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Overexpression of MAP4 inhibits organelle motility and trafficking in vivo

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SUMMARY

We previously prepared cell lines that inducibly overexpress MAP4, a microtubule (MT)-associated protein widely expressed in non-neuronal cells. Overexpression of either the full-length MAP4 molecule or its MT-binding domain, MTB, stabilized MTs and retarded cell growth, suggesting that overexpressed MAP4 impacts on MT-dependent functions in vivo. To test this hypothesis, we examined MT-based vesicle movements in living cells, using high resolution DIC microscopy. Overexpression of either MAP4 or MTB yielded a dose-dependent reduction in the frequency of MT-dependent organelle movements, relative to control cells. At steady state, both MAP4- and MTB-overexpressing cells showed unusual distributions of transferrin, LDL, dextran, and Golgi elements, as compared to control cells. MAP4 preferentially inhibited receptor-dependent uptake and degradation of LDL, and repositioning of Golgi elements after disruption by the drug, brefeldin A. L-MOCK cells treated with Taxol to stabilize the MTs to an extent equivalent to MAP4 overexpression did not show similar inhibition of vesicle motility or organellar trafficking, suggesting that deficits in organelle movements in vivo represent a direct effect of the presence of MAP4 or MTB, rather than an indirect effect of the stabilization of MTs by overexpressed MAP constructs. Our results show that MAP4 has the capacity to affect transport along MTs in vivo; these findings suggest a potential mechanism by which MAP4 could contribute to polarization or morphogenesis of cells.

Key words: Assembly-promoting MAP, Kinesin, Dynein, Motor protein, Transfectant

INTRODUCTION

To explore the in vivo functions of MAP4, a microtubule-associated protein (MAP) bound along the surface of MTs in all vertebrate non-neuronal cells (Bulinski and Borisy, 1980; Parysek et al., 1984; Murofushi et al., 1986), we previously prepared stably transfected clonal mouse L^{tk-} cell lines expressing full-length human MAP4 (L-MAP4 cells) (Nguyen et al., 1997). We also prepared cells that overexpressed only the MT-binding domain (MTB) of MAP4 (L-MTB cells), in order to determine which, if any, functions of MAP4 could be mimicked by MTB. From its in vitro properties, MAP4 had been postulated to function as an in vivo stabilizer of MTs. Indeed, analysis of the transfectant cells showed that their MTs exhibited a heightened stability. However, an equally exciting finding was that overexpression of MAP4 or MTB altered the growth parameters of the transfectants. For example, we noted that we were unable to isolate transfectant colonies that constitutively overexpressed MAP4 or MTB at a high level, that induction of MAP synthesis lengthened cell doubling time significantly, as compared to control cells, and that the longer doubling time was attributable to a lengthening of all phases of the cell cycle rather than to a lengthening of the mitotic period or to cell killing. These results suggested that excess MAP4 impacted on cell growth by interfering with some MT-dependent function(s), either as a direct consequence of the heightened level of MAP4 or as an indirect effect of MT stabilization by MAP4.

MTs serve as 'tracks' for a variety of vesicle movements that are important for cell growth, morphogenesis, mitosis, and axonal transport. MAPs that carry out these movements are classified as MT motor MAPs, that is, MT-dependent ATPases of the kinesin and dynein families (Barton and Goldstein, 1996; Vallee and Sheetz, 1996). Either kinesin or dynein family members, or both, have been implicated in a variety of MT-dependent movements in vivo. For example, evidence has accumulated for the motor- and MT-dependence of radial movement of lysosomes (Hollenbeck and Swanson, 1990), endocytosis in lysed cell models of polarized cells (Bomsel et al., 1990), vesicle trafficking from Golgi to ER (Lippincott-Schwartz et al., 1995), fusion of early endosomes with late endosomes (Bomsel et al., 1990; Aniento et al., 1993), and movement of mitochondria toward the cell periphery (Nangaku et al., 1994).

The fact that MTs in living cells interact not only with assembly-promoting MAPs such as MAP4, but also with kinesin and dynein, raised the question of how these two disparate types of MAPs might affect the activity of one another. Lopez and Sheetz (1993, 1995) previously demonstrated that dynein- and kinesin-powered movements along MTs were inhibited in the presence of physiological levels of the assembly-promoting neuronal MAP, MAP2. In these studies, only 25% of the motility seen along MAP-free MTs was observed in preparations con-

taining as little as one MAP2 molecule per 69 tubulin dimers. This observation may be physiologically significant for MAP4, since a variety of wild-type human cell lines contain about one MAP4 molecule per 50 dimers (Bulinski and Borisy, 1979), a concentration that would appear to be sufficient to confer a similar limitation in MT-based motility.

MAP2, like MAP4, is organized into two domains; namely a C-terminal MT-binding domain that contains all MT-interactive portions of the MAP, and a projection domain that protrudes from the wall of the MT. The experiments of Lopez and Sheetz (1993) showed that full-length MAP2 potently inhibits motility, unlike either the MT-binding fragment of MAP2 or the full-length tau molecule, which has both a MT-binding domain and a small projection domain. In addition to this evidence, which suggests that assembly-promoting MAPs sterically inhibit movement along MTs, results from other groups demonstrated that very high levels of either MAP2 or tau could inhibit kinesin movement by competing for binding sites on the MT (Paschal et al., 1989; von Massow et al.,1989). The MAP4 and MTB transfectant cell lines that were selected for high levels of MAP expression contain as much as one MAP4 molecule per 7 tubulin dimers, or one MTB molecule per two tubulin dimers (Nguyen et al., 1997); thus any steric or binding inhibition of MT motor molecules might be observable in these cells. In vitro tests of MAP4's effect(s) on dynein- or kinesin-based movements have not yet been performed; likewise, the possibility that any of the assembly-promoting MAPs affect the activity of MT motor MAPs in vivo has not been explored.

In addition to direct effects MAP4 might have on dynein- and kinesin-based intracellular movements, MT-based transport could be affected if MAP4 and other MAPs that are not themselves motors, such as CLIP-170 (Rickard and Kreis, 1990) or $p150_{glued}$ (Waterman-Storer et al., 1995), compete with one another for binding sites on the MT surface. There are many proteins that possess MT-binding domains, and occupancy of MT-binding sites by MAP4 molecules could disrupt the amount or organization of binding of these molecules to the MTs. Further, MAP4 might compete for kinases or other regulatory enzymes, causing dramatic changes in MT-based transport activity. If, indeed, expression of MAP4 were shown to alter vesicle trafficking, we could determine which MAP4 domains were involved and obtain clues about the molecular basis of the altered vesicle trafficking.

That occupancy of MT-binding sites might affect MT-based transport in vivo had been suggested by the studies of Robbins et al. (1995), who observed deficits in MT-based trafficking events in cells overexpressing glyceraldehyde 3-phosphate dehydrogenase, an enzyme known to bind to MTs. The inhibition of certain endocytic events observed by Robbins et al. (1995) suggested that it would be useful to test the effects of a more physiologically relevant MT-binding protein, MAP4, on MT-based vesicle movements and distributions.

