Characterization of the Microtubule-binding Domain of Microtubule-associated Protein 1A and Its Effects on Microtubule Dynamics*

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To determine how MAP1a interacts with microtubules we expressed several 6myc-tagged MAP1a fragments in P19 EC and HeLa cells. Confocal immunofluorescence microscopy showed that the fragment consisting of amino acids (aa) 1–281 of MAP1a did not bind while the fragment consisting of aa 1–630 did, indicating that the region of MAP1a between aa 281 and 630 contains a microtubule-binding domain. Deletion of the basic repeats from aa 336–540 did not result in loss of microtubule binding, suggesting that the regions flanking the basic repeats can bind MAP1a to microtubules. These observations were confirmed using an in vitro microtubule binding assay. The levels of acetylation and detyrosination of polymerized microtubules were assessed by quantitative dot blotting in cells expressing MAP1a fragments or MAP2c. Compared with untransfected cells, the polymerized tubulin in cells expressing full-length MAP1a was more acetylated and detyrosinated, but these increases were smaller than those seen in cells expressing MAP2c. Consistent with this, the microtubules in MAP2c expressing cells were more resistant to colchicine than those in cells overexpressing MAP1a. These data implicate aa 281–336 and/or 540–630 of MAP1a in microtubule binding and suggest that MAP1a is less able to stabilize microtubules than MAP2c.

In neurons, microtubules become increasingly resistant to microtubule depolymerizing drugs (1), and less dynamic (2) with time of differentiation. This increasing stability of microtubules is believed to be necessary for neuronal morphogenesis. One of the mechanisms for increasing microtubule stability is the interaction of microtubules with microtubule-associated proteins (MAPs).

MAP1a and MAP1b are structurally related MAPs which may play complementary roles in regulating microtubule dynamics during neuronal development (3, 4). Inhibition of MAP1b expression in cultured neurons using antisense oligodeoxynucleotides leads to the loss of neurite outgrowth (5). Analyses of MAP1b knockout transgenic mice have demonstrated that MAP1b is involved in normal neuronal development (6, 7). In adult brain, MAP1b remains only in regions where growth and plasticity still occur (8, 9), suggesting it also plays a role in neuronal growth in the adult. The function of MAP1a is less clear. The pattern of MAP1a expression in developing brain suggests that it plays a role in regulating the stability of the neuronal cytoskeleton and, consequently, in the transition of growing neurons to the mature, static state (3).

In this study, we have characterized a microtubule-binding domain of MAP1a by expressing several different 6myc-tagged fragments of MAP1a in undifferentiated P19 embryonal carcinoma (EC) cells and in HeLa cells. The effects of MAP1a and MAP2c on microtubule dynamics were assessed by measuring the acetylation, detyrosination, and resistance to colchicine-induced depolymerization. Our results show that MAP1a contains a microtubule-binding domain similar to that of MAP1b and that MAP1a increases microtubule stability, but to a lesser extent than MAP2c.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**

Three overlapping cDNA clones spanning the entire mRNA for MAP1a (4) were used to make all expression constructs (see Fig. 1). pKJ1ΔF-6myc and pPOP (a gift from Dr. M. McBurney, University of Ottawa) are pUC19-based vectors containing the constitutively active mouse phosphoglycerate kinase (PGK) promoter. PKJ1ΔF-6myc drives the expression of 6 repeats of a 9-amino acid (aa) epitope from the human c-MYC protein. These vectors were used to express MAP1a fragments epitope tagged at the N terminus. When necessary, sequencing was performed to ensure that all MAP1a fragments were expressed in-frame with the 6myc tag, confirm ligations, and determine the location of in-frame stop codons in the cDNA. All sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer). All reactions were run on an ABI model 373A automated sequencer. PGK-MAP2cmyc was constructed by C. Addison in our laboratory.

PGK-6myc—This is the unmodified pKJ1ΔF-6myc vector. It ex-

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1 F-6myc vector. It ex-

1 The abbreviations used are: MAP, microtubule-associated protein; aa, amino acid; EC, embryonal carcinoma; MAB, microtubule assembly buffer; PGK, phosphoglycerate kinase; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; mAb, monoclonal antibody; PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; 6myc, six repeats of 9-amino acid epitope from human c-MYC.
presses the 6myc tag followed by a 25-aa tail (NSCPGDPLVLRPPRRYSSDDPCRN).

PGK-6mycN1a—A Ncol-HindIII fragment from clone p19a was blunt-end ligated into SmaI-SacI cut pKJ1ΔF-6myc. This vector expresses the 6myc tag followed by 5-aa (NSCP), followed by aa 1–2016 of MAP1a, and a 2-aa tail (IL).

