Stable Expression of Heterologous Microtubule-associated Proteins (MAPs) in Chinese Hamster Ovary Cells: Evidence for Differing Roles of MAPs in Microtubule Organization

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Abstract. To study the effects of microtubule-associated proteins (MAPs) on in vivo microtubule assembly, cDNAs containing the complete coding sequences of a Drosophila 205-kD heat stable MAP, human MAP 4, and human tau were stably transfected into CHO cells. Constitutive expression of the transfected genes was low in most cases and had no obvious effects on the viability of the transfected cell lines. High levels of expression, as judged by Western blots, immunofluorescence, and Northern blots, could be induced by treating cells with sodium butyrate. High levels of MAPs were maintained for at least 24–48 h after removal of the sodium butyrate. Immunofluorescence analysis indicated that all three MAPs bound to cellular microtubules, but only the transfected tau caused a rearrangement of microtubules into bundles. Despite high levels of expression of these exogenous MAPs and the bundling of microtubules in cells expressing tau, transfected cells had normal levels of assembled and unassembled tubulin. With the exception of the tau-induced bundles, microtubules in transfected cells showed the same sensitivity as control cells to microtubule depolymerization by Colcemid. Further, all three MAPs were ineffective in reversing the taxol-dependent phenotype of a CHO mutant cell line. The absence of a quantitative effect of any of these heterologous proteins on the assembly of tubulin suggests that these MAPs may have different roles in vivo from those inferred previously from in vitro experiments.

Microtubules are involved in multiple cellular processes including mitosis, organelle transport, and determination of cell morphology. Many studies have demonstrated that these filaments are highly dynamic both in interphase and mitosis (reviewed in Gelfand and Bershadsky, 1991; McIntosh and Hering, 1991; McIntosh, 1988; Salmon, 1989). A number of mechanisms exist by which this equilibrium might be regulated. One prediction is that the amount of polymer would be dictated by the total tubulin pool, and in mammalian cells, tubulin mRNA stability and tubulin synthesis have been found to be regulated in response to the pool of free subunits (reviewed in Cleveland, 1989). Other factors, however, have also been found to influence the dynamics of individual microtubules. For example, posttranslational modifications such as glutamylation (Edde et al., 1990), detyrosination (Gundersen et al., 1984), and acetylation (Piperno et al., 1987) correlate with increased stability of microtubules. In addition, microtubule-associated proteins (MAPs) have been demonstrated to affect in vitro dynamics (Pryer et al., 1992), and they have been implicated in the stabilization of microtubules in vitro and in vivo (for reviews see Olmsted, 1986; Wiche et al., 1991).

There are two major classes of MAPs: the motor MAPs and the structural MAPs. While the motor MAPs influence events such as organelle transport (reviewed in Bloom, 1992; Schroer and Sheetz, 1991; Vallee and Shpetner, 1990), there is little to suggest that this class of MAPs is important in the regulation of microtubule distribution and maintenance. In contrast, there are several lines of evidence that demonstrate structural MAPs might influence microtubule stability and assembly in vivo as well as in vitro. The two most extensively studied neural MAPs, MAP2 and tau, are both synthesized during neural differentiation (Drubin et al., 1985), and it has been suggested that they are important in maintaining the microtubule array characteristic of neural morphology (reviewed in Matus, 1990). This hypothesis has been supported by experiments in which inhibition of either tau (Caceres and Kosik, 1990) or MAP 2 (Caceres et al., 1992; Dinsmore and Solomon, 1991) expression with antisense constructs suppresses neurite formation. Conversely, transfection analyses have demonstrated that expression of these proteins in non-neural cells causes stabilization and bundling of microtubule arrays (Kanai et al., 1989; Lee and Rook, 1992; Lewis et al.,...
indeed, in some nonneural cells where introduced tau is highly expressed, neurite-like processes with typical neuronal microtubule arrays are formed (Knops et al., 1991). While these experiments provide a strong argument that neural MAPs influence overall microtubule stability and organization, less dramatic effects have been reported for transfection of a ubiquitous structural MAP, MAP lb (Noble et al., 1989; Takemura et al., 1992). MAP 2 and tau share a similar microtubule-binding domain consisting of 18-mer repeats (Lewis et al., 1988), whereas the more ubiquitous MAP lb has a unique binding domain (Noble et al., 1989). Therefore, one postulate is that the ability of these different structural MAPs to affect microtubule equilibrium or stability might be influenced primarily by the site that binds to microtubules.

Recently, two additional structural MAPs have been cloned. MAP 4 is a widely distributed thermostable protein of approximately 200 kD (reviewed in Olmsted, 1991). A portion of the microtubule-binding domain of MAP 4 shares the 18-mer repeats characteristic of this domain in MAP 2 and tau, but other portions of this protein share no sequence similarities to either of the neuronal-specific MAPs (Aizawa et al., 1990; Chapin and Bulinski, 1991; West et al., 1991). The Drosophila 205-kD protein is another thermostable structural MAP (Goldstein et al., 1986). Although originally postulated to be a homologue of MAP 4, it possesses a unique microtubule-binding domain (Irminger-Finger et al., 1990), and sequence comparisons indicate that these two proteins only share general biochemical characteristics of charge distribution and thermostability. In the experiments described here, stably transfected cell lines expressing MAP 4, the Drosophila 205-kD MAP, or tau were generated to compare the influence of these MAPs on the expression and organization of tubulin, and to assess whether these MAPs affect the distribution of tubulin monomer–polymer pools.

Materials and Methods

Construction and Transfection of Vectors Containing Genes that Encode MAPs

All constructs were cloned into the plasmid pRC/CMV (Invitrogen, San Diego, CA), a vector carrying a neo gene and the promoter from cytomegalovirus (CMV), using procedures described in (Sambrook et al., 1989).

