Identification and Molecular Characterization of E-MAP-115, a Novel Microtubule-associated Protein Predominantly Expressed in Epithelial Cells

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Abstract. A novel microtubule-associated protein (MAP) of Mr 115,000 has been identified by screening of a HeLa cell cDNA expression library with an antiserum raised against microtubule-binding proteins from HeLa cells. Monoclonal and affinity-purified polyclonal antibodies were generated for the further characterization of this MAP. It is different from the microtubule-binding proteins of similar molecular weights, characterized so far, by its nucleotide-insensitive binding to microtubules and different sedimentation behavior. Since it is predominantly expressed in cells of epithelial origin (Caco-2, HeLa, MDCK), and rare (human skin, A72) or not detectable (Vero) in fibroblastic cells, we name it E-MAP-115 (epithelial MAP of 115 kD). In HeLa cells, E-MAP-115 is preferentially associated with subdomains or subsets of perinuclear microtubules. In Caco-2 cells, labeling for E-MAP-115 increases when they polarize and form blisters.

The molecular characterization of E-MAP-115 reveals that it is a novel protein with no significant homologies to other known proteins. The secondary structure predicted from its sequence indicates two domains connected by a putative hinge region rich in proline and alanine (PAPA region). E-MAP-115 has two highly charged regions with predicted α-helical structure, one basic with a pI of 10.9 in the NH₂-terminal domain and one neutral with a pI of 7.6 immediately following the PAPA region in the acidic COOH-terminal half of the molecule. A novel microtubule-binding site has been localized to the basic α-helical region in the NH₂-terminal domain using in vitro microtubule-binding assays and expression of mutant polypeptides in vivo. Overexpression of this domain of E-MAP-115 by transfection of fibroblasts lacking significant levels of this protein with its cDNA renders microtubules stable to nocodazole. We conclude that E-MAP-115 is a microtubule-stabilizing protein that may play an important role during reorganization of microtubules during polarization and differentiation of epithelial cells.

Epithelial cells exhibit a structural and functional asymmetry referred to as apical-basal polarity. The establishment of this polarity has been studied in epithelial cell models such as MDCK and Caco-2, and microtubules have been implicated in this process (for reviews see Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990). Microtubules are nucleated by pericentriolar material in the perinuclear region of the microtubule-organizing center (MTOC) in sparse MDCK cells (Bré et al., 1990). Microtubules are nucleated by pericentriolar material in the perinuclear region of the microtubule-organizing center (MTOC) in sparse MDCK cells (Bré et al., 1990). As the cells form contacts, junctions assemble and polarization is induced (Gumbiner and Louvard, 1985).

During establishment of cell polarity, the centrioles split, migrate towards the apical pole of the cell, and the pericentriolar material disperses; simultaneously, the microtubules reorganize into bundles running parallel to the apical-basal axis, apparently no longer originating from the perinuclear region (Bacallao et al., 1989; Buendia et al., 1990; Gilbert et al., 1991).

Formation of cell–cell contacts is accompanied by stabilization of microtubules in epithelial cells, but not in fibroblasts (Pepperkok et al., 1990; Wadsworth and McGrail, 1990). This stabilization appears to occur along the entire microtubule length (Bré et al., 1990). Stable microtubules often contain posttranslationally modified tubulin, but it has been suggested that acetylation and deetyrosination are rather the result than the cause of microtubule stability (Webster et al., 1990; Bré et al., 1987). Thus, other factors, such as microtubule-associated proteins (MAPs), must be involved in the initial events of microtubule stabilization.
Despite extensive studies, performed predominantly with neuronal MAPs, little is known about the precise function of MAPs in mammalian nonneuronal cells. Apart from the well-characterized microtubule-based molecular motor proteins kinesin and cytoplasmic dynein, which are not expected to have structural functions, relatively few MAPs have been characterized so far in these cells. MAP4 designates a group of ubiquitous proteins of ~200 kD (Olmsted et al., 1986), initially identified as 210-kD HeLa MAP (Bulinski and Borisy, 1979), murine MAP4 (Olmsted and Lyon, 1981), and bovine MAP-U (Murofushi et al., 1986). They belong to a family of proteins originating from alternatively spliced mRNA which are differentially expressed in various tissues (West et al., 1991). MAP3 (180 kD), first identified in astroglia and neurons (Huber et al., 1985) and subsequently detected in various nonneuronal rat tissues, might also be a member of the MAP4 family (Huber and Matus, 1990). Chartins constitute another complex family of MAPs (Duerr et al., 1981; Magendanz and Solomon, 1985). The functions of these two protein families are unknown. Several additional “MAPs” (~200, 151, 125, 114, ~100, and 68 kD) have been identified in HeLa cells (Bulinski and Borisy, 1979; Weatherbee et al., 1980); they are so far only poorly characterized.

