Journal of Cell Science 110, 2373-2380 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS7112

Expression of a minus-end-directed motor protein induces Sf9 cells to form axon-like processes with uniform microtubule polarity orientation

David J. Sharp[1], Ryoko Kuriyama[2], Russell Essner[2] and Peter W. Baas[1,*]

[1]Department of Anatomy and Program in Neuroscience, The University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, USA
[2]Department of Cell Biology and Neuroanatomy, The University of Minnesota Medical School, Minneapolis, MN 55455, USA

*Author for correspondence (e-mail: pwbaas@facstaff.wisc.edu)

SUMMARY

Neurons extend two types of processes with distinct morphologies and patterns of microtubule polarity orientation. Axons are thin cylindrical processes containing microtubules that are uniformly oriented with their plus-ends-distal to the cell body while dendrites are stout tapering processes that contain nonuniformly oriented microtubules. We have proposed that these distinct microtubule patterns are established by molecular motors that transport microtubules into each type of process with the appropriate orientation. To test the feasibility of this proposal, we have embarked on a series of studies involving the expression of vertebrate motors in insect Sf9 cells. We previously focused on a kinesin-related protein termed CHO1/MKLP1, which localizes to the midzone of the mitotic spindle, and which has been shown to have the appropriate properties to transport microtubules of opposite orientation relative to one another. Expression of a fragment of CHO1/MKLP1 containing its motor domain induces Sf9 cells to extend processes with a stout tapering morphology and a nonuniform microtubule polarity pattern similar to dendrites. Here we focus on a minus-end-directed kinesin-related motor protein termed CHO2, which localizes to the nonoverlapping regions of the mitotic spindle, and which has been shown to have the appropriate properties to transport microtubules with plus-ends-leading. Sf9 cells induced to express a fragment of CHO2 containing its motor domain extend processes with a long cylindrical morphology and a uniformly plus-end-distal microtubule polarity pattern similar to axons. These results show that motor proteins have the capacity to organize distinct patterns of microtubule polarity orientation during process outgrowth, and that these patterns are intimately related to the unique morphological characteristics of the processes. Moreover, mutation of three amino acids corresponding to the ATP binding site necessary for motor function suppresses the capacity of the CHO2 fragment to induce process formation and microtubule reorganization, indicating that at least in the case of CHO2, the transport properties of the motor are essential for it to elicit these effects.

Key words: Microtubule, Kinesin, Motor, Axon, Neuron, Mitosis, Spindle

INTRODUCTION

Neurons generate two different types of processes, long cylindrical axons and stout tapering dendrites. The microtubules (MTs) within axons and dendrites differ with regard to their patterns of polarity orientation. Axonal MTs are uniformly oriented with their plus-ends-distal to the cell body (Heidemann et al., 1981), whereas dendritic MTs are nonuniformly oriented (Baas et al., 1988). These distinct MT polarity patterns provide a structural basis for the transport of different complements of cytoplasmic constituents into these processes (Black and Baas, 1989), and in theory could contribute to the unique morphological specializations of axons and dendrites. We have proposed that the polarity orientation of an individual MT within these processes is determined by the molecular machinery that transports it from the cell body into the process (Baas and Ahmad, 1993; Sharp et al., 1995). In this view, specific motor proteins interact with MTs within the cell body and transport them either with plus-ends-leading into axons and dendrites or minus-ends-leading exclusively into dendrites. Evidence for motor proteins with the appropriate properties to transport MTs with each of these orientations derives from the mitotic spindle, the formation and functioning of which involves a number of different molecular motors.

To test the feasibility of our proposal, we have embarked on a series of studies in which vertebrate motor proteins are ectopically expressed in normally rounded insect ovarian Sf9 cells. The goal of these studies is to ascertain whether motor-driven MT transport can support the outgrowth of cellular processes, and to determine whether the specific transport properties of the motors establish distinct patterns of MT polarity orientation. In the first of these studies, we focused on CHO1/MKLP1 (Sellitto and Kuriyama, 1988), a mitotic kinesin-related motor that has been shown to transport MTs with minus-ends-leading against other MTs of opposite orientation in vitro (Nislow et al., 1992). When expressed in Sf9 cells, an N-terminal fragment of CHO1/MKLP1 containing the motor domain of the molecule induces the formation of

processes containing MTs of nonuniform polarity orientation (Kuriyama et al., 1994; Sharp et al., 1995). Interestingly, these processes have a stout tapering morphology similar to dendrites, suggesting a causal relationship between MT polarity pattern and morphology. In the present study, we have focused on another mitotic motor protein with very different transport properties. This motor, termed CHO2, is an unusual kinesin-related protein in that its motor domain is located at its C terminus rather than at its N terminus (Kuriyama et al., 1995). Moreover, unlike most kinesin-related proteins, CHO2 is a 'minus-end-directed' motor that has been shown to transport MTs with their plus-ends-leading in vitro. Our studies demonstrate that a fragment of the CHO2 molecule containing its motor domain also induces the formation of MT-rich processes when expressed in Sf9 cells. Comparison of the processes induced by these two different motors affords a unique opportunity to evaluate the merits of our proposal that the transport properties of the motor determine the polarity orientation of MTs within the processes that it generates, and in turn whether the polarity pattern of the MTs contributes to the unique morphological characteristics of the processes.

MATERIALS AND METHODS

Cell culture and baculovirus infection

Sf9 cells were maintained in TNM-FH medium, which consists of Grace medium purchased from Gibco/BRL (Grand Island, NY), 0.33% yeastolate, 0.33% lactalbumin hydrolysate, and 10% fetal bovine serum, pH 6.2 (Kuriyama et al., 1994). The cells were maintained in plastic tissue culture flasks, and then subcultured onto plastic tissue culture dishes for electron microscopy or glass coverslips pre-treated with polylysine (Sigma Chemical Company, St Louis, MO) for immunofluorescence analyses. Baculovirus constructs encoding the entire CHO2 molecule or roughly 3/4 of the molecule including the C-terminal region were generated as previously described (fragment Δ1 described by Kuriyama et al., 1995). An additional baculovirus construct termed Δ1′ was generated by site-directed mutagenesis as described below. Medium containing the viral particles was added directly to plated cells.