The plasmid bearing a full-length copy of the Drosophila 205-kD MAP (pRC205) was constructed in two steps. First, the 4.3-kb KpnI-SspI fragment containing the full coding sequence was subcloned from plasmid pBluescript II SK (Stratagene, La Jolla, CA) to generate plasmid pBSK 205. In the second step, pBSK 205 was digested with KpnI and XbaI. The resulting 1.2-kb fragment was gel purified and ligated to the HindIII (filled in)-XbaI sites of pRC/CMV.

Construction of the plasmid containing the full length human MAP 4 (pRC/MAP 4) required first cloning the CMV promoter/enhancer region of the plasmid pRC/CMV (KpnI-SspI) into the plasmid pBluescript II SK (KpnI-Sapl) to expand the polylinker sites available for cloning. This initial construct was named Whitescr ipt. Next, the plasmid H2 (West et al., 1991) was digested with Sall and NotI to release a 5-kb fragment containing the full coding sequence of human MAP 4. The fragment was gel purified and ligated to the Xbol-BgIII fragment from Whitescr ipt and the BgIII-NotI fragment from pRC/CMV.

To construct the plasmid bearing human tau (pRC/Tau), the plasmid pEN 1234c (Lee and Rook, 1992) containing the full coding of human tau was digested with SacI, filled in with Klenow, and digested with XbaI. The resulting 1.2-kb fragment was gel purified and ligated to the HindIII (filled in)-XbaI sites of pRC/CMV.

Transfections of wild-type CHO cells or the taxol-dependent CHO cell line, Tax 18 (Cabral, 1983; Cabral et al., 1983) were carried out using a modified calcium phosphate protocol as described by Chen and Okayama (1987). Approximately 2 × 10^5 cells were seeded onto 100-mm tissue culture dishes containing coverslips and grown overnight as described below. A 1 ml solution of buffered DNA was then prepared by adding 1 vol of 50 μM CaCl₂, 20 μg plasmid DNA, and 20 μg sheared herring sperm DNA dropwise with agitation to an equal volume of 2× BBS (50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, and 1.5 mM Na₂HPO₄, pH 6.95). After incubation at room temperature for 25 min, the DNA solution was added to the dish of cells containing 10 ml of growth medium (see next section for composition). After overnight incubation at 37°C, a very fine precipitate covered the cells. The medium was then removed, and cultures were incubated 2.5 min at room temperature with 4 ml of growth medium containing 10% glycerol. The glycerated medium was then diluted with 8 ml of PBS, and the cells were rinsed three times with PBS and then grown another 24 h with normal growth medium. The coverslips were aseptically removed and prepared for immunofluorescence as described below. The remaining cells were trypsinized, and 2 × 10^5 cells were resuspended onto 100-mm tissue culture dishes for 24 h in complete medium. The neomycin analogue, G418 (Gibco Laboratories, Grand Island, NY), was then added at a concentration of 2 mg/ml to select for stably transfected cells.

Isolation, Growth, and Induction of Stably Transfected Cell Lines

Clones that survived in G418 were isolated and maintained at 37°C in 5% CO₂ in alpha modification of minimal essential medium containing 5% fetal bovine serum (both from Gibco Laboratories), 2 mM glutamine, 50 μM of penicillin, and 50 μg/ml of streptomycin (all from ICN Biomedicals, Inc., Costa Mesa, CA). The growth medium also contained 2 mg/ml G418 to ensure retention of the DNA segment containing the transfected MAP. Stable transfection efficiencies were varied from 10⁻³ to 5 × 10⁻³ depending on the experiment. To induce a higher expression of the transfected MAP, cells were treated with various concentrations (0.5-5 mM) of sodium butyrate (Sigma Immunochemicals, St. Louis, MO) overnight (16 h) in normal growth medium.

Immunofluorescence Analyses

Transfected cell lines were grown on glass coverslips and fixed in methanol at -20°C for ≥6 min. The cells were then incubated at 37°C in a humid chamber with a rabbit polyclonal antibody against the Drosophila 205-kD MAP (1:20 dilution; gift from Dr. Larry Goldstein, Harvard University, Cambridge, MA), a rat polyclonal antibody raised to a fusion protein containing the KDM repeat of human MAP 4 (1:100 dilution, West, 1992), or a mouse monoclonal antibody to tau (1:40 dilution; Boehringer Mannheim Biochemicals, Indianapolis, IN). For double-label experiments, a mouse monoclonal antibody to α-tubulin (1:100 dilution of DMI; Accurate Chemical & Scientific Corp., Westbury, NY) was paired with the rabbit antibody to the Drosophila MAP followed by affinity-purified secondary antibodies. To induce a higher expression of the transfected MAP, cells were treated with various concentrations (0.5-5 mM) of sodium butyrate (Sigma Immunochemicals, St. Louis, MO) overnight (16 h) in normal growth medium.
Purification of Human MAP 4

Microtubules were prepared from a 500-ml spinner culture of HeLa cells using taxol as previously described (Vallee, 1982). The microtubule pellet was resuspended in 50 ml of PME buffer (80 mM Pipes-Na, pH 6.8, 1 mM ethylenediamine-N,N,N',N'-tetraacetic acid, 1 mM MgCl2) containing 0.5 M NaCl and 10 μM taxol, kept on ice for 30 min, and centrifuged at 12,000 g for 10 min to separate MAPs from the microtubules. Dithiothreitol (1 mM) was added to the supernatant, which then boiled for 5 min and cooled on ice before centrifugation at 12,000 g for 10 min to remove denatured protein. The resultant supernatant contained purified MAP 4.