Microtubule-associated proteins were initially defined and purified by their ability to copurify with microtubules during cycles of polymerization and depolymerization. Many of them promote tubulin polymerization in vitro. They usually interact rather stably with microtubules and have therefore been implicated in regulation of microtubule stability. Overexpression of neuronal MAP2 and tau, but not of the microtubule-based motor protein kinesin (Navone et al., 1992), leads to bundling and stabilization of microtubules in nonneuronal cells (Lewis et al., 1989; Lee and Rook, 1992; Kanai et al., 1992). Microinjection of purified tau into fibroblasts also stabilizes microtubules (Drubin and Kirschner, 1986). Additional approaches have recently been used to identify novel microtubule-binding proteins. A doublet of 95 and 105 kD proteins has been identified in CHO cells by raising antibodies against isolated mitotic spindles (Selitto and Kuriyama, 1988), and an antigenically cross-reacting doublet of 100 and 115 kD was found in HeLa cells (Nislow et al., 1990). Although these proteins do not display the typical nucleotide-sensitive microtubule-binding characteristics (Nislow et al., 1990), one of them has recently been proposed to belong to the class of molecular motors (Nislow et al., 1992). A 170-kD protein (CLIP-170) has been identified in HeLa cells by its cosedimentation with taxol-polymerized microtubules (Rickard and Kreis, 1990, 1991). CLIP-170 is involved in the interaction of endosomes with microtubules (Pierre et al., 1992). It also accumulates at desmosomal plaques in polarizing epithelial cells (Wacker et al., 1992). CLIP-170 may thus link microtubule plus-ends to other cytoplasmic structures and thereby play a role in regulating microtubule stability.

Several neuronal MAPs are expressed differentially during development, probably correlating with their functional properties (Matus, 1988). It might also be expected that specific MAPs are involved in microtubule stabilization during epithelial cell polarization, and in the (re)organization of cytoarchitecture, but so far, no epithelial cell-specific MAP has been identified. Here we characterize a novel MAP with an apparent molecular weight of 115,000 (E-MAP115), predominantly expressed in cells of epithelial origin, which may be a candidate for a protein regulating microtubule stability and reorganization during cell polarization.

**Materials and Methods**

**Preparation of HeLa Microtubule-binding Proteins**

HeLa-S3 cells were grown in suspension in Joklik's MEM supplemented with 1% glutamine, penicillin and streptomycin, as well as 5% newborn calf serum, at 37°C in air to a density of 6 × 10⁶ cells/ml. Cells were spun for 10 min at 500 g, washed twice in Hank's buffer at 4°C, and once in 100 mM KPi, 1 mM EGTA, 1 mM MgSO₄, pH 6.8 (PB). The washed cells were resuspended in 1 ml/ml of pellet in PB with 1% Triton X-100, 1 mM DTT and 1 mM PMSF and lysed for 30 min on ice. The lysate was spun for 10 min at 4°C at 12,000 g in a SS-34 rotor (Sorvall Instrs., Norwalk, CT) and the supernatant centrifuged for 1 h at 150,000 g in a Ti-70 rotor (Beckman Instrs., Fullerton, CA) at 4°C. This high-speed supernatant was incubated for 20 min at 37°C with 30 μg/ml of purified calf brain tubulin, prepared as described (Mitchison and Kirschner, 1984), and 20 μM taxol (a generous gift of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) to promote microtubule polymerization. 1 U/ml apyrase was added for an additional 20 min at 37°C to increase binding of nucleotide-sensitive microtubule-binding proteins. Microtubules from this solution were then sedimented through a cushion of 4 M glycerol in PB with 2 μM taxol and 1 mM DTT for 30 min at 30,000 g at 30°C in a SW27 rotor (Beckman Instrs.), and washed once in PB with 10 μM taxol and 1 mM DTT. The microtubule-binding proteins were eluted by resuspending the pellet in PB with 0.8 M NaCl, 10 mM MgATP, 10 μM taxol, and 1 mM DTT. This suspension was finally spun at 80,000 g for 30 min at 30°C in a TL-A100 rotor (Beckman Instrs.). The supernatant containing the crude microtubule-binding proteins was used to immunize rabbits.

**cDNA Expression Library Screening with Antisera**

A mixture of antisera from eight different bleedings of two rabbits immunized with HeLa cell microtubule-binding proteins was used to screen ~200,000 colonies of a random-prime HeLa cell cDNA library prepared in the expression plasmid pUEX (Bressan and Stanley, 1987). The fusion proteins expressed by the positive clones were used to affinity-purify antibodies from the mixture of sera on nitrocellulose blots as described (Rickard and Kreis, 1990).

**Antibodies**

An E-MAP115 fusion protein (produced by clone pl/125, corresponding to aa 245-749) was purified by SDS-PAGE. The acrylamide gel piece containing the protein was washed with PBS, homogenized, and emulsified into an equal volume of complete Freund's adjuvant (GIBCO BRL, Gaithersburg, MD). Soluble fusion protein, for intravenous injections of mice, was obtained by electro-elution from the gel as described (Bhown et al., 1980). Immunization of mice was done according to Allan and Kreis (1986). Mouse spleen cells were used for production of mAbs and positive clones were identified in a first round by immunofluorescence on HeLa cells (Allan and Kreis, 1986) and in a second round by immunoblotting to HeLa cell lysates. Rabbits were immunized with peptides and antibodies were affinity-purified as described (Duden et al., 1991).

**Cell Culture**

Vero, HeLa, Caco-2, and MDCK cells, as well as human skin fibroblasts, were grown in MEM. A72 tumor-derived dog fibroblasts (Beit et al., 1980) were grown in L-15 (Leibovitz) medium. All media were supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, as well as 5% (MDCK, Vero), 10% (HeLa, A72, human skin fibroblasts), 20% (Caco-2) FCS. The HeLa, Caco-2, and human skin fibroblast media also contained 1% non-essential amino acids. Cells were grown with 5% CO₂ at 37°C in humidified incubators.

