Identification of a Microtubule-associated Motor Protein Essential for Dendritic Differentiation

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Abstract. The quintessential feature of the dendritic microtubule array is its nonuniform pattern of polarity orientation. During the development of the dendrite, a population of plus end–distal microtubules first appears, and these microtubules are subsequently joined by a population of oppositely oriented microtubules. Studies from our laboratory indicate that the latter microtubules are intercalated within the microtubule array by their specific transport from the cell body of the neuron during a critical stage in development (Sharp, D.J., W. Yu, and P.W. Baas. 1995. *J. Cell Biol.* 130:93–104). In addition, we have established that the mitotic motor protein termed CHO1/MKLP1 has the appropriate properties to transport microtubules in this manner (Sharp, D.J., R. Kuriyama, and P.W. Baas. 1996. *J. Neurosci.* 16:4370–4375). In the present study we have sought to determine whether CHO1/MKLP1 continues to be expressed in terminally postmitotic neurons and whether it is required for the establishment of the dendritic microtubule array. In situ hybridization analyses reveal that CHO1/MKLP1 is expressed in postmitotic cultured rat sympathetic and hippocampal neurons. Immunofluorescence analyses indicate that the motor is absent from axons but is enriched in developing dendrites, where it appears as discrete patches associated with the microtubule array. Treatment of the neurons with antisense oligonucleotides to CHO1/MKLP1 suppresses dendritic differentiation, presumably by inhibiting the establishment of their nonuniform microtubule polarity pattern. We conclude that CHO1/MKLP1 transports microtubules from the cell body into the developing dendrite with their minus ends leading, thereby establishing the nonuniform microtubule polarity pattern of the dendrite.

During the differentiation of the neuron, distinct patterns of microtubule polarity orientation are established within developing axons and dendrites. In the axon the microtubules are uniformly oriented with their plus ends distal to the cell body (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987), while in the dendrite the microtubules have a nonuniform or mixed polarity orientation (Baas et al., 1988, 1991; Burton, 1988). We have proposed that these microtubule polarity patterns are established by motor proteins that transport microtubules from the cell body of the neuron into axons and dendrites specifically with either the plus end or the minus end of the microtubule leading (Baas and Ahmad, 1993; Sharp et al., 1995; Baas and Yu, 1996). The immature processes that give rise to both axons and dendrites contain uniformly plus end–distal microtubules (Baas et al., 1989), suggesting that a common motor is responsible for the transport of microtubules with this orientation into both axons and dendrites. As one of these processes develops into the axon, this polarity pattern is preserved. As the other processes develop into dendrites, a second population of oppositely oriented microtubules is intermingled among the plus end–distal microtubules. What is the motor protein responsible for the transport of minus end–distal microtubules into developing dendrites?

At least one known kinesin-related motor protein appears to have the appropriate properties to establish microtubule arrays of nonuniform polarity orientation. This motor, termed CHO1 or MKLP1, is present in the midzone region of the mitotic spindle where it is thought to transport oppositely oriented microtubules relative to one another (Sellitto and Kuriyama, 1988). While not unequivocally proven, this view is strongly supported by studies showing that a function-blocking antibody to this motor prevents spindle elongation (Nislow et al., 1990), and by studies demonstrating the capacity of CHO1/MKLP1 to transport oppositely oriented microtubules relative to one another in vitro (Nislow et al., 1992). In addition, expression of a portion of the CHO1/MKLP1 molecule in insect

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ovarian Sf9 cells causes these normally rounded cells to extend processes with a thick tapering morphology and nonuniform microtubule polarity pattern similar to neuronal dendrites (Kuriyama et al., 1994; Sharp et al., 1996).

In a recent study we examined the distribution of CHO1/MKLP1 in cultured mouse neuroblastoma cells that are mitotic but also extend axon-like and dendrite-like processes during interphase. Similar to bona fide axons and dendrites, these processes display uniform and nonuniform microtubule polarity patterns, respectively. We found that CHO1/MKLP1 is present in the spindle midzone during mitosis, and it appears throughout the cell body and within the dendrite-like but not the axon-like processes during interphase (Yu et al., 1997). In addition, we found that inhibition of the expression of CHO1/MKLP1 suppresses the formation of the dendrite-like but not the axon-like processes. These results indicate that CHO1/MKLP1 redistributes during the cell cycle, and that this redistribution is essential for the formation of the primitive dendrites extended by these cells. With regard to bona fide postmitotic neurons, it is possible that CHO1/MKLP1 continues to be expressed after the last mitotic division and is used to establish nonuniform microtubule polarity orientation within developing dendrites. Another possibility is that neurons no longer express CHO1/MKLP1 after they cease dividing, and instead express another kinesin-related motor protein with properties similar to CHO1/MKLP1 that can be used for dendritic development. In the present study we have addressed this issue in studies on primary rat sympathetic and hippocampal neurons.

Materials and Methods

Cell Culture

For the generation of cultures of rat sympathetic neurons, superior cervical ganglia were dissected from 18-d fetal rats, treated with trypsin and collagenase as previously described (Baas and Ahmad, 1993), dissociated by trituration, and plated in serum-free medium on glass coverslips that had been treated with polylysine and laminin as previously described (Higgins et al., 1991). Cultures of rat hippocampal neurons were generated as previously described (Goslin and Banker, 1991; Sharp et al., 1995). Continuous cultures of CHO cells were maintained in plastic tissue culture flasks as previously described (Sellitto and Kuriyama, 1988), and the cells were plated on glass coverslips for experimental analyses.

In Situ Hybridization Analyses

In situ hybridization was used to determine whether CHO1 mRNA is expressed within primary rat sympathetic and hippocampal neurons. CHO cells were used as a positive control. For detection of CHO1 mRNA within these cells, sense and antisense riboprobes, respectively, were prepared from a 1-kb and 815-bp hamster cDNA fragment cloned into pBluescript vector flanked by T3 and T7 promoters. The 1-kb fragment of cDNA corresponds to the NH_2-terminal region of the CHO1/MKLP1 protein. The sense riboprobe was transcribed in vitro from an NsiI-linearized plasmid using T3 RNA polymerase (Ambion, Austin, TX). The PstI 815-bp fragment spans bases 2,424–3,239 of the hamster cDNA and encodes a part of the carboxy-terminal region of the CHO1/MKLP1 protein (amino acids 809–953) and the entire 3′ untranslated region (Kuriyama et al., 1994). This 815-bp fragment shows no homology with any known proteins including other kinesin-related proteins. The antisense riboprobe was transcribed from a PstI-linearized plasmid using T7 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Both sense and antisense riboprobes were transcribed in the presence of digoxigenin-UTP (Boehringer Mannheim Biochemicals) according to the manufacturer's protocol. The specificity of the antisense CHO1 probe was assessed by Northern blot analysis, which revealed a single band (data not shown) at the size (3.6 kb) described by Kuriyama et al. (1994).