Thus, one reasonable explanation for the altered growth of cells overexpressing MAP4 or MTB is that vesicle movements along MTs are inhibited by the presence of excess assembly-promoting MAPs in vivo. The MAP4 and MTB transfectant cell lines selected for high levels of MAP expression provided ideal material with which we could test the hypothesis that content of MAP4 impacts upon in vivo movements of vesicles in cultured cells. Our results show that movements of bulk vesicles, as well as specific endocytic and recycling movements, are inhibited by the presence of extra MAP4 or MTB along cellular MTs. These data provide in vivo evidence that MAP4 has the potential to regulate MT-dependent transport and localization of membranous organelles in vivo; this capacity could be utilized by the cell to modulate transport along particular MTs in order to polarize cells or differentiate regions of cytoplasm.

MATERIALS AND METHODS

Materials

Except as noted, all chemicals were purchased from Sigma (St Louis, MO) or from Fisher Scientific (Springfield, NJ). All tissue culture materials were from Gibco Life Sciences (Gaithersburg, MD). All fluorescently conjugated ligands were obtained from Molecular Probes, Inc. (Eugene, OR).

Culture and induction of stable cell lines overexpressing MAP4

The five transfectant cell lines used in this study; L-MOCK-#2-E, L-MAP4-#13-2, L-MAP4-#18-1, L-MTB-#3-6, and L-MTB-#8-1, were transfected, cloned, and characterized as described previously (Nguyen et al., 1997) and were compared to naive L^{tk-} cells in all experiments. Cells were grown on coverslips or in multi-well dishes in culture medium consisting of DMEM with 10% calf serum and antibiotics. All cells were induced with dexamethasone either 24 or 48 hours prior to use.

In vivo movement of vesicles

Living cells were observed and videotaped under high resolution DIC, at 37°C. Vesicle movements were quantified in an area 1.5 μm^2 during a 1.5 minute interval, following the methods of Hamm-Alvarez et al. (1993); vesicle movements in transfectant cell lines were normalized to L-MOCK cells, which contain plasmid alone, without a MAP4 insert. All cells were treated with 1 μM dexamethasone for 24 hours prior to recording. To ascertain that the movements we were measuring were MT-dependent, we depolymerized all cellular MTs with nocodazole (10 μM) for 2 hours before videotaping and quantifying vesicle movement. Nocodazole treatment reduced vesicle movement to less than 1% of that observed in untreated cells of each line.

Assays of transferrin (Tf), LDL, and dextran distribution using fluorescent ligands

For measurements of Tf and LDL, L-MOCK, L-MTB, and L-MAP4 cells were pretreated with dexamethasone for 24 hours, and then preincubated in 10 μg/ml FITC-labeled Tf, for 4 hours. At the start of the experiment (t=0) they were washed and placed in serum-free medium containing 10 μg/ml di-I-labeled LDL. At the indicated times, cells were fixed in 3.7% formaldehyde in phosphate buffered saline, transferrin and LDL fluorescence were viewed, and random fields were photographed using a Micromax cooled CCD camera (Princeton Instruments, Trenton, NJ) with a Kodak KAF1400 chip (Kodak, Rochester, NY). Images were processed with the MetaMorph system (Universal Imaging Corp. West Chester, PA) and printed on a Kodak dye sublimation printer (Speed Graphics, New York, New York).

For observation of trafficking to lysosomes, L-MOCK, L-MTB, and L-MAP4 cells on coverslips were pretreated with dexamethasone for 24 hours and preincubated in 10 μg/ml FITC-labeled dextran, overnight. At t=0, they were washed twice and placed in serum-free medium lacking dextran. Cells were fixed, viewed, and images were captured as above for Tf and LDL analysis.

The effects of Taxol on the trafficking of fluorescent ligands were monitored by treating L-MOCK cells on coverslips with the desired Taxol concentration (50-800 nM) subsequent to the 24 hour dexamethasone treatment, and then incubating with each fluorescent ligand exactly as above, still in the presence of Taxol.

Assays of transferrin recycling and LDL degradation

Human transferrin (Tf) was purchased from Sigma (St Louis, MO), and was purified on a sizing column (Sephacryl S-300) before being ^{125}-I-labeled as described by McGraw et al. (1987). Assays of the timing of approach to steady-state ^{125}-I-Tf uptake, the ratio of Tf receptor $(Tr)_{surface}$: $(Tr)_{internal}$ ratio, and the rate of Tf efflux were each performed as described by Johnson et al. (1994).

LDL was purified from human blood plasma and ^{125}-I-labeled as described by Bottalico et al. (1991); these materials were the generous gifts of Dr Ira Tabas and Nan Beatini (Columbia University). The protocol for the LDL degradation assay was described previously by Basu et al. (1981).

Positioning and movement of Golgi elements

L-cell transfectants were grown on coverslips, induced with dexamethasone for 1-2 days, fixed in 2% formaldehyde in PBS for 20 minutes, and permeabilized with 200 μg/ml saponin in PBS containing 1% calf serum. Coverslips were immunolabeled with antibody to mannosidase-II (Man-II; the generous gift of Dr Jennifer Lippincott-Schwartz, NCI), at a dilution of 1/200 to detect medial-Golgi elements, either without or with pretreatment with brefeldin A (BFA; 1 μg/ml, 15 minutes; BFA was obtained from Sigma). For monitoring recovery from BFA, coverslips were washed five times in serum-free medium, and then placed in BFA-free culture medium in a tissue culture incubator for 1-4 hours before fixation and staining. Images of Golgi staining were viewed and captured as described above for images of fluorescent ligands. Golgi distribution was scored as ‘dispersed’ or ‘aggregated’ by a naive observer at the microscope by through-focussing in each field and ascertaining how far Golgi elements lay from the periphery of the nuclear envelope. If all were clustered around the nucleus, no further than one-third nuclear diameter away, cells were scored as having aggregated Golgi, while if some elements were more distant, the cells were tallied among those with dispersed Golgi. A minimum of 200 cells were scored for each cell type and each experimental condition.

RESULTS

We previously noted that overexpression of MAP4 or its MT-binding domain, MTB, slows cell growth. Overexpression of MAP did not cause cell death, permanent withdrawal from the cell cycle, or inhibition of mitosis; instead, it appeared to slow progression through all phases of the cell cycle (Nguyen et al., 1997). We hypothesized that excess MAP4 might manifest this effect by inhibiting MT-dependent transport of membranous vesicles necessary for delivering growth signals, metabolites, or new plasma membrane components, or for delivering newly synthesized components to cytoplasmic sites of post-translational processing. Accordingly, we examined in vivo organelle motility in L-MAP4 and L-MTB cells, by observing living cells under high resolution DIC. As shown in Fig. 1A, in both high- and low-expressing L-MAP4 and L-MTB cells, in vivo organelle motility was inhibited, as compared to control cells, either L^{tk-} naive cells or L-MOCK cells transfected with plasmid alone. Within the limits of clonal variation in the cell populations, and accuracy of the measurements, the inhibition appeared to be dose-dependent; that is, less vesicle motility was observed in cell lines manifesting higher total content of MAP4 and higher ratios of MAP to tubulin. MTB was not as potent as the full-length MAP4 molecule on a mol/mol basis, since L-MTB-#3-6 cells, which express approximately three times as much MTB as exogenous MAP4 in L-MAP4-#13-2 cells (Nguyen et al., 1997) showed an inhibition of motility that was similar to that in L-MAP4-#13-2 cells.