Site-directed mutagenesis

To ascertain whether the results obtained with the Δ1 fragment of CHO2 are dependent upon its transport properties, we generated a mutant identical to Δ1 with the exception of three amino acids corresponding to part of the ATP binding site necessary for motor function. Specifically, amino acids 365, 366, and 367 were changed from G, K, and T into alanine residues, and the resulting mutant was termed Δ1′. It has been established that, as expected, mutation of the ATP binding site of a motor protein obliterates its MT transport properties (Nakata and Hirokawa, 1995). The mutant CHO2 fragment was generated by PCR using the ExSite PCR-based site-directed mutagenesis kit (Strategene, La Jolla, CA). Briefly, the cDNA of the CHO2 fragment was subcloned into pBluescript and amplified with Taq polymerase and taq extender (Strategene) by the following two primers:

(1) 5′-GcC gcG gCC TTC ACT ATG GAG GGC A-3′ (small letters indicate mutated bases);

(2) 5′-ACT GCC GGT CTG TCC GTA GGC AAA-3′.

The primer #1 corresponds to nucleotide positions 1,093-1,116 and the primer #2 is complementary to nucleotide positions 1,069-1,092 of the CHO2 open reading frame. After digestion of the parental template and hybrid DNA by *Dpn*I, end-polished mutation-containing DNA was synthesized with *pfu* DNA polymerase. The site of the mutation was confirmed by DNA sequence analysis, and the mutated CHO2 fragment was further cloned into pVL1392.

Immunofluorescence microscopy

As in our previous study on CHO1/MKLP1 (Sharp et al., 1996), immunofluorescence staining of Sf9 cells was performed using one of two methods. In the first method, cells were fixed for 5 minutes in cold methanol (–20°C), transferred to cold absolute acetone (–20°C) for 5 minutes, air-dried, and then rehydrated in PBS containing 0.05% Tween-20 (Kuriyama et al., 1994). The cells were then exposed either to a mouse monoclonal β-tubulin antibody alone (Amersham, Arlington Heights, IL) or together with a rabbit polyclonal antiserum that recognizes CHO2 (Kuriyama et al., 1995) or CHO1/MKLP1 (see Sharp et al., 1996) in double label experiments. In the second method, cells were pre-extracted under conditions that remove free tubulin but stabilize existing MTs, and then fixed as previously described (Sharp et al., 1995). The cells were then exposed to the mouse monoclonal β-tubulin antibody noted above. At this point, cultures prepared by these methods were rinsed free of primary antibodies, and exposed to appropriate fluorescent second antibodies as previously described (Kuriyama et al., 1994; Sharp et al., 1995). For quantitative analyses on MT levels in the pre-extracted cells, images were captured with the Zeiss LSM 410 laser confocal microscope (Carl Zeiss Incorporated, Thornwood, NY). The pinhole was opened minimally to allow the highest resolution of fluorescently labeled proteins, serial optical sections were taken through the entire cell, and the images were then reconstructed by Zeiss system software. This resulted in complete visualization of fluorescently labeled material in a single high-resolution image. Fluorescence intensities were quantified using NIH Image software (provided free of charge from the National Institutes of Health, Bethesda, MD). Fluorescence intensities were calculated for uninfected cells, infected process-bearing cells, and infected cells that grew processes in the presence of vinblastine sulfate (see below), and expressed in arbitrary fluorescence units (AFU). Ten cells were analyzed for each of these conditions.

Electron microscopy

Cultures were prepared for electron microscopy by conventional means. Briefly, they were fixed for 20 minutes as indicated in 0.1 M cacodylate containing 2% glutaraldehyde, rinsed twice for 5 minutes each in 0.1 M cacodylate, postfixed for 10 minutes in 1% OsO_4, rinsed twice for 2 minutes each in NaCl, rinsed twice for 2 minutes each in water, contrasted for 30 minutes in 5% aqueous uranyl acetate, dehydrated in ethanols, and embedded in LX-112 (Ladd Research Industries, Burlington VT). After curing overnight, plastic tissue culture dishes were removed. Cells of interest were circled with a diamond marker objective and their images were recorded using a thermal video-printer (Sony, Japan). Thin sections of a uniform thickness of 100 nm were obtained with an Ultracut S Ultramicrotome (Reichert-Jung, Vienna, Austria), picked up on Formvar coated slot grids, stained with uranyl acetate and lead citrate, and observed with a CX-100 electron microscope (JEOL USA, Inc., Peabody, MA).

Microtubule polarity studies

As in our previous study on CHO1/MKLP1 (Sharp et al., 1996), MT polarity analyses were performed by a modification of a previously described method (Euteneuer and McIntosh, 1982; Heidemann, 1991). This method involves the decoration of existing MTs with exogenous brain tubulin using a buffer that promotes the formation of lateral protofilament sheets. These sheets appear as curved appendages termed 'hooks' on the MTs when viewed in cross-section under the electron microscope. A clockwise hook indicates that the plus-end of the MT is directed toward the observer, while a counterclockwise hook indicates that the minus-end is directed toward the observer. Cultures were rinsed briefly in PBS and then incubated at 37°C for 20 minutes in a solution containing 0.25% saponin, 0.5 M Pipes, 0.1 M EGTA, 0.01 mM EDTA, 0.1 mM $MgCl_2$, 2.5% DMSO, 0.5 mM GTP, and 1.2 mg/ml brain tubulin. Cultures were then fixed by the addition of an equal quantity of 4% glutaraldehyde, and then processed and embedded for electron microscopy by conventional methods. Video-

print images were obtained prior to sectioning, and these were used to precisely document the points along the lengths of processes at which cross-sections were made. The sections were visualized and photographed using a JEOL CX100 electron microscope (JEOL USA, Inc., Peabody, MA).

Pharmacologic studies

In one set of experiments, MT assembly was suppressed by the addition of vinblastine sulfate (Sigma Chemical Co., St Louis, MO) to cultures at a final concentration of 10 nM. Vinblastine was added at the time of baculovirus infection.