Cells that had been grown on glass coverslips were fixed for 15 min at room temperature in 4% paraformaldehyde in 1× PBS, rinsed three times for 5 min in 1× PBS, and dehydrated in graded alcohols (30, 50, 70, 85, 95, and 100%). Hybridization was carried out overnight at 50°C with 250 μl of a hybridization mixture containing 50% deionized formamide, 4× SSC, 5× Denhardt's solution, 10% dextran sulfate, 250 μg/ml yeast tRNA, 250 μg/ml salmon sperm DNA, and 100 mM DTT. For each coverslip, 5 ng of digoxigenin RNA probe (sense or antisense) was used. After hybridization, cells were rinsed twice for 15 min at room temperature in 4× SSC and twice for 15 min in 2× SSC, treated with 20 μg/ml RNase A for 30 min at 37°C, and washed twice for 5 min in 2× SSC at 37°C. The cells were then subjected to high stringency washes once for 15 min in 0.5× SSC and once in 0.1× SSC, both at 60°C. All rinse and wash buffers contained 0.158 g/ml sodium thiosulfate. The cells were then incubated with sheep anti-digoxigenin alkaline phosphatase antibody (Boehringer Mannheim Biochemicals) diluted 1:1,000 in Tris-HCl buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma Chemical Co., St. Louis, MO) overnight at 4°C. The coverslips were rinsed twice for 10 min in Tris-HCl buffer and then for 5 min in color development buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), after which they were incubated with Tris-HCl buffer substrate solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) containing nitroblue tetrazolium (NBT; 340 μg/ml) and bromochloroindolylyl phosphate (BCIP; 170 μg/ml). To reduce the endogenous phosphatase activity, 5 mM levamisole was added to the color development buffer. The color signal was monitored by microscopy and the reaction was stopped when a strong cellular signal was developed against a low background. After transferring them to 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, the coverslips were washed twice for 10 min in distilled water, air-dried, and mounted in Histomount solution (Oncogene Science, Cambridge, MA). Cells were visualized and photographs were taken using differential interference contrast optics to reveal simultaneously cellular morphology and the reddish alkaline phosphatase reaction product.

Oligonucleotides Used to Inhibit CHO1/MKLP1 Expression

As in our previous study on neuroblastoma cells (Yu et al., 1997), translation of CHO1/MKLP1 was suppressed by treatment of cultures with phosphorothioate-substituted DNA oligonucleotides (Research Genetics, Huntsville, AL). An antisense oligonucleotide consisting of the sequence 5′-CTTAGCTTTCGCTGGTTTCATG-3′, which is the inverse complement of the coding sequence −1 + 18 of hamster CHO1/MKLP1 transcript (Kuriyama et al., 1994), was used in all experimental conditions. This sequence was termed AS2. Dose–response experiments were performed to determine the lowest concentration of oligonucleotide that induced the maximal inhibition of CHO1/MKLP1 expression. Oligonucleotides were stored in serum-free medium, aliquoted, and frozen at −80°C. 2 d after the cells were plated, a portion of the plating medium was removed and new medium containing oligonucleotides was added at final concentrations of 0.1, 1, and 5 μM. This medium was changed every 12 h for the remainder of the experiment. Dishes were fixed at 2, 4, and 6 d and prepared for quantitative immunofluorescence microscopy. On the basis of the results of the dose–response analyses, a concentration of 1 μM was used for morphometric and microtubule polarity analyses. For a positive control, the antisense oligonucleotide consisting of the sequence 5′-CAGGTTTCCTGGGCATCTT-3′, which is the inverse complement of the sequence + 19 + 37 of hamster CHO1/MKLP1 transcript, was added to cultures at a concentration of 0.1, 1, and 5 μM and prepared for dose–response analyses as described above. We termed this sequence AS1. For negative controls, two oligonucleotides consisting of the sequences 5′-CATGAAACCAGCGAAAGCT-3′ and 5′-AAGATGCCCAGGAAACCTG-3′, which are the inverse complements of the two antisense oligonucleotides mentioned above, respectively, were used at 1 μM. These sequences were termed S2 and S1. BLAST searches indicated no other matches for the antisense or sense sequences.

Immunofluorescence Microscopy

For immunofluorescence analyses, the cultures were fixed for 6 min in cold methanol (−20°C), rinsed three times for 5 min each in PBS, and incubated for 1 h in a blocking solution containing 5% normal goat serum in PBS. The cells were then exposed overnight at 4°C to mouse mAbs that

specifically recognize either CHO1/MKLP1 (used at 1:5,000; Sellitto and Kuriyama, 1988), β-tubulin (used at 1:500; Amersham Corp., Arlington Heights, IL), microtubule-associated protein-2 (MAP2) (used at 1:100; provided as a kind gift from Dr. I. Fischer, Medical College of Pennsylvania, Philadelphia), or the cytoplasmic dynein intermediate chain (used at 1:1,000; provided as a kind gift from Dr. K.K. Pfister, University of Virginia, Charlottesville; see Pfister et al., 1996). The cells were then rinsed extensively in PBS, treated again for 1 h in blocking solution, and exposed for 1 h at 37°C to an appropriate fluorescent secondary antibody used at 1:100 (Jackson Immunoresearch Laboratories, West Grove, PA). Images were captured with the LSM 410 Laser Confocal Microscope (Carl Zeiss Inc., Thornwood, NY). For quantitative analyses on the levels of CHO1/MKLP1 within individual cells, the pinhole on the confocal system was opened maximally to allow the complete visualization of fluorescently labeled cells in a single 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 cells treated with antisense oligonucleotides, sense oligonucleotides, or neither. Intensities were expressed in arbitrary fluorescence units (AFU).[1] For the determination of the levels of β-tubulin, cytoplasmic dynein, and tau, fluorescence images of numerous fields from within the culture were acquired, each containing four to five neuronal cell bodies, and the total protein levels were determined for each field. From this analysis, the average protein level per cell was obtained. 100 cells were analyzed for each condition.

Microtubule Polarity Analyses

The polarity orientation of microtubules within processes extended under the various experimental conditions was determined using our recent modification (Sharp et al., 1996) of a previously described method (Heidemann and McIntosh, 1980). This method involves the decoration of existing microtubules 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 microtubules when viewed in cross-section under the electron microscope. A clockwise hook indicates that the plus end of the microtubule 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 min 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, processed, and embedded for EM by conventional methods. Video-print images were obtained before sectioning, and these were used to document precisely the points along the lengths of processes at which cross-sections were made. The sections were visualized and photographed using an electron microscope (CM 120; Phillips, The Netherlands). As in our previous work, a single section taken in the midregion of each process was used for quantification.

Results

The protein referred to as CHO1/MKLP1 was first identified as a centrosomal component in CHO cells by an mAb termed CHO1 raised against isolated mitotic spindles (Sellitto and Kuriyama, 1988). Subsequently, cDNAs coding for the antigen recognized by this antibody were isolated from CHO cells (Kuriyama et al., 1994) and from HeLa cells (termed MKLP1; Nislow et al., 1992), and it was determined that the encoded protein is a kinesin-related motor involved in mitosis (Nislow et al., 1990, 1992). Here we refer to the mAb and the riboprobe used in our in situ hybridization analyses as CHO1, and to the motor protein itself as CHO1/MKLP1.