PGK-6mycN1a-2—A Ncol-BamHI fragment from PGK-6mycN1a (see below) was blunt-ended and religated. This vector expresses the 6myc tag, the NSCSP linker, aa 1–630 of MAP1a followed by a 6-aa tail (TDPCRN).

PGK-6mycN1a-3—A partial Ncol-BamHI fragment (7800 bp) from PGK-6mycN1a-4 (see below) was blunt-ended and religated. The expressed protein contains the 6myc tag, a 7-aa linker (NSREFLH), aa 1–1310 of MAP1a, and a 2-aa tail (IL).

PGK-6mycN1a-4—A ClaI-BamHI fragment from PGK-6myc1a (see below) was blunt-ended ligated into Smal cut pPOP. The expressed protein contains the 6myc tag, a 7-aa linker (NSREFLH), aa 1–2016 of MAP1a, and a 14-aa tail (RGSSRVDLQLFMIY).

PGK-6mycN1a—A Ncol-EcoRI fragment from clone p19a was blunt-end ligated into SmaI-HindIII cut pKJ1ΔF-6myc. A partial ApaI fragment (1595 bp) from clone p14 was then ligated into this vector at the ApaI site. Into this a 6070-bp EcoRI-EcoRV fragment from clone p19 was blunt-end ligated into the Eco site. Finally a short oligonucleotide containing a Smal site (5‘-AATTCTCCGGG-3‘, New England Biolabs) was inserted into the EcoRI site between the 6myc tag and the MAP1a cDNA to bring the cDNA for MAP1a in-frame with the 6myc tag. The expressed protein contains the 6myc tag, a 7-aa linker (NSREFLH), and the full-length cDNA for MAP1a. The predicted molecular weight for the tagged protein is based on the predicted molecular weight for the MAP1a heavy chain as reported in Ref. 4.

Expression vectors were introduced into P19 EC and HeLa cells by calcium phosphate-mediated transfection (12). For protein extraction, cells were plated onto 60-mm dishes (Corning) at 1.25 × 10^5 cells/dish at 1.25 × 10^5 cells/dish (P19 EC) or 2 × 10^4 cells/dish (HeLa) in 5 ml of medium (see above). For immunofluorescence microscopy, cells were plated onto 18-mm round coverslips (Corning) at 1.25 × 10^4 cells/coverslip (P19 EC) or 2 × 10^4 cells/coverslip (HeLa) in 1 ml of medium. Cells were allowed to settle for 24 h. 40 μg of DNA in 500 μl of 0.25 M CaCl₂ was then gently mixed with 500 μl of 2 × BES buffer (50 mM BES, pH 6.86, 280 mM NaCl, 1.5 mM Na₂HPO₄), followed by 500 μl of 2 × BES buffer (50 mM BES, pH 6.86, 280 mM NaCl, 1.5 mM Na₂HPO₄) to a final volume of 1 ml and allowed to sit for 20 min. The solution of calcium phosphate-DNA precipitate was then gently added to the cells (1 ml for dishes, 200 μl for coverslips) without removing the medium and allowed to sit for 8 h in the incubator. The DNA/media solution was then aspirated and replaced with fresh medium. Cells were incubated a further 48 h before processing.

Primary Antibodies

Anti-MAP1a mouse monoclonal IgG (clone 1A-1, a gift from Dr. R. Vallee, Ref. 13) was diluted 1:1000 for Western blotting and 1:500 for immunofluorescence microscopy. Anti-α-tubulin rat monoclonal IgG

MAP1a cDNA to bring the cDNA for MAP1a in-frame with the 6myc tag, a 7-aa linker (NSREFLH), and the full-length cDNA for MAP1a. The predicted molecular weight for the tagged protein is based on the predicted molecular weight for the MAP1a heavy chain as reported in Ref. 4.
(clone YOL 1/34, from Serotech) was diluted 1:15 for immunofluorescence microscopy. Anti-c-MYC mouse monoclonal IgG (clone 9E10, a gift from Dr. C. Garner) was used at 1:2 for Western blotting and quantitative dot blotting. Anti-acetylated α-tubulin monoclonal IgG (clone 6–11B-1 (Sigma)) was diluted 1:1000 for quantitative dot blotting. Polyclonal anti-detyrosinated α-tubulin (anti-E, Ref. 14) was provided by Dr. T. MacRae and diluted 1:250 for quantitative dot blotting.