Inhibition of in vivo vesicle motility could be a direct effect of the presence of exogenous MAP4, or an indirect effect of the stabilization of MTs by excess MAP4 or MTB. Therefore, we quantified the extent of MT stabilization that was conferred upon MTs by exogenous MAP4 or MTB; in the highest expressing cell line, L-MAP4-#13-2, in which we previously showed the

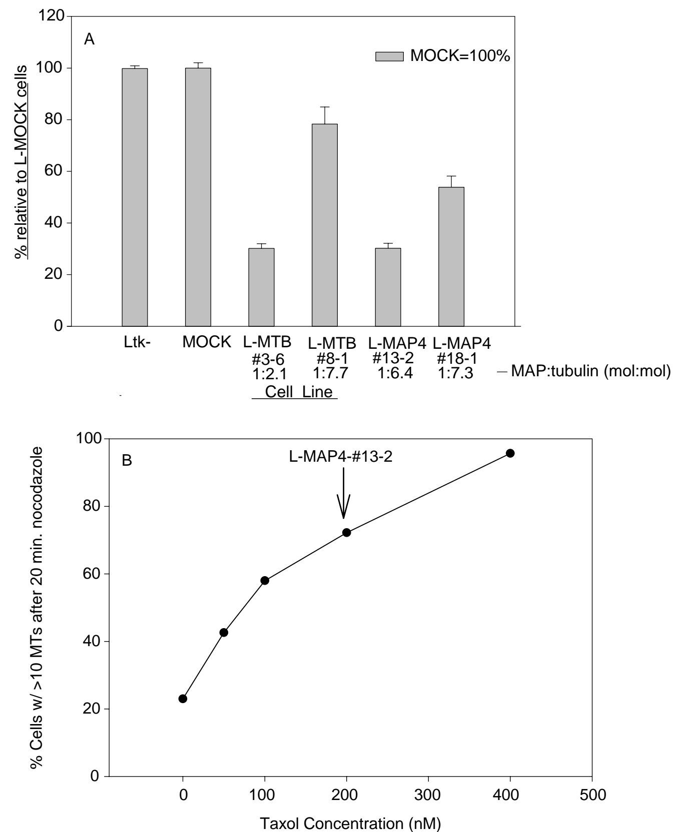

Fig. 1. (A) Overexpression of MAP4 or MTB inhibits vesicle motility in living cells. Living cells were observed and videotaped under high resolution DIC, as described in Materials and Methods. Vesicle movements in cells of the L^{tk-} parental line, L-MOCK-#2-E cells stably transfected with plasmid alone, and cells expressing high levels (L-MTB-#3-6, and L-MAP4-#13-2) or low levels (L-MTB-#8-1, and L-MAP4-#18-1) of MTB or full-length MAP4, respectively, were quantified and normalized to L-MOCK-#2-E cells (=100%). For each cell line, the level of expressed MAP is shown as a ratio (MAP:tubulin), with quantification data taken from Nguyen et al. (1997). Note that L-MAP4-#13-2 and L-MAP4-#18-1 have very similar ratios of MAP:tubulin; although L-MAP4-#13-2 cells expresses almost twice as much full-length MAP4 as L-MAP4-#18-1 cells they also express a higher level of tubulin. Error bars indicate s.e.m. for each set of measurements; note that at least three different experiments were performed for each cell line, and at least 20 areas of each of 7-10 cells were videotaped and scored for each data point shown. (B) Stabilization of MTs in MAP-expressing transfectants. Stability of MTs in L-MAP4-#13-2 cells, the MAP-overexpressing cells with the highest level of MT stability (Nguyen et al., 1997; MT stability is shown as an arrow in the figure), was compared to stability of MTs in L-MOCK-#2-E cells that had been pretreated with the indicated concentrations of Taxol for 4 hours. All cells were treated with 1 μM dexamethasone for 24-48 hours prior to the experiment. Error bars (s.e.m.) are not shown since they were smaller than the symbols used for graphical representation in each set of measurements.

maximum MT stabilization (Nguyen et al., 1997), MT stability conferred by MAP4 overexpression was roughly equivalent to stability conferred by treatment of cells with 200 nM Taxol (Fig. 1B). Hamm-Alvarez et al. (1993) previously demonstrated that stabilization of MTs by Taxol decreased vesicle movement, and Masurovsky et al. (1981) observed vesicles that appeared to be trapped along MTs in Taxol-treated neuronal cells. In agreement with the results of Hamm-Alvarez et al. (1993) on kidney epithelial cells, we found that high concentrations of Taxol (~800 nM) had to be applied to L-MOCK cells before there was a significant effect on vesicle transport, while a Taxol concentration of 200 nM inhibited in vivo vesicle motility only slightly (<13% inhibition; data not shown). The fact that very high concentrations of Taxol were required to produce effects similar to that of overexpressing MAP4 or MTB suggested that inhibition of vivo vesicle movement was caused by the overexpressed MAP, per se, rather than by MT stabilization alone.

In these studies, we had no means of measuring the amount of MTB or MAP4 that was bound to the surface of MTs in vivo. However, MTB and MAP4 were shown previously to stabilize MTs with the same efficacy in vitro (Aizawa et al., 1991). Therefore, it is reasonable to assume that the same molar level of MAP4 or MTB is bound to the MTs when the MTs show the same stability in vivo. The L-MAP4-#13-2 and L-MTB-#3-6 cells exhibit identical levels of MT stability, even though the L-MTB-#3-6 cells express threefold more MTB than L-MAP4-#13-2 cells express of full-length MAP4; Nguyen et al., 1997). Although we were surprised by the result that, in vivo, MTB is less potent than full-length MAP4 in binding to and stabilizing MTs (Nguyen et al., 1997), a focus on these two cell lines for the current study was convenient, since the results we report here are consistent with the notion that a similar level of coating of the MT surface occurs in cells of either line. Thus, when we compared the effects of MAP4 and MTB at similar levels of binding, we found a similar degree of inhibition of transport by MAP4 and MTB.

In order to determine what sort of vesicles were impeded in their movement by the increased cellular level of MAP4, we examined the behavior of ligands whose trafficking in cells is well understood. First, we incubated cells with fluorescein-conjugated transferrin (Tf) for 4 hours, a period sufficient to allow equilibration throughout the cell, then we released cells into fresh (chase) medium. Since Tf remains complexed with the Tf receptor (Tr) throughout its internalization and intracellular trafficking to the lysosome (Hannover and Dickson, 1985), images of fluorescein-Tf captured following chase provide an image of the trafficking of the Tr. As seen in Fig. 2, at steady state (t=0; a-c), in all cell types, including L-MOCK control cells, L-MTB and L-MAP4 cells, the Tf-Tr