CHO1/MKLP1 Is Expressed in Neurons

In situ hybridization analyses were performed on dissociated cultures of rat sympathetic and hippocampal neurons obtained from 18-d fetal rats to determine whether CHO1/MKLP1 is expressed within these cells. At the time of their dissection from the animal, the vast majority of the neurons had ceased cell division and had begun to form processes. Variable numbers of fibroblastic and glial cells were also present in the cultures, and these were distinguishable from the neurons on the basis of their more flattened morphology. CHO1 mRNA was detected using a digoxigenin-labeled CHO1 antisense probe 3–4 d after plating, by which time the cells had differentiated axons and were in the early stages of dendritic differentiation. Hybridization of the probe with native mRNA is indicated by the presence of a reddish reaction product within the cell. Fig. 1 shows differential interference contrast (DIC) images of cultures of rat sympathetic neurons and CHO cells. The CHO cells were used as a positive control. After hybridization with the CHO1 antisense probe, CHO1 labeling was prominent within the cell bodies of the sympathetic neurons (Fig. 1 *A*). Labeling was also apparent within the nonneuronal cells in the cultures. No labeling was detected with the sense CHO1 probe in the neurons or in the nonneuronal cells within the sympathetic neuron cultures (Fig. 1 *B*). Similar results were obtained in analyses on the cultured hippocampal neurons (data not shown). As in the neuron cultures, labeling was observed in the CHO cells after hybridization with the antisense probe (Fig. 1 *C*), but not the sense probe (Fig. 1 *D*).

Localization of CHO1/MKLP1 in Neurons

Immunofluorescence analyses using the monoclonal CHO1 antibody were performed to reveal the localization of CHO1/MKLP1 within the cultured hippocampal and sympathetic neurons. This antibody stains CHO1/MKLP1 in the midzone of the mitotic spindle in a variety of cell types (Sellitto and Kuriyama, 1988; Nislow et al., 1990, 1992; Yu et al., 1997) and in the cell body and dendrite-like processes of cultured neuroblastoma cells (Yu et al., 1997). The results on cultured hippocampal neurons are shown in Fig. 2, in which the immunofluorescence images are shown both overlaid on their corresponding DIC images and on their own, and in Fig. 3, in which immunofluorescence images are shown at a higher magnification. In freshly plated neurons that had not yet grown processes, diffuse staining was present within the cell body but not within the lamellipodia (Fig. 2, *A* and *A′*). After the formation of immature processes (the common precursors of both axons and dendrites; see Dotti et al., 1988), the staining remained diffuse and restricted to the cell body (Fig. 2, *B* and *B′*). The staining became slightly less diffuse and more patchy after one of the early processes had differentiated into the axon (Fig. 2, *C* and *C′*). Minute levels of staining sometimes extended for a few microns into the processes at this stage. Major alterations in both the distribution and appearance of CHO1 staining occurred as the remaining immature processes began to differentiate into dendrites. Specifically, the staining was no longer restricted to the cell body, but now extended into the newly differentiating dendrites (Fig. 2, *D* and *D′*). Within the dendrite and the cell body, the staining lost its diffuse quality and instead appeared as discrete elongate patches that varied somewhat in their

1. *Abbreviations used in this paper*: AFU, arbitrary fluorescence unit; DIC, differential interference contrast.

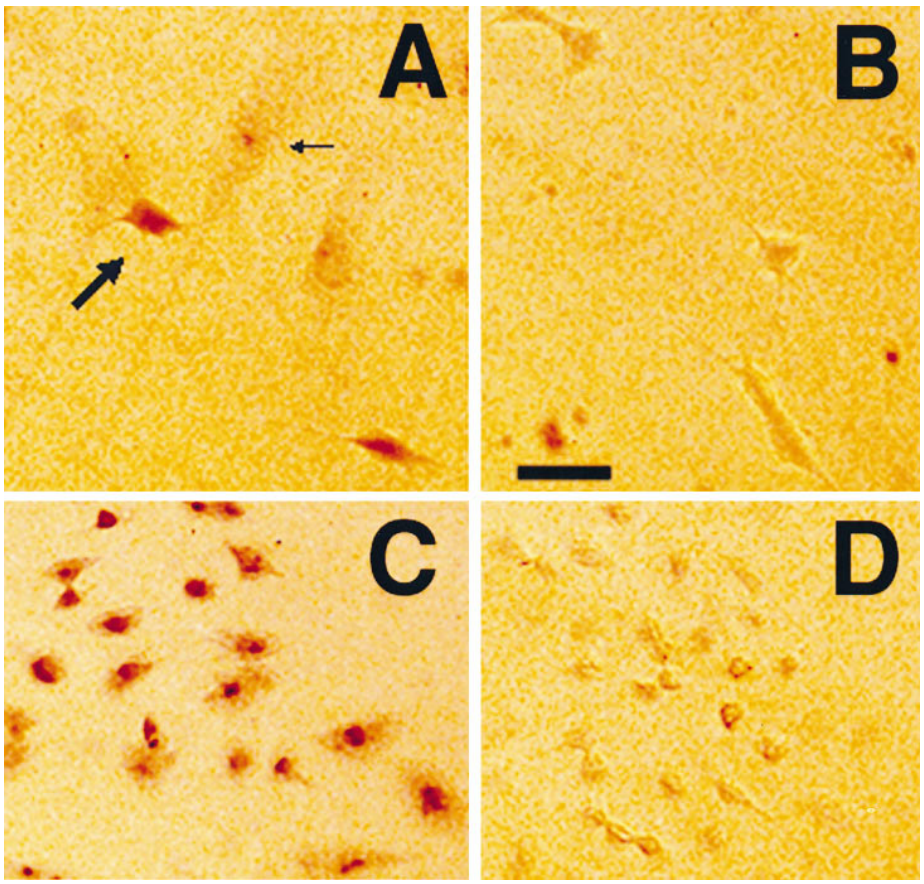

Figure 1. Analysis of CHO1 mRNA expression in cultured sympathetic neurons and CHO cells by in situ hybridization. Hybridization of the digoxigenin-labeled probe with native mRNA is indicated by the presence of a reddish reaction product within the cell. (*A* and *C*) Hybridization with antisense probe on sympathetic neurons and CHO cells, respectively. (*B* and *D*) Hybridization with sense probe on sympathetic neurons and CHO cells, respectively. CHO1 mRNA hybridization is prominent within the cell bodies of the sympathetic neurons (*A*, *large arrow*). Labeling is also apparent within the cytoplasm of the nonneuronal cells (*A*, *small arrow*) within the cultures. No labeling was detected with the sense CHO1 probe in the neurons or the nonneuronal cells within these cultures (*B*). As in the neuron cultures, labeling was observed in the CHO cells after hybridization with the antisense probe (*C*) but not with the sense probe (*D*). In both cases, the labeling was more intense in the nucleus than in the cytoplasm. Bar, 30 μm.

size and shape. Sometimes these patches, particularly in the dendrite, appeared filamentous in nature. When viewed at higher magnifications, and particularly in neurons that were flatter against the substratum, this staining pattern is more pronounced (Fig. 3, *A* and *B*). The patches varied in length but were generally 0.5–7 μm.

Similar findings were obtained in cultured sympathetic neurons. Staining for CHO1/MKLP1 remained diffuse and confined to the cell body during axonal development, and then acquired a somatodendritic localization as dendrites developed. Fig. 4, *A* and *B*, shows the DIC and immunofluorescence images, respectively, of a field containing two neurons that had developed dendrites. The staining within the dendrites was composed of discrete patches, but these were somewhat shorter than the patches in the hippocampal dendrites. Given that CHO1/MKLP1 is a microtubule-associated motor protein that is not involved in organelle transport, it seems reasonable that the patches of CHO1 staining may correspond to regions of the microtubule array itself. Unfortunately, the CHO1 antibody is not amenable to immuno-EM because antigenicity is not preserved in cells fixed with glutaraldehyde (Nislow et al., 1990) and, for reasons that are unclear, the antibody did not perform well in any standard double-labeling immunofluorescence preparations. For these reasons, an indirect approach was taken to address this issue. Neurons were treated for 30 min with 10 μg/ml nocodazole to depolymerize a substantial portion of their microtubule polymer. As shown in Fig. 4, *C* and *D*, the CHO1/MKLP1 staining became diffuse in neurons treated in this manner, indicating that the staining pattern observed in dendrites is dependent upon an intact microtubule array. Similar results were obtained on cultured hippocampal neurons (data not shown).