**Immunofluorescence Microscopy**

Cells plated on glass coverslips were briefly rinsed in PEM (80 mM PIPES, pH 6.9, 5 mM EGTA, 1 mM MgCl₂, all from Sigma) and fixed at room temperature by two different protocols.

**Precipitation** (15)—1 h incubation in Zamboni’s fixative (14% picric acid (Fisher), 4% paraformaldehyde (J. B. EM Services Inc.) in 0.5 M Na₂HPO₄, 0.5 M NaH₂PO₄, pH 7.1) followed by a 3 × 5-min phosphate-buffered saline (PBS, 130 mM NaCl, 5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) wash and 5-min extraction with 0.5% Triton X-100 (Pierce) in PBS.

**Extraction/Fixation** (16)—2-min pre-extraction with 0.2% Triton X-100 in PEM (80 mM PIPES, pH 6.9, 10 mM EGTA, 5 mM MgSO₄) followed by a 10-min fixation with 3.7% formaldehyde (BDH), 0.25% glutaraldehyde (J. B. EM Services Inc.), and 0.5% Triton X-100 in PEM, followed by a 3 × 5-min PEM wash and quenching 3 × 5 min with 1 mg/ml sodium borohydride (BDH) in PBS.

Cells prepared by either fixation procedure were again rinsed 3 × 5 min in PBS. All antibody incubations were for 1 h. Protein concentrations were determined using a 1-ml pipetter in the same volume of MAB2 (0.1 M PIPES, pH 6.4, 1 mM EGTA, 1 mM MgCl₂, 4 M glycerol, and 0.1 mM GTP, and 20 mM taxol) for in vitro MAP binding assay. Steps indicated with an asterisk (*) contain extracts from P19 EC cells expressing various MAP1a fragments.

**Protein Extraction**

Whole cell protein for Western blotting was extracted from 100-mm dishes according to Ref. 17, but using higher concentrations of protease inhibitors in the extraction buffer: 40 μM benzamidine HCl (Sigma), 4 mM p-aminomethylbenzenesulfonyl fluoride (Centrichem Inc.), 1 mM 1,10-phenanthroline (Sigma), 40 μg/ml each of aprotonin, peptatin A, and leupeptin (all from Sigma).

To obtain extracts for the in vitro assembly assay, cells were rinsed briefly in cold MAB1 (0.1 M MES, pH 6.4, 2.5 mM EGTA, 0.1 mM EDTA, 0.5 mM MgCl₂, Ref. 18) and then 250–500 μl of extraction buffer (MAB1 + 4 mM PEFA, 1 mM 1,10-phenanthroline, and 40 μg/ml each of aprotonin, peptatin A, and leupeptin) was added. Cells were immediately scraped into an Eppendorf and sonicated 15 s at 94 watts with a Braun sonicator, then immediately spun for 10 min at 10,000 rpm and 4 °C. The supernatant was removed and stored at −80 °C.

To prepare extracts for analysis of α-tubulin modifications, cells were processed according to Ref. 19. Protein concentrations were determined using the bichinchoninic acid assay (Pierce) using BSA (Pierce) as a standard.

**In Vitro MAP Binding Assay**

Parts of this procedure (see Fig. 2) were derived from the taxol-dependent purification of MAPs described in Ref. 20. 1 ml (approximately 13 mg) of 3 × cycled bovine brain microtubule preparation (21, 22) in MAB2 (0.1 M PIPES, pH 6.4, 1 mM EGTA, 1 mM MgCl₂, 4 mM glycerol, and 0.1 mM GTP) was brought to 1.8 mM GTP and 20 μM taxol, assembled for 30 min and 37 °C, and then spun 15 min at 36,000 × g and 37 °C (18,000 rpm using a Sorval SS-20 rotor). The pellet was gently resuspended using a 1-ml pipette in the same volume of MAB2 (+400 mM NaCl, 1.8 mM GTP, and 20 μM taxol). The suspension was then spun for 15 min at 36,000 × g and 37 °C. The pellet was then resuspended in MAB1 + 1.8 mM GTP and 20 μM taxol, and spun for 15 min at 36,000 × g and 37 °C. The pellet was then resuspended in MAB1 at 37 °C in MAB1.