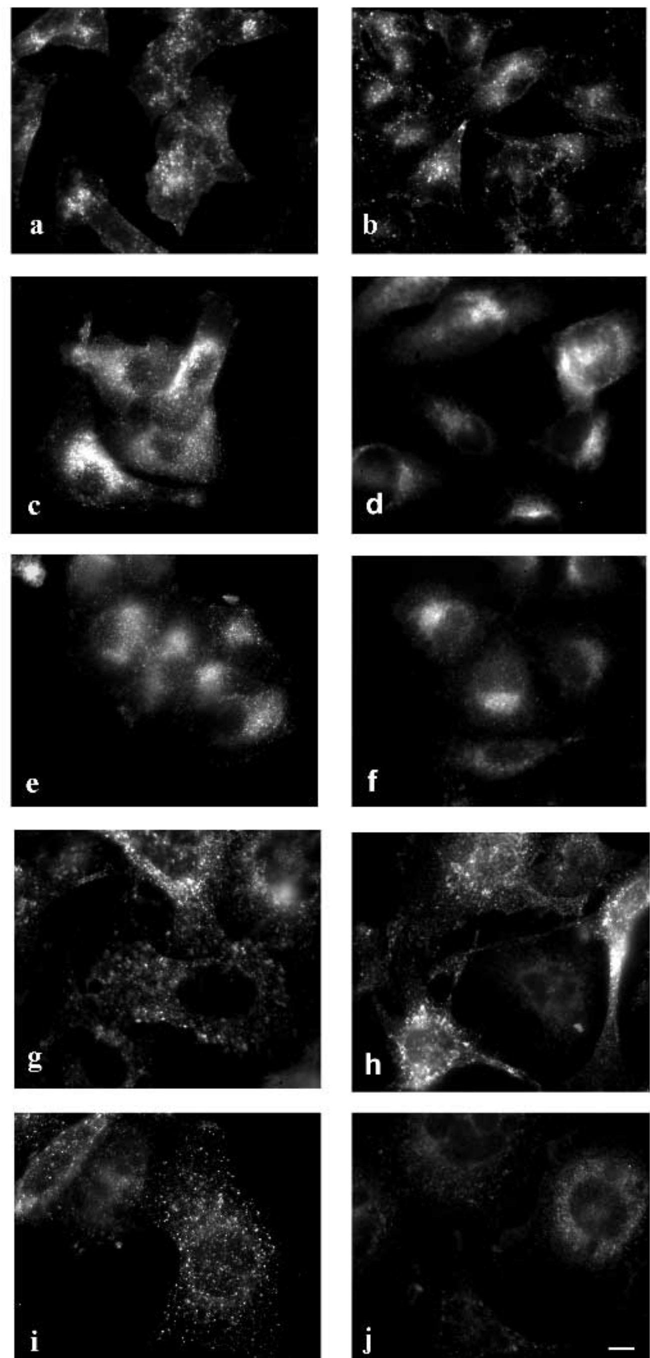

Fig. 2. Transferrin distribution is altered during recycling in cells overexpressing MAP4 or MTB. L-MOCK-#2-E control cells (a,b, g-j), cells overexpressing MTB (L-MTB-#3-6 (c,d), and cells overexpressing full-length MAP4 (L-MAP4#13-2 (e,f) were grown on coverslips, treated with dexamethasone for 24 hours, and preincubated in FITC-labeled transferrin (FITC-Tf), for 4 hours. Prior to incubation in FITC-Tf, L-MOCK cells whose images are shown in g,h and i,j were pretreated with Taxol for 4 hours (200 nM for g,h and 800 nM for i,j). At the start of the experiment (t=0, a,c,e,g,i) coverslips were washed and placed in medium lacking Tf for 60 minutes (t=60 minutes; b,d,f,h,j). Images of random fields of each cell type at each time point are shown, and the dim fluorescence at the 60 minutes time point has been enhanced so that the distribution of fluorescent label remaining could be assessed. Note that extensive fluorescence adjacent to the nucleus is present at t=0 and t=60 in all cell types, though the distribution of Tf fluorescence is more notably perinuclear in MAP-overexpressing cells than in L-MOCK cells at each time point (compare c and e to a, and d and f to b). Notice that, unlike MAP-overexpression, treatment with Taxol does not yield a significant clustering of Tf fluorescence near the nucleus (compare g-j with c-f). Tf distribution in Taxol-treated cells does appear abnormal (compare g-i with the control cells shown in a and b); however, the fact that Taxol-treatment does not have the same effect on Tf distribution as does MAP overexpression demonstrates that the effects MAP overexpression manifests on Tf distribution cannot be attributed to MT stabilization alone. Bar, 10 μm.

complex showed a roughly perinuclear distribution, with Tf fluorescence clustered around the MTOC. This result is consistent with the results of Yamashiro et al. (1984); however, in L-MTB and L-MAP4 cells the Tf fluorescence is more intensely focussed on the pericentriolar recycling compartment than it is in the L-MOCK control cells. Following release of Tf-loaded cells into fresh medium, the difference in distribution of Tf-Tr between MAP-overexpressing and control cells became more dramatic. As shown in Fig. 2D-F, the L-MOCK cells showed a uniform, dispersed distribution of fluorescent spots due to remaining Tf, with pericentriolar clustering observed in less than 10% of the cells. In contrast, the Tf that persisted showed a tightly clustered fluorescence around the nucleus in most L-MTB and L-MAP4 cells (>85% and >90%, respectively), suggesting that Tf persisted in the pericentriolar recycling compartment. These results suggested that distribution and movement of Tf-Tr are compromised in MAP4-overexpressing cells.

The abnormal distribution of Tf shown in Fig. 2 raised the possibility that there might be *quantitative* differences in such parameters as Tr number, distribution, or rate of recycling. To test this possibility, we assayed the uptake and trafficking of ^{125}-I Tf. We determined that the timing of approach to steady-state level of uptake of ^{125}-I Tf (≤1 hour), as well as the amount of surface labeling of Tf per cell ($6.16\pm0.80\times10^{-3}$ cpm/cell; proportional to the number of Tf-Tr on the cell surface) were both nearly identical for L-MOCK, L-MTB, and L-MAP4 cells. Next, we measured the distribution of Tr on the cell surface and inside the cell. Here again, the ratio $(Tf)_{surface}$:$(Tf)_{internal}$ was identical or within experimental error for all three cell lines (ratio was 0.51±0.019). Finally, since the observed clustering in the pericentriolar compartment following removal of extracellular Tf could conceivably retard Tf efflux, we measured the rate of recycling of Tf. Fig. 3 shows a pulse-chase experiment of cells that had been preloaded with ^{125}-I Tf. Within experimental error, the rate of recycling of ^{125}-I Tf was the same in L-MOCK, L-MTB, and L-MAP4 cells.

As in the case of the in vivo vesicle motility experiments (Fig. 1), we envisioned that the alteration in Tf distribution in cells expressing extra MAP4 (Fig. 2) might either be attributed to a MAP-induced alteration in MT stability, or to the presence of extra MAP4 or MTB per se. We determined that the presence of MAP4 or MTB, and not simply stabilization of MTs, was required in order to cause deficits in Tf distribution in vivo. Fig. 2g,h, shows that the distribution of Tf in L-MOCK cells treated with 200 nM Taxol, that is, a concentration of Taxol that effected MT stability equivalent to that measured in L-MTB and L-MAP4 transfectants, did not have the same effect on distribution or trafficking of Tf as did MAP overexpression. Even in cells treated with larger concentrations of Taxol (~800 nM; Fig 2i and j), in which Tf distribution looks markedly aberrant, we did not observe persistent juxtanuclear clustering of Tf, similar to the clustering we observed in MAP4-overexpressing cells (Fig. 2e,f).