Inhibition of CHO1/MKLP1 Expression Inhibits Dendritic Development

The localization and staining pattern of CHO1/MKLP1 are consistent with the possibility that it regulates an important feature of the dendritic microtubule array. To investigate this possibility, we used antisense oligonucleotides to inhibit the expression of CHO1/MKLP1 in primary neurons. In a previous study neuroblastoma cells were cultured in the presence of two different antisense oligonucleotide 19-base sequences. These oligonucleotides (but not their corresponding sense controls) lowered the levels of CHO1/MKLP1 and inhibited the formation of dendrite-like processes (Yu et al., 1997). A concern with performing antisense studies of this kind on primary neurons is that they have a stronger tendency than neuroblastoma cells to exhibit nonspecific ill effects in response to oligonucleotides. Another concern, specifically related to CHO1/MKLP1, is that inhibiting the expression of this protein may have deleterious effects on the nonneuronal cells that provide necessary growth factors for the neurons. For the latter reason, we used sympathetic neurons for the antisense analyses rather than hippocampal neurons because sympathetic neurons do not require such growth factors if cultured in the presence of OP1, a mor-

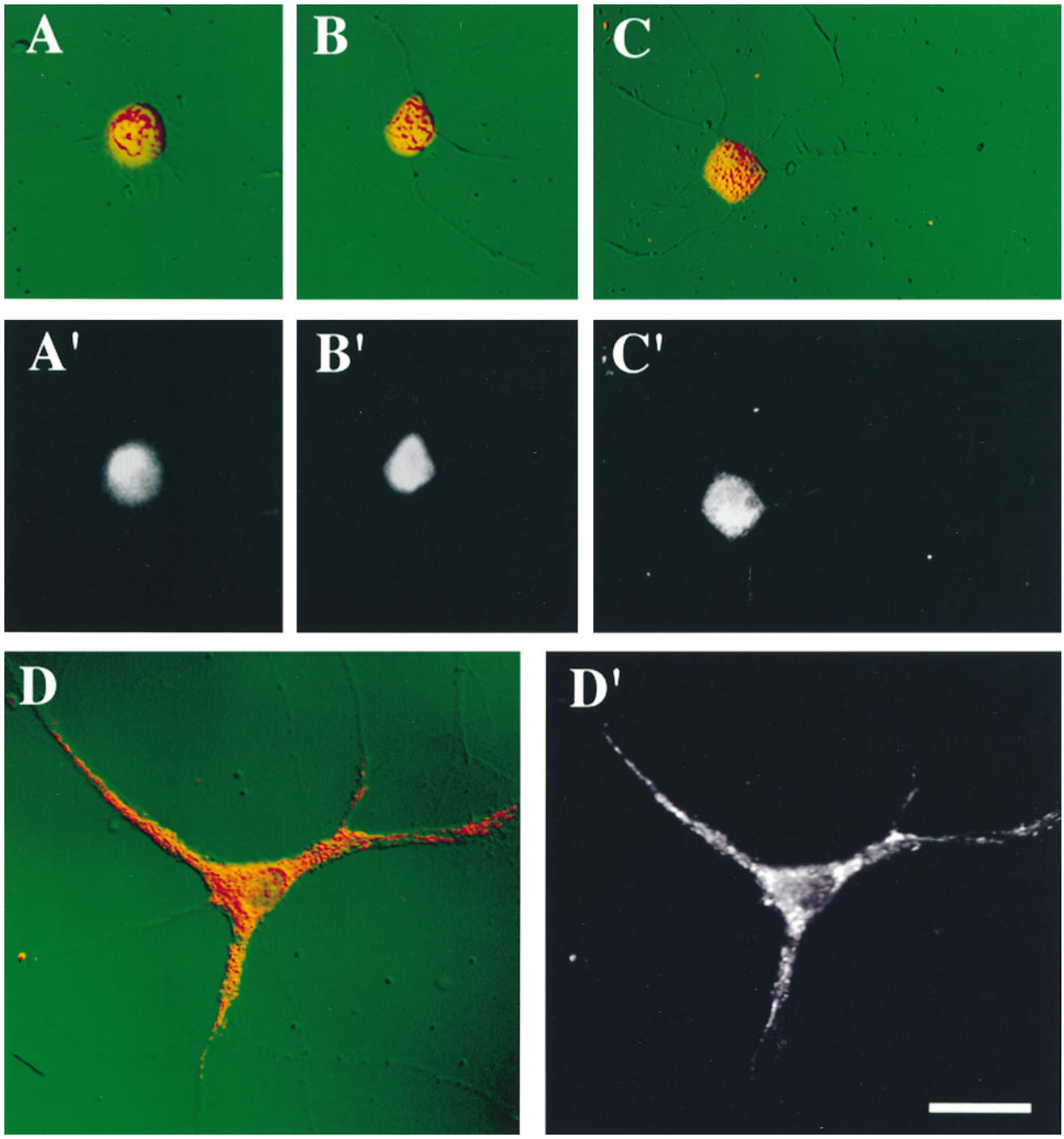

Figure 2. Localization of CHO1/MKLP1 within developing hippocampal neurons in culture. Immunofluorescence images are shown both overlaid on their corresponding DIC images (*A–D*) and on their own (*A′–D′*). (*A* and *B*) In freshly plated neurons that had not yet grown processes, diffuse staining is present within the cell body but not within the lamellipodia. (*A′* and *B′*) After the formation of immature processes, the staining remains diffuse and restricted to the cell body. (*C* and *D*) After one of the early processes had differentiated into the axon, the staining is somewhat more patchy but still almost completely confined to the cell body. (*C′* and *D′*) As the remaining minor processes differentiated into dendrites, the staining takes on the appearance of discrete elongated patches. Staining is present both within the cell body and the developing dendrites, but it remains absent from the axon. Bar, 20 μm.

phogen that promotes the robust and rapid differentiation of dendrites (Lein et al., 1995). Experimental conditions were optimized as follows. Neurons were cultured for 4 d without serum or OP1, during which time a dense mat of axons but few or no dendrites developed. Antisense or sense oligonucleotides were then added to the culture medium for an additional 2 d and were replenished every 12 h. After these 2 d, OP1 was added in concert with the oligonucleotides for an additional 3 d. Both the OP1 and the oligonucleotides were replenished every 12 h. The same antisense (termed AS1 and AS2) and sense (S1 and S2) oligonucleotide sequences were used as in our previous study (Yu et al., 1997; see Materials and Methods). In the absence of oligonucleotides, many of the neurons began to differentiate dendrites within the first day in OP1 and, by day 3, nearly all of the neurons displayed at least one ro-