**Electron Microscopy**

Samples were fixed 1 min at 37 °C in a equal volume of fixative (4% paraformaldehyde, 0.2% glutaraldehyde in MAB1 or MAB2, depending when the sample was taken). 5 μl of the sample was allowed to settle onto parlodion-coated 400 mesh copper grids (J. B. EM Services Inc.) for 1 min. Grids were then washed with 5 drops of Photoform solution (2 drops of Photoform (Kodak) in 100 ml of ddH₂O, followed by 5 drops of ddH₂O and then stained with 4 drops of 1% uranyl acetate. Samples were then examined using a Philips 201 transmission electron microscope. Images were recorded on Kodak Electron Image Film SO-163 and printed on Ilford multigrade III paper.

**SDS-PAGE and Western Blotting**

Equal amounts of protein in sample buffer (23) were placed in a boiling water bath for 5 min, loaded onto 12% polyacrylamide gels and separated using the Bio-Rad minigel apparatus. Proteins were electroblotted onto nitrocellulose in 20% methanol and the blots were rinsed in PBS. Immunodetection of Western blots was performed as follows: block for 1 h in 5% skim milk in PBS, 1 h incubation in primary
antibody diluted in 2% skim milk in PBS, 1 h incubation in biotinylated horse anti-mouse IgG (Vector) diluted 1:1000 in 2% skim milk in PBS, and 1 h of incubation in biotinylated streptavidin horseradish peroxidase (Amersham) diluted 1:5000 in PBS. A 3 × 5-min PBS wash was done between all antibody incubations (with 2% milk added between the primary and secondary). Antibody binding was detected by enhanced chemiluminescence (ECL) (Amersham) using Hyperfilm-ECL (Amersham). All steps were performed at room temperature.

Quantitative Dot Blotting

Protein samples were diluted in PBS and 200 μl/well was passed through nitrocellulose in a 96-well Minifold apparatus (Schleicher & Schuell) which had been pre-wetted with 200 μl/well of PBS. After the entire sample had been passed through the nitrocellulose by a gentle vacuum, an additional 400 μl of PBS/well was passed through the nitrocellulose. The nitrocellulose was then removed from the apparatus, equilibrated in PBS and processed as for Western blotting. The resulting films were scanned at 200 dpi with an 8 bit dynamic range using a Hewlett-Packard 4c scanner. The chemiluminescent signal from each dot in the digitized image was quantified using SigmaGel v1.0 (Jandel Scientific). These values were then imported into Excel 97 (Microsoft). Standard curves for all antibodies were established with bovine brain extract to ensure that signals fell within the linear response of the antibody used.

RESULTS

Expression of MAP1a Fragments in P19 EC and HeLa Cells—All MAP1a fragments used in this study are presented in Fig. 1. To ensure that all fragments were being expressed correctly, they were analyzed by Western blotting using mAb 9E10 (see Fig. 3a, arrows) and HeLa (Fig. 3b, arrows) cells of the fragments displayed mobilities within 5–10 kDa of their predicted molecular masses, except for PGK-6myc1a, which had an apparent mobility of ~360 kDa (compared with its predicted size of 312 kDa). However, this is not unexpected since endogenous MAP1a migrates at ~350 kDa and has a predicted molecular mass of 299 kDa (4). The amounts of individual fragments decreased as the size of the fragments increased. The bands seen in all lanes (P19 and HeLa cells) at 118 and 70 kDa are nonspecific gel artifacts that we have observed with a variety of monoclonal antibodies. In HeLa cell extracts the endogenous human c-MYC was detected by the 9E10 antibody (see Fig. 3b, *). Additional bands present between 60 and 30 kDa are degradation products from exogenously expressed MAP1a fragments.

Detection of MAP1a Fragments with mAb 1A-1—To see if any of the expressed fragments could be detected with mAb 1A-1, Western blots from transfected P19 EC were probed with mAb 9E10 (Fig. 4a) and mAb 1A-1 (Fig. 4b). In all lanes probed with mAb 1A-1, the endogenous MAP1a could be detected. A second band detected in the extract from PGK-6mycN1a-a transfected cells displayed mobility identical to the MAP1a fragment present.

Confocal immunofluorescence microscopy was used to detect MAP1a in P19 EC and HeLa cells expressing 6myc1a. In all P19 EC cells, a weak diffuse staining was seen (Fig. 5a’) which represents the normal staining pattern of endogenous MAP1a in undifferentiated cells. In P19 EC cells expressing 6myc1a, there was an increase in the signal compared with untransfected cells and microtubule colocalization was easily seen (Fig. 5b’). In HeLa cells no MAP1a was detected, except in 6myc1a-transfected cells in which MAP1a colocalized with microtubules (Fig. 5b’). This shows that the full-length MAP1a was expressed in-frame.

