

# Nonuniform Microtubular Polarity Established by CHO1/MKLP1 Motor Protein Is Necessary for Process Formation of Podocytes

Naoto Kobayashi,\* Jochen Reiser,\* Wilhelm Kriz,\* Ryoko Kuriyama,‡ and Peter Mundel§

\*Department of Anatomy and Cell Biology, University of Heidelberg, D-69120 Heidelberg, Germany; ‡Department of Cell Biology and Neuroanatomy, University of Minnesota Medical School, Minneapolis, Minnesota 05545; and §Department of Medicine and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

**Abstract.** Podocytes are unique cells that are decisively involved in glomerular filtration. They are equipped with a complex process system consisting of major processes and foot processes whose function is insufficiently understood (Mundel, P., and W. Kriz. 1995. *Anat. Embryol.* 192:385–397). The major processes of podocytes contain a microtubular cytoskeleton. Taking advantage of a recently established cell culture system for podocytes with preserved ability to form processes (Mundel, P., J. Reiser, A. Zúñiga Mejía Borja, H. Pavenstädt, G.R. Davidson, W. Kriz, and R. Zeller. 1997b. *Exp. Cell Res.* 36:248–258), we studied the functional significance of the microtubular system in major processes. The following data were obtained: (a) Microtubules (MTs) in podocytes show a nonuniform polarity as revealed by hook-decoration. (b) CHO1/MKLP1, a kinesin-like motor protein, is associated with MTs in podocytes. (c) Treatment of differentiating

podocytes with CHO1/MKLP1 antisense oligonucleotides abolished the formation of processes and the nonuniform polarity of MTs. (d) During the recovery from taxol treatment, taxol-stabilized (nocodazole-resistant) MT fragments were distributed in the cell periphery along newly assembled nocodazole-sensitive MTs. A similar distribution pattern of CHO1/MKLP1 was found under these circumstances, indicating its association with MTs. (e) In the recovery phase after complete depolymerization, MTs reassembled exclusively at centrosomes. Taken together, these findings lead to the conclusion that the nonuniform MT polarity in podocytes established by CHO1/MKLP1 is necessary for process formation.

**Key words:** glomerular podocyte • process formation • microtubular polarity • CHO1/MKLP1 • microtubule-organizing center

**P**ODOCYTES are highly organized cells with thick major processes and delicate foot processes (Mundel and Kriz, 1995; Kobayashi and Mundel, 1998). The major processes contain a well-developed cytoskeleton composed of microtubules (MTs)<sup>1</sup> and intermediate filaments (IFs), while foot processes contain a core cytoskeleton of actin filaments (AFs). A recently established cell culture

model of conditionally immortalized podocytes allows process formation to be analyzed in an inducible system (Mundel et al., 1997a,b). The mechanism(s) governing process formation have so far been studied mainly in neurons, where MTs and their associated proteins play a crucial role in the development and maintenance of cell processes. In nonneuronal cells, including podocytes, the functional relevance of the microtubular cytoskeleton during process formation remains to be elucidated (Kobayashi and Mundel, 1998).

MTs are polarized polymers of tubulin heterodimers with fast-growing plus ends and slow-growing minus ends. Under physiological conditions, the microtubule-organizing center (MTOC) is necessary to initiate nucleation of MTs. The  $\gamma$ -isoform of tubulin appears to be necessary for MT nucleation (Joshi et al., 1992; Moritz et al., 1995); so far, the centrosome is accepted as the most significant  $\gamma$ -tubulin-containing MTOC in mammalian cells. MTs are generally nucleated at the centrosome, which also serves

Naoto Kobayashi's current address is Department of Anatomy, School of Medicine, University of Ehime, Ehime 791-0295, Japan.

