensconsin. MT-bound ensconsin and tubulin sedimented in assays such as that described in Fig. 3 were quantified by densitometry and the ratio of ensconsin to tubulin in polymer was quantified for each sample. Data from four different MT-binding assays performed with purified ensconsin, in the absence (open symbols) or presence (filled symbols) of 0.6 M NaCl have been plotted. A molecular mass of 110 kDa was used in calculations of the molar amount of both tubulin and ensconsin.

whether ensconsin was a distinct molecular species, or whether we were, in fact, discovering heretofore unknown properties of a MAP previously described by others. For example, we tested the ability of antibodies reactive with high molecular mass form of tau protein (~100 kDa), kinesin heavy chain (~116 kDa), STOP (~110 kDa), CLIP-170 (~170 kDa), and MAP4 (~210 kDa) to recognize ensconsin. An identical experiment was performed on our behalf with antibodies against dynamin (~100 kDa) by Dr Chris Burgess, of the Worcester Foundation. We were also interested in the reciprocal possibility; that is, that antibodies prepared against ensconsin would be reactive with one or more of these other MAPs. However, since we determined that anti-ensconsin antisera exhibited human-specific reactivity, anti-ensconsin reactivity with brain MAPs, which we isolated from rat or bovine tissue, could not be assessed. Some representative blots are shown in Fig. 5. None of the antibodies tested showed any reactivity against ensconsin, nor was anti-ensconsin reactive with any rat MAPs tested. Although MAP4 antibodies were unreactive with ensconsin, a small amount of MAP4 was detectable at the 210 kDa position of electropherograms of purified ensconsin, either by anti-MAP4 immunostaining (data not shown) or by Coomassie staining of heavily loaded gels (Fig. 2A, lane d). The absence of antibody cross-reactivity between ensconsin and other MAPs, taken together with the unusual purification strategy we used for ensconsin, which is not amenable to the purification of other known MAPs, suggest that ensconsin is, indeed, a novel MAP, rather than a MAP previously characterized by others.

While this manuscript was being prepared, Masson and Kreis (1993) reported the characterization of HeLa E-MAP-115, a MAP of molecular mass similar to ensconsin. While we have not performed reciprocal antibody cross-reaction tests for the direct comparison of E-MAP and ensconsin, we have obtained the sequence of two peptides proteolytically cleaved

from ensconsin. Although one of the peptides derived from ensconsin is rich in proline residues, as is a large region within E-MAP, neither ensconsin-derived peptide shows significant homology to E-MAP. Therefore, on the basis of the information available to date, we believe that ensconsin is a previously uncharacterized MAP, different from E-MAP and other MAPs present in human cells.

Detection of ensconsin in cell extracts, fixed cells and cytoskeletons

The next question we addressed was whether ensconsin is a MAP unique to the HeLa MT cytoskeleton or if it is present in other types of cells in humans, other primates, or even non-primates. Because HeLa is a human epithelial tumor line, we examined two other lines of epithelial cells (MCF-7 and DU-145), as well as a human fetal muscle cell strain (H271), and human fibroblasts (HuT and H356). Fig. 6 shows an immunoblot in which extract proteins from several types of human cells have been probed with anti-ensconsin antisera. In each lane, a band or characteristic doublet of bands is visible; each band corresponds to a molecular mass of approximately 110 kDa. Preimmune antisera from the same mice, subsequently immunized with ensconsin or antisera preabsorbed with acrylamide pieces containing 5 µg of pure ensconsin (not shown), did not label any species in the western blots, providing confidence in the specificity of our anti-ensconsin antibodies, and demonstrating that ensconsin is present in human cells derived from several types of tissues. To date, we have not detected ensconsin in any nonhuman cell types or tissues with any of 13 mouse antisera; for example, monkey