Analysis of 6myc-tagged MAP1a Fragment Binding—To determine which fragments of MAP1a bound to microtubules, transfected P19 EC and HeLa cells were observed by confocal
immunofluorescence microscopy. Cells were fixed by precipitation so that expression of 6myc-tagged MAP1a fragments could be monitored even if they did not bind microtubules. The extraction/fixation method was used to determine if a particular fragment was bound to microtubules.

Cells expressing 6myc prepared by precipitation fixation showed a diffuse 6myc staining in the cytoplasm (Fig. 6, a' and e'). The microtubules in transfected cells showed a normal cytoplasmic interphase organization that appeared identical to that of the untransfected cells in the population (Fig. 6, a and e). Extraction/fixation of cells expressing 6myc showed no 6myc labeling (Fig. 6, b' and f'). Similar results were obtained for cells expressing 6mycN1a-1 (data not shown) showing that the tagged protein did not remain bound to microtubules.

Microtubule organization was unaffected in cells expressing 6mycN1a-2 (Fig. 6, c and g) prepared by precipitation fixation and this fragment showed diffuse cytoplasmic staining, similar to 6myc and 6mycN1a-1 (Fig. 6, c' and g'). However, extraction/fixation of 6mycN1a-2 expressing cells showed the 6myc tag colocalized with microtubules (Fig. 6, d, d', h, and h'). Similar results were obtained for cells expressing 6mycN1a-3, 6mycN1a-4, 6myc1a, and 6mycN1a-2ABR (data not shown) showing that all of these fragments bound to microtubules and that the microtubule distribution was unchanged compared with untransfected cells. These results are summarized in Table I.

In a few P19 EC cells expressing high levels of the 6myc1a, as judged by the intensity of 6myc labeling, process outgrowth was observed (Fig. 7). No process outgrowth was observed in transfected HeLa cells with any of the fragments of MAP1a.

**Taxol Treatment of Transfected P19 EC Cells**—We have previously shown that taxol treatment enhances the detection of low levels of microtubule-bound MAP1a in undifferentiated P19 EC cells by concentrating the microtubule-bound protein (24). To see if the lack of detection of the 6myc tag and 6mycN1a-1 was due to low levels of fragments present after extraction/fixation, transfected P19 EC cells were treated with taxol to induce microtubule bundles.

The microtubules in taxol-treated cells were arrayed in thick bundles running through the cytoplasm (Fig. 8, a and b). Microtubule association of 6myc (Fig. 8c') or 6mycN1a-1 (data not shown) after extraction/fixation of taxol-treated, transfected cells was never observed. As expected, extraction/fixation of cells expressing 6mycN1a-2 showed MYC labeling which colocalized with the microtubule bundles present (Fig. 8, b and b'). Similar results were obtained for 6mycN1a-3, 6mycN1a-4, 6myc1a, and 6mycN1a-2ABR (data not shown).

In Vitro Microtubule Binding of MAP1a Fragments—To confirm the microscopic analysis of MAP fragment binding, an assay was devised to test the binding of MAP fragments to assembled microtubules in vitro (see “Experimental Procedures” and Fig. 2). Immunoblot analysis indicated that tubulin was present throughout the procedure (Fig. 9a) and electron microscopy showed this tubulin was assembled into microtubules (Fig. 9, c, d, and e). MAP1a, although at very high levels after the first assembly step (Fig. 9b, lane 1), was mostly removed by the salt washes (Fig. 9b, lanes 2–7). Some MAP1a still remained bound to microtubules at the end of the procedure.

6myc and 6mycN1a-1 were absent from microtubules following the final sucrose wash while 6mycN1a-2, 6mycN1a-3, and 6mycN1a-2ABR were bound (Fig. 10, lane 14 in all gels). To control for the effects of any cellular proteins normally present, equal volumes of P19 extract were loaded, regardless of the level of exogenous MAP1a fragments present. As 6mycN1a-2ABR was expressed at a much higher level than any of the other fragments, a much higher amount of 6mycN1a-2ABR was added to the microtubules. The 6mycN1a-2ABR in the supernatant (fraction 11) reflects excess 6mycN1a-2ABR left over after saturation of available binding sites on microtubules. 6mycN1a-4 and 6mycN1a were not analyzed due to the very low levels of tagged protein available from culture extracts. As stated above, bands seen at 118 and 70 kDa result from nonspecific gel artifacts. These results are summarized in Table I.