Address correspondence to Dr. Peter Mundel, Division of Nephrology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Tel.: (718) 430-3158. Fax: (718) 430-8963. E-mail: mundel@acom.yu.edu

1. *Abbreviations used in this paper:* AF, actin filament; AS, antisense; IF, intermediate filament; MT, microtubule; MTOC, microtubule-organizing center; RT-PCR, reverse transcription PCR; S, sense; TEM, transmission electron microscopy.

as the MTOC during interphase (Pereira and Schiebel, 1997; Hyman and Karsenti, 1998). The MT nucleation activity of the centrosome can determine the polarity of MTs; a uniform arrangement of MTs can be established by nucleation of MTs from the perinuclear centrosome, leading to the generation of a plus end–distal polarity of MTs. However, in dendrites (Baas et al., 1988, 1989; Wang et al., 1996) and in glial cell processes (Kidd et al., 1994; Lunn et al., 1997), the polarity of MTs is nonuniform. MTs are oriented both in plus end–distal and minus end–distal fashions. The generation of this mixed MT polarity requires an additional mechanism, since MTOCs other than centrosomes are unlikely to exist in neurons (Baas and Joshi, 1992; Yu et al., 1993). Currently, the most likely hypothesis explaining the nonuniform orientation of dendritic MTs is as follows: after assembly at centrosomes, MTs are released from centrosomes and transported in an antiparallel (i.e., minus end–distal fashion) along preexisting plus end–distal MTs (Baas, 1997). This hypothesis requires a motor protein that mediates the antiparallel transport of MTs; conventional MT-associated proteins establish only uniform MT polarity in a plus end–distal fashion (Baas et al., 1991; Chen et al., 1992; Takemura et al., 1995).

CHO1/MKLP1 was originally defined by a monoclonal antibody called “CHO1,” which stained the midbody of mitotic spindles in CHO cells (Selitto and Kuriyama, 1988). Later, it was characterized as a plus end–directed motor protein enabling antiparallel sliding of MTs (Nislow et al., 1992). Molecular cloning of CHO1/MKLP1 revealed that it is a member of the kinesin superfamily (Nislow et al., 1992). Recent evidence suggests that CHO1/MKLP1 motor has a dual function: force generation in mitosis (Nislow et al., 1990, 1992) and transport of MTs in dendrites (Sharp et al., 1997; Yu et al., 1997). The latter function implies that CHO1/MKLP1 conveys MTs in a minus end–distal fashion along preexisting plus end–distal MTs to establish the nonuniform polarity of MTs.

Since all cells with nonuniform MT polarity described so far are arborized cells with prominent processes, we were interested to see whether podocytes are equipped with both plus and minus end–distal MTs, and if so, whether this nonuniform MT polarity plays a role in process formation of these cells. To clarify this issue, we analyzed the polarity of MTs in a differentiated podocyte cell line and revealed its nonuniformity. Next, we analyzed the expression of CHO1/MKLP1 motor protein in podocytes and its relevance for the nonuniform polarity of MTs and process formation. We also demonstrated that the centrosome is the major  $\gamma$ -tubulin-containing MTOC in cultured podocytes. Based on these findings, we discuss the mechanism generating the nonuniform polarity of MTs and the functional significance of this mixed polarity for the process formation of podocytes.

## Materials and Methods

### Cell Culture of Mouse Podocytes

The generation and initial characterization of conditionally immortalized mouse podocyte clones (hereafter referred to as “podocytes”) has previously been described (Mundel et al., 1997b). Podocytes were maintained in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% FCS (Boehringer Mannheim, Mannheim, Germany),

100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Life Technologies) in a humid atmosphere with 5% CO<sub>2</sub>. To propagate podocytes, the culture medium was supplemented with 10 U/ml recombinant mouse  $\gamma$ -interferon (Sigma, Munich, Germany) to enhance expression of T-antigen, and cells were cultivated at 33°C (permissive conditions). To induce differentiation, podocytes were cultured on type I–collagen (Biochrom, Berlin, Germany) at 37°C without  $\gamma$ -interferon (nonpermissive conditions). Since it takes at least 10 d to induce differentiation, podocytes maintained for less than 1 wk under nonpermissive conditions are referred to as “differentiating cells” or “process-forming cells,” while those cultivated for 2–3 wk at nonpermissive conditions were termed “differentiated cells” or “process-bearing cells.” In the present study, podocytes of clone 5 (MPC-5; Mundel et al., 1997b) between passage 15 and 25 were used in all experiments.