**Immunofluorescence**

Cells grown on coverslips were fixed for 4 min in methanol at −20°C, in
Immunogold Labeling for Electron Microscopy

HeLa cells grown on glass coverslips were fixed for 4 min in methanol at -20°C and incubated twice for 2 h with an anti-E-MAP-115 mAb (D9C or D9G). Three washes of 10 min with 0.5% BSA in PBS were followed by incubation with goat anti-mouse antibodies coupled with 10-nm colloidal gold (AuroProbe EM GAR G10; Amersham Corp., Arlington Heights, IL) for 2 h at 37°C. Additional washes included three times 10 min in 0.5% BSA in PBS and three times 5 min in PBS. After fixation and further processing, the specimen was done as described (Wacker et al., 1992).

Electrophoresis and Immunoblotting

Gel electrophoresis and immunoblotting was done as described (Rickard and Kreis, 1990).

Sedimentation of E-MAP-115 with Microtubules

200 μl of HeLa cytosol (∼8 mg/ml) was incubated at 37°C for 30 min with different additions. The samples were loaded on a 480 μl 10% sucrose cushion at 30,000 g at 30°C and incubated twice for 2 h with either 20 μM taxol or 40 μM nocodazole. The different templates in BLUESCRIPT were linearized at convenient restriction sites and RNAs were produced by in vitro transcription using T3 polymerase and an in vitro transcription kit (Promega Corp., Madison, WI) according to the manufacturer's instructions. RNAs were subsequently used for translation using a rabbit reticulocyte lysate (Promega Corp.) for 60 min at 30°C using 35S-methionine (Sambrook et al., 1989).

Immunoprecipitation

[35S]Methionine-labeled protein was obtained either by in vitro transcription/translation with clone pl/49 as template or overnight labeling of HeLa cells with 100 μCi [35S]methionine and 20 μM Tris, 100 mM NaCl, 0.4% SDS, 1 μM PMSF, pH 7.4 (TX-100) was added to a final concentration of 0.5% to dilute the translation reaction and the lysate were spun at 150,000 g for 1 h and at 15,000 g for 15 min, respectively, at 4°C. The supernatants were used for immunoprecipitation with the mAb D9C according to Rickard and Kreis (1991). The immunoprecipitates were analyzed by 8% SDS-PAGE. The gel was incubated in 1 M sodium salicylate for 30 min, dried, and exposed to X-Omat AR films (Eastman Kodak).

Generation of Mutant cDNA Clones

The cDNA coding for mutant Mal cDNA was constructed by digestion of clone pl/49 with Nhel and Hpal, treatment by nuclease S1 and religation. Clones were sequenced, and one with the same reading frame as the original clone was selected for transcription and subsequent in vitro translation. In this clone, base 227 (numbering corresponds to the sequence shown in Fig. 6 B) is ligated to base 784 giving rise to a protein lacking residues 77-228. To construct the Mb mutant, a novel Nhel site was introduced at base 1,382 by PCR and used for ligation with the original Nhel site. To generate the Nhel site, the oligonucleotides 5'CCTCCGCTGCGCTAGCCATGG3' (bases 1,369-1,388; substituted bases underlined) and 5'GATATCCGCTGCGCTAGCCATGG3' corresponding to the antisense oligonucleotide of bases 2,500-2,516, were used as primers with clone pl/44 as a template. PCR product was cut by Nhel and Xbal and ligated with clone pl/49 digested by Nhel and Xbal. The resulting cDNAs were used as primers with clone pl/49 as a template. The PCR product was cut by Nhel and Xbal and ligated with clone pl/49 digested by Nhel and Xbal. The resulting cDNAs were sequenced and analyzed by 8% SDS-PAGE. The gel was incubated in 1 M sodium salicylate for 30 min, dried, and exposed to X-Omat AR films (Eastman Kodak).

In Vitro Microtubule-binding Assays with In Vitro Translated Polypeptides

27 μl of in vitro translation reaction containing 30 μCi of [35S]methionine were diluted in PB in a final volume of 210 μl with a final concentration of 1 mM cold methionine and 1 mM PMSF. After 30 min on ice, the reaction mixture was spun at 55,000 rpm for 60 min at 4°C in a TLA-100 rotor (Beckman Instrs.). 100 μl of the supernatant were incubated with or without 30 μg of taxol-polymerized microtubules and the supernatant was diluted in PB in a final volume of either 20 μM taxol or 40 μM nocodazole and loaded on 560 μl of 10% sucrose in PB. After 30 min centrifugation at 30,000 g at 30°C in a SW50 rotor (Beckman Instruments), equal amounts of the supernatants and pellets were analyzed by 8% SDS-PAGE and the gel subsequently treated for fluorography as above.

Sequence Analysis and Structure Prediction

The secondary structure prediction, the molecular weight, and amino acid composition of E-MAP-115 were obtained using the GCG-programs, PEPTIDE-STRUCTURE and PEPTIDESORT (Devereux et al., 1984). FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) were used to search the GenEMBL nucleotide data library (release 34.0) and the SwissProt protein data library (release 24.0) for sequences homologous to E-MAP-115. Comparison of E-MAP-115 with the so far characterized microtubule-interacting proteins was also done using the Sensitive Sequence Comparison program (ISSC; Rechid et al., 1989).
Figure 1. Immunofluorescence localization of E-MAP-115. HeLa cells (A and B) and human skin fibroblasts (C and D) were fixed in methanol after detergent preextraction. Double immunofluorescence labeling was performed with mAb D9C1 against E-MAP-115 (A and C) and a rabbit-tubulin antiserum (B and D). Images were taken as described in Materials and Methods with exposure times for E-MAP-115 labeling of 8 s for HeLa cells (A) and 25 s for human skin fibroblasts (C). Staining for E-MAP-115 is observed on segments or subsets of microtubules near the nucleus (A and C). Only a small subset of microtubules is positive for E-MAP-115 in fibroblasts (C). Bar, 20 μm.