RESULTS

Sf9 cells are small rounded mitotic cells that typically do not extend processes. A small number of the cells within a culture (<1%) extend short processes, <20 μm in length, presumably due to spontaneous mutation. In our previous studies, we established that cultures exposed to a baculovirus construct encoding the entire CHO1/MKLP1 molecule do not show an incease in the frequency of process generation, nor do they show marked changes in MT organization. However, when the cells are exposed to a construct encoding roughly half of the molecule containing its N-terminal motor domain, the proportion of cells that extend processes increases to 10-40% (Kuriyama et al., 1994; Sharp et al., 1996). In the present study, we have found comparable results with CHO2. Expression of the full-length molecule did not induce an increase in the frequency of process formation, while expression of a fragment of the molecule (termed Δ1) containing its C-terminal motor domain resulted in process formation from 10-40% of the cells. In this case, the fragment corresponded to roughly 3/4 of the molecule containing the motor domain and a portion of the central stalk (see Kuriyama et al., 1995). These results were obtained in analyses of over 1,000 cells from four separate experiments. Immunostain analyses with antibodies to the motor proteins indicate that the proportion of cells expressing detectable levels of the motor fragments is generally high (>90%), but that some of the variability in the proportions of cells extending processes probably relates to variability among different experiments in the proportion of cells expressing the motor protein fragments and possibly the levels of the protein expressed.

Processes induced by the CHO1/MKLP1 fragment and the CHO2 fragment differed from one another in both their rates of development and their morphology. The processes induced by the CHO1/MKLP1 fragment began to develop within the first 2 days of infection, and generally stopped growing by the third or fourth day. In contrast, the processes induced by the CHO2 fragment generally did not begin to develop until the fourth day of infection, and appeared to continue growing nonstop until the death of the culture roughly seven to eight days postinfection. (Baculovirus infection causes the cells to stop dividing, reduce normal protein synthesis, and die typically by the eighth day postinfection.) In terms of morphology, the earliest processes generated by either motor fragment showed a similar short, thin, non-tapering morphology. Fig. 1A shows a newly initiated process induced by the CHO2 fragment which illustrates this morphology. With time, however, the processes generated by the CHO1/MKLP1 and the CHO2 fragments showed markedly different morphological characteristics. The processes generated by the CHO2 fragment remained thin and cylindrical and grew to lengths often exceeding 300 μm (Fig. 1B,C). In contrast, the processes induced by the CHO1/MKLP1 fragment were typically conical; they were thick at the cell body and tapered with distance from it. Moreover, these processes were significantly shorter than processes generated by the CHO2 fragment, rarely exceeding 80 μm in length (Fig. 1D-F). It was our impression that the processes generated by the CHO2 fragment stopped growing as a result of the death of the culture, whereas the processes generated by the CHO1/MKLP1 fragment stopped growing after reaching a certain length, and that this was independent of the death of the culture. No additional efforts were made to quantify the lengths of the processes beyond these observations, because it would be difficult to make meaningful comparisons (beyond maximum length) given the different timetables by which the two different motor fragments induced process outgrowth.

Microtubule distribution and organization in motor-induced processes

A key finding in our previous studies was that expression of the N-terminal fragment of CHO1/MKLP1 induces a redistribution of MTs. In control Sf9 cells, MTs are distributed throughout the cell body, resulting in a diffuse immunofluorescence staining pattern (Knops et al., 1991; Kuriyama et al., 1994; see Fig. 2A).

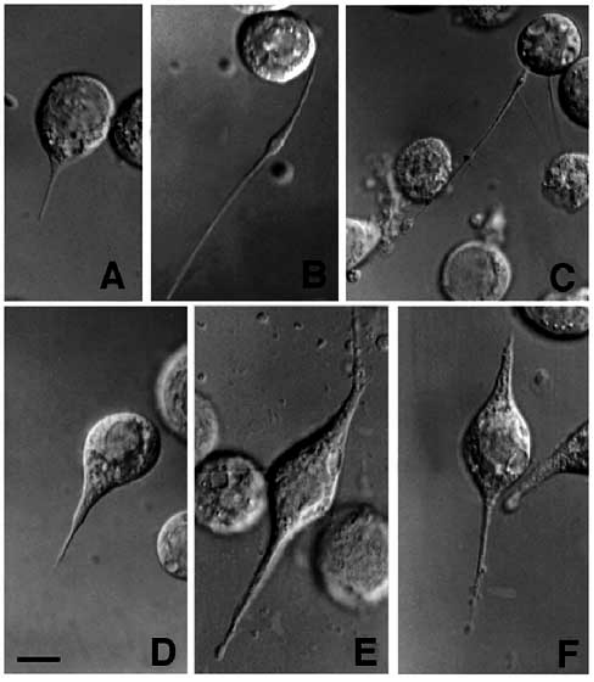

Fig. 1. DIC micrographs of Sf9 cells induced to express either the C-terminal fragment of CHO2 (A-C) or the N-terminal fragment of CHO1/MKLP1 (D-F) as described in Results. (A) A newly initiated process induced by the CHO2 fragment. The process has a thin nontapering morphology, and this same morphology is characteristic of newly initiated processes induced by the CHO1/MKLP1 fragment (not shown). With time, the processes induced by the CHO2 fragment remained thin and cylindrical, but grew extensively to obtain lengths often exceeding 300 μm. (Capturing the full length of a CHO2-induced process in a single micrograph was difficult because these processes tended to weave into different planes of focus.) In contrast, the process induced by the CHO1/MKLP1 fragment began to thicken proximally (see D) and take on a conical tapering morphology. These processes grew somewhat longer, but rarely exceeded 80 μm. Bar, 10 μm.

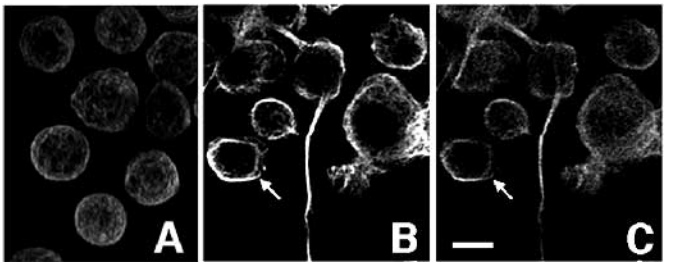

Fig. 2. Immunofluorescence analyses on uninfected Sf9 cells (A), and Sf9 cells induced to express the CHO2 fragment (B,C). Uninfected Sf9 cells stain diffusely for tubulin (A). Cells expressing the CHO1fragment display more filamentous staining patterns for tubulin, and the staining is concentrated either at cell periphery or within the processes of process-bearing cells (B). Double-label analyses confirm that the CHO2 fragment is expressed in these cells. (C) The CHO2 staining co-localizes with the tubulin staining both in the cell body and processes. The staining for the CHO2 fragment is generally grainy, but in some cases appears more filamentous as it coincides with MTs (arrows, B and C). Bar, 15 μm.