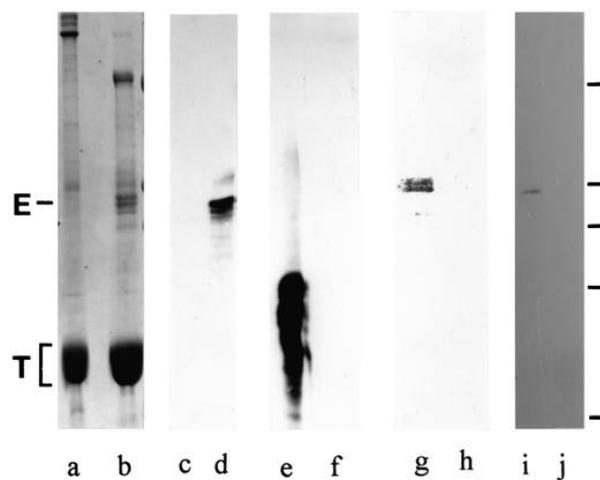


Fig. 5. Immunological cross-reactivity of ensconsin (E) with other MAPs. Replicate samples of a MT fraction polymerized from rat brain extract in the absence of nucleotide (40 µg; lanes a,c,e,g and i) and thrice-cycled MTs purified from HeLa extract (50 µg; lanes b,d,f,h and j) were electrophoresed, and the electropherograms were stained with Coomassie Brilliant Blue (a,b) or immunoblotted and immunostained with antibodies prepared against ensconsin (c,d), tau (e,f), dynamin (g,h), and kinesin (i,j). Only the mouse polyclonal antibodies prepared against ensconsin were reactive with ensconsin (lane d); no cross-reactivity was observed between ensconsin and any other MAPs of similar molecular mass. Molecular mass markers are given on the right as for Fig. 1. T (on left), tubulin.

TC-7 cells, mouse 3T3, rat NRK, Chinese hamster CHO cells, or bovine or porcine brain samples (data not shown). Although it is formally possible that ensconsin is a MAP expressed only in humans, our results are more likely to reflect species specificity of the ensconsin antisera. We suspect that species specificity may be a general property of the immunized animal's response to ensconsin, as has been found for MAP4 (Bulinski and Borisy, 1980c; Chapin and Bulinski, 1991).

Tenacious association of ensconsin with stable MTs in vivo

As shown in Fig. 7, we used our well characterized mouse anti-ensconsin antisera to confirm and extend previous, more cursory results on the immunolocalization of ensconsin (Bulinski and Borisy, 1980a). As shown in the micrographs in Fig. 7, anti-ensconsin labeled all or most MTs in epithelial (a,b, and g,h), muscle (c,d), and fibroblast (e,f) cells. In these methanol-fixed preparations, ensconsin staining appeared to be identical to tubulin immunostaining (b,d,f,h). Control experiments used to document the specificity of anti-ensconsin immunofluorescence included staining HeLa, MCF-7 and H271 cells with preimmune sera (from the same mice subsequently immunized with ensconsin) and staining with sera preabsorbed with ensconsin acrylamide gel pieces; both gave only dim diffuse staining patterns (not shown). In addition, when anti-ensconsin sera were used to stain nonhuman cell types,

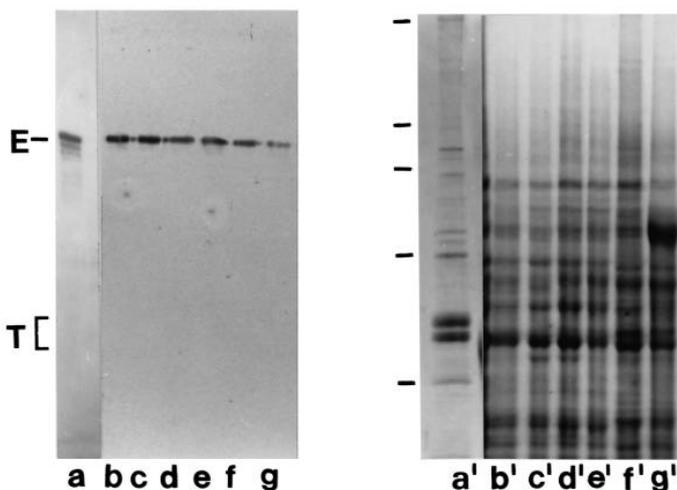


Fig. 6. Detection of ensconsin in extracts prepared from human cell lines. Samples of 12 μ g purified HeLa MTs (twice-cycled MTs, lanes a, a'; and 50 μ g each of extracts of HeLa cells (b,b'); differentiated (c,c') and undifferentiated (d,d') human muscle cells, strain H271; HuT fibroblasts (e,e'), human fibroblasts, strain 356 (f,f'), and MCF-7 mammary epithelial cells (g,g') were electrophoresed in triplicate blots. In lanes a-g, samples were electrophoresed, electroblotted, and the blot was immunostained with a 1/2000 dilution of mouse anti-ensconsin serum. In lanes a'-g', samples were electrophoresed as before, and then the electropherogram was stained with Coomassie Brilliant Blue. The third set of samples was electrophoresed and blotted, and the blot (not shown) was stained with a 1/2000 dilution of preimmune serum obtained from the same mouse used in immunostaining the blot depicted in a-g; the preimmune serum did not detectably label any bands. E and T indicate the electrophoretic mobility of ensconsin and tubulin, respectively; small bars at the left side of a'-g' indicate molecular mass standards, as described for Fig. 1.

such as monkey TC-7, mouse 3T3, and rat NRK cells, only a low level of diffuse staining was observed (data not shown).