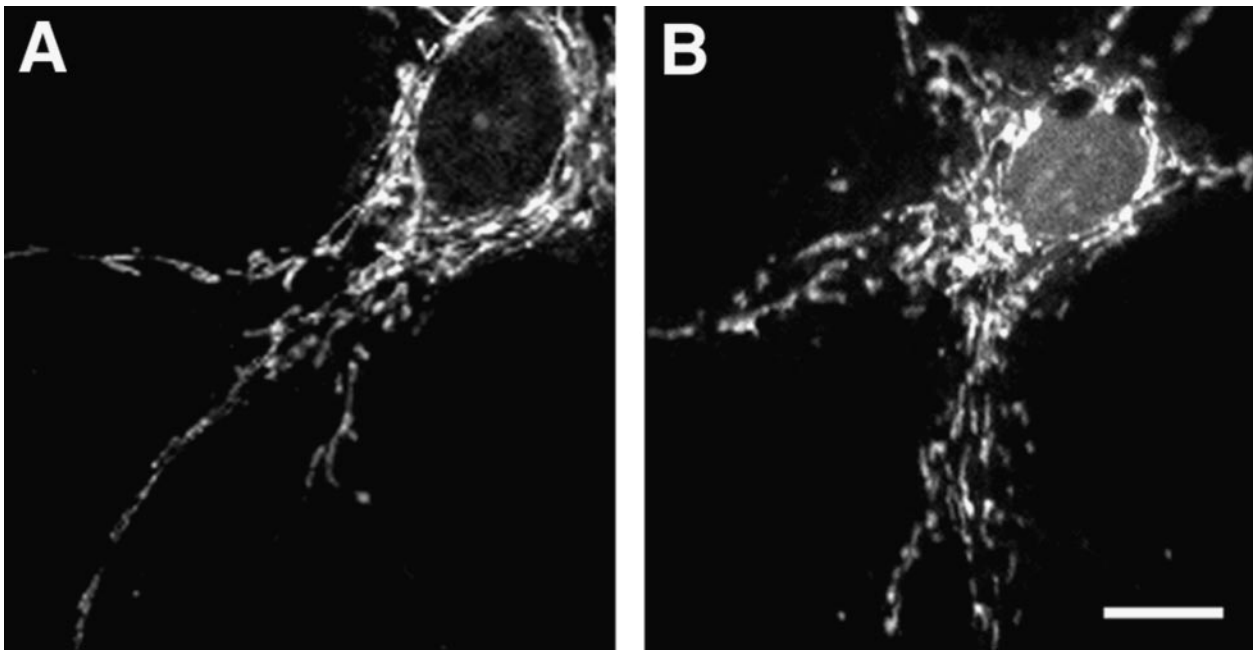

Figure 3. The localization of CHO1/MKLP1 within flatter hippocampal neurons in culture shown at higher magnification. Staining is particularly patchy and sometimes filamentous. Bar, 10 μm.

bust dendrite. Under these conditions, neither the sense nor the antisense oligonucleotides (at concentrations ranging from 0.1–5 μM) caused the axons to stop growing nor did they induce signs of nonspecific damage to the neurons such as beading or blebbing of the cell bodies or axons.

Quantitative immunofluorescence analyses indicate that the antisense oligonucleotides reduced the levels of CHO1/MKLP1 within the neurons in a dose- and time-dependent manner (Fig. 5, *left two panels*). By 2 d in either 1 or 5 μM of either AS1 or AS2, the levels of CHO1/MKLP1 had diminished to <10% of the levels within parallel oligonucleotide-free cultures. A concentration of antisense of 0.1 μM resulted in a reduction in protein levels to ~30% of the levels within the oligonucleotide-free cultures. Diminution in CHO1/MKLP1 levels persisted through day 4, with the levels falling to <5% of control levels in neurons exposed

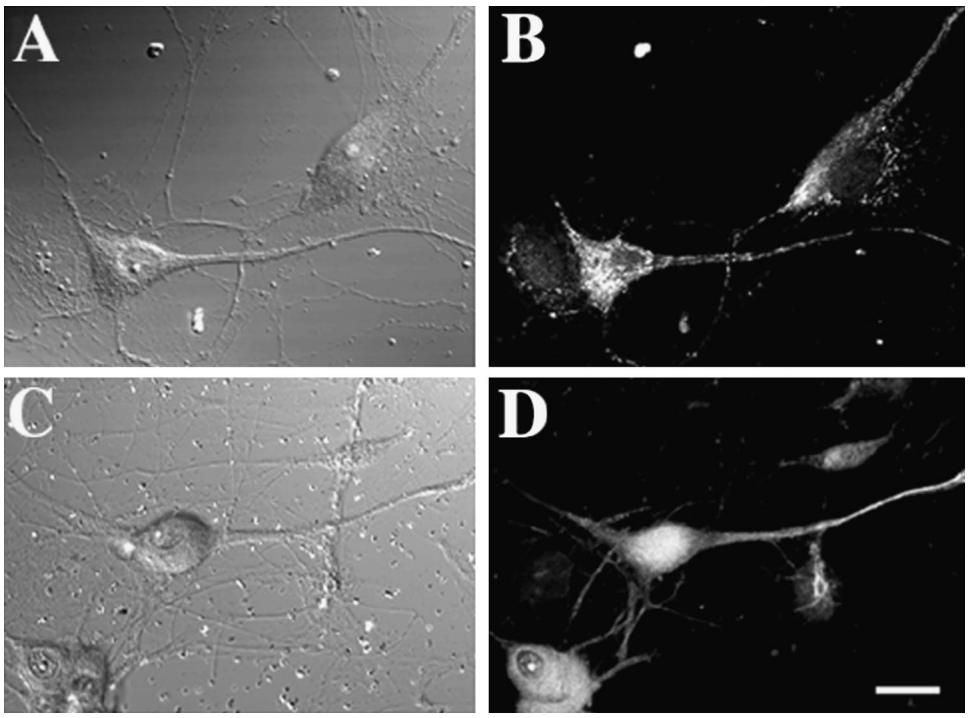

Figure 4. Localization of CHO1/MKLP1 within cultured sympathetic neurons and its association with the microtubule array. (*A* and *B*) DIC and CHO1 immunofluorescence images, respectively, of a field containing two neurons that had developed robust dendrites. The staining within the dendrites is patchy in nature, but the individual patches are somewhat shorter than those in the hippocampal dendrites. (*C* and *D*) DIC and CHO1 immunofluorescence images of a neuron treated for 30 min with 10 μg/ml nocodazole to depolymerize a substantial portion of the microtubule polymer. The staining becomes diffuse. Bar, 15 μm.