Western Blot Analyses

Transfected cells that were either uninduced or induced overnight in various concentrations of sodium butyrate were washed twice with PBS, lysed directly with hot (100°C) SDS sample buffer (Laemmli, 1970), and analyzed by SDS polyacrylamide gel electrophoresis. 6% polyacrylamide gels were used to analyze the high molecular weight MAPs (Drosophila 205-kD MAP and human MAP 4), and 7.5% gels were used for cells transfected with human tau. The gel-separated proteins were electrophoretically transferred to nitrocellulose as described (Towbin et al., 1979), except that the transfer buffer used for the high molecular weight MAPs contained 0.2% SDS. For visualization of the transfected MAP proteins, the nitrocellulose blots were incubated with a 1:1,000 dilution of rabbit anti-Drosophila MAP (gift from Dr. Larry Goldstein), 1:1,000 dilution of rat anti–MAP 4 (West, 1992), or 1:500 dilution of mouse anti-tau (tau-1; Boehringer Mannheim Biochemicals) followed by 1:2,000 dilutions of the appropriate peroxidase-conjugated secondary antibodies (Cappel Laboratories, Cochranville, PA). The blots were then stained with 4-chloronaphthol as previously described (Hawkes et al., 1982). In those cases in which more quantitative results were needed, the immunostaining was visualized using the ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL), and various exposures to x-ray film (Hyperfilm MP; Amersham Corp.) were made to ensure that band densities were in the linear range of the film. Relative intensities of the bands on the film were measured using whole band densitometry software running on a SPARC station IPC (Sun Microsystems, Mountain View, CA) equipped with an image analysis system (Bio Image, Ann Arbor, MI).

Northern Blot Analyses

RNA was isolated from stably transfected cell lines using previously described procedures (West et al., 1991). Total RNA was fractionated on a denaturing 0.8% agarose gel and then blotted onto Gene Screen Plus (New England Nuclear, Boston, MA). Blots were hybridized (50% formamide, 0.75 M NaCl at 42°C for 12-16 h) and washed (0.1x SSC/0.1% SDS at 65°C) at high stringency. P-Tubulin transcripts were detected using a probe made from a clone of Sullivan and Cleveland, 1986, gift from Dr. D. W. Cleveland, Johns Hopkins University School of Medicine, Baltimore, MD). Actin transcripts were detected using a putative actin clone isolated from a mouse melanoma library by hybridization with an authentic Drosophila actin clone (Tenbarge, K. M., and J. B. Olmsted, unpublished observation).

Measurement of Monomer and Polymer Pools

Procedures for measuring monomer/polymer pools in cultured cells and the verification of the methodology have been previously described (Minotti et al., 1991). Briefly, cells were incubated with [3H]methionine overnight to label the proteins uniformly. The cells were then lysed with a microtubule stabilizing buffer (20 mM Tris-HCl, pH 6.8, 0.5% Nonidet P-40, 1 mM MgCl2, 2 mM EGTA, 0.14 M NaCl, 4 μg/ml taxol), and the cytoskeleton was separated from the soluble proteins by centrifugation. A constant amount of [35S]-labeled cell extract was added to each pellet and supernatant fraction, and the proteins were resolved by two-dimensional gel electrophoresis. After staining and drying, the tubulin spots and actin spots were excised from the gels, and the radioactivity in each spot was measured by liquid scintillation counting. The [3H]/[35S] ratio for tubulin (or actin) in the pellet was divided by the total [3H]/[35S] ratio for pellet and supernatant fractions to obtain the percentage of tubulin (or actin) that appeared in the cytoskeleton.

To measure total relative tubulin levels in various cell lines, a modification of a previously published method (Boog and Cabral, 1987) was used. Briefly, the [3H]/[35S] ratios for tubulin in the supernatant and pellet fractions were added for each sample and then divided by the sum of the [3H]/[35S] ratios for actin in the same fractions. The resulting quotients for the control cells was arbitrarily set at 100% in each experiment, and the quotients for all other samples were expressed as fractions of the control value times 100%.

Measurement of Drug Resistance

To measure drug resistance of transfected cells, 100 cells were seeded into replicate wells of a 24-well tissue culture dish, and the cells were treated overnight with 2 mM sodium butyrate in normal growth medium to induce expression of the transfected gene. The butyrate was then washed out and the medium was replaced with varying concentrations of Colcemid for a 24-h period (previous experiments show that MAP expression persists for >24 h, see Results). After the 24-h exposure to the drug, the cells were rinsed in medium to remove the drug and then incubated another 5 d in medium to allow the formation of visible colonies. After removal of the medium, the cells were stained with 0.03% methylene blue as previously described (Cabral et al., 1980).

Results

Isolation of CHO Transfectants

To explore a possible role for MAPs in promoting microtubule assembly in living cells, we have carried out experiments involving the transfection and stable expression of exogenous MAPs in CHO cells. CHO cells represent a good system in which to carry out such experiments because they appear to contain low levels of endogenous MAPs. Previous studies indicated that CHO cells express a heat-stable 210-kD MAP believed to be analogous to MAP 4 of other species (Brady, R. C., and F. Cabral, 1985. J. Cell Biol. 101:30a). Our observations suggested that this CHO MAP is expressed at very low levels (unpublished data), and this is supported by the Northern blot data presented here (see Fig. 3). Furthermore, we have been unable to identify other CHO MAPs in taxol prepared microtubules by biochemical methods or by cross-reaction with a variety of MAP antibodies borrowed from a number of different laboratories. We therefore concluded that tubulin polymerization in CHO cells could potentially be influenced by the overexpression of exogenous MAPs.

To test this prediction, cDNAs containing the full coding sequences of a Drosophila 205-kD heat-stable MAP (Irninger-Finger et al., 1990), human MAP 4 (West et al., 1991), and human tau (Lee and Rook, 1992) were cloned into a pRC/CMV expression vector that places the cloned cDNA under the control of a powerful cytemegolovirus promoter and that contains a neomycin resistance gene for the isolation of stable transfectants. Transfection experiments were carried out in parallel using the pRC/CMV vector alone or the pRC/CMV vector plus inserted MAP cDNA. Approximately equal numbers of transfectants were isolated regardless of the presence or absence of the inserted MAP cDNA, indicating that expression of the exogenous MAP did not seriously reduce the viability of CHO cells. Furthermore, the frequency at which transient expression was seen by immunofluorescence appeared to parallel the number of stable transfectants isolated in each experiment.