Transient Transfection of Vero Cells

Clone p1/49 and the clones encoding polypeptides P3, Mal, and Mb were subcloned into an SV-40 expression vector pSG5 (Green et al., 1988). Plasmid DNA used for transfection was purified on a QiaGen column and dialyzed against 1 mM EDTA, 10 mM Tris, pH 7.5. Vero cells were split 24 h before transfection so that they were ~80% confluent on the day of transfection. The cells were washed twice with serum-free medium and transfected with DOTAP (Boehringer, Mannheim) according to the manufacturer's instructions with the following modifications. The transfection-reagent-DNA mixture was added directly to cells for 2 min and then diluted with serum-free medium. After 4 h, the medium was replaced by medium containing 5% FCS. Cells were processed for immunofluorescence staining after 20 h.

Results

Identification of E-MAP-115

Antisera raised against crude microtubule-binding proteins from HeLa cells were used to screen a HeLa cell cDNA expression library to identify and clone novel MAPs. Clones encoding fragments of already characterized proteins likely to be recognized by the sera were identified in a first round using a mixture of murine mAbs against tubulin, MAP4, CLIP-170, vimentin, and actin. Approximately 500 clones were detected and not further characterized. 95 new clones were identified in the second round with a mixture of the rabbit antisera against HeLa microtubule-binding proteins. Antibodies were affinity-purified on fusion proteins expressed by the different clones and used to group the clones into immunologically related families, to localize the corresponding cellular proteins by immunofluorescence and to determine their Mr by immunoblotting of cell lysates. Four families were obtained by this approach: 52 clones encoding fragments of a protein with an apparent Mr of 115,000. This latter protein (termed E-MAP-115) was characterized further using monoclonal and affinity-purified polyclonal antibodies prepared against a fusion protein corresponding to a fragment of E-MAP-115 (clone p1/125).

E-MAP-115 Is Associated with Subdomains of Microtubules

E-MAP-115 colocalizes with microtubules in HeLa cells and human skin fibroblasts (Fig. 1). E-MAP-115 is preferentially localized on microtubules in the perinuclear region in HeLa cells (Fig. 1 A). In the skin fibroblasts, labeling for E-MAP-115 is significantly weaker and restricted to only a few microtubules (Fig. 1 C). No labeling was detected in Vero (monkey fibroblast) cells. The intensity of labeling for E-MAP-115 along microtubules is gradual and diminishes towards their peripheral ends. This labeling pattern is independent of the method of cell fixation (para-formaldehyde, glutaraldehyde, or methanol, with or without detergent pre-extraction; not shown). The distribution of E-MAP-115 is in contrast to that of MAP4 and the 125-kD HeLa MAP, which are present on virtually all microtubules and label continuously along their entire length (Bulinski and Borisy, 1980b). Furthermore, labeling for E-MAP-115 is heterogenous within the same population of cells (Fig. 3 A). In the fibroblasts, some of the microtubules in the perinuclear area containing E-MAP-115 are curvy. Such microtubules frequently correspond to a subset of stabilized microtubules enriched in dehydroglutaryl tubulin (Schulze et al., 1987; Webster et al., 1987; Kreis, 1987). Labeling for Glu-tubulin to correlate E-MAP-115 localization with microtubules enriched in Glu-tubulin revealed that only few Glu-microtubules existed in the fibroblasts; they were all positive for E-MAP-115, but not all E-MAP-115-positive microtubules were labeled for Glu-tubulin (not shown). Thus, E-MAP-115 could be in-
volved in stabilizing microtubules and rendering them more susceptible to the slowly acting carboxypeptidase (Argarana et al., 1978). This hypothesis is consistent with previous data suggesting that microtubules are stabilized before detyrosination (Webster et al., 1990; Bré et al., 1987).

The specific association of E-MAP-115 with microtubules in vivo was confirmed by immuno-electron microscopy using a preembedding labeling procedure on methanol-fixed HeLa cells (Geuens et al., 1983). ~85% (95) of the colloidal gold particles were within 50 nm of microtubules (arrowheads in Fig. 2, A and B); the remaining particles were associated with unidentified structures. No E-MAP-115 was detected on intermediate or actin filament bundles.

The association of E-MAP-115 with microtubules in HeLa cells was further analyzed using microtubule-active drugs. Prolonged treatment of HeLa cells with nocodazole induces depolymerization of most microtubules and concomitant dispersal and solubilization of E-MAP-115 (Fig. 3, A and B); preextraction of these cells with detergent before fixation results in the disappearance of soluble E-MAP-115 and tubulin and reveals remaining clusters of randomly nucleated microtubules which always contain E-MAP-115 (Fig. 3, C and D). Interestingly, it appears that the number of microtubules resistant to treatment of cells with nocodazole to depolymerize microtubules correlate with the amount of E-MAP-115 expressed in the cells (Vero < HeLa < Caco-2, MDCK; not shown).