The MTs in cells induced to express the CHO1/MKLP1 fragment coalesced into organized bundles, and these bundles tended to form either a circumferential ring of MTs just beneath the plasma membrane of non-process-bearing cells or a dense array of MTs within the processes of process-bearing cells. In both cases, there was a notable depletion of MTs from the central region of the cell, and in the case of the process-bearing cells, there was a partial or sometimes nearly complete depletion of MTs from all regions of the cell body (Kuriyama et al., 1994; Sharp et al., 1996). Immunofluorescence analyses on cells induced to express the CHO2 fragment also showed marked alterations in MT distribution and organization. As with the CHO1/MKLP1 fragment, the MT array of cells expressing the CHO2 fragment was more filamentous than controls, and cells that did not grow processes displayed a circumferential ring of MTs beneath the plasma membrane (Fig. 2B). In cells elaborating processes, very little MT polymer remained in the cell body (Fig. 2B), and the amount of MTs remaining in the cell body appeared to be inversely proportional to the length of the processes and levels of MTs within it. These results strongly suggest that CHO2, like CHO1/MKLP1, induces both the bundling of MTs and their transport from the cell body into the growing processes. Immunostains with an affinity purified polyclonal antibody raised against bacterial fusion protein CHO2-17a (Kuriyama et al., 1995) showed staining in >90% (in most experiments) of the cells exposed to the virus (Fig. 2C). This staining pattern overlapped with MTs, both in the cell body and in the processes, sometimes appearing filamentous but more often appearing grainy (arrows, Fig. 2B,C). Uninfected Sf9 cells did not stain for CHO2 (data not shown). The redistribution of MTs to cell periphery was consisently noted in roughly 80% of the cells shown to be positive for each of the two motor fragments (over 100 cells examined under each condition).

Electron micrographs reveal that the MTs within the processes induced by the CHO2 fragment are aligned relatively paraxially. However, the density of MTs and spacing between individual MTs varied significantly from process to process and at different points along the length of an individual process. In processes shorter than 50 μm, the MTs were generally tightly packed, with little space between individual MTs (Fig. 3A). Longer processes generally contained more widely spaced MTs, and spacing and density of the MTs tended to decrease with distance from the cell body. A good example of such a process, 160 μm in length, is shown in Fig. 3B-D. The MTs are most concentrated proximally (Fig. 3B), and become progressively less concentrated in the middle (Fig. 3C) and distal regions (Fig. 3D) of the process.

Microtubule polarity analyses

To determine the pattern of MT polarity orientation within the processes induced by the Δ1 CHO2 fragment, we used the standard assay for determining MT polarity patterns (see Materials and Methods). In this method, cells are extracted in the presence of exogenous brain tubulin in a buffer that promotes the formation of lateral appendages on the MTs. When these appendages are viewed in cross section they appear as hooks, with the directionality of the hook indicating the polarity orientation of the MT. All hooks were viewed from the vantage point of the distal tip of the process such that clockwise hooks indicate MTs with their plus-ends distal to the cell body and counterclockwise hooks indicate MTs oriented with their minus-ends distal to the cell body. Given the error involved with this procedure, a percentage of hooks greater than 90% indicates uniformity (Heidemann, 1991). We analyzed 12 processes ranging in length from 100 to 300 μm near their midpoints, and 5 of these were analyzed at additional points along their lengths. Fig. 4A and B show a tracing of one of these cells and the MT hooking pattern at the point indicated, respectively. Ninety eight per cent of the MT hooks at this point were clockwise. At all points of all processes analyzed, between 95-100% of MT hooks were clockwise. Based on these results we conclude that, similar to axons, the MTs of processes induced by the CHO2 fragment are uniformly oriented with their plus-ends distal to the cell body. This contrasts with the results obtained with the processes induced by the CHO1/MKLP1 fragment in which MTs are nonuniformly oriented in their midregions with higher proportions of minus-end-distal MTs in their more proximal regions (see Sharp et al., 1996).

Process formation in the absence of net MT assembly

Several studies have shown that both MAP2 and tau, which function by stimulating MT bundling, assembly, and stabilization but have no known transport properties, generate processes when expressed in Sf9 cells and these processes contain a uniformly plus-end-distal MT array (Baas et al., 1991; Chen et al., 1992; LeClerc et al., 1993). This is presumably the result of endogenous motor proteins within the Sf9 cells that are able to orient the MTs as they are assembled, stabilized, and bundled by the MAPs. Thus one possibility is that the uniform MT polarity orientation in the CHO2-induced processes is not the result of the transport properties of the motor but rather to other effects that it may have on the MTs that permit them to better interact with an endogenous motor. For reasons that are unclear to us, expression of either the CHO1/MKLP1 and CHO2 fragment resulted in a net increase in total MT mass within the cells. To determine whether this increase in MT mass is the critical factor in inducing process outgrowth (as opposed to the transport properties of the motor), we infected cells in the presence of 10 nM vinblastine to suppress net MT assembly during process formation. This same strategy was used in our previous study on CHO1/MKLP1 (Sharp et al., 1996). Over