Biochemical Characterization of Gene Expression in Stable Transfectants

To identify stable transfectants, colonies able to survive in the neomycin analogue, G418, were isolated at random and
screened for expression of the transfected gene products by immunoblot analysis. Initial results suggested that few of the G418 resistant colonies had measurable expression of the heterologous MAP proteins. To enhance expression, cells were treated with sodium butyrate, a compound others have found to greatly enhance expression of genes that are otherwise poorly expressed (Gorman and Howard, 1983; Kruh, 1982). When assayed after overnight treatment with varying amounts of sodium butyrate, CHO cells transfected with each of the MAPs exhibited moderate to high levels of expression in ~50% of the G418 resistant clones. The dose responses to sodium butyrate for three G418-resistant cell lines exhibiting good induction of expression are shown in Fig. 1. In some cases (e.g., Fig. 1 B), there was little or no detectable production of the heterologous protein in the absence of sodium butyrate, while in other cases (e.g., Fig. 1, A and C), heterologous protein was evident before treatment with sodium butyrate, but expression was enhanced after treatment.

To obtain a better estimate for the level of exogenous MAP that accumulates, the transfected cell line shown in Fig. 1 B was grown overnight in 2 mM sodium butyrate, and the MAP

Figure 2. Quantitation of MAP 4 expression. A purified MAP 4 preparation and SDS cell lysates of HeLa cells and MAP 4-transfected CHO cells were run on a 6% SDS polyacrylamide gel and then transferred to nitrocellulose. Proteins were detected using a rat anti-human MAP 4 antibody and a mouse anti-α-tubulin antibody to demonstrate equivalent loading of the cell lysates. Visualization was carried out using peroxidase-conjugated secondary antibodies and a chemiluminescence substrate followed by exposure to X-ray film. The MAP 4-transfected CHO cells were treated overnight with 2 mM sodium butyrate before cell lysis. (Lanes a–c) 2.5, 5, and 10 ng of purified MAP 4; (lanes d–f) 2.5, 5, and 10 μg HeLa cell extract; (lanes g and h) 2 and 5 μg of cell lysate from MAP 4-transfected CHO cells. (Arrow) position of MAP 4; (T) position of α-tubulin. Note the absence of any residual tubulin in the MAP preparation.

4 content was compared to HeLa cells and quantitated using purified MAP 4 and Western blot analysis with chemiluminescence detection. The results shown in Fig. 2 indicate that the transfected CHO cells accumulate more MAP 4 than HeLa cells express normally. Based on densitometric analysis of the corresponding autoradiograms, MAP 4 was calculated to make up ~0.06% of the total protein in HeLa cells. If one assumes that tubulin is 4% of the total protein, a tubulin:MAP 4 weight ratio of 68:1 is obtained. This value is similar to the 40:1 ratio reported by others based on copurification of MAP 4 with microtubules (Bulinski et al., 1980). By contrast, MAP 4 makes up 0.56% of the total protein in transfected CHO cells. This corresponds to a tubulin:MAP 4 ratio of 7:1, assuming an equivalent amount of tubulin in both cell lines. Therefore, the transfected CHO cells have approximately nine times more MAP 4 than HeLa cells, a level that should be sufficient to produce effects on microtubule assembly.

The patterns of mRNA expression in the transfected CHO cells were also examined. In initial experiments, the abundance of endogenous mRNA for MAP 4 was examined by comparing the presence of transcripts in CHO cells with that in another hamster cell line, BHK. Because sequence divergence precluded detection of any CHO sequence with the human MAP 4 probe, these data were obtained using a mouse MAP probe (M7; West et al., 1991). As shown in Fig. 3 A, levels of transcripts in BHK cells (lane 3) are comparable to that seen in mouse L cells (lane 2). However, only low levels of transcripts were detected in CHO cells (lane 4). The results from these studies are consistent with our previous immunological and biochemical observations that CHO cells express only low levels of an endogenous MAP 4 related protein. Expression of the heterologous MAPs was also measured with each of the respective probes in the transfected

Figure 1. Effects of sodium butyrate on expression of exogenous MAPs in CHO cells. Cells were grown to 50–70% confluence in individual wells of a 24-well tissue culture dish and were then treated with 0, 0.5, 1, 2, or 5 mM sodium butyrate for 16 h. After lysis of the cells in SDS, cellular proteins were resolved on 6% (A and B) or 7.5% (C) polyacrylamide gels and were transferred onto nitrocellulose. (A) A CHO cell line transfected with the Drosophila 205-kD MAP; (B) a CHO cell line transfected with human MAP 4; (C) a CHO cell line transfected with human tau. In each case, the blot was stained with an antibody specific for the transfected MAP (arrow) and with an antibody to tubulin (T) or actin (A) to control for protein loading. Actin was used as a control in C because tubulin comigrated with tau. Lanes labeled C are samples from a cell line transfected with the pRC/CMV vector alone.
Figure 3. Northern blot analysis of various cell lines and of CHO cells transfected with exogenous MAPs. Total RNA was isolated from each cell line and 10 µg was loaded onto each lane of an agarose gel. After electrophoresis, the RNA was transferred onto nitrocellulose and probed for the presence of MAP. (A), endogenous MAP expression in various cultured cell lines. (Lane 1) HeLa RNA probed with human MAP 4; (lane 2) mouse L cell RNA probed with mouse MAP 4; (lane 3) BHK RNA probed with mouse MAP 4; (lane 4) CHO RNA probed with mouse MAP 4. (B) Exogenous MAP expression in wild-type and MAP-transfected CHO cells. (Lane 1) CHO cells transfected with pRe/CMV and probed with human MAP 4; (lane 2) CHO cells transfected with human MAP 4 and probed with human MAP 4; (lane 3) CHO cells transfected with Drosophila 205-kD MAP and probed with Drosophila MAP; (lane 4) CHO cells transfected with human tau and probed with human tau. (Upper arrows) MAP 4 or Drosophila 205-kD MAP; (lower arrow) human tau.