In nocodazole wash-out experiments, E-MAP-115 initially labeled the regrowing microtubules along their length (not shown). This observation suggests that E-MAP-115 is present at non-saturating levels in HeLa cells since it labels microtubules uniformly when they are reduced in number and total length. This is supported by the observation that E-MAP-115 cannot be detected in HeLa cytosol by immuno-

Comparison of E-MAP-115 with Other Microtubule-binding Proteins

The microtubule-binding characteristics and biochemical properties of E-MAP-115 were investigated to compare it with previously described proteins of similar molecular weight. HeLa cytosol was incubated with taxol to polymerize endogenous tubulin in the presence of either ATP, GTP, AMP-PNP, or an ATP-depleting system, and the microtu-
Figure 3. Effects of microtubule-active drugs on the distribution of E-MAP-115. HeLa cells were incubated with 10 μM nocodazole for 90 min at 37°C (A–D), or with 10 μM vinblastine for 3 h at 37°C (E and F). Cells were fixed in methanol (A, B, E, and F) or in methanol after detergent preextraction (C and D). Double immunofluorescence labeling was performed with mAb D9Cl against E-MAP-115 (A, C, and E) and a rabbit-anti-tubulin antiserum (B, D, and F). The few stable remaining microtubules in nocodazole- and the tubulin paracrystals in vinblastine-treated cells are heavily labeled for E-MAP-115. Bar, 20 μM.
bules were sedimented through sucrose. E-MAP-115 cosediments with microtubules under all these conditions, showing that its binding to microtubules is nucleotide-insensitive (Fig. 4). This indicates that E-MAP-115 is distinct from kinesin (120 kD; Brady, 1985; Vale et al., 1985) and dynamin (100 kD; Shpetner and Vallee, 1989), proteins of similar molecular weight. E-MAP-115 appears to interact tightly with microtubules since concentrations of >0.5 M NaCl had to be used to elute it, whereas MAP4 is completely eluted at <0.5 M NaCl (not shown). When HeLa cytosol was incubated with nocodazole to prevent tubulin polymerization several proteins (including actin) sedimented through the sucrose cushion, but E-MAP-115 did not, indicating that its sedimentation is microtubule-dependent.

To further compare the sedimentation properties of these microtubule-binding proteins, HeLa cytosol was sedimented through a sucrose gradient and the fractions were analyzed by immunoblotting (not shown). E-MAP-115 (and MAP4,
CLIP-170, and tubulin; not shown) sediments at ~6 S, clearly different from the 125-kD HeLa MAP (14 S; Bulinski and Borisy, 1980a) and the mitotic spindle-associated protein doublet of ~110 kD (11 S; Selito and Kuriyama, 1988; 100 and 115 kD, Nislow et al., 1990), and kinesin (9.5 S; Bloom et al., 1988). These results indicate that E-MAP-115 differs in molecular weight and sedimentation properties from the microtubule-binding proteins described so far. Furthermore, E-MAP-115 is heat-denatured under conditions that leave members of the MAP4 family apparently intact (Kotani et al., 1988) (not shown).

**E-MAP-115 Is Predominantly Expressed in Cells of Epithelial Origin**

Since E-MAP-115 appeared more abundant in HeLa epithelial cells compared to fibroblasts, its presence and distribution was studied in different cell lines of epithelial and fibroblastic origin to obtain more information on the potential function of this protein. By immunoblotting (Fig. 5), E-MAP-115 is detected in the human (HeLa, Caco-2) and canine (MDCK) epithelial cells, but not in primary human skin fibroblasts, monkey Vero cells, and A72 dog fibroblasts. E-MAP-115 could not be detected in porcine brain extract (not shown).

Antibodies against E-MAP-115 brightly stain microtubules in Caco-2 (Fig. 6, A and C) and MDCK cells (not shown). Although human and dog fibroblasts appear negative for E-MAP-115 by immunoblotting (Fig. 5, lanes 3 and 5), weak labeling (threelfold longer exposure of film compared to epithelial cells) is detected on a few microtubules by immunofluorescence (Fig. 1 C). Furthermore, no significant labeling for E-MAP-115 was found in rat hippocampal neurons (not shown). When Caco-2 and MDCK cells start to polarize upon cell-cell contact formation, they detach from the substrate and form blisters (Fig. 6, C and D). There is a strong increase in labeling for E-MAP-115 in these polarized cells, suggesting that E-MAP-115 expression levels increase with cell polarization (Fig. 6 C). In contrast, staining for tubulin in these cells is only slightly brighter compared to isolated cells, presumably because the cells are thicker (Fig. 6 D). This again demonstrates a correlation between stabilized microtubules (abundant in polarized cells) and the presence of E-MAP-115.

**cDNA Cloning of E-MAP-115**

A family of 19 overlapping clones (encoding bases 576–2,840 of the final cDNA for E-MAP-115) was obtained in the first screening of a HeLa cell cDNA expression library with antisera against HeLa microtubule-binding proteins. The missing 5' cDNA was obtained by further screenings of the same and another cDNA library with restriction fragments from the 5' ends of the clones obtained initially (143 clones were analyzed and partially or fully sequenced). The longest sequence obtained by the overlap of the two most extreme clones is shown in Fig. 7, A and B. No stop codon has been found at the 5' end of the longest ORF (bases 1–2,347). However, the sequence surrounding the first ATG in the ORF, AGCACCATGG, conforms well with the consensus sequence, GCC(A/G)CCATGG, for eukaryotic translation initiation (Kozak, 1984). For this reason, and because among the many clones analyzed none was found to extend further in the 5' direction, we expected that this cDNA encoded the full-size protein. In vitro transcription and translation of clone 49 (bases 69–3,120) generated a polypeptide of Mr 115,000 (the major high molecular weight translation product) which comigrated with immunoprecipitated cellular E-MAP-115 (Fig. 7 C), suggesting that, indeed, this cDNA codes for the full-size protein. The protein with its first methionine encoded by the first in frame ATG codon has 749 residues and a calculated molecular weight of 84,051. The reason for the anomalous migration of E-MAP-115 on SDS-PAGE (apparent molecular weight of 115,000) is unclear so
A Novel MAP Predominantly Expressed in Epithelial Cells