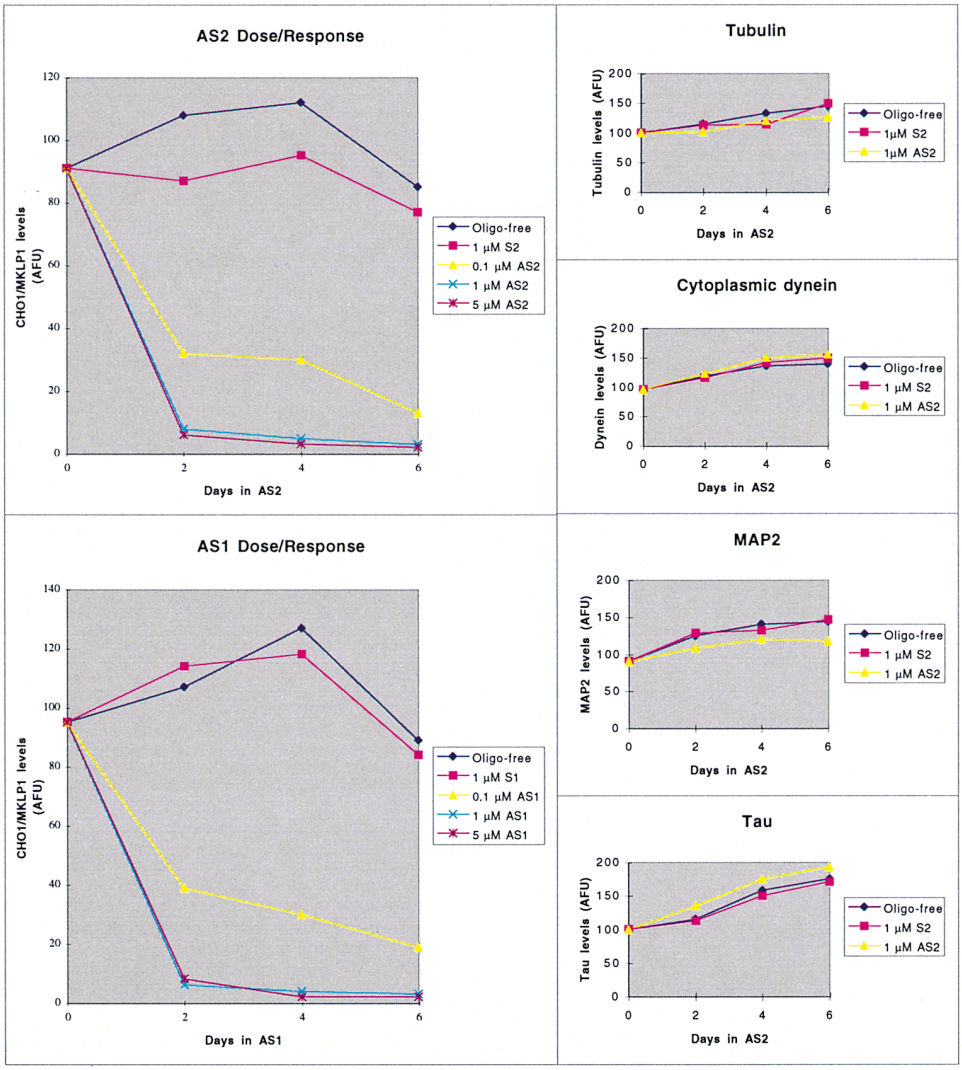

Figure 5. Quantitative analyses on the levels of CHO1/MKLP1 (*left two panels*) and β-tubulin, cytoplasmic dynein, MAP2, and tau (*right four panels*) in sympathetic cultures treated with antisense, sense, and no oligonucleotides. Protein levels were determined using quantitative immunofluorescence microscopy and were expressed in arbitrary fluorescence units (*AFU*). In all of the analyses shown, 100 cells were examined from each condition and at each time point. Standard deviations were <20 AFU and error bars were not included because they would be short and blur together.

to 1 or 5 μM antisense and holding at ~30% in neurons exposed to the 0.1 μM concentration. By day 6, CHO1/MKLP1 was virtually undetectable in neurons exposed to 1 or 5 μM antisense, and the levels had fallen to ~20% of control levels in the cultures exposed to 0.1 μM. Cultures treated with sense oligonucleotides showed no significant reductions in CHO1/MKLP1 compared to oligonucleotide-free cultures. To determine the specificity of these effects, we also performed quantitative immunofluorescence analyses on the levels of β-tubulin, cytoplasmic dynein, MAP2, and tau (Fig. 5, *right four panels*). For these analyses, we used the 1 μM concentration of AS2, the lowest concentration that produced a maximal diminution of CHO1/MKLP1 levels. No significant alterations in the levels of any of these four proteins were observed. For all data points shown in Fig. 4, 100 cells were randomly selected, and a mean ± SD was obtained for neurons from each condition.

The antisense treatments also resulted in marked morphological effects on the neurons. Neurons grown in the absence of oligonucleotides or in the presence of the sense oligonucleotides responded to the addition of OP1 with the rapid formation of dendrites, confirmed by their cytochemical composition (see Lein et al., 1995), thick tapering morphology, and the obtuse angle at which they extend from the cell body (Fig. 6 *A*). In contrast, neurons grown in the presence of 1 or 5 μM AS1 or AS2 did not demonstrate this same responsiveness to OP1. The vast majority of the processes extended by these neurons had the thin cylindrical morphology characteristic of axons (Fig. 6 *B*). A small number of the processes had an ambiguous morphology that was slightly thicker than a typical axon but not as broad calibered as the processes clearly identifiable as dendrites within control cultures (Fig. 6 *C*). Neurons treated with the sense oligonucleotides were entirely similar in appearance to neurons from oligonucleotide-free cultures (not shown). To quantify these effects, morphometric analyses were performed on 100 neurons from cultures exposed to either 1 μM AS2, 1 μM S2, or no oligonucleotides (Fig. 7). Before the addition of OP1, <5% of the neurons in all conditions had differentiated at least one process identifiable as a dendrite by morphology. After a 2-d exposure to OP1, 87% of the neurons from cultures grown in the absence of oligonucleotides, and 82% of the neurons grown in S2, had differentiated at least one dendrite. By contrast, only 5% of the neurons exposed to