Immunofluorescence Analysis of MAP-transfected CHO Cells

Cells that appeared to express high levels of exogenous MAP by the Western blot assay were screened by immunofluorescence for the uniformity of expression among the individual cells in the population. Wide variability in response was noted. In some cases, few cells (<10%) had appreciable levels of fluorescence in the absence of sodium butyrate treatment but had a strong response (>80% of the cells) after treatment. Others had a good response, even before sodium butyrate treatment, but increased further (to essentially 100%) after treatment. Cell lines showing the best uniformity of response were chosen for further study and representatives are shown in Fig. 4. MAP expression in the absence of sodium butyrate was not homogeneous, and only a subset of the cells had visible staining. However, all three cell lines showed good uniformity of staining when an overnight treatment with 2 mM sodium butyrate was included before the immunofluorescence assay. In all three cases, the exogenous MAPs retained microtubule binding activity, as judged by the immunolocalization of the proteins on microtubules. However, of the three transfected genes, only tau caused a distinctive reorganization of microtubules into thick linear or circular bundles (Fig. 4, G--I); this is reminiscent of patterns reported previously for tau (Kanai et al., 1989; Knops et al., 1991; Lee and Rook, 1992; Lewis et al., 1989). Based on qualitative estimates of relative fluorescence intensity, the degree of bundling appeared to correlate with the level of tau expression. Cells with weak tau fluorescence exhibited little evidence of microtubule bundling, but the microtubules themselves appeared straighter than in nontransfected cells. Cells with intermediate levels of expression had various extents of microtubule bundling, including thick linear arrays of microtubules that had no obvious association with the centrosome. Finally, many of the cells with the highest tau expression had circular bundles of microtubules around the nucleus or periphery of the cell. These cells tended to round up and were easily lost from the coverslips during the processing for immunofluorescence, resulting in a probable underestimation of cellular expression.

In double-label experiments with antibodies specific for tubulin, it was apparent that the exogenous MAPs interacted with all cytoplasmic microtubules in interphase cells. In the cells transfected with Drosophila 205-kD MAP or human MAP 4, the microtubule patterns observed using the MAP-specific or tubulin-specific antibodies were identical (Fig. 4, B, C and E, F). However, in the cells transfected with human tau, diffuse background staining with the tau-specific antibody was more prominent than what was observed with the tubulin antibody (Fig. 4, H and I), suggesting that the free tau protein is better retained by the fixation conditions or is present in great excess over available microtubule binding sites. An interesting and unexpected phenomenon was observed when the mitotic figures in transfected cells were examined. As shown in the insets in Fig. 4, both Drosophila 205-kD MAP and human MAP 4 showed typical microtubule-like patterns within the spindle, whereas tau staining was never observed on the mitotic apparatus. These data suggest that the interaction of tau with microtubules in mitotic cells is regulated differently than the other transfected MAPs.

Effects of Exogenous MAP Expression on In Vivo Microtubule Assembly

The idea that MAPs might enhance microtubule assembly in vivo, as they have been shown to do in vitro, was tested using an assay that was recently shown to be of sufficient sensitivity to measure subtle effects on microtubule assembly resulting from mutations in tubulin genes (Minotti et al., 1991). To carry out these experiments, cells were metabolically labeled overnight in [3H]methionine while being induced to express MAP with 2 mM sodium butyrate, and the tubulin...
Figure 4. Immunofluorescence of exogenous MAPs. CHO cell lines transfected with *Drosophila* 250-kD MAP (A–C), human MAP 4 (D–F), or human tau (G–I) were fixed in methanol before (A, D, and G) or after (B, C, E, F, H, and I) treatment with 2 mM sodium butyrate for 16 h. The cells were then stained with MAP-specific antibodies (A, D, and G) or simultaneously stained with a MAP-specific antibody (B, E, and H) and an antibody to tubulin (C, F, and I). Insets show mitotic cells from the same slides. Note that in the absence of sodium butyrate induction, many cells in the background fail to stain with the MAP-specific antibodies. However, after induction, most of the cells react positively with the MAP antibodies. Also note that cells transfected with the *Drosophila* 205-kD MAP or the human MAP 4 have normal appearing microtubules arrays, but that cells transfected with human tau have highly bundled microtubules.

distribution between cytoskeletal and free forms was then quantified as described previously (Minotti et al., 1991). The data are summarized in Table I. Little, if any, increase in tubulin polymerization was associated with expression of the three exogenous MAPs, but a large increase in tubulin polymerization was measured in cells treated with taxol. However, the values measured for the extent of tubulin polymerization in all of the cell lines, including the control, are lower than were previously reported for wild-type CHO cells (Minotti et al., 1991). This reduced level of polymerized
tubulin appears to be caused by the butyrate treatment. Experiments were therefore carried out to assess the reversibility of the butyrate. See Materials and Methods for details of the assay.

Results are consistent with the other observations presented in this paper indicating that exogenous MAP expression does not appreciably affect the extent to which tubulin assembles in the cell.

**Effect of Exogenous MAPs on Tubulin Expression**

A number of previous studies have shown that tubulin synthesis in a variety of cell lines is an autoregulated process (for review see Cleveland, 1989). This autoregulation is mediated by changes in tubulin polysomal mRNA degradation that are sensitive to the state of microtubule polymerization. In cells treated with microtubule-disrupting agents, tubulin synthesis is greatly decreased, while in taxol treated cells, tubulin synthesis is somewhat elevated. Thus, if exogenous MAP expression in CHO cells were increasing tubulin assembly, one might expect to measure an increase in the amount of cellular tubulin. To test this prediction, cells were labeled overnight with [3H]methionine while being induced to maximize their expression of MAP with sodium butyrate. After cell lysis and resolution of the proteins on two-dimensional gels, tubulin was quantified by liquid scintillation counting as previously described (Boggs and Cabral, 1987). The results shown in Table II are expressed as a percentage relative to cells transfected with vector alone and arbitrarily set at 100%. Cells transfected with each of the three MAP cDNAs had similar levels of tubulin as cells transfected with vector alone within the experimental error of the procedure. Only cells treated with taxol demonstrated a modest elevation in the tubulin content. Northern analyses of control and MAP-transfected cell lines also showed that there was no MAP-dependent increase in tubulin or actin mRNA levels (data not shown). This lack of effect of MAP expression on tubulin levels is consistent with our data indicating that there is no appreciable effect on microtubule assembly.