Figure 7. cDNA sequencing of E-MAP-115. (A) Schematic representation of the E-MAP-115 cDNA, constructed by the overlap of clones pII/4 and pII/49, showing the restriction sites used and the open reading frame coding for E-MAP-115 (boxed region). N, NheI; S, Sad; H, HpaI; X, XbaI; E, EcoRI. (B) Nucleotide and deduced amino acid sequence of E-MAP-115. The stop codon is marked with an asterisk. The PAPA-region, rich in alanine and proline, is boxed. The two putative 18-amino acid repeats are indicated with superscript dashed lines. Underlined amino acids correspond to peptides used to raise antibodies. These sequence data are available from EMBL/GenBank/DDBJ under accession number X73882. (C) Comparison of the in vitro translation product of clone pII/49 with cellular E-MAP-115. [35S]Methionine-labeled E-MAP-115, translated in vitro using the cDNA clone pII/49 (1) or from HeLa cells (2), was immunoprecipitated with mAb D9C1 and separated by SDS-PAGE. The autoradiogram shows that both proteins migrate with identical apparent molecular weights. Molecular weights are indicated (×10^5).

Additional evidence supports the conclusion that this cDNA codes for full-size E-MAP-115 and that the first ATG initiates translation. The size of E-MAP-115 mRNA is ~3.5 kb (not shown), ~400 bases longer than the 3.120 bases of the cloned cDNA. No poly(A) tail is present in the cDNA sequence suggesting that a 3' extension is lacking. Furthermore, if the start methionine were further upstream to the one identified here, the polypeptide would increase in size by at least 33 aa, giving a difference in molecular weight between the in vitro-translated and the HeLa protein which is abolished by preincubating the sera with the corresponding peptides. Staining of cells with affinity-purified rabbit antibodies raising against one of the peptides (aa 667–682) far. A similar behavior has, however, also been observed for tau (Lee et al., 1988), MAP2 (Lewis et al., 1988), and MAP4 (West et al., 1991).

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Polyclonal antibodies were raised against four peptides deduced from the cDNA sequence (Fig. 7 B, underlined); all react by immunoblotting with E-MAP-115. This reaction is abolished by preincubating the sera with the corresponding peptides. Staining of cells with affinity-purified rabbit antibodies raised against one of the peptides (aa 667–682)
A) Polypeptide

B) Polypeptide

Figure 8. Domain structure and charge distribution prediction of E-MAP-115 and deletion mutants. (A) The secondary structure predictions and sequence analysis suggest that E-MAP-115 can be divided into six main domains (top). The pI and percentage of charged amino acids were calculated for each of these domains. (B) Full-size and mutant E-MAP-115 constructs (names of constructs are indicated on the left) used to localize its microtubule-binding site.

gives the same microtubule pattern as the mAb (recognizing a different epitope, localized between aa 427 and 550) (not shown). These results confirm that the ORF used to predict the amino acid sequence is correct.

Sequence Analysis and Comparison

Searching the EMBL and SWISSPROT data libraries with the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) did not reveal significant sequence similarities between E-MAP-115 and the proteins present in these data banks. Pairwise comparisons were also performed to search for similarities with already characterized MAPs using the sensitive homology search matrices (Argos, 1987). This analysis established that tau (Lee et al., 1988), MAPIB (Noble et al., 1989), MAP2 (Lewis et al., 1988), MAP-U (Aizawa et al., 1990), 205 kD Drosophila MAP (Irminger-Finger et al., 1990), kinesin heavy chain from squid (Kozik et al., 1990), CLIP-170 (Pierre et al., 1992), and dynamin (Obar et al., 1990) share no homology with E-MAP-115.

The secondary structure and charge display of E-MAP-115 were predicted using the program PeptideStructure (not shown). E-MAP-115 can be divided into six main regions according to this analysis (Fig. 8 A): the nearly neutral (pI 7.7) NH2-terminal 59 amino acids, a basic (pI 10.9) predicted α-helical region (aa 60-149) which is highly charged (66% charged residues), a poorly structured basic (pI 11.6) region (aa 150-416), an alanine/proline-rich region (PAPA region, aa 417-457, with 36.6% A, 34.1% P) which is acidic (pI 3.7), a second highly charged (65% charged aa) predicted α-helical region (aa 477-619) which is nearly neutral (pI 7.6), and the acidic (pI 4.1) COOH terminus (aa 620-749).

Table I. Interaction of E-MAP-115 and Derived Mutants with Microtubules

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<th>Polypeptide</th>
<th>Deletions (aa)</th>
<th>MT binding</th>
<th>MT stabilization</th>
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<tr>
<td>P1</td>
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<td>Mb</td>
<td>81-426</td>
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Binding of E-MAP-115 polypeptides to microtubules (MTs) was analyzed in vitro using a MT-binding assay (see also Fig. 8) and in vivo by transfection of Vero cells (see also Figs. 9 and 10). MT stabilization was tested by treating transfected cells with 10 μM nocodazole for 1 h (for details see Materials and Methods). For the in vitro binding assays, "+" indicates 100%, "±" ~50%, and "-" no binding; nd, not determined.
microtubules. This result is consistent with the notion that MAPs interact with microtubules via their basic domains. We further analyzed the role of this region in binding of E-MAP-115 to microtubules by constructing mutant Mal with amino acids 77-228 deleted. This polypeptide partially binds to microtubules in vitro (~50%), whereas Mal2 (Mal lacking aa 265 onward) does not. Mal transfected into Vero cells (details see below), however, does not bind to microtubules in vivo (Fig. 11, Table I). We assume that microtubule binding of mutant polypeptides expressed in Vero cells better reflects its native properties, than when assayed in vitro, where a large excess of tubulin polymer over in vitro translated protein is used. To test whether the other highly charged region (pI 7.6) with a predicted α-helix interacts with microtubules, polypeptide Mb lacking residues 81-426 (complete basic part of E-MAP-115) was constructed. This polypeptide clearly does not interact with microtubules, neither in vitro (Fig. 9) nor in vivo (not shown). We concluded therefore that a microtubule-binding site of E-MAP-115 is in the NH2-terminal basic domain containing the highly charged region with predicted α-helical structure. We cannot exclude at this stage, however, that additional (weak) microtubule-binding site(s) are present in other regions of E-MAP-115. Obviously, mutation of E-MAP-115 may result in (partial) denaturation of the corresponding polypeptide and thus “inactivate” functional domains.