To gain further insight into vesicle transport in L-cell transfectants, we tested uptake, movement, and degradation of another ligand, LDL. As shown in Fig. 4, probing the L-MAP4 and L-MTB transfectants with di-I-labeled LDL revealed that, even though the pattern of LDL fluorescence appeared to be normal in L-MOCK cells (Fig. 4a; compare to Tabas et al., 1990), the LDL was more clustered around the nucleus and somewhat brighter in both MAP4- and MTB-overexpressing cells (Fig. 4b,c). The perinuclear concentration of ligand observed in Fig. 4b,c, compared to the more homogeneous distribution of spots in control cells (Fig. 4a) was reminiscent of the altered distributions observed for Tf in the presence of either overexpressed MAP4 or MTB. As was the case for Tf, the differences in distribution in LDL could not be attributed to alterations in MT stability, as treatment with Taxol to impart equivalent or greater stability to cellular MTs did not give rise to significant clustering of LDL near the nucleus or, indeed, to an LDL distribution that resembled that in MAP4-overexpressing cells (Fig. 4d).

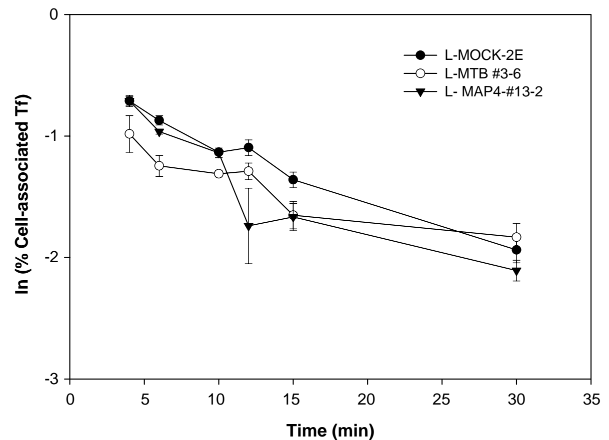

Fig. 3. Overexpression of MAP4 or MTB does not significantly affect the rate of transferrin recycling in L-MOCK cells. L-MOCK-#2-E control cells (●), cells expressing a high level of MTB and full-length MAP4 (L-MTB-#3-6 (○) and L-MAP4#13-2 (▼) cells, respectively) were incubated to steady-state level in ^{125}-I-labeled Tf, and then in Tf-free medium for an additional period, in order to measure Tf recycling, as described by Johnson et al. (1984). Recycling of Tf was plotted as the log of the percentage cell-associated Tf remaining at each time point. Measurements are shown with error bars indicating standard deviation. The rate of Tf recycling was not significantly altered in the presence of increased intracellular levels of MAP4 or MTB.

Differences in LDL distribution observed with fluorescent LDL, especially the decreased abundance of peripherally located spots of LDL fluorescence, suggested to us that the *rate* of LDL degradation might be detectably changed in L-MAP4 or L-MTB cells. Fig. 5 shows that this was the case: L-MAP4 cells exhibiting increased expression of MAP4 degraded approximately one-fourth as much LDL as control L-MOCK cells at 2 hours, while LDL degradation in L-MTB cells was inhibited to a more modest extent. Thus, in two types of receptor-mediated endocytic events we examined, the recycling of transferrin (Tf) and the uptake and degradation of low density lipoprotein (LDL), the presence of a larger than normal complement of MAP4 significantly affected the distribution of ligand, while only in the case of LDL was the rate of uptake and degradation significantly affected.

We also examined lysosomal sorting of dextran beads in the presence of overexpressed MAP4. Fig. 6 shows that cells preincubated in medium containing fluorescein-labeled dextran showed myriad bright spots coalesced near the nucleus (Fig. 6a), which spread throughout the cytoplasm after the dextran

had been washed out of the culture medium and had been targeted intracellularly to the lysosomes (Fig. 6b). Similar to what was observed with the fluorescent ligands, Tf or LDL, after preincubation in fluorescein-dextran, the fluorescence was slightly more concentrated near the nucleus in L-MTB (Fig. 6c) and L-MAP4 (Fig. 6e) than in L-MOCK cells (Fig. 6a). 2 hours after washout of the dextran, the perinuclear distribution of fluorescent dextran in lysosomes was more pronounced in L-MTB (Fig. 6d) and L-MAP4 (Fig. 6f) cells than it was in control cells (Fig. 6b). Thus, either trafficking or intracellular distribution of lysosomes, a third pathway involving vesicle movements that have been hypothesized to be MT-dependent (Hollenbeck and Swanson, 1990), appeared to be compromised in cells that overexpressed MAP4 or MTB.

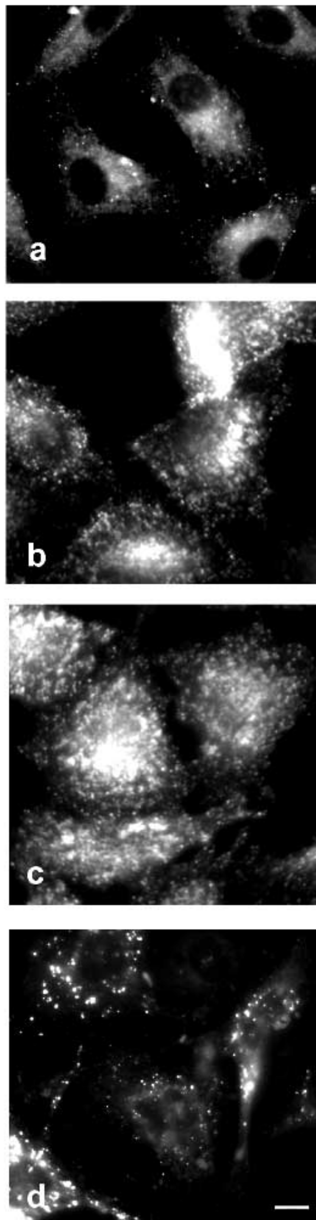

Fig. 4. LDL uptake and degradation in cells overexpressing MAP4 or MTB. L-MOCK-#2-E control cells (a), cells expressing a high level of MTB or full-length MAP4 (L-MTB-#3-6 (b), L-MAP4#13-2 (c), cells, respectively) and L-MOCK-#2-E cells pretreated with 200 nM Taxol (d) were incubated for 60 minutes in serum-free medium containing di-I-labeled LDL. Micrographs of random fields are shown. Bar, 10 μm. Note that extensive fluorescent spots adjacent to the nucleus are present after 60 minutes in MAP-overexpressing cells (b,c), as compared to the weaker, more dispersed dots of LDL fluorescence in L-MOCK cells (a). Stabilization of MTs by Taxol (d) does not have as dramatic an effect on LDL distribution as does overexpression of MAP4 or MTB (b,c); therefore, overexpression of MAP has effects beyond merely increasing stability of cellular MTs.