**Drug Resistance of MAP-transfected Cells**

As another measure of potential effects of MAP expression on microtubule assembly, the sensitivity of MAP-transfected cell lines to agents that are known to bind tubulin and alter microtubule assembly was examined. The basis for these experiments comes from recent work, indicating that cell lines resistant to drugs that disrupt microtubules have higher basal levels of microtubule assembly, while cell lines that are resistant to taxol, a microtubule stabilizing drug, have lower levels of assembly (Minotti et al., 1991). If MAPs serve to enhance microtubule assembly and stabilize microtubules in vivo, cell lines expressing higher levels of MAPs should be relatively resistant to depolymerizing drugs such as Colcemid. Since sodium butyrate enhances expression of MAPs in the transfected cells but is inhibitory to cell growth, a modified assay to measure drug resistance was developed. In this assay, the cells were incubated overnight in 2 mM sodium butyrate to induce MAP expression and the medium was then replaced with medium containing varying amounts.

**Table I. Effect of Exogenous MAP Expression on Extent of Microtubule Assembly**

<table>
<thead>
<tr>
<th>MAP</th>
<th>+2 mM sodium butyrate</th>
<th>+2 mM sodium butyrate + 2 h reverse</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>34.6 ± 2.0</td>
<td>38.8 ± 2.3</td>
</tr>
<tr>
<td>pRc/CMV</td>
<td>31.7 ± 2.4</td>
<td>38.9 ± 2.3</td>
</tr>
<tr>
<td>Drosophila MAP</td>
<td>35.9 ± 1.2</td>
<td>39.3 ± 3.3</td>
</tr>
<tr>
<td>MAP 4</td>
<td>32.8 ± 4.0</td>
<td>37.9 ± 2.2</td>
</tr>
<tr>
<td>Tau</td>
<td>34.0 ± 3.4</td>
<td>41.4 ± 3.5</td>
</tr>
<tr>
<td>Wild type + taxol</td>
<td>56.6 ± 3.4</td>
<td>59.1 ± 2.0</td>
</tr>
</tbody>
</table>

The values represent the percentage of total tubulin in the cytoskeletal fraction for each of the transfected cell lines measured after induction of the cells with 2 mM sodium butyrate for 16 h, or measured 2 h after the removal of sodium butyrate. See Materials and Methods for details of the assay.

**Table II. Tubulin Levels in Cells Expressing Exogenous MAPs (Percent of Control)**

<table>
<thead>
<tr>
<th>MAP</th>
<th>+2 mM sodium butyrate</th>
<th>+2 mM sodium butyrate + 2 h reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRc/CMV</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Drosophila MAP</td>
<td>113 ± 9</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>MAP 4</td>
<td>100 ± 8</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>Tau</td>
<td>110 ± 10</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>Wild type + taxol</td>
<td>131 ± 6</td>
<td>134 ± 11</td>
</tr>
</tbody>
</table>

The amount of tubulin in each cell line was measured as the ratio of methionine incorporation into β-tubulin divided by the methionine incorporation into actin in the same sample. Because the actual numbers vary greatly from experiment to experiment, depending on the ratio of [3H] and [35S] added to each sample, the results are expressed as a percentage relative to cells transfected with pRc/CMV run as a control in each experiment. See Materials and Methods for details of the assay.
of Colcemid. After a 24-h exposure to the drug, a length of time during which MAP levels remain high (Fig. 5), the drug was washed out and the cells were allowed to recover for ~5 d until visible colonies were seen. By this assay, there was no discernable change in the sensitivity to Colcemid in any of the transfected cell lines when compared to either untransfected cells or to cells transfected with vector alone (data not shown).

To get a better estimate of individual cellular response to Colcemid treatment, immunofluorescence analysis was carried out on cells that had been pretreated for 2 h with 0.05 μg/ml Colcemid, the minimum concentration that was previously found to eliminate virtually all cytoplasmic microtubules in wild-type CHO cells. Using these conditions, microtubules were lost to an equivalent extent in cells transfected with Drosophila 205-kD MAP, human MAP 4, or pRc/CMV vector. The results with the Drosophila MAP are shown in Fig. 6, C and D. In the case of tau-transfected cells, only the microtubule bundles appeared to be resistant to this treatment (Fig. 6, E and F). To determine whether these microtubule bundles are resistant to depolymerization at a dose of 0.05 μg/ml Colcemid, or simply require more time to depolymerize, the incubation in drug was extended to 6 h (Fig. 6 G) or 16 h (Fig. 6 H). The residual microtubule bundles were progressively lost with time, consistent with the lack of change in the dose response to Colcemid determined by the clonogenic assay already discussed. These data extend the conclusion reached by the direct biochemical measurements summarized in Table I that exogenous MAP expression does not alter the extent of assembly of the microtubules in the transfected cell lines.

Transfection of a Taxol-dependent Cell Line

As a final independent assay for the ability of transfected MAPs to influence microtubule assembly, attempts were made to rescue the taxol-dependent phenotype of a mutant cell line by transfecting genes for the same three MAPs into Tax-18. As previously reported, this cell line is unable to assemble spindle microtubules or divide into daughter cells unless taxol is present in the growth media (Cabral, 1983; Cabral et al., 1983). The properties of this and other drug resistant cell lines suggested that the taxol-dependent phenotype results from a reduced ability of the cell to form microtubules (Cabral and Barlow, 1989, 1991). In support of this conclusion, it has been reported that agents other than taxol can rescue the cells. For example, dimethyl sulfoxide, an agent previously reported to enhance stability of microtubules in vitro (Himes et al., 1977), can partially alleviate the dependence on taxol (Barlow et al., 1986), and other studies have shown that microinjection of bovine brain tubulin can also rescue Tax-18 (Saxton and McIntosh, 1987). Furthermore, the ability of taxol analogues to rescue Tax-18 correlated directly with the ability of those agents to increase microtubule assembly in vitro (Cabral F., unpublished experiments). It would thus appear that any agent capable of increasing the levels of microtubule assembly in Tax-18 should remove the requirement for taxol and allow the cells to grow normally.