**Colchicine Treatment of Transfected P19 EC Cells**—Transfected P19 EC cells were treated with colchicine to ensure that the pattern of 6myc labeling in cells prepared by extraction/fixation was really due to microtubule association and also to see if the colchicine resistance of microtubules was altered by any of the MAP1a fragments. As a positive control, MAP2c was expressed in P19 EC cells (using PGK-MAP2cmyc, provided by C. Addison). MAP2c is a juvenile form of MAP2 and, like high

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**FIG. 5.** Detection of tubulin (mAb YOL1/34, a and b) and MAP1a (mAb 1A-1, a' and b') in 6myc1a expressing P19 EC (a and a') and HeLa cells (b and b') by confocal double immunofluorescence microscopy. Cells were fixed by the extraction/fixation method. Endogenous MAP1a was detected in P19 cells and 6myc1a expressing cells showed a more intense staining that colocalized with microtubules (a and a'). In HeLa cells, no MAP1a was detected except in 6myc1a expressing cells in which it colocalized with microtubules (b and b'). Scale bar = 20 μm.

**FIG. 6.** Detection of tubulin (YOL 1/34, a–h) and 6-MYC-tagged MAP1a fragments (mAb 9E10, a'–h') in transfected P19 EC (a–d') and HeLa (e–h') cells by confocal double immunofluorescence microscopy. Cells were fixed by precipitation (2 left columns) or by extraction/fixation (2 right columns). 6-MYC and 6mycN1a-1 were present following precipitation (a, a' and e, e') but absent after extraction/fixation (b, b' and f, f'). 6mycN1a-2 was present after precipitation (c, c', g, g', and h, h'). The microtubules in taxol-treated cells were arrayed in thick bundles running through the cytoplasm (Fig. 8, a and b). Microtubule association of 6myc (Fig. 8c') and HeLa (b'). Scale bars = 20 μm.
molecular weight MAP2, can bundle microtubules and render them resistant to drug-induced depolymerization (25).

MAP2cmyc expressing cells showed the presence of thick bundles of microtubules in the cytoplasm (Fig. 11a) and 6myc labeling was found colocalized with these bundles (Fig. 11a). After 30 min of colchicine treatment almost all microtubules were completely depolymerized, except for the MAP2c-bundled microtubules that remained polymerized (Fig. 11b). In contrast, with any of the MAP1a fragments no polymerized microtubules were observed after 30 min of colchicine treatment. Fig. 11c–d shows an example of cells transfected with 6mycN1a-2ΔBR untreated and treated with colchicine for 30 min.

Effect of MAP1a Fragments on α-Tubulin Modifications—The results of the colchicine experiments showed that MAP2c was a stronger microtubule stabilizer than MAP1a, but did not allow us to determine if MAP1a could also alter microtubule stability. As another test of the effect of MAP1a fragments on microtubule dynamics, the extent of α-tubulin acetylation and detyrosination in transfected cultures was determined by quantitative dot blotting. These post-translational modifications of tubulin have been shown to be biochemical markers of neuronal microtubules that have a decreased rate of turnover (26). As a positive control, levels of α-tubulin acetylation and detyrosination were also determined in cells expressing MAP2cmyc.

The relative changes in the levels of acetylation and detyrosination per microgram of polymerized tubulin compared with untransfected cells (control cells) were determined (Fig. 12, a and b). Values are reported for fragments that gave changes in acetylation or detyrosination that were markedly different from changes induced by the 6myc tag alone or 6mycN1a-1, which do not bind to microtubules. There was an increase relative to control cells in acetylation and detyrosination in cells expressing 6myc1a, however, the increases seen with MAP2c were 2-fold higher than with MAP1a. There were marked decreases in acetylation and detyrosination, relative to control cells, in cells expressing 6mycN1a-4 and 6mycN1a-2ΔBR.

We then determined the relative changes in acetylation and detyrosination per unit of 6myc-tagged MAP per μg of polymerized tubulin compared with untransfected cells (Fig. 12, c and d). This allowed us to assess how each MAP fragment affected

### Table I

| MAP1a fragment       | P19 EC cells | HeLa cells | Binds in vitro |
|----------------------|--------------|------------|---------------|
|                      | Present in cytoplasm | Microtubule colocalization | Present in cytoplasm | Microtubule colocalization | Binds in vitro |
| 6myc                 | Yes          | No         | Yes           | No                  | No            |
| 6mycN1a-1            | Yes          | No         | Yes           | No                  | No            |
| 6mycN1a-2            | Yes          | Yes        | Yes           | Yes                 | Yes           |
| 6mycN1a-3            | Yes          | Yes        | Yes           | Yes                 | Yes           |
| 6mycN1a-4            | Yes          | Yes        | Yes           | Yes                 | ND*           |
| 6myc1a               | Yes          | Yes        | Yes           | Yes                 | Yes           |
| 6mycN1a-2ΔBR         | Yes          | Yes        | Yes           | Yes                 | Yes           |

*ND, not determined.