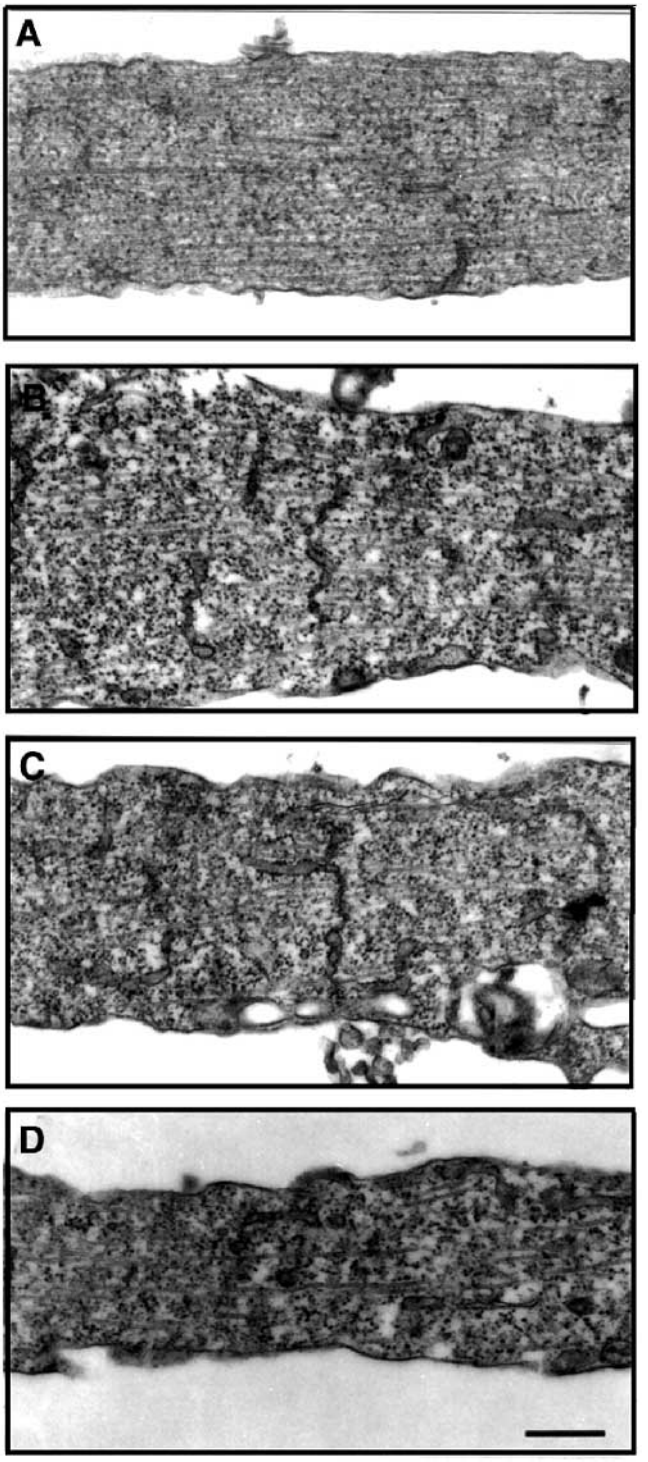

Fig. 3. Electron micrographs of processes induced by expression of the CHO2 fragment in Sf9 cells. (A) The midregion of a relatively short (50 μm) process. (B,C,D) The proximal, middle, and distal regions, respectively, of a longer process approximately 160 μm in length. The shorter process shows tightly packed MTs, while the longer process shows less tightly packed MTs. There is a gradient along the length of the longer process, with progressively fewer MTs with distance from the cell body. Vesicular organelles appear throughout the length of the process shown in B-D, suggesting that the MTs contained within these processes are competent substrates for organelle transport. Bar, 0.33 μm.

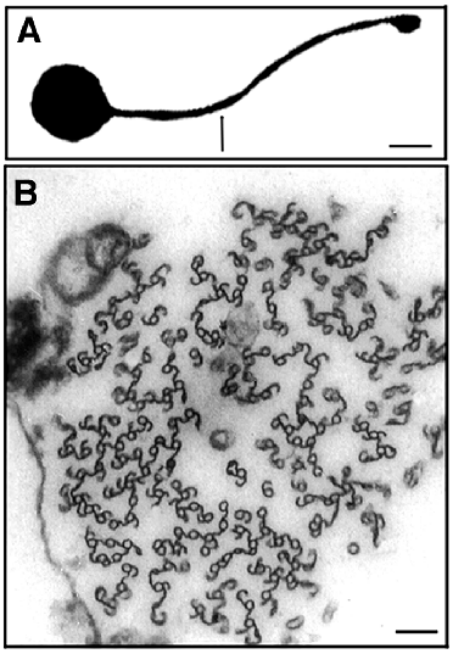

Fig. 4. MT polarity analyses on the processes of Sf9 cells induced to express the CHO2 fragment. (A) A tracing of a process-bearing cell. (B) An electron micrograph of the MT hooking pattern at the point indicated by arrow in A. Clockwise hooks indicate MTs oriented with their plus-ends distal to the cell body, while counterclockwise hooks indicated MTs with their minus-ends distal to the cell body. 98% of the hooks on the MTs were clockwise at this point, indicating a uniformly plus-end-distal MT polarity pattern. Similar results were obtained at every point of every process assessed. Bar, 0.15 μm.

the same time frame as infected cells not exposed to the drug, the vinblastine-treated infected cells extended processes. These processes were somewhat shorter in length but otherwise indistinguishable from those extended by cells infected in the absence of the drug. Quantitative fluorescence analyses were performed to determine the MT mass within 10 representative cells from each condition. The results of these analyses, group mean ± standard deviation in arbitrary fluorescence units (AFUs), were as follows: Control cells, 1,200±400; infected cells without vinblastine, 2,250±800; infected cells with vinblastine, 800±150. Thus cells infected in the absence of the drug contained nearly twice as much MT polymer as uninfected cells. However, cells infected in the presence of vinblastine contained about 2/3 as much polymer as uninfected cells, indicating that this increase is not necessary for the process formation stimulated by the fragment. MT polarity analyses were performed at the midregions of 5 processes generated in the presence of vinblastine, and in each of these greater than 95% of the MTs were oriented with their plus-ends distal to the cell body. Thus the increase in polymer mass that results from the expression of the CHO2 fragment is not necessary for process formation, for the accumulation of MTs within the processes, or for the establishment of the uniformly plus-end-distal polarity pattern within the processes.