To ascertain whether transfection of exogenous MAPs would rescue the taxol-dependent phenotype in Tax-18, experiments were carried out in which parallel transfections were made in the absence of taxol, in the presence of various levels of taxol, or in the presence of taxol but with subsequent removal of taxol at various times after the transfection. Stable G418-resistant colonies were only obtained if taxol was present throughout the procedure, indicating that expression of MAPs at constitutive levels could not rescue the taxol-dependent phenotype. To determine whether higher levels of expression might succeed in rescuing Tax-18, stable transfectants were induced to express the transfected gene by overnight treatment with sodium butyrate. As was previously demonstrated for wild-type CHO cells, transfected Tax-18 cells were able to express high levels of exogenous MAPs; again, only transfected tau caused an extensive bundling of the cellular microtubules (Fig. 7, B, D, and F). When butyrate and taxol were removed from cells transfected with pRc/CMV alone (Fig. 7, A, C, and E) or with vector plus tau (Fig. 7, B, D, and F) for 1 d (Fig. 7, C and D) or 2 d (Fig. 7, E and F), the cells displayed the typical taxol starvation morphology reported previously (Cabral, 1983; Schibler and Cabral, 1986). The cells became larger and flatter, and the nuclear size and morphology became increasingly heterogeneous with time. There were no apparent differences between transfected and nontransfected cells in these characteristics, indicating that expression of the exogenous MAPs was ineffective in reversing the taxol dependent phenotype. Finally, the reversion frequency of Tax-18 in nontransfected cells was compared to the same cell line transfected with various MAPs, and no differences were found. Thus, by a third independent measure, expression of exogenous MAPs in CHO cells appeared unable to influence the extent of microtubule assembly intracellularly.

Discussion

The results presented here demonstrate that stably transfected lines of CHO cells expressing high levels of structural MAPs can be isolated, and that the exogenous MAPs bind to all cytoplasmic microtubules. However, the immunofluorescence patterns indicate that the various structural MAPs examined here differ in fundamental properties. For example, although the proteins are not homologous in other regions, one might have postulated that MAPs that share common microtubule-binding domains (MAP 2, tau, and MAP 4) would all have similar effects on microtubule organization. The general phenotypes of cells transfected with MAP 2 (Edson et al., 1993; Lewis et al., 1989; Weisshaar et al., 1992) or tau (Kanai et al., 1989; Knops et al., 1991; Lee and Rook, 1992; data presented here) are similar. In our experiments, both tau and MAP 4 contained the conserved microtubule binding motif represented by four 18-mer repeats, and both were expressed under the same set of conditions. However, the absence of bundling and stabilization in MAP 4-transfected cells and the ability of the MAP 4 protein to colocalize with spindle microtubules contrast sharply with the results found with tau constructs. This phenomenon is not peculiar to CHO cells, as transient transfections carried out with the same MAP 4 construct have also shown no reorganization of microtubules in a variety of other cell lines, including mouse fibroblasts (Olson, K. R., and J. B. Olmsted, unpublished results). Furthermore, transfection of the Drosophila 205-kD MAP into HeLa cells also failed to produce microtubule bundles, but transfection of the tau construct did produce bundles (Barlow, S., and F. Cabral, unpublished re-
Figure 6. Immunofluorescence of MAP-transfected CHO cell lines treated with Colcemid. Wild-type cells (A and B), cells transfected with *Drosophila* 205-kD MAP (C and D), or cells transfected with human tau (E-H) were seeded onto glass coverslips and induced to express MAPs with a 16-h treatment in 2 mM sodium butyrate. The cells were then treated (B, D, and F-H) with 0.05 μg/ml Colcemid in medium without sodium butyrate for 2 (B, D, and F), 6 (G), or 16 h (H). The cells shown in A, C, and E were washed free of sodium butyrate for 2 h but received no Colcemid. At the end of each time period, the cells were fixed in methanol and stained with antibodies to tubulin. Only tau-induced bundles remained after 2 h in Colcemid, but even these disappeared after 6–16 h in the drug. Bar, 10 μm.

The differences in results with MAP 4 and tau are unlikely to reflect low expression of the MAP 4 construct because quantitation indicates that the transfected CHO cells accumulate nine times as much MAP 4 as HeLa cells normally express. These data suggest that the basic domains lying outside the 18-mer repeats, which are not conserved between these MAPs, may be as important as the conserved repeat motif in influencing interaction with microtubules. For example, Aizawa et al. (1991) have demonstrated that MAP 4 contains an additional basic region that also interacts...
strongly with microtubules. Clearly, the results obtained here suggest that the interaction of MAP 4 with the microtubule surface differs significantly from that of tau, and that these differences could arise from regulation by the nonhomologous portions of the basic domain or perhaps even the projection domain of these two molecules.