**FIG. 7. Confocal immunofluorescence microscopy of process outgrowth in 6myc1a expressing P19 EC cells.** Cells were fixed by extraction/fxiation. Tubulin was detected using mAb YOL1/34 (a) and 6myc1a was detected using mAb 9E10 (a'). Scale bar = 20 μm.

**FIG. 8. Detection of tubulin (YOL 1/34, a and b) and 6-MYC-tagged MAP1a fragments (mAb 9E10, a'-b') in transfected, taxol-treated P19 EC cells by confocal double immunofluorescence microscopy.** Cells were fixed by extraction/fxiation. 6myc was not detectable after extraction/fxiation (a and a'), but 6mycN1a-2 was co-localized with microtubules (b and b'). Scale bar = 20 μm.

**FIG. 9. In vitro MAP binding assay.** Equal volumes of sample were taken from each step during the protocol (see “Experimental Procedures”) and separated by SDS-PAGE on 12% (a) or 7.5% (b) gels. Immunodetection of tubulin with DM1B (a) or MAP1a with 1A-1 (b) on Western blots shows the prevalence of tubulin and MAP1a during the procedure. Whole cell extracts from P19 EC cells expressing 6myc-tagged MAP1a fragments were added to microtubules at step 10. Samples were negatively stained for transmission electron microscopy at steps 1 (c), 7 (d), and 14 (e) to show the presence of intact microtubules throughout the entire procedure. Scale bar = 100 nm.
acetylation and detyrosination of microtubules on a per molecule basis. This showed that, per molecule, MAP1a and MAP2c caused similar increases in acetylation and detyrosination relative to control cells. 6mycN1a-4 caused marked decreases in both modifications, while 6mycN1a-2ΔBR had almost no effect.

Analysis of the levels of 6myc-tagged fragment bound per mg of polymerized tubulin (Fig. 12e) showed that all fragments which bound microtubules in vitro could be detected associated with microtubules in P19 EC cells. The binding of 6mycN1a-2ΔBR to microtubules was 10-fold higher than for the other MAP1a fragment. In addition, approximately twice as much MAP2c was bound compared with MAP1a.

DISCUSSION

Microtubule Binding of 6myc-tagged MAP1a Fragments—In both P19 EC and HeLa cells the 6myc tag was expressed, but was not detected bound to microtubules even in taxol-treated cells. 6mycN1a-1 also did not bind microtubules, showing that aa 1–281 of MAP1a were not sufficient for microtubule binding. 6mycN1a-2 did bind to microtubules, suggesting that aa 282–630 of MAP1a are involved in microtubule binding. Removal of the basic repeats from 6mycN1a-2, to produce 6mycN1a-2ΔBR, did not prevent its binding to microtubules, indicating the presence of microtubule-binding domain(s) within aa 281–355 or aa 451–630 or both. Langkopf et al. (4) have reported a region of protein similarity flanking the basic repeat domain in MAP1a and MAP1b, and in MAP1b, these flanking domains can bind microtubules in the absence of the basic repeats (10). The larger fragments of MAP1a, 6mycN1a-3, and 6mycN1a-4, and the full-length MAP1a (6myc1a) also bound microtubules. The ability of these fragments to bind microtubules in vitro confirmed the microscopical observations.

Cravchik et al. (27) failed to detect binding of a MAP1a fragment consisting of aa 1–1300 to microtubules in HeLa cells. They also showed that a region near the middle of MAP1a (aa 1307–1606) consisting of self-similar acidic elements could bind microtubules and that this domain induced formation of short, nocodazole-resistant perinuclear microtubules. In contrast, we find that a microtubule-binding domain is located in a fragment consisting of aa 1–1310 of MAP1a. Also, we observed neither a perinuclear distribution of microtubules nor resistance to drug-induced depolymerization in cells expressing MAP1a fragments. It is possible that the NH2-terminal MAP1a fragment used in Ref. 27 was not expressed in the correct translational frame, thus abolishing its microtubule binding activity, or that the fixation procedure used did not preserve its association with microtubules.

The basic repeats of MAP1b alone can bind microtubules (10). One then might hypothesize that the similar domain in MAP1a could also bind to microtubules; we were unable, however, to test this hypothesis in the present study.