Movement of components from the endoplasmic reticulum (ER) to the Golgi, as well as movement between elements of the Golgi, is carried out by vesicles, and those Golgi elements that move in an anterograde direction (i.e. Golgi back to ER) have been shown to require kinesin for their translocation along MTs (Lippincott-Schwartz et al., 1995). Accordingly, we addressed the question of how distribution and movement of Golgi elements might be affected by overexpressed MAP4 or MTB. Fig. 7 shows the steady-state distribution of medial Golgi elements, visualized by labeling with mannosidase-II antibody in L-MAP4, L-MTB and L-MOCK cells. Note that the Golgi elements appear to be less clustered around the nucleus and more tubular in appearance in L-MTB and L-MAP4 cells than in L-MOCK control cells (compare Fig. 7a,b, to f,g, and k,l). We analyzed the Golgi distribution quantitatively, as shown in Table 1. Significantly more L-MOCK control cells (53.8%) than L-MTB or L-MAP4 cells (38.7%, 39.6%, respectively) showed Golgi clustered near the MTOC (visualized in preparations double-stained for tubulin, data not shown).

To examine the distribution and motility of Golgi elements, we treated all three cell lines with the drug, brefeldin A (BFA), which causes the Golgi to become tubular and to merge with or extend out along elements of the ER (Lippincott-Schwartz et al., 1989). We examined the distribution of medial Golgi elements (Fig. 7) and compared this to MT distribution (not shown). The Golgi elements of all three cell lines were similarly affected by a 15 minute BFA treatment (Fig. 7, compare c,h,m);

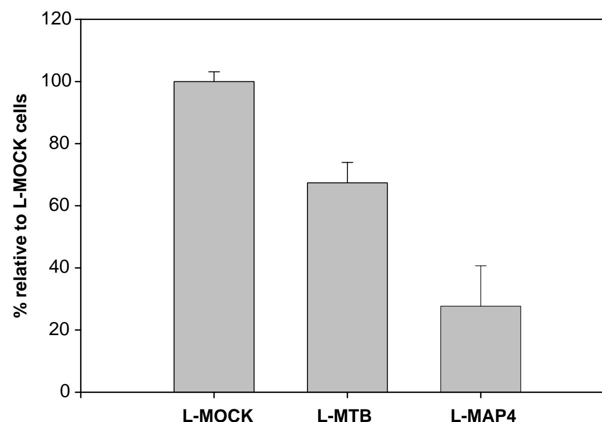

Fig. 5. Quantification of LDL uptake and degradation in cells overexpressing MAP4 or MTB. Uptake and degradation of LDL was measured in control cells (L-MOCK-#2E) and cells expressing a high level of MTB or full-length MAP4 (L-MTB-#3-6 and L-MAP4-#13-2 cells, respectively) as described by Basu et al. (1981). Note that the degradation of ^{125}-I-labeled LDL in each culture, normalized for cell number, was significantly inhibited in those cells overexpressing MAP4, and to a lesser extent, in those expressing MTB. Error bars indicate standard deviation for each set of measurements.

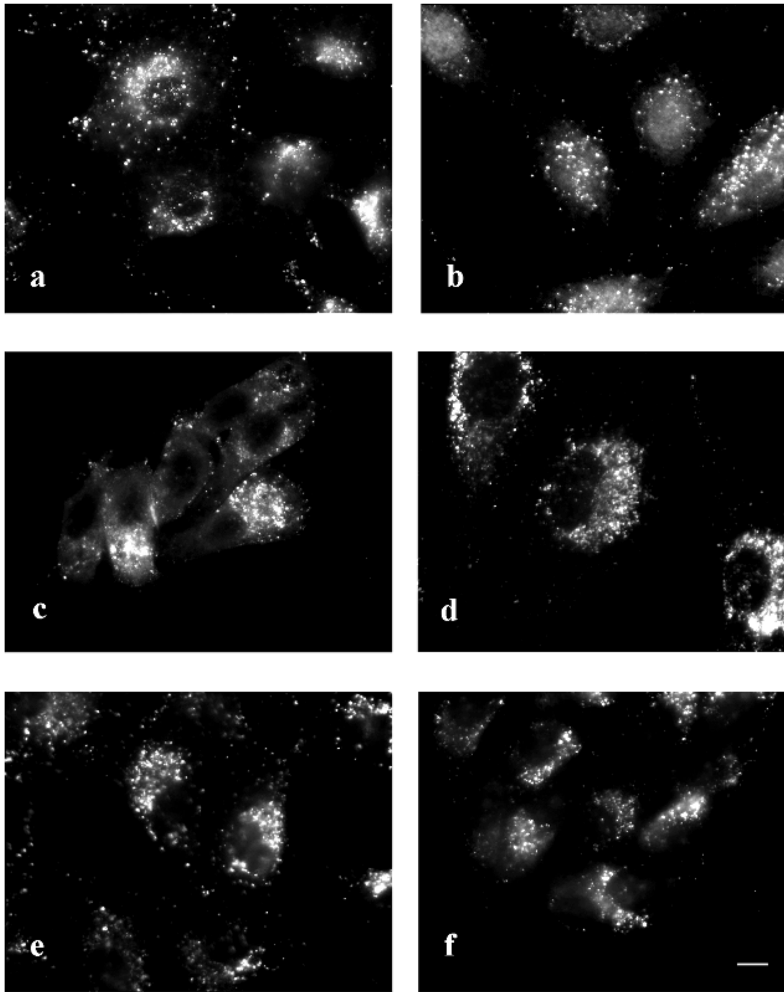

Fig. 6. Recycling to lysosomes is altered in MAP-overexpressing cells. L-MOCK-#2-E (a,b), L-MTB-#3-6 (c,d), and L-MAP4#13-2 cells (e,f), pretreated with dexamethasone for 24 hours, were preincubated in 10 μg/ml FITC-labeled dextran, overnight. At time=0 (a,c,e), they were washed twice and placed into serum-free medium without dextran for 2 hours (b,d,f). At the indicated times, cells were fixed in 3.7% formaldehyde and dextran fluorescence was photographed. Note that more extensive fluorescence adjacent to the nucleus is present at both time points in MAP-overexpressing cells, as compared to the more dispersed lysosomal-associated fluorescence in L-MOCK cells. Bar, 10 μm.

tubulation and dispersion occurred, and the Golgi elements appeared as dots or strands rather than as the dense aggregated structure more commonly observed in untreated control cells (Fig. 7a,b). When we examined cells that had been allowed to recover from BFA treatment for 1.5 hours, we found that the Golgi recovered slowly and/or less completely in the L-MTB and L-MAP4 cells, as opposed to the efficient recovery of normal Golgi morphology in the L-MOCK control cells (compare Fig. 7d,i,n). Recovery for 3 hours allowed reclustering of Golgi to the same level as was observed before treatment (compare e,j,o to b,g,l). Results of the BFA treatment experiments are compiled in Table 1. Our results clearly show that the presence of more than the normal complement of full- or partial-length MAP4 inhibits the normal and BFA-induced movement of Golgi elements along MTs in vivo. Thus, defects in one or all of four pathways of vesicle movement and sorting may explain the decreased movement of vesicles that we originally observed in MAP4 transfectants (Fig. 1A).

Table 1. Distribution and movement of Golgi elements in response to brefeldin A treatment

	Clustered Golgi elements (%)		
Treatment	L-MOCK-#2E	L-MTB-#3-6	L-MAP4-#13-2
Control	53.8	38.7	39.6
BFA-treated	30.7	18.9	20.5
BFA-treated+1.5 hour recovery	62.2	30.0	10.8
BFA-treated+3 hour recovery	64.4	40.3	41.6

Cells with clustered Golgi elements were defined as those cells in which no distinct Golgi staining further than one-third nuclear diameter away was detected in a through-focus series, while if some elements were more distant, the cells were tallied among those with dispersed Golgi. A minimum of 200 cells were scored for each cell line and each experimental condition.