### Determination of Microtubular Polarity

The polarity of MTs in podocytes was determined by the “hook” decoration method (Heidemann, 1991). MT proteins (C<sub>2</sub>S fraction) were enriched from porcine brains by two cycles of temperature-dependent depolymerization and polymerization in the presence of glycerol (Shelanski et al., 1973). Tubulin was purified from the C<sub>2</sub>S fraction by column chromatography (Vallee, 1986). Differentiated podocytes that had been cultured for 2–3 wk under nonpermissive conditions were rinsed briefly with PBS and permeabilized by incubation with prewarmed PEMG buffer (0.5 M Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, and 1 mM GTP) containing 0.1% saponin (Sigma) and 2 mg/ml tubulin for 2 min. Subsequently, permeabilized cells were incubated with PEMG buffer containing 2 mg/ml tubulin, but without saponin, at 37°C for 30 min. Both preparations, C<sub>2</sub>S fraction and purified tubulin, gave identical results for hook decoration. Samples were fixed with 4% glutaraldehyde in PBS and further processed for transmission electron microscopy (TEM). Sequences of serial ultrathin sections, including the profiles of centrosomes, were obtained to confirm the proximal–distal axis of examined podocytes and observed by TEM. The polarity of each MT (either plus end– or minus end–distal) was determined by “hook-handedness” as described by Heidemann (1991). Viewed from the cell periphery, MTs with clockwise hooks were judged as plus end–distal, whereas MTs with counterclockwise hooks were judged as minus end–distal. This evaluation was done in two series of ultrathin sections, each containing part of a cell starting at the centrosome and extending into the periphery of a process as reported previously (Takemura et al., 1995). The numbers of MTs identified either as plus end– or as minus end–distal on each electron micrograph were summarized among the sections selected from several independent sequences (Table I).

### Immunocytochemistry

The characterization of the antibodies against CHO1/MKLP1 motor protein were previously reported (Selitto and Kuriyama, 1988; Nislow et al., 1992; Kuriyama et al., 1994). Differentiated podocytes were fixed with absolute methanol at –20°C for 5 min and immunostained with polyclonal CHO1/MKLP1 antibody at 1:50 dilution. In some experiments, double labeling immunofluorescence with polyclonal anti-CHO1/MKLP1 and monoclonal  $\alpha$ -tubulin (Oncogene Science, Hamburg, Germany) was carried out. Antigen–antibody complexes were visualized with fluorochrome (Cy2, Cy3)-conjugated secondary antibodies (BioTrend, Cologne, Germany). CHO cells were used as a positive control of immunostaining.

### Detection of CHO1/MKLP1 mRNA Expression by RT-PCR

Total RNA was isolated from differentiated and undifferentiated podocytes using the RNeasy kit (QIAGEN, Hilden Germany). Reverse transcription (RT) of cDNA and PCR were performed according to standard protocols using the following two primers: GCACACCTGTCAATGT–

Table I. MT Polarity in Oligonucleotide-treated Podocytes as Determined by Hook Decoration

	CW	CCW	% CCW
Nontreated ( <i>n</i> = 2)	89	50	36
Antisense-treated ( <i>n</i> = 3)	82	17	17
Sense-treated ( <i>n</i> = 3)	63	44	41

CW, clockwise hooks; CCW, counter-clockwise hooks.

CACC (sense primer; nucleotide number +2213 to +2232 of hamster CHO1/MKLP1 cDNA; Kuriyama et al., 1994) and TGATCTACCCAT-CTGCTCCC (antisense primer; +2425 to +2444). These two primers are identical between hamster and mouse CHO1/MKLP1. After 40 cycles of amplification, PCR products were analyzed by agarose gel electrophoresis.