Effect of MAP1a Fragments on Colchicine Resistance of Microtubules—Cells expressing MAP2cmyc displayed bundled arrays of microtubules that were resistant to depolymerization by 30 min of colchicine treatment. This is consistent with the strong microtubule bundling and stabilizing activity of MAP2c (25). In contrast, microtubules in cells expressing MAP1a fragments were less resistant to colchicine. This does not preclude a microtubule stabilizing effect of MAP1a, but indicates that the ability of MAP1a to render microtubules resistant to colchicine is small in comparison to MAP2c.

Effect of MAP1a Fragments on Acetylation and Detyrosination—...
The map of MAP1a fragments tested, only 6myc1a showed marked increases in the levels of acetylation and detyrosination per μg of polymerized tubulin relative to control cells. These increases were approximately half those seen in cells expressing MAP2cmyc. This suggests that an increase in the amount of MAP1a bound to microtubules causes these microtubules to turn over more slowly than in untransfected cells, but that these effects are weaker than those seen with MAP2c. On a per molecule basis, MAP1a and MAP2c were almost equivalent in their ability to affect microtubule turnover; however, approximately twice as much MAP2c was bound to microtubules compared with MAP1a. These levels of binding occurred under saturating conditions, since only a fraction of the available MAP expressed was found associated with microtubules (data not shown). Pedrotti et al. (28) have shown that the rates of assembly and disassembly in vitro are 2–3-fold greater for MAP1a than for MAP2, which is in agreement with our in vivo data on α-tubulin modifications. In addition, they observed that more MAP2 could bind to the microtubule surface than MAP1a under saturating conditions. This also is consistent with our in vivo observations on the relative amounts of these two MAPs bound to microtubules.

MAP1a is associated with three light chains (LC1, LC2, and LC3), which associate near the microtubule-binding domain (4, 29) and are thought to alter the ability of MAP1a to bind microtubules and to regulate microtubule dynamics (30, 31). The marked increases in acetylation and detyrosination seen for 6myc1a compared with the smaller MAP1a fragments may be due to the inclusion of the coding sequence for light chain 2 only in 6myc1a. In cells expressing any of the fragments of MAP1a, light chain 2 would not be synthesized, and its absence might reduce the ability of MAP1a to stabilize microtubules.

Both 6mycN1a-2ΔBR and 6mycN1a-4 fragment resulted in decreased acetylation and detyrosination of microtubules relative to control cells. The reasons for this are unclear. The increase in microtubule turnover seen with 6mycN1a-2ΔBR may be attributed to the much higher levels of this fragment bound to microtubules. This could result in the replacement of endogenous MAP1a by 6mycN1a-2ΔBR, which has less microtubule stabilizing ability.

**Regulation of MAP1a Affinity for Microtubules**—As noted above, the amount of 6mycN1a-2ΔBR bound per μg of polymerized tubulin was greater than for any other MAP1a fragment, including 6myc1a. This higher binding of 6mycN1a-2ΔBR was not due solely to the higher expression of this fragment as the proportion of total cellular 6mycN1a-2ΔBR bound to microtubules was also much greater than for all other fragments (data not shown). This suggests that the microtubule-binding regions that flank the basic repeats in MAP1a show higher affinity for microtubules in the absence of the basic repeats. The presence of the basic repeats in the other fragments resulted in “reduced” or normal binding. These observations suggest that the affinity of MAP1a for microtubules may be modulated by the basic repeats. This type of cooperativity has already been demonstrated for tau, in which regions flanking the basic repeats have a strong microtubule binding activity that is modulated by the presence of the basic repeats (32).

**MAP1a Function**—MAP1a expression in developing brain continually increases during development (3, 30) to become one of the predominant MAPs in the adult brain. As this increase in MAP1a is concomitant with an increase in microtubule stability (1, 2) it has been proposed that MAP1a belongs to the group of MAPs that stabilizes microtubules during the maturation of neurons (33).

However, MAP1a is found in regions of the brain where neuronal growth persists in the adult (30, 34). Also, we have previously shown that during the differentiation of P19 EC neurons, MAP1a is found at its highest levels during the growth phase (24). These studies suggest a growth-related function for MAP1a. Our observations of the effects of MAP1a and MAP2 on acetylation, detyrosination, and colchicine resistance of microtubules shows that MAP1a is weaker than MAP2 in reducing microtubule turnover and in stabilizing microtubules to drug-induced depolymerization. We suggest that the role of MAP1a in the growth of neurons is to render microtubules stable enough to support process outgrowth, but still moderately dynamic so growing neurites remain plastic. The
stabilization of microtubules by MAP1a without bundling microtubules may be critical for neuritic plasticity.

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