DISCUSSION

The inhibition of vesicle movements we have observed in the presence of overexpressed MAP4 can best be interpreted in light of the three mechanisms of inhibition suggested by previous studies; steric inhibition, competitive inhibition, and alteration of MT dynamics. In the in vitro motility system of Lopez and Sheetz (1993), inhibition of vesicle transport by physiological concentrations of MAP was steric, since neither the MTB fragment of MAP2, which lacks a projection domain, nor the entire tau molecule, which has a very short projection domain, inhibited motility significantly, as full-length MAP2 did. In contrast, the

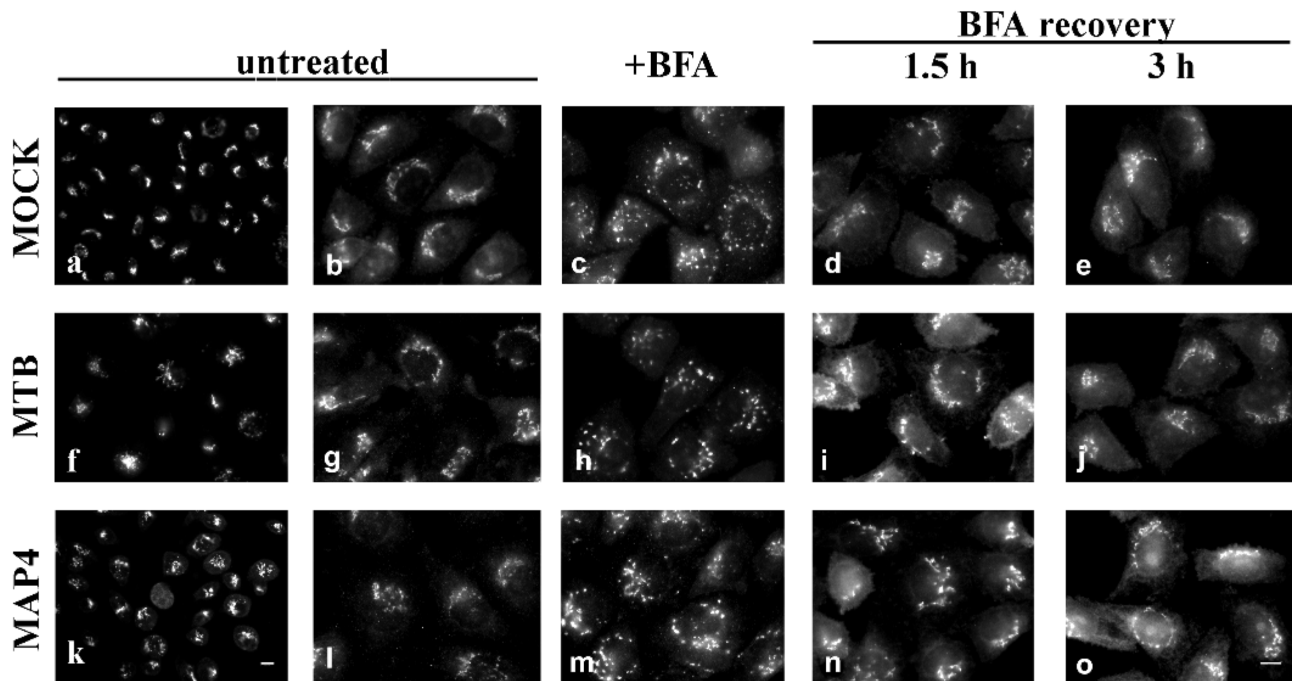

Fig. 7. Golgi elements show unusual steady-state distribution and recovery from BFA in MAP-overexpressing cells. Immunolocalization of medial Golgi elements with anti-mannosidase-II antibody in L-MOCK-#2-E (a-e), L-MTB-#3-6 (f-j), and L-MAP4#13-2 (k-o), either without BFA (a,b,f,g,k,l), following pretreatment with 1 μg/ml BFA for 15 minutes (c,h,m), or pretreatment with BFA and washout from BFA for 1.5 hours (d,i,n) or 3 hours (e,j,o). Note that, in MAP4 and MTB-overexpressing cells, Golgi elements appear to be less clustered at steady state, and recovery from BFA also appears to be reduced or retarded. Bars: in k, for a,f and k, 10 μm; in o, for remaining panels, 10 μm.

studies from the Vallee and Mandelkow labs (Paschal et al., 1989; von Massow et al., 1989) suggested that, at much higher concentrations, even small domains of MAPs could limit motility, perhaps by inhibiting binding interactions of motor proteins. Other experiments (Hamm-Alvarez et al., 1993), in which Taxol treatment of cells reduced vesicle transport, suggested that alterations in MT dynamics could also decrease vesicle motility.

In our wild-type and transfectant cells, respectively, steric inhibition would be expected to occur as vesicles travelling along a MT encountered the projection domain of 50-350 molecules of full-length MAP4 in each micron of transit. In contrast, MTB at either high or low concentrations would not be expected to yield significant steric inhibition. Binding of MAP4 or MTB to MTs could equally inhibit MT-binding of other molecules involved in vesicle transport, such as CLIP-170 (Rickard and Kreis, 1990) or $P150_{glued}$ (Waterman-Storer et al., 1995) if the MT binding sites overlapped. Whether the MT-binding sites of these molecules physically overlap that of MAP4 is unknown. Although binding of motors or other molecules might also be inhibited in the presence of MTB, with negligible steric inhibition brought about by the smaller MTB molecule, inhibition of MT-dependent transport might only occur at extremely high ratios of MTB to tubulin, such as the 800 molecules that are present per micron of MT in the L cell line expressing the highest level of MTB (L-MTB-#3-6 cells; Nguyen et al., 1997). Finally, since MT binding of MTB and MAP4 affect MT dynamics, alterations equally dependent upon the overexpression of MTB or MAP4 could result from effects on MT dynamics, similar to the inhibition brought about by Taxol. Comparisons of the effects of MAP4 and MTB overexpression versus effects of Taxol addition provide a means of differentiating whether access to MTs, dynamic behavior of MTs, or a specific binding interaction are critical to the MT-based motility events in question. Our results, demonstrating that overexpression of MAP4 and MTB both inhibit MT-based vesicle motility in vivo, provide insight concerning the mechanism of this inhibition. For example, some dynamic processes, such as uptake and degradation of LDL (Figs 4 and 5), and reformation of the Golgi apparatus upon recovery from BFA treatment (Fig. 7), appear to be affected more acutely by full-length MAP4 than by MTB. This is most consistent with the steric inhibition mechanism proposed earlier from in vitro work (Lopez and Sheetz; 1993), and is inconsistent with competitive inhibition of MT binding sites or MT stabilization mechanisms.