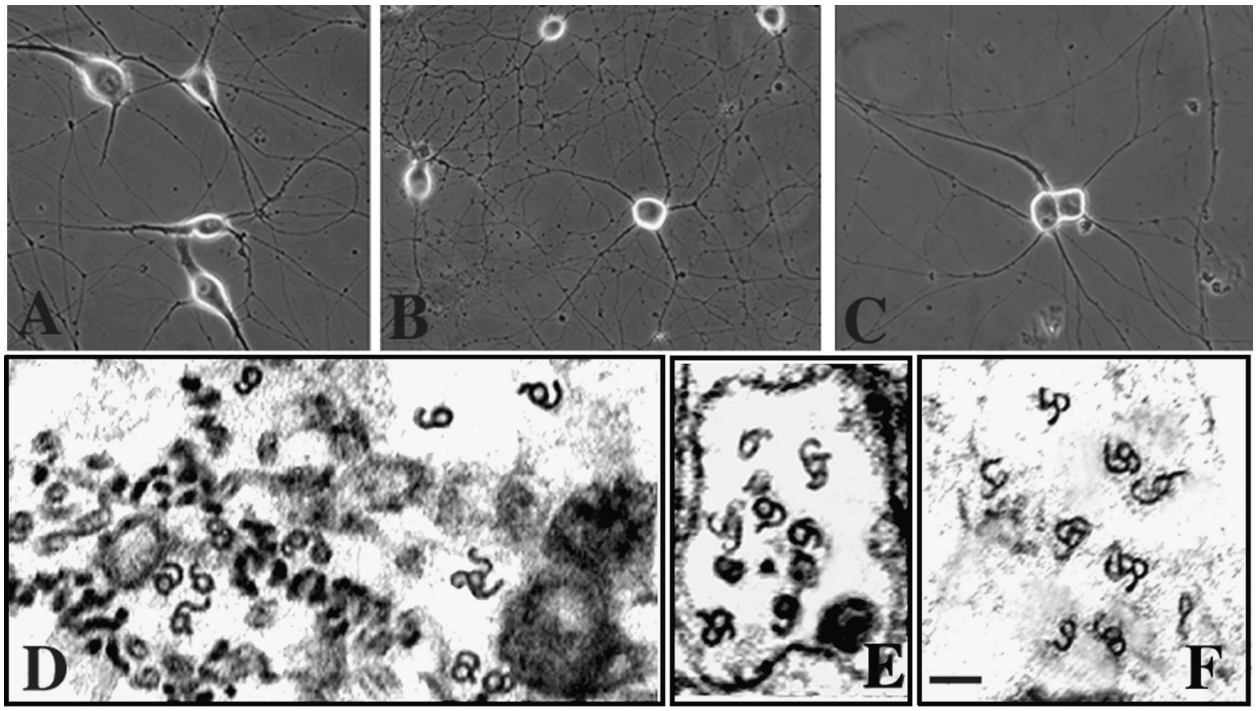

Figure 6. Phase micrographs and microtubule polarity analyses on sympathetic neurons cultured with no oligonucleotides (*A*) or 1 μM AS2 (*B* and *C*). (*A*) Neurons respond to the addition of OP1 to the culture medium by differentiating robust dendrites as indicated by their thick tapering morphology. (*B* and *C*) Most neurons grown in antisense form only processes with the thin cylindrical morphology of axons (*B*), but a small number form the processes with an ambiguous morphology that is slightly thicker than typical axons (*C*). In microtubule polarity analyses, hooked appendages on the microtubules indicate the polarity orientation of the microtubule. Plus end–distal microtubules show clockwise hooks and minus end–distal microtubules show counterclockwise hooks. (*D*) In dendrites from oligonucleotide-free cultures, ~60% of the microtubules show clockwise hooks and 40% show counterclockwise hooks. (*E*) In the thin cylindrical processes that form in AS2, ~99% of the microtubules show clockwise hooks. (*F*) In the processes with an ambiguous morphology, ~94% of the microtubules show clockwise hooks. Bars: (*A–C*) 25 μm; (*D–F*) 60 nm.

AS2 had differentiated at least one dendrite. 90% of the neurons had only axons and 5% had extended processes with the ambiguous morphology. After 4 d in OP1, nearly all of the neurons from oligonucleotide-free and sense-treated cultures had differentiated at least one distinct dendrite, 97% and 92%, respectively. In antisense-treated cultures, the percentage of neurons with distinct dendrites was 6%, essentially the same as on day 4. 89% of the neurons had only axons, and 5% had at least one process with an ambiguous morphology. Essentially these same results were observed after 2 wk in antisense, during which time the cultures continued to appear healthy. Removal of antisense from the cultures resulted in the reappearance of CHO1/MKLP1 and a resumption of dendritic development (data not shown).

These results indicate that suppression of CHO1/MKLP1 inhibits the capacity of neurons to form morphologically distinct dendrites. On the basis of the transport properties of the motor and its specific association with the dendritic microtubule array, it seems reasonable that CHO1/MKLP1 is necessary for the establishment of the nonuniform microtubule polarity pattern essential for dendritic development. To investigate this possibility, we performed microtubule polarity analyses using the standard hooking protocol. In this technique, cells are extracted in a special microtubule assembly buffer in the presence of exogenous brain tubulin. The exogenous tubulin adds onto existing microtubules in the form of lateral protofilament sheets that, when viewed in cross-section, appear as hooked appendages on the microtubules. As viewed from the distal tip of the process, a clockwise hook indicates that the plus end of the microtubule is directed away from the cell body while a counterclockwise hook in-

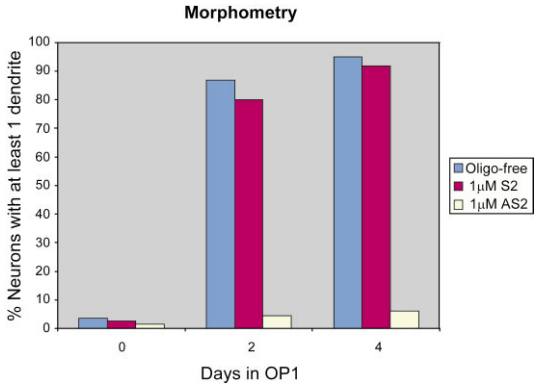

Figure 7. Morphometric analyses performed on neurons from cultures treated with 1 μM AS2, 1 μM S2, or no oligonucleotides. 100 neurons from each condition were analyzed. The number of cells bearing at least one process morphologically distinguishable as a dendrite was tabulated from each condition.

dicates the opposite. In cultures free of oligonucleotides and in cultures exposed to S2, over 98% of the hooks on the axonal microtubules were clockwise, indicating that these axons contain uniformly plus end–distal microtubules. Approximately 60% of the hooks on the dendritic microtubules were clockwise, indicating that these dendrites contain nonuniformly oriented microtubules (Fig. 6 *D*). In neurons exposed to 1 μM AS2, over 98% of hooks on the microtubules within the axons were clockwise (Fig. 6 *E*). In processes with the ambiguous morphology, 94% of the hooks on the microtubules were clockwise, indicating that these processes had not acquired the nonuniform microtubule polarity pattern characteristic of dendrites. In the rare processes that exhibited dendritic morphological characteristics, 82% of the hooks on the microtubules were clockwise, indicating that these processes had acquired minus end–distal microtubules but in significantly lower numbers than in control dendrites. The data from these analyses are presented in Table I.

Discussion

There has been a great deal of controversy in recent years concerning the means by which neurons generate their microtubule arrays. The first comprehensive model invoked microtubule transport as a key principle (Lasek, 1982; Black, 1994; Baas and Yu, 1996), but this idea has met with repeated challenges over the years from authors who contend that microtubules are stationary structures within axons and dendrites and that tubulin is transported as free subunits or small oligomers (Bamburg et al., 1986; Okabe and Hirokawa, 1989; Funakoshi et al., 1996). During the course of this debate, significant progress has been made on the role of microtubule transport in other cellular events, most notably mitosis. Strong evidence now suggests that motor proteins are essential for driving apart microtubules attached to the duplicated centrosomes during prophase (Vaisberg et al., 1993; Blangy et al., 1995), for moving together microtubules from opposite poles into a bipolar spindle through metaphase (Heald et al., 1996; Echeverri et al., 1996; Endow et al., 1994), and for sliding antiparallel microtubules originating from opposite poles apart during anaphase B (Nislow et al., 1990, 1992). Microtubule transport has also been directly observed in interphase cells as the movement of microtubules from the centrosome to the cell periphery (Keating et al., 1997). We find it attractive that neurons, once terminally postmitotic, would use motor-driven transport events in a modified fashion to establish the microtubule arrays of the axon and the dendrites. It seems less reasonable to us that the neuron would abandon microtubule transport as a key principle, cease microtubule movements, and develop entirely novel mechanisms for the establishment of the axonal and dendritic microtubule arrays.

Table I. Summary of Microtubule Polarity Analyses

	Axons*	Dendrites*	Ambiguous*
Control	99 ± 1% (n = 10)	58 ± 7% (n = 5)	NA
Sense (S2)	97 ± 2% (n = 10)	61 ± 9% (n = 5)	NA
Antisense (AS2)	98 ± 1% (n = 100)	82 ± 8% (n = 2)	95 ± 4% (n = 6)

*Percentage of hooked microtubules with clockwise curvature as viewed from the distal tip of the process (mean ± SD).
n, number of processes examined.