The transport properties of the CHO2 fragment are required for it to stimulate dramatic microtubule redistribution and process formation

The pharmacologic studies do not eliminate the possibility that

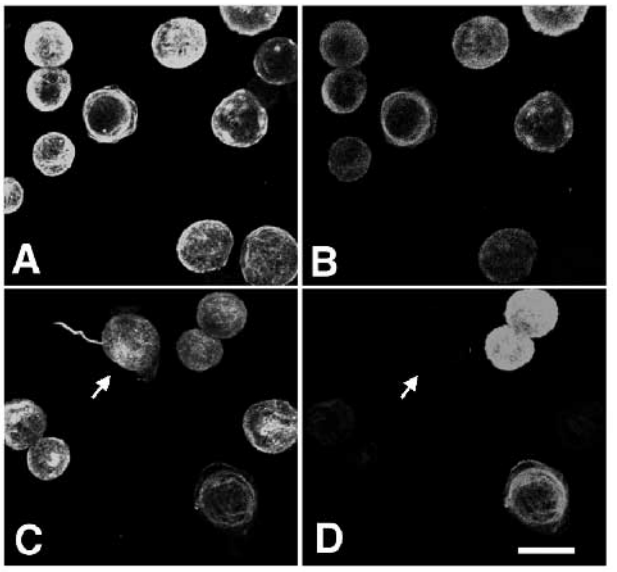

Fig. 5. Two pairs of immunofluorescence images from Δ1′-expressing cultures double-labeled with antibodies to tubulin (A and C) and CHO2 (B and D). Most of the cells expressing the mutated CHO2 fragment do not show marked alterations in MT distribution or bundling, but a few of the cells show some peripheral MT bundling and some degree of MT depletion from cell center (see text for more details). Virtually all of the cells that extended short processes in the cultures infected with the mutant CHO2 fragment were among the small number of cells that were actually not expressing the Δ1′ fragment (see arrows in C and D). Bar, 15 μm.

it is the bundling properties of the CHO2 fragment and not its transport properties that provide the basis for its capacity to stimulate process outgrowth and the reorganization of MTs into uniformly plus-end-distal arrays. To investigate this possibility with appropriate rigor, we mutated the ATP binding site of the CHO2 molecule that is necessary for it to transport MTs. The mutated fragment was termed Δ1′. Details on its preparation and altered amino acid sequence are described in Materials and Methods. As noted previously, a small proportion of cells in uninfected cultures (<1%) extended short 'spontaneous' processes (generally no longer than 20 μm), while 10-40% of the cells from the cultures expressing the Δ1 fragment extended long slender processes. The cells in the cultures expressing the Δ1′ fragment were morphologically similar to the uninfected cells. They were generally round, with fewer than 1% displaying short processes no longer than 20 μm. Fig. 5 shows two pairs of images from Δ1′-expressing cultures double-labeled with antibodies to tubulin (A,C) and CHO2 (B,D). Many of the cells expressing the mutated CHO2 fragment do not show marked alterations in MT distribution or bundling, but a few of the cells (roughly 15% of over 100 cells examined) showed some degree of peripheral MT bundling and some depletion of MTs from the central region of the cell. However, none of the cells showed the kind of near-complete depletion of MTs from the central region of the cell and a corresponding concentration of MTs near the periphery that was observed with the unmutated CHO2 fragment. Notably, virtually all of the cells that extended short processes were among the small number of cells that were actually not expressing (or expressing undetectable levels of) the Δ1′ fragment. These results indicate that the mutated CHO2 fragment is able to bundle MTs and shift their distribution to some degree in a small number of the cells, but is not able to induce the kind of dramatic relocation of the vast majority of the MTs to cell periphery nor the formation of MT-rich processes observed with the unmutated fragment. Thus the transport properties of the molecule are essential for it to elicit these results.

Table 1. Summary of data on morphology and MT distribution

	% Cells with processes	Maximum process length (μm)	% Cells with MT redistribution
Control	<1	<20	0
CHO1 fragment	10-40	<80	~80
CHO2 Fragment	10-40	>300	~80
Mutant CHO2 fragment	<1	<20	~15

Table 1 shows a summary of observations on the morphology and MT distribution of cells exposed to baculovirus endoding the CHO1 fragment, the CHO2 fragment, and the mutated CHO2 fragment.

DISCUSSION

This study and its predecessor document the capacity of molecular motors to drive the formation of processes containing MT arrays with distinct polarity patterns. In our previous study, we established that ectopic expression in Sf9 cells of a fragment of the kinesin related motor protein termed CHO1/MKLP1 results in the formation of processes containing a nonuniform MT polarity pattern (Sharp et al., 1996). This pattern is established by the sequential addition of plus-end-distal MTs followed by minus-end-distal MTs, the same sequence of events that occurs during the establishment of nonuniform MT polarity orientation in developing dendrites (Baas et al., 1989). Here we report that the expression of a fragment of a kinesin-related-protein termed CHO2 induces Sf9 cells to extend processes containing uniformly plus-end-distal MTs, the pattern characteristic of axons. The fragment of each protein expressed in these studies contains its motor domain, and the distinct MT polarity pattern induced by the expression of each fragment is consistent with the expected transport properties of the individual motor. In vitro analyses have shown that CHO1/MKLP1 transports MTs with minus-end-leading against MTs of the opposite orientation, while CHO2 transports MTs with plus-end-leading. Thus the simplest interpretation of the findings is that the motor protein fragments transport MTs from the cell body into the developing processes and do so in a manner that organizes the MTs into distinct polarity patterns.

In contemplating this idea, however, it is necessary to consider the fact that expression of other proteins, namely the fibrous microtubule-associated proteins (MAPs) tau and MAP2, cause Sf9 cells to extend MT-rich processes. These proteins stimulate MT assembly, stabilization, and bundling, but have no intrinsic transport properties (see Brandt and Lee, 1984). Nevertheless, the MTs within the processes induced by these proteins are uniformly oriented with their plus-ends distal to the cell body (Baas et al., 1991; Chen et al., 1992; LeClerc et al., 1993). We suspect that an endogenous motor within the Sf9 cells orients the MTs as the MAP bundles them, and that

this motor also organizes the uniformly plus-end-distal bundle of MTs within the initial processes that form during CHO1/MKLP1 expression. It is clear that the introduction of minus-end-distal MTs into the MT bundle in cells induced to express the CHO1/MKLP1 fragment cannot be explained in terms of either MT bundling or this endogenous motor activity. The case for MT transport is particularly compelling with regard to CHO1/MKLP1 because the introduction of minus-end-distal MTs has not been observed with any other molecule expressed in these cells, and because the results are entirely consistent with the known transport properties of the molecule. However, the fact that the CHO2 fragment produces the same uniform MT polarity pattern as molecules with no transport properties renders the results obtained with the CHO2 fragment more difficult to interpret. Like CHO1/MKLP1, CHO2 has the capacity to bundle MTs and our data show that its expression in Sf9 cells somehow results in an increase in MT assembly. Thus the possibility remained that the results obtained with the CHO2 fragment may not relate to its transport properties.