### **Inhibition of CHO1/MKLP1 Expression by Antisense Oligonucleotides**

Based on the cDNA sequence of CHO1/MKLP1, two antisense (AS) and two corresponding sense (S) oligonucleotides (AS1:CAGGTTTC-TGGGCATCTT, AS2:AGCTTTCGCTGGTTTCATG, S1:AAGATG-CCTCAGGAAACCTG, and S2:CATGAAACCAGCGAAAGCT) were designed as described previously (Sharp et al., 1997; Yu et al., 1997). AS1 corresponds to the sequence between +19 and +37 of the hamster CHO1/MKLP1 cDNA, and AS2 corresponds to the sequence between -1 and +18; S1 and S2 are the inverse complements of AS1 and AS2, respectively. Purified phosphorothioated oligonucleotides (Research Genetics Inc., Huntsville, AL) were resolved in serum-free medium and kept at -80°C until use. Podocytes were cultured for 4 d under nonpermissive conditions before they were plated onto collagen-coated coverslips; these postmitotic podocytes begin to develop processes (Mundel et al., 1997b). Starting 1 d after plating, podocytes were cultivated for up to 7 d in the presence of oligonucleotides at 0.5–10  $\mu$ M by daily renewal of oligonucleotide-containing medium and observed by phase contrast microscopy. Cells were immunostained for CHO1/MKLP1 and MTs or processed for hook decoration. In some experiments, podocytes were further cultured in the absence of oligonucleotides after antisense treatment to confirm the reversibility of the observed effects.

### **Visualization of Taxol-stabilized Microtubules during Process Formation**

Podocytes cultured for 4 d under nonpermissive conditions were plated onto collagen-coated coverslips. 6 h after plating, cells were treated with 0.1–10  $\mu$ M taxol (paclitaxel; Sigma) for 3 d and immunostained for MTs and AFs as described above. After taxol treatment, podocytes were further cultured for 3 d in the absence of taxol to permit recovery of MT arrays, followed by immunostaining for  $\alpha$ -tubulin and CHO1/MKLP1. Some cells recovering from taxol treatment were treated with 10  $\mu$ M nocodazole for 2 h before immunostaining.

### **Localization of the MTOC**

The MTOC was visualized by observation of MT arrays along the time course during recovery from nocodazole treatment (Takemura et al., 1995). Differentiated podocytes were incubated with 10  $\mu$ M nocodazole (methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate; Sigma) for 2 h; afterwards, nocodazole was washed out with normal medium. At several time points during the recovery phase (0, 1, 2, 5, 10, 30, 60, and 120 min), cells were fixed with a prewarmed (37°C) MT-stabilizing fixative (0.1 M Pipes, pH 6.9, 5 mM EGTA, 1 mM MgCl<sub>2</sub>, 4% polyethylene glycol 6000, 0.5% Triton X-100, 2% paraformaldehyde, and 4% sucrose). This fixative extracts free tubulin subunits but leaves polymerized MTs intact (Okabe and Hirokawa, 1988). Immunostaining of paraformaldehyde-fixed cells was performed as previously described (Mundel et al., 1997b) using  $\alpha$ -tubulin mAb and fluorochrome-conjugated phalloidin (Sigma). Some cells were stained with vimentin polyclonal antibody (ProGen, Heidelberg, Germany) to visualize the distribution of IFs. To label centrosomes, podocytes were fixed in the recovery phase after nocodazole treatment with methanol at -20°C for 5 min, rinsed with PBS, and stained with affinity-purified  $\gamma$ -tubulin antiserum raised against a *Xenopus*  $\gamma$ -tubulin fusion protein (Stearns et al., 1991; Reinsch and Karsenti, 1994; courtesy of S. Reinsch, EMBL, Heidelberg, Germany).

## **Results**

### **The Polarity of Microtubules in Podocytes Is Nonuniform**

To characterize the MT arrays of cultured podocytes, we first examined their intracellular distribution and polarity.