**Microtubules in Cells Transfected with E-MAP-115 Are Stable to Nocodazole Treatment**

Since E-MAP-115 could not be detected in Vero fibroblasts, we used these cells for transfection experiments with (mutant) E-MAP-115 to further characterize its microtubule-binding domain and to study its effect on microtubule organization in vivo. At low expression of transfected polypeptides (PI, P3), its distribution is very similar to the pattern of endogenous E-MAP-115 in HeLa cells; microtubules are positive predominantly near the nucleus and staining is weaker towards their peripheral ends (Fig. 10.4). In cells expressing high levels of E-MAP-115, all the microtubules are positive for the protein along their entire length and a cytosolic pool of soluble protein appears (Fig. 10 C). Saturation of binding of E-MAP-115 to microtubules impairs immunolabeling of tubulin (Fig. 10 D). Microtubules appear bundled to some extent and no longer predominantly centrosome nucleated. Microtubule bundling has also been observed with expression of MAP2 and tau in nonneuronal cells (Lewis et al., 1989; Lee and Rook, 1992; Kanai et al., 1992) and it has been proposed that bundling is the general result of microtubule stabilization (Lee and Brandt, 1992).

Since binding of E-MAP-115 appears to correlate with stabilized microtubules, we investigated the possibility that E-MAP-115 stabilizes microtubules by treatment of transfected Vero cells with nocodazole (Figs. 10 and 11; Table I). Nocodazole completely depolymerizes interphase microtubules in the nontransfected cells, whereas in cells expressing intact E-MAP-115 (PI, Fig. 10), or the microtubule-binding site of E-MAP-115 (P3, Fig. 11) numerous (individual or bundled) microtubules remain which are always positive for E-MAP-115.

Incubation of epithelial cells with nocodazole is alone not sufficient to depolymerize all microtubules; additional cold-treatment is necessary (Bacallao et al., 1989). Transfected cells were therefore, in parallel experiments, incubated at 0°C to depolymerize microtubules. No significant difference in microtubule stability could be detected in the transfected and non-transfected cells (not shown), indicating that E-MAP-115 stabilizes microtubules to nocodazole but not to cold treatment. E-MAP-115 is therefore unlikely to be related to the STOPs (stable tubulin only polypeptides) which make microtubules resistant to cold, Ca2+, and microtubule active drugs (Job et al., 1987).

**Discussion**

We have characterized E-MAP-115, a novel microtubule-associated protein, at the molecular level. It is distinct from previously described microtubule-interacting proteins and has no homology to any other protein in the data banks. The organization of the charged regions of E-MAP-115 is reminiscent of, yet inverse to, that of several other MAPs. The NH2-terminal half of the protein has a high pI and is separated from the rather acidic COOH-terminal half by a...
Figure 10. Transfection of E-MAP-115 into Vero cells stabilizes microtubules to depolymerization with nocodazole. Vero cells were transfected with cDNA coding for full-length E-MAP-115. After 20 h, cells were either directly fixed in methanol (A-D) or first treated with 10 μM nocodazole for 1 h to depolymerize the microtubules (E and F) and double labeled for E-MAP-115 with mAb D9C1 (A, C, and E) and tubulin with a rabbit polyclonal antiserum (B, D, and F). Nontransfected Vero cells are negative for E-MAP-115. At low expression levels (A and B), E-MAP-115 is found on microtubules near the nucleus, similar to what is observed for endogenous E-MAP-115 in HeLa cells. At higher levels of expression (C and D), E-MAP-115 labels the whole length of all microtubules (accessibility of antibodies to tubulin appears reduced in these cells). Single or bundled microtubules in Vero cells expressing E-MAP-115 are resistant to depolymerization with nocodazole (E and F). Bar, 20 μm.

stretch of amino acids rich in proline and alanine (PAPA-region). MAP2 (Lewis et al., 1988), the proteins belonging to the MAP4 family (Aizawa et al., 1990; West et al., 1991; Chapin and Bulinski, 1991), tau (Lee et al., 1988), and Drosophila 205 kD MAP (Irminger-Finger et al., 1990) have acidic NH₂-terminal and basic COOH-terminal regions; MAP2, tau, and MAP4 also have a proline rich region upstream of the basic microtubule-binding site. Proline-rich regions are predicted to be poorly structured and may correspond to flexible regions separating functionally different domains.