Three initial sets of observations argued against this possibility. First, in the case of both motor fragments, there is a depletion of MTs from cell center that occurs concomitantly and progressively as MTs accumulate around the periphery of the cell body, and this depletion continues as the MTs accumulate within developing processes. Such a dramatic depletion of MTs from the cell body has not been observed in the studies involving MAP expression in Sf9 cells (Baas et al., 1991; LeClerc et al., 1996). Second, in cells bearing longer processes induced by the CHO2 fragment, the cell body is further depleted of MTs and the processes show a gradual decrease in MT levels with distance from the cell body and a lower density of MTs compared to shorter processes from other cells. This result is consistent with the ongoing transport of relatively short MTs down these processes. Third, the observed redistribution of MTs and establishment of distinct MT polarity patterns in the developing processes occur unhindered when MT assembly is pharmacologically inhibited during expression of the CHO1/MKLP1 or CHO2 fragments, indicating that the MT assembly stimulated by the motor expression is not required for the motor to induce the documented results.

Despite these observations, it remained possible that the bundling properties of the motor were sufficient to elicit the observed results, and therefore we wished to investigate with more rigor whether the transport properties of the CHO2 fragment are indeed required. To accomplish this, we performed site-directed mutagenesis on the CHO2 fragment. Specifically, we altered three amino acids within the ATP binding site necessary for the transport properties of the motor. When cultures were infected with baculovirus containing the mutant CHO2 fragment, some of the expressing cells (roughly 15%) showed some degree of MT bundling beneath the cell periphery and some degree of MT depletion from cell center. However, none of the cells showed these effects as dramatically as cells expressing the unmutated fragment. Roughly 80% of the cells expressing the unmutated fragment showed more complete depletion of MTs from cell center and more pronounced peripheral bundling of MTs. In addition to these differences in MT distribution, the cells expressing the mutant CHO2 fragment did not extend processes. On the basis of these results, we conclude that the CHO2 fragment is not as potent at bundling MTs as the fibrous MAPs or the CHO1/MKLP1 fragment (at least when expressed in Sf9 cells) and that the transport properties of the motor are essential for the more dramatic degree of MT redistribution and the process outgrowth observed in cells expressing the unmutated fragment.

MT polarity patterns directly influence process morphology

During the course of these studies, we observed morphological similarities between the processes induced by the CHO2 and CHO1/MKLP1 fragments and their neuronal counterparts with the same MT polarity patterns. As previously reported, the processes generated by the CHO1/MKLP1 fragment are generally short, not longer than 80 μm, and have a conical shape, tapering with distance from the cell body (Sharp et al., 1996). These processes appear to reach a maximum length and then stop growing prior to signs of morbidity in the culture. In contrast, the processes generated by the CHO2 fragment are generally thin and cylindrical in shape and, although somewhat slower to start growing, obtain lengths that often exceed hundreds of microns before the death of the culture. While we cannot dismiss the possibility that other properties of the motor proteins might be responsible for generating these morphological differences, it seems reasonable to speculate that it is the distinct MT polarity patterns generated by the motors that account for the differences in length and shape displayed by these processes. Like the processes induced by the CHO1/MKLP1 fragment, dendrites contain a nonuniform MT polarity pattern, and have a short tapering morphology. Like the processes induced by the CHO2 fragment, axons contain a uniformly plus-end-distal MT polarity pattern, and have a long thin cylindrical morphology. Interestingly, during the early stages of process formation driven by either the CHO1/MKLP1 or the CHO2 fragment, the processes contain uniformly plus-end-distal MTs and are generally thin and cylindrical in shape. Similarly, studies on cultured hippocampal neurons indicate that both axons and dendrites develop from thin cylindrical immature processes that contain uniformly plus-end-distal MTs (Baas et al., 1989). Collectively, these results suggest a direct link between the specific MT polarity pattern within a cellular processes and fundamental features of its morphology such as length, thickness, and taper.

The means by which MT polarity patterns might regulate features of morphology is unclear. Regarding process length, one possibility is that the uniform orientation of assembly favored plus-ends toward the direction of growth might assist a process in growing longer than a process containing nonuniformly oriented MTs. However, this seems unlikely in that processes containing a uniformly plus-end-distal MT array are still longer than those containing a nonuniformly oriented array when both are grown under conditions that inhibit MT assembly (present results and Sharp et al., 1996). Another possibility is that the length differences relate directly to the transport of MTs. Specifically, it may be that after MTs of opposite orientation intercalate, their ability to continue moving is diminished, whereas MTs of the same orientation move unabated. A third and perhaps most likely possibility is that these differences in MT polarity patterns affect the vectorial delivery of membranous elements required for the distal expansion of the processes. The means by which nonuniform MT polarity orientation results in taper are also unclear, but may relate to the enrichment of minus-end-distal MTs proximally in both the processes induced by the CHO1/MKLP1 fragment (Sharp et al., 1996) and in bona fide dendrites (Baas et al., 1989). Whatever the case may be, other

factors undoubtedly contribute to the morphology of axons and dendrites, and principal among these may be the fibrous MAPs. The studies mentioned above on MAP expression in Sf9 cells indicate that the presence of tau or various isoforms of MAP2 can have an enormous impact on process morphology without inducing different MT polarity patterns. We would surmise that motors and MAPs function in concert to establish the distinct morphologies of axons and dendrites.

Mechanisms for generating axonal and dendritic microtubule patterns

The results of our analyses raise the possibility that the motor proteins involved in transporting MTs into axons and dendrites may be the same motors that drive MT transport during the formation and functioning of the mitotic spindle. Strong support for this idea has recently emerged in the case of CHO1/MKLP1. We have shown that this motor continues to be expressed in postmitotic neurons, where it localizes within developing dendrites and is essential for their differentiation (Yu et al., 1997; Sharp et al., 1997). Presumably, the role of this motor protein is to transport minus-end-distal MTs among plus-end-distal MTs during the differentiation of these processes. We have speculated that the dual functions of CHO1/MKLP1 may be regulated by modifications within its C-terminal domain, the presence of which suppresses the capacity of the molecule to induce process formation when expressed in Sf9 cells (Sharp et al., 1996). Whether or not CHO2 plays a role in transporting MTs into the axon is unknown, but if it does, then modifications within its N-terminal domain might similarly determine whether it functions in mitosis or process formation. At present, we suspect that cytoplasmic dynein may be a better candidate than CHO2 for the motor protein that transports MTs into the axon. The Pfister laboratory has recently shown that almost 80% of the cytoplasmic dynein that is transported anterogradely down the axon is transported at the same rate as actin filaments and the cytomatrix (Dillman et al., 1996). They speculated that this dynein might somehow interact with actin filaments via its cargo domain, leaving its motor domain available to transport MTs with plus-ends-leading down the axon. While more work is needed to test these various ideas, it is clear from the present studies that MT transport by motor proteins is a feasible means by which the distinct MT polarity patterns of axons and dendrites can be established. In addition, we suspect that this same principle might apply to other cell types that must deploy their MTs toward the cell periphery and organize them into distinct patterns of polarity orientation.