Because of ensconsin's unusually tenacious binding to MTs in vitro, we tested whether avid binding was also characteristic of ensconsin in the in vivo context. Accordingly, as shown in Fig. 8, we extracted H271 cells with mild detergent prior to methanol fixation. This treatment had no effect on ensconsin immunostaining (compare Fig. 8a and c), although it completely abolished MAP4 staining (compare Fig. 8b and d). The ability of MAP4 to be extracted from cytoskeletons by mild detergent treatment has been reported previously (Schliwa et al., 1981), although the biochemical basis of this phenomenon has not yet been elucidated. We also determined that ensconsin remained bound to MTs even when cells were extracted with detergent for very long periods (>25 minutes; data not shown). Ensconsin's association with the MT cytoskeleton was resistant to several other detergent treatments (including Triton, Nonidet, and *N*-octylglucoside) in other human cell lines, as well (HeLa, MCF-7 and HuT), even though MAP4 rapidly dissociated from the MTs under each of these extraction conditions (data not shown).

We also tested the capacity of ensconsin to bind to stable MTs in drug-treated cells. Fig. 8g,h shows H271 cells treated with sufficient nocodazole to depolymerize most cellular MTs. Ensconsin remained tightly bound to the remaining MTs, demonstrating that it was also ensconced on the subset of stable MTs that are resistant to both drug depolymerization and detergent extraction. Thus, our data are consistent with the hypothesis that ensconsin is associated very firmly with MTs in vivo. The tenacity of this association is a property in which ensconsin differs from MAP4, since MAP4's association with MTs is abrogated by detergent treatment.

DISCUSSION

In this paper, we have revitalized the study of a prominent species of HeLa MAP originally identified by Bulinski and Borisy (1979) and Weatherbee et al. (1980). Because we have purified the protein and gained insight into its biochemical properties in vitro and in vivo, we have renamed it ensconsin. Ensconsin's former name, 125 kDa MAP, was in need of replacement because it reflected none of the MAP's features except its molecular mass, and modern data suggested that even this was inaccurate (assignment of ensconsin's molecular mass relative to the electrophoretic mobility standards now available yield a value of ~110 kDa). In any case, a name based on molecular mass alone could prove to be problematic, since as studies of ensconsin progress, we might expect to discover variations in ensconsin's molecular mass in other organisms, or with other electrophoresis systems. With identical reasoning, MAP4's original name, 210 kDa MAP, a name connoting only molecular size, was previously abandoned in favor of the name MAP4 (discussed by Parysek et al., 1984). We chose the name ensconsin for the MAP we are investigating for two reasons: first, we wanted to choose a name that reflected the unusual biochemical properties of the MAP; these properties might also be related to the in vivo function of ensconsin, which has yet to be determined. The word *ensconce* means to settle snugly or securely, and the tenacious binding of ensconsin to stable MTs in vitro and in vivo connotes this