If our perspective on this issue is correct, the question arises as to whether the motor proteins that transport microtubules into axons and dendrites are novel motors expressed only in postmitotic neurons or whether they are are in fact the same motors that transport microtubules during mitosis. Several lines of evidence suggest that the motor protein studied here, CHO1/MKLP1, has the appropriate properties to participate both in mitosis and in the formation of processes from postmitotic cells. In mitosis, CHO1/MKLP1 is involved in spindle elongation during late anaphase and is thought to function by transporting the minus ends of microtubules from one pole toward the plus ends of microtubules from the other pole (Sellitto and Kuriyama, 1988; Nislow et al., 1990, 1992). The capacity of CHO1/MKLP1 to participate in process formation was first indicated by studies in which ectopic expression of a fragment of the protein was shown to induce insect ovarian cells to extend dendrite-like processes with nonuniform microtubule polarity orientation (Kuriyama et al., 1994; Sharp et al., 1996). More recently, it was shown that endogenously expressed CHO1/MKLP1 is essential for both mitosis and the formation of dendrite-like processes from neuroblastoma cells (Yu et al., 1997). In the present study we have documented that CHO1/MKLP1 is expressed in primary sympathetic and hippocampal neurons that have been terminally postmitotic for several days. We do not feel that the continued expression of CHO1/MKLP1 in these cells is an artifact of cell culture because we have also demonstrated the expression of this protein in postmitotic neurons from the brain (Ferhat, L., R. Kuriyama, G.E. Lyons, and P.W. Baas, manuscript in preparation).

The localization of CHO1/MKLP1 within developing neurons provides a first line of evidence for a functional role of the protein in dendritic development. Before the development of dendrites, the protein is present only within the cell body of the neuron and is diffuse in appearance. The protein does not enter immature processes or developing axons to any appreciable degree at any stage of development, indicating that it is not freely diffusible despite its appearance. As dendrites develop, CHO1/MKLP1 begins to appear within the developing dendrites and also takes on a less diffuse appearance. The staining pattern within the cell body and dendrites manifests as elongated patches that vary somewhat in their size and shape. Sometimes the patches appear as thin filaments, but this is not common. Given this staining pattern, it is unlikely that the patches of CHO1/MKLP1 correspond to entire microtubules undergoing transport into the dendrite. However, the discrete patchy nature of the staining is almost completely lost after a brief treatment with a microtubule-depolymerizing drug, indicating that the patches correspond to some feature of the microtubule array. We suspect that the patches do not correspond to regions of the microtubules per se, but to overlapping regions of oppositely oriented microtubules sliding relative to one another. This interpretation is consistent with the fact that the staining pattern observed in cell bodies and dendrites is reminiscent of the staining for CHO1/MKLP1 in the midzone region of the

mitotic spindle where it is thought to bridge antiparallel microtubules (Sellitto and Kuriyama, 1988; Nislow et al., 1990). Even so, it is likely that at least one of these sliding regions corresponds specifically to a more labile microtubule domain given the loss of the patches after such a brief drug treatment. Interestingly, the staining pattern for CHO1/MKLP1 is less patchy in neuroblastoma cells, which also require the motor for the establishment of nonuniform microtubule polarity orientation in their dendrite-like processes (Yu et al., 1997). One possibility is that the patchy staining pattern reflects a tighter, less transient association of the motor with microtubules in primary neurons compared with neuroblastoma cells. This may account for the fact that minus end–distal microtubules never extend as far into the neuroblastoma processes as they do within the bona fide dendrites of primary neurons.

A second line of evidence indicating an essential role for CHO1/MKLP1 in dendritic development is provided by studies in which we documented the effects of inhibiting CHO1/MKLP1 expression in developing primary neurons. For these analyses we found that two nonoverlapping antisense oligonucleotides were effective in diminishing the levels of CHO1/MKLP1 in these neurons and in suppressing their capacity to develop dendrites. As with any study involving the use of antisense oligonucleotides, it is important to establish that the observed effects are specific and not due to a diminution in the health of the cultures. The fact that the same results were obtained with both of the antisense oligonucleotide sequences but not their corresponding sense sequences is strong support for the specificity of the effects. Further support derives from the fact that the antisense treatments decreased the levels of CHO1/MKLP1 in a time- and dose-dependent manner and did not result in alterations in the levels of any of the four other cytoskeletal proteins that we examined. In addition, there was a return in CHO1/MKLP1 expression and a resumption in dendritic development upon removal of the antisense. Finally, these results are consistent with those obtained previously in studies in which the same antisense oligonucleotides were shown to inhibit both mitotic division and the formation of dendrite-like processes from neuroblastoma cells (Yu et al., 1997).

Authors who favor the view that tubulin is transported in neurons as oligomeric structures and not as microtubules might argue that CHO1/MKLP1 transports such oligomers rather than minus end–distal microtubules. These authors might suggest that the patches of CHO1/MKLP1 staining observed in dendrites correspond to such oligomers. We feel that this is an unlikely possibility, given that the patches are lost during treatments that specifically depolymerize microtubule polymer. In addition, the view that CHO1/MKLP1 transports microtubules themselves is attractive in that it is entirely consistent with the demonstrated transport properties of the motor (Nislow et al., 1992). Most importantly, it is difficult to imagine how the transport of tubulin oligomers along plus end–distal microtubules could result in the acquisition of minus end–distal microtubules within these processes. Thus, while microtubule transport has not yet been directly observed within developing dendrites, we contend that the requirement for CHO1/MKLP1 in dendritic differentiation is strong indirect evidence that such transport occurs.

Collectively, the results presented here strongly suggest that neurons use an already identified motor protein for the transport of minus end–distal microtubules into developing dendrites. Thus, not only is motor-driven microtubule transport a common theme between mitosis and neuronal process formation but, at least in this case, it appears that the same motor protein is used for both. This strategy is economical, particularly in light of the similar functions that CHO1/MKLP1 performs in both, but the question arises as to how the motor is assigned one task during mitosis and the other task in postmitotic neurons. Virtually all animal cells use CHO1/MKLP1 (or a homologous motor) for spindle elongation during cell division, but only neurons extend dendrites. Some insight into the manner by which CHO1/MKLP1 is assigned one or the other of these tasks may be indicated by our previous studies in which we ectopically expressed fragments of the motor in insect ovarian cells. The protein did not induce the formation of dendrite-like processes when expressed in its full length, but only when expressed in a truncated form that did not include a substantial portion of its carboxy-terminal region (Kuriyama et al., 1994; Sharp et al., 1996). One possibility is that neurons express a variant of CHO1/MKLP1 in which some of this region is spliced out. If this is the case, then the spliced region must be relatively short, given the results of our in situ hybridization and Northern blot analyses in which we obtained a positive result and a single band, respectively, using a probe corresponding to the carboxy-terminal region of the molecule. An interesting possibility is that the carboxy-terminal region contains a nuclear localization signal that is spliced out in neurons, and that this event is critical to the capacity of the motor to participate in nonmitotic events. It is also possible that the function of CHO1/MKLP1 is regulated by posttranslational truncation, posttranslational modifications such as phosphorylation, or conditions within the cell such as the presence of neuron-specific microtubule-associated proteins. Additional studies on the CHO1/MKLP1 molecule within developing neurons will be required to distinguish among these possibilities.

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