The microtubule-binding site of E-MAP-115 has been localized to the positively charged NH₂-terminal domain. It contains the first highly charged region with predicted α-helical structure. Most microtubule-binding sites characterized so far are confined to positively charged regions (e.g., MAP1B [Noble et al., 1989], MAP2/tau [Lewis et al., 1988], MAP4 [Aizawa et al., 1990], CLIP-170 [Pierre et al., 1992], with the exception of MAP1A [Hemphill et al., 1992]). The microtubule-binding site of E-MAP-115 shows no homology to any of the previously characterized sites of other microtubule-interactive proteins including the microtubule-based motor proteins. Interestingly, E-MAP-115 has two highly charged predicted α-helical domains. These α-helices show resemblance due to their high contents of charged amino acids. They contain several clusters of charged amino acids reminiscent of the KKE motif which is involved in binding of MAP1B (Noble et al., 1989) and presumably MAP1A (Langkopf et al., 1992) to microtubules. Overall, however, the charged amino acids in the predicted
α-helices of E-MAP-115 appear to be randomly arranged and no clear motif can be identified. Both predicted α-helical domains begin with homologous stretches of 18 amino acids. Although identical in size to the MAP2/tau repeats which are involved in microtubule-binding (Lewis et al., 1988; Lee et al., 1988), we could not detect significant similarities between these sequences. The basic NH2 terminus, including the predicted α-helix, contains the microtubule-binding site, while the second is nearly neutral and appears not to interact with microtubules. The physiological role of this second charged region is unclear at the moment. It may be speculated, however, that these two domains may interact with each other upon posttranslational modifications; such an interaction might regulate the microtubule-binding and stabilization properties of E-MAP-115. In fact these two α-helical domains are connected via a putative hinge in the PAPA-region.

Transient overexpression of E-MAP-115 (and its microtubule-binding site) in Vero cells, which lack significant levels of this protein, leads to stabilization of microtubules and the formation of noncentrosomal microtubules. In addition, its localization and expression in tissue culture cells correlates with the presence of stable microtubules. In all cells analyzed, E-MAP-115 is most abundant on microtubule segments proximal to the MTOC. These domains are more stable than the peripheral plus ends, which show alternating phases of growth or rapid shrinkage (Soltys and Borisy, 1985). Expression of E-MAP-115 is significantly increased in epithelial cells, where it has been shown that microtubules become stabilized (in contrast to fibroblasts) upon establishment of cell-cell junctions and polarization (Pepperkok et al., 1990; Wadsworth and McGrail, 1990; Bré et al., 1990). This stabilization could occur by the formation of stable segments which subsequently serve as seeds for growth of longer (non-centrosomal) microtubules. E-MAP-115 could indeed be directly involved in the stabilization and reorganization of microtubules.

Several mechanisms may lead to the (local) stabilization of interphase microtubules; their dynamic ends may be "capped" by interaction with other cytoplasmic structures (e.g., the MTOC or domains at the plasma membrane; Kirschner and Mitchison, 1986; Mogensen et al., 1989), and MAPs may laterally associate with the tubulin polymer. It is unlikely that subclasses of microtubules with different stabilities arise from polymerization of different tubulin iso- types (Lewis et al., 1987; Lopata and Cleveland, 1987; for review see Murphy, 1991). Posttranslational modifications of tubulin correlate with microtubule stability but are rather the result than the cause of stability (Webster et al., 1990; Bré et al., 1987). This is consistent with our observation that microtubules with detyrosinated tubulin are usually positive for E-MAP-115, but not vice versa, indicating that E-MAP-115 associates with microtubules before modification of the tubulin subunits. Transient interactions with cytoplasmic membrane domains, for example, may initially render subsets of microtubules more stable and thus favor association of MAPs. Subsequent stabilization by MAPs will be most specific if these proteins are present at subsaturating levels. Several lines of evidence suggest that E-MAP-115 is indeed nonsaturating with respect to polymerized tubulin in HeLa cells and fibroblasts. On the one hand, E-MAP-115 is associated with subdomains of microtubules, and labeling is not continuous along a single microtubule. On the other hand, E-MAP-115 labels microtubules uniformly when they are reduced in number (by drug treatment). Furthermore, since no E-MAP-115 can be detected by immunoblotting in the supernatant after sedimentation of taxol-polymerized HeLa microtubules, it is unlikely that a significant pool of soluble E-MAP-115 exists. Finally, E-MAP-115 appears to have high affinity for polymerized tubulin. Exchange and redistribution of the MAP to newly polymerized, dynamic microtubules will thus be slow, giving rise to the establishment of subdomains or subsets of microtubules. A higher ratio of the microtubule-stabilizing MAPs to tubulin should result in an increased number of stabilized (non-centrosomal) microtubules. Whether higher expression of E-MAP-115 in epithelial cells is the trigger for microtubule stabilization or a consequence of cellular polarization remains to be investigated.

The physiological role of a putative stabilization of micro-
microtubules by E-MAP-115 is unclear. Subsets of few, apparently stabilized microtubules have been implicated in the movement of intracellular organelles (Matteoni and Kreis, 1987; Ho et al., 1988; Miller et al., 1987). Interestingly, E-MAP-115 is associated with only few microtubules in fibroblasts. Whether organelle movement occurs preferentially along these microtubules remains to be shown. Stabilization of microtubules by E-MAP-115 may also lead to rearrangement of microtubules in polarizing epithelial cells, from a preferentially centrosome-nucleated pattern in sparse cultures to a predominantly noncentrosome-nucleated organization in polarized cells (Bré et al., 1990). More experiments, including analysis of the distribution of E-MAP-115 in tissues and during development, will be necessary to understand the precise role of E-MAP-115 in the organization of the cytoplasm. Our data so far suggest that E-MAP-115 is a novel microtubule-associated protein, whose expression may be involved in regulating microtubule stability.

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