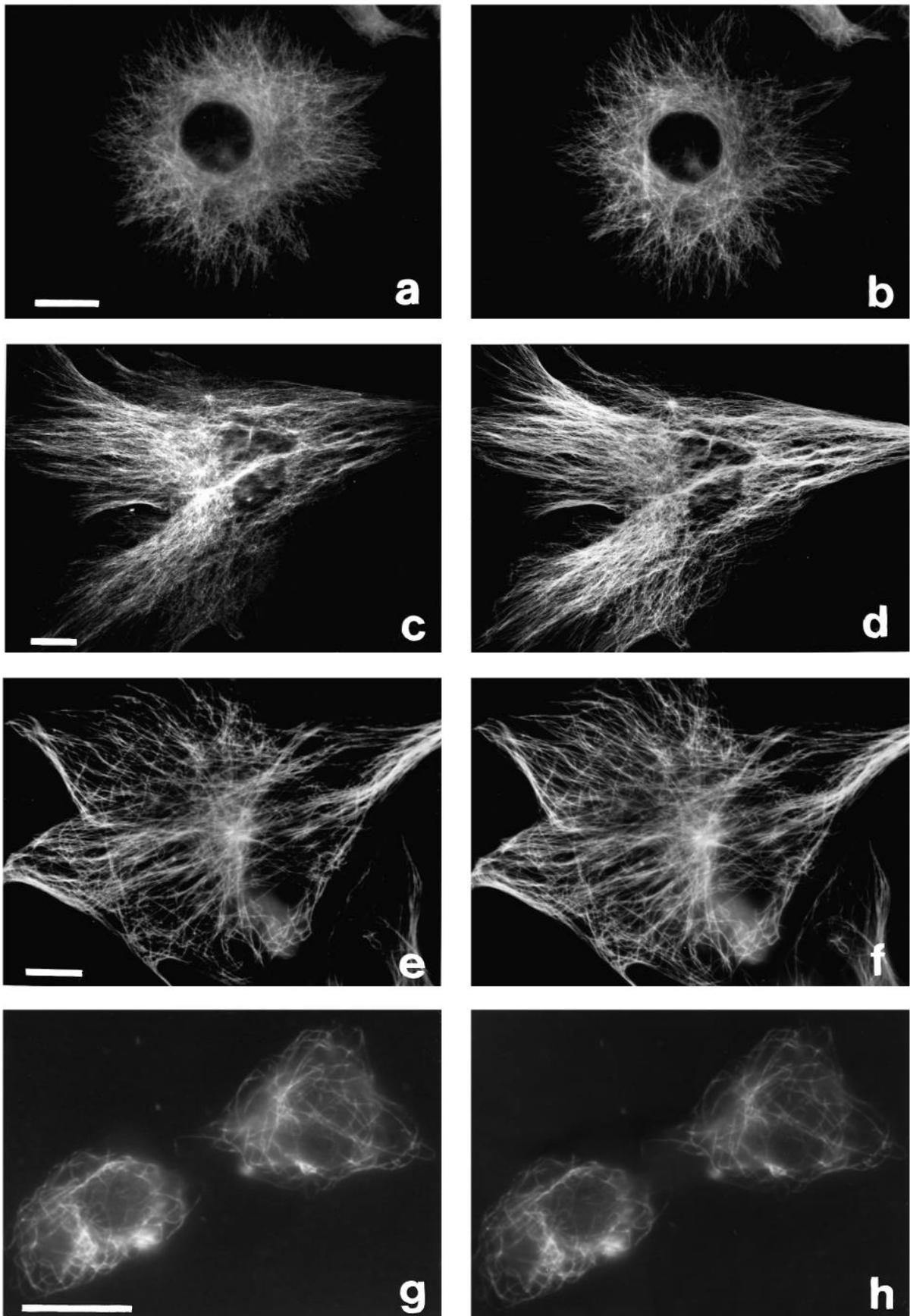


Fig. 7. For legend see p. 2848

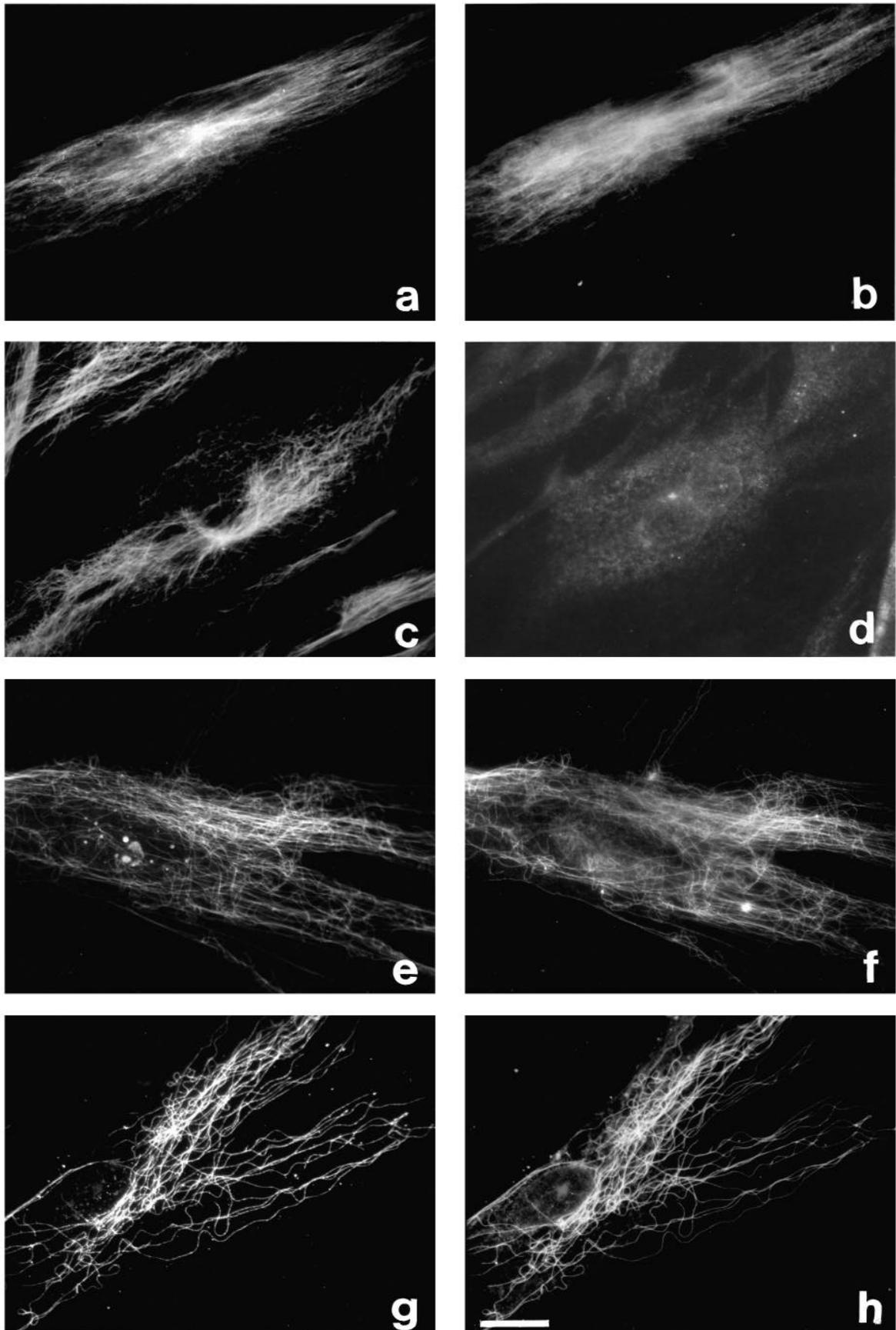


Fig. 8. For legend see p. 2848

Fig. 7. Association of ensconsin with MTs in vivo. Double-label immunofluorescence of human cells with anti-ensconsin (a,c,e,g) and anti-tubulin (b,d,f,h). Cells stained are MCF-7 (a,b); differentiated H271 human muscle cells (c,d), 356 fibroblasts (e,f), and HeLa cells (g,h). Note that in all ensconsin images most or all MTs appear to be labeled with ensconsin antibody, except perhaps in the myotube shown in c and d. Controls in which cells were immunostained with preimmune sera and sera preabsorbed with ensconsin verified the specificity of immunostaining (not shown). Bars, 10 μ m. All cells shown were methanol-fixed, except in g and h, which were first extracted in saponin, and then fixed in methanol, as described in Materials and Methods.

Fig. 8. Ensconsin localization on stable MT subsets in extracted or drug-treated cells. Undifferentiated H271 human muscle cells in the double immunofluorescence images shown were immunostained with anti-ensconsin antiserum (a,c,e and g) and anti-MAP4 polyclonal antibody (b,d), or anti-tubulin antibody (f,h). All micrographs show cells that were methanol-fixed; however, in c-h, the cells were extracted with the detergent saponin prior to methanol fixation. In g and h, cells were first treated with 10 μ M nocodazole for 10 minutes, before detergent extraction and fixation. Note that ensconsin immunostaining (c,e), unlike MAP4 immunostaining (d), is not abrogated by detergent extraction prior to cell fixation; also, ensconsin is found in association with the few stable MTs that remain following nocodazole depolymerization and extraction with detergent (g). All micrographs are the same magnification; bar, 10 μ m.

secure type of association. The name ensconsin is additionally suitable because ensconce also means to cover, hide or protect; these meanings arise from its root, sconce, which is a fortification. Ensconsin is also a reasonable name for the HeLa MAP we have described, since its presence on MTs appears to protect or fortify them against depolymerization by cold and calcium. Finally, the name ensconsin also contains a mnemonic for Wisconsin; it was at the University of Wisconsin that ensconsin was first discovered (Bulinski and Borisy, 1979). A similar mnemonic for a university was employed in naming another cytoskeletal protein, ezrin, whose name is meant to allude to Cornell University's founder, Ezra Cornell (Bretscher, 1994).