The most surprising result from these studies was lack of effect of MAP overexpression on microtubule assembly. Numerous in vitro studies have demonstrated that structural MAPs enhance microtubule assembly (for review see Olimsted, 1986). Few studies, however, have attempted to demonstrate that MAPs play such a role in vivo. Neither the Drosophila 205-kD MAP nor human MAP 4 has been previously transfected into heterologous cell types, and little information on their effects on in vivo assembly is therefore available. On the other hand, several reports have appeared on the effects of tau in heterologous cells in which either microinjection (Drubin and Kirschner, 1986) or transfection (Kanai et al., 1989; Knops et al., 1991; Lee and Rook, 1992) was used. In the microinjection experiments, morphometric analysis of paired sister cells in which only one member of the pair was microinjected with tau indicated that tau increased microtubule assembly an average of 44% and stabilized the microtubules to the microtubule-disrupting drug, nocodazole. However, no bundling of microtubules occurred, and the injected tau appeared to degrade relatively rapidly. The results of this study seem clear, but they differ somewhat from our own results. In agreement with the earlier study, we find that tau expression stabilizes microtubules to Colcemid, but we see that stabilization is limited to the tau-induced bundles. As reported in the previous study, this stabilization is a time-dependent phenomenon in which longer incubations in low Colcemid concentrations eventually lead to complete depolymerization of the microtubules in tau transfected cells. This interpretation is consistent with our observation that there is no dose-dependent shift in the sensitivity of the tau transfectants to Colcemid in cloning efficiency assays. However, our data contrast with that obtained previously on the induction by tau of microtubule assembly because we see no effect on the monomer-polymer equilibrium, and tau expression does not reverse the taxol-dependent phenotype of a mutant CHO cell line. It remains to be established whether the nature of the cell lines that were used or the state of the tau that accumulates in the cells can account for the differences in these two studies. However, our unpublished findings (Minotti, A. M., and F. Cabral) indicate that mitotic cells assemble microtubules to the same extent as interphase cells, despite fivefold differences in microtubule dynamics (Saxton et al., 1984), suggesting that stability and extent of assembly may not be synonymous parameters.

Our results also differ somewhat from those reported for tau transfections into mouse L cells (Kanai et al., 1989), ST3 cells (Lee and Rook, 1992), and insect SF9 cells (Knops et al., 1991) in that high level expression of tau in the CHO cell line produces circular bundles of microtubules rather than the neuritic-like processes described in the other studies. This is likely to reflect a difference among the cell lines used in the various studies because all used the identical cDNA construct. In support of this suggestion, a recent report has demonstrated that the ability of MAP 2-transfected cells to form neuritic-like processes is related to the strength of the subcortical microfilament network because treatment with cytochalasin B greatly enhanced the ability of the cells to express such processes (Edson et al., 1993). We have obtained similar results with the tau-transfected CHO cells (data not shown). In agreement with previous studies, CHO cells, judged by immunofluorescence to be expressing higher levels of tau, frequently exhibited microtubule bundles that were not associated with the microtubule-organizing center. Cells expressing lower levels of tau displayed more normal arrays of microtubules weakly decorated with the MAP; but in many cases, the microtubules appeared straighter than in nontransfected cells.

Although levels of microtubule assembly were not measured in previous transfection studies, Kanai et al. (1989) reported an increase in tubulin expression, as judged by densitometry of tubulin bands on SDS gels, in stably transfected mouse L cells. However, because the authors were unable to identify a unique band for tau on their gels, and in our hands tau and tubulin comigrate on one-dimensional gels (data not shown), it is possible that the increase in tubulin expression reported for the tau-transfected cells actually reflects an increase in band density caused by the comigration of tau at that position in the gel. Our own studies failed to measure an increase in either tubulin protein or mRNA in tau-transfected CHO cells. Because the autoregulatory model for tubulin expression (Cleveland, 1989) predicts that any tau-dependent increase in microtubule polymer would cause an increase in tubulin expression, our data are consistent with a lack of perturbation of the monomer-polymer pool by the heterologous MAP.

The lack of effect of structural MAP overexpression on microtubule assembly in our experiments could potentially be caused by the presence of other unidentified MAPs that already maximally promote microtubule assembly. Although we cannot formally rule out this possibility, it seems an unsatisfying explanation. CHO cells are relatively simple, nondifferentiated cells that have lost many nonessential genes during their evolution in tissue culture. In fact, this cell line was chosen for these experiments, in part, because they appear to express only low levels of a heat-stable MAP 4-like protein. Thus, these cells might be expected to be more sensitive rather than less sensitive to the effects of MAP overexpression compared to other cell lines. The fact that we do not see an increase in microtubule assembly does not

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Figure 7. Immunofluorescence of a tau-transfected taxol dependent mutant. Tax-I 8, a taxol-dependent CHO cell line, was stably transfected with the vector pRc/CMV or with the same vector containing a cDNA insert encoding human tau. Both transfected cell lines were treated with 2 mM sodium butyrate in taxol containing medium for 16 h and then switched to medium lacking both butyrate and taxol for 2 (A and B), 24 (C and D), or 48 h (E and F). At each time point, the cells were fixed in methanol and stained with a tubulin antibody to visualize the microtubules and propidium iodide to visualize the nucleus. Cells expressing tau are easily identified by the presence of bundled microtubules. Note that with increasing time of taxol deprivation there is an increase in cell size and degree of multinucleation for both tau-expressing and -nonexpressing cells. Bar, 10 μm.
conflict with data from other transfection studies because this is the first time that effects on in vivo assembly have been biochemically quantitated.

Thus, we favor the possibility that structural MAPs, while affecting microtubule assembly in vitro, may play a different role in living cells. Consistent with this interpretation, disruption of the gene for the 205-kD MAP in Drosophila has been found to have no effect on the growth or development of that organism (Pereira et al., 1992). Our own results indicate that overexpression of the same protein in CHO cells fails to alter the extent of microtubule assembly and further suggests that this MAP does not play a critical role in microtubule assembly in vivo. In separate experiments, a number of laboratories have attempted to identify MAPs as genetic suppressors of mutations that destabilize microtubules. The only successful study reported to date identified a new member of the tubulin gene family, γ-tubulin (Oakley and Oakley, 1989), that localizes to the centrosome and appears to influence microtubule nucleation rather than as a structural protein that copolymerizes with tubulin (Oakley et al., 1990; Zheng et al., 1991). Thus, genetic analysis has also, thus far, failed to confirm a role for MAPs in regulating microtubule assembly in vivo. Finally, in contrast to the lack of effects of these MAPs on tubulin expression or microtubule assembly in vivo, we could measure clear effects of taxol under similar conditions. While it is apparent that neural MAPs are involved in neurogenic processes, we suggest that it may be incorrect to think of all structural MAPs as “taxol-like” substances, and that tissue context and still-unknown regulatory factors may dramatically affect the cellular roles of these proteins.

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