We thank Dr Wenqian Yu for her valuable assistance, and Drs Gary Borisy, Jon Scholey, J. R. McIntosh, and Lotfi Ferhat for helpful suggestions and discussions. We thank Drs Javed Siddiqi and Jun Wang of DNA Technologies, Inc. (Gaithersburg, MD) for assistance with the site-directed mutagenesis. This work was supported by grants from the National Institutes of Health and the National Science Foundation to P. W. Baas, and grants from the National Institutes of Health and the Council for Tobacco Research to R. Kuriyama. P. W. Baas is the recipient of a Research Career Development Award from the National Institutes of Health.

REFERENCES

Baas, P. W., Deitch, J. S., Black, M. M. and Banker, G. A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc. Nat. Acad. Sci. USA* **85**, 8335-8339.

Baas, P. W., Black, M. M. and Banker, G. A. (1989). Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J. Cell Biol.* **109**, 3085-3094.

Baas, P. W., Pienkowski, T. P. and Kosik, K. S. (1991). Processes induced by tau expression in Sf9 cells have an axon-like microtubule organization. *J. Cell Biol.* **115**, 1333-1344.

Baas, P. W. and Ahmad, F. J. (1993). The transport properties of axonal microtubules establish their polarity orientation. *J. Cell Biol.* **120**, 1427-1437.

Black, M. M. and Baas, P. W. (1989). The basis of polarity in neurons. *Trends Neurosci.* **12**, 211-214.

Brandt, R. and Lee, G. (1994). Orientation, assembly and stability of microtubule bundles induced by a fragment of tau protein. *Cell Motil. Cytoskel.* **28**, 143-154.

Chen, J., Kanai, Y., Cowan, N. J. and Hirokawa N. (1992). Projection domains of MAP-2 and tau determine spacings between microtubules in dendrites and axons. *Nature* **360**, 674-677.

Dillman, J. F., Dabney, L. P. and Pfister, K. K. (1996). Cytoplasmic dynein is associated with slow axonal transport. *Proc. Nat. Acad. Sci. USA* **93**, 141-144.

Euteneuer, U. and McIntosh, J. R. (1982). Structural polarity of kinetochore microtubules in PTK2 cells. *J. Cell Biol.* **98**, 338-345.

Heidemann, S. R., Landers, J. M. and Hamborg, M. A. (1981). Polarity orientation of axonal microtubules. *J. Cell Biol.* **91**, 661-665.

Heidemann, S. R. (1991). Microtubule polarity determination based on the formation of protofilament hooks. *Meth. Enzymol.* **196**, 469-477.

Knops, J., Kosik, K. S., Lee, G., Pardee, J. D., Cohen-Gould, L. and McConlogue, L. (1991). Overexpression of tau in a nonneuronal cell induces long cellular processes. *J. Cell Biol.* **114**, 725-733.

Kuriyama, R., Dragas-Granoic, S., Maekawa, T., Vassilev, A., Khodjakov, A. and Kobayashi, H. (1994). Heterogeneity and microtubule interaction of the CHO1 antigen, a mitosis-specific kinesin-like protein. Analysis of subdomains expressed in insect Sf9 cells. *J. Cell Sci.* **107**, 3485-3499.

Kuriyama, R., Kofron, M., Essner, R., Kato, T., Omoto, C. K. and Khodjakov, A. (1995). Characterization of a minus-end-directed kinesin-like motor protein from cultured mammalian cells. *J. Cell Biol.* **129**, 1049-1059.

LeClerc, N., Kosik, K. S., Cowan, N., Pienkowski, T. P. and Baas, P. W. (1993). Process formation in Sf9 cells induced by the expression of a microtubule-associated protein 2c-like construct. *Proc. Nat. Acad. Sci. USA* **90**, 6233-6227.

LeClerc, N., Baas, P. W., Garner, C. C. and Kosik, K. S. (1996). Juvenile and mature MAP2 induce distinct patterns of process growth. *Mol. Biol. Cell* **7**, 443-455.

Nakata, T. and Hirokawa, N. (1995). Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. *J. Cell Biol.* **131**, 1039-1053.

Nislow, C., Lombillo, V. A., Kuriyama, R. and McIntosh, J. R. (1992). A plus-end-directed motor that moves anti-parallel microtubules in vitro localizes in the interzone of mitotic spindles. *Nature* **359**, 543-547.

Sellitto, C. and Kuriyama, R. (1988). Distribution of a matrix component of the midbody during the cell cycle in Chinese hamster ovary cells. *J. Cell Biol.* **106**, 431-439.

Sharp, D. J., Yu, W. and Baas, P. W. (1995). Transport of dendritic microtubules establishes their nonuniform polarity orientation. *J. Cell Biol.* **130**, 93-104.

Sharp, D. J., Kuriyama, R. and Baas, P. W. (1996). Expression of kinesin related protein induces Sf9 cells to form dendrite-like processes with nonuniform microtubule polarity orientation. *J. Neurosci.* **16**, 4370-4375.

Sharp, D. J., Yu, W., Ferhat, L., Kuriyama, R., Rueger, D. C. and Baas, P. W. (1997). Identification of a microtubule-based motor protein essential for dendritic differentiation. *J. Cell Biol.* **138**, 833-843.

Yu, W., Sharp, D. J., Kuriyama, R., Mallik, P. and Baas, P. W. (1997). Inhibition of a mitotic motor protein compromises the formation of dendrite-like processes from neuroblastoma cells. *J. Cell Biol.* **136**, 659-668.

(Received 9 June 1997 – Accepted 23 July 1997)