Both the biochemical properties of ensconsin and the antibody cross-reaction tests we have performed with antibodies reactive with other MAPs suggest that ensconsin is a novel MAP. The ensconsin purification scheme is unlike any used to purify tau, STOP, MAP4, dynamin or kinesin, and ensconsin does not exhibit nucleotide-sensitive binding to MTs, as the latter two MAPs do. Further evidence that ensconsin is unrelated to other known MAPs of similar molecular mass is derived from the failure of antibodies reactive with other MAPs to recognize ensconsin, as well as negative results in the reciprocal experiments with anti-ensconsin antibodies. In fact, our experiments with antibodies to other MAPs were performed with a high stringency; that is, each blot was performed with samples containing an amount of ensconsin (~1 μ g) adequate to allow us to detect even MAPs present as low level contaminants (5-10%, relative to ensconsin) within our preparations. Thus, the presence of small amounts of these other MAPs of molecular mass ~100 kDa in our preparations of HeLa MTs or purified ensconsin is extremely unlikely. Our ensconsin preparations do contain a low level of MAP4; however, since the size and behavior of the two proteins are quite different, we can effectively rule out

the possibility that MAP4 contamination would influence the properties of ensconsin that we measured, and confound our MT-binding results. Taken together, our experiments demonstrate that ensconsin is a novel protein with biochemical properties distinct from those of other MAPs.

The relationship of ensconsin to E-MAP (Masson and Kreis, 1993), which was also obtained from HeLa cells, is not yet well established. However, several results suggest that the two MAPs are unrelated proteins. The sequence of E-MAP was published, and it shows no homology to the sequence of two proteolytic peptides that we obtained from ensconsin (Bossler and Bulinski, unpublished results). In addition, Masson and Kreis (1993) named their MAP E-MAP (i.e. epithelial MAP) because they determined that E-MAP was not abundant in fibroblasts. In contrast, ensconsin appears to be equally abundant in epithelial, fibroblast, and even myoblast cells. Also, unlike ensconsin, E-MAP was reported to be heat-labile, and to exhibit a perinuclear distribution. Some properties of E-MAP and ensconsin are intriguingly similar, though; especially the association each MAP shows with MTs in detergent extracted cytoskeletons and in nocodazole-treated cells (compare Fig. 3c of Masson and Kreis (1993) with Fig. 8e,f in this paper). Either molecular cloning of ensconsin or biochemical characterization of E-MAP will be necessary in order to compare these two molecules definitively.

Although ensconsin exhibits several characteristics common to the so-called assembly-promoting MAPs (AP-MAPs; Vallee, 1990), such as thermostability, protease sensitivity and saturable binding to MTs, it also manifests some novel properties. Ensconsin's most striking properties are its specific association with taxol MTs that are highly resistant to depolymerization by cold and calcium treatment, as well as its capacity to bind to MTs even in the presence of moderate concentrations of salt (0.6 M NaCl). These properties, which have not been previously demonstrated for other MAPs, suggest that ensconsin's mode of binding to MTs is likely to be different from that of the AP-MAPs. Tau, MAP4 and MAP2 each contain repeated sequence elements within their positively charged MT-binding domains, and the repeated elements, surrounding sequence and net charge are all thought to contribute to MT binding (Chapin and Bulinski, 1992). It is likely that ensconsin possesses one or more MT binding sites structurally distinct from the MT binding sites of these other MAPs. It is curious that ensconsin shows saturation of its binding sites on MTs at a molar ratio similar to that of the AP-MAPs, tau, MAP2 and MAP4 (Kim et al., 1986; Chapin and Bulinski, 1994). This similarity may be mere coincidence; perhaps, instead, ensconsin and the AP-MAPs bind by different mechanisms, but utilizing equivalent sites on the MT. Competition experiments between ensconsin and MAP4 could be performed in order to address this question.

Our experiments have demonstrated that ensconsin differs from MAP4 in its MT association in vivo. For unknown reasons, MAP4 is released from MT cytoskeletons when cells are extracted with mild detergent (Schliwa et al., 1981; also see Fig. 8c,d). In contrast, we found that ensconsin is not extractable, even in cells in which only a few stable MTs have been preserved (Fig. 8a,b,e,f). The tenacious binding of ensconsin to MTs suggests that in vivo, as well as in vitro, ensconsin's MT-binding differs from that of MAP4, and raises the possibility that ensconsin may be a potent effector of MT

stabilization in vivo. Further studies will be needed to elucidate the role ensconin may play in the generation and/or maintenance of stable MTs in vivo.

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