In contrast, some processes involving vesicle distribution were affected similarly in cells exhibiting high level expression of either full-length MAP4 or MTB. For example, several ligands, including transferrin, LDL, and dextran, showed similar pericentriolar clustering of ligands in cells whose MTs were stabilized equivalently by similar quantities of exogenous bound MAP4 or MTB (Figs 2, 4, and 6). These data are most consistent with an inhibition of MT-binding of these ligands by MAP4; inhibition of binding could compromise the anchoring of vesicles to MTs. The similar effects of MTB and MAP4 on total level of vesicle motility (Fig. 1A) were perplexing initially. Most likely the similarity in effects is due to a rate-limiting step that requires MT-binding and is therefore affected equivalently by a comparable density of MAP (MTB or MAP4) covering the MT surface. Although we have no direct measure of the fraction of MAP4 or MTB actually bound to the MTs in the highest overexpressing cells, our working assumption that there is a comparable *density* of MAP4 or MTB bound to the MTs in the L-MAP4-#13-2 and

the L-MTB-#3-6 cells is a reasonable one, since in each of these cell lines, the MTs are stabilized to an equivalent degree. Equal occupancy of MT-binding sites by MAP4 or MTB is also consistent with our current findings: in no case was there a greater effect of MTB than MAP4 (even though the total level of MTB expressed in L-MTB-#3-6 cells was threefold higher than the level of MAP4 in L-MAP4-#13-2 cells), and there were several instances in which the effects of MTB and MAP4 were virtually identical. The total vesicle transport measurements we reported in Fig. 1A involve the visualization of many different membrane traffic events, of which receptor recycling represents only a small fraction. The process of membrane traffic in cells shows a linkage (particularly in rate) between the two directions of transport and even the processing steps that couple them (Sheetz and Yu, 1996). Thus, other steps in the cycle, such as the processing of membranes to switch direction of movement, could be rate-limiting and would be expected to show inhibition upon coating of the MT surface with MAP molecules.

Alternatively, MT dynamics itself could be involved in inhibiting vesicle movement, if Taxol treatment had a similar effect on motility or distribution of any ligand. However, we found no evidence that alterations in MT dynamics, that is, stabilization by Taxol, manifested effects analogous to effects of MAP4 overexpression.

What implications does MAP4's ability to inhibit receptor recycling have for cell function or behavior? First, previous studies have indicated that recycling of many types of receptors and redistribution of Golgi elements are MT-dependent processes. In particular, Golgi reformation after BFA treatment requires the presence of MTs (Lippincott-Schwartz et al., 1995). Other seven transmembrane domain receptors also show MT-dependence of recycling, including the bradykinin receptor (Hamm-Alvarez et al., 1994) and atrial natriuretic factor (ANF; Iida et al., 1988). The recycling rate of each of these is known to be decreased by two- to fourfold upon inhibition of MT-dependent organelle transport. Thus, inhibition of vesicle motility by MAP4, that is, increasing recycling times for any of these receptors, would be expected to change cell behavior by reducing ligand responsiveness of the cell significantly.

The changes noted in the equilibrium distribution of the Golgi and the receptor compartments, which were impacted upon similarly by both full-length MAP4 and MTB are, to a milder degree, the same changes observed in previous studies, in cells that had been subjected to more drastic treatments; i.e. cells treated with concentrations of nocodazole or Taxol that completely depolymerized or completely stabilized MTs, respectively (e.g. Achler et al., 1989; Eilers et al., 1989; Hamm-Alvarez et al., 1993; Thatte et al., 1994; Rossignol and Raymond, 1994; Fath et al., 1993). Our MAP4 inhibition results could be physiologically significant, since the inhibition of vesicle transport or trafficking events we have observed occurred in the presence of a MT array that was quite normal, both in appearance and dynamics. In contrast, many studies in which inhibition of vesicle transport has been reported were performed in the absence of MTs or at the expense of all dynamics of MTs (e.g. Thatte et al., 1994; Rossignol and Raymond, 1994).

Our finding that the Golgi apparatus is partially disrupted in cells that overexpress MAP4 or MTB is consistent with the notion that the Golgi compartment is stabilized within the pericentriolar space by attachment to MTs. Similarly, receptors such as Tf, LDL, and ANF are inhibited in their movement from the central compartment to peripheral recycling compartments, perhaps because those peripheral compartments have also been stabilized by binding to MTs. These alterations in the equilibrium distribution of compartments may also affect the efficiency of recycling and Golgi function. Assuming that the Golgi distribution has been optimized in wild-type cells in order to maximize efficiency of Golgi function, then alteration of that distribution would be expected to compromise function.

The distinction between dynamic processes that are preferentially inhibited by full-length MAP4 and equilibrium distributions that are altered nearly equally by MTB and MAP4 is useful for defining different aspects of MT function. Of course, another reason differential effects might be observed on different endocytic and sorting pathways is that the activity of dynein, kinesin, members of the kinesin superfamily, or non-motor molecules such as CLIP-170 and $p150_{glued}$ might be differentially affected by the presence of MAP4. Further work would have to be performed to determine whether MAP4 and MTB affect the interaction of each of these molecules with MTs to a similar degree.

Second, our results are consistent with the notion that the cell growth inhibition we measured in MAP4- and MTB-overexpressing cells (Nguyen et al., 1997) is caused by hampered MT-based transport, that is, an effect of *covering* the MT with MAP. Growth inhibition suggests that defects may be most pronounced in transport of those vesicles carrying nutritionally necessary substances such as LDL. Interestingly, the growth rates are similarly slowed in the cells whose MTs contain high levels of either MAP4 or MTB bound to them (Nguyen et al., 1997). This correlation suggests a connection between cell growth and overall level of vesicle motility. At this point, however, we do not know whether alterations in distribution or transport of vesicles by excess MAP4 are sufficient to explain the deficits in cell growth.

Finally, MAP4's inhibition of vesicle motility has implications for cell behavior: our results suggest a mechanism by which cells could achieve polarization, if MAP4 or other MAPs actually functioned to limit transport along individual MTs or groups of MTs in a single cell. In this model, MT subsets with locally high or low concentrations of MAP4 along different fibers would impact upon transport of membranous vesicles, and even more dramatically upon the recycling of receptors along those MTs. In previous work, fibers depleted in MAP4 were detected (Chapin and Bulinski, 1994), and these actually predominated within cellular processes. At least two possibly related kinases capable of releasing MAPs from MTs, called embryonic brain kinase (Lopez and Sheetz, 1995) and MARK (Illenberger et al., 1996), have been described. Each of these is capable of removing MAPs from MTs, potentially generating fibers with heterogeneous levels of MAP4 on them. In fact, transfection of CHO cells with MARK results in breakdown of cellular MTs, presumably by removal of MAPs (Drewes et al., 1997). Alternatively, extensive end dynamics, in which MTs depolymerize near their ends and MAP4 rebinds at higher concentration to the shortened MT, could provide a stochastic heterogeneity in MAP4 composition that would also be capable of altering transport efficiency on individual MTs. In either case, heterogeneous MAP4 content along individual MTs or zones of MTs could be utilized by cells undergoing polarization or shape changes. For example, depleting MAP4 from particular MTs would be predicted to increase vesicle transport along that MT and could be a necessary step in generating a MT-filled process or neurite. This mechanism is now

amenable to a direct test. The results we have presented here show that MAP4 can affect transport along MTs, particularly in receptor recycling, and suggest that it could play such a role in vivo, perhaps in altering vesicle motility leading to plasma membrane growth, or in sprouting or elongation of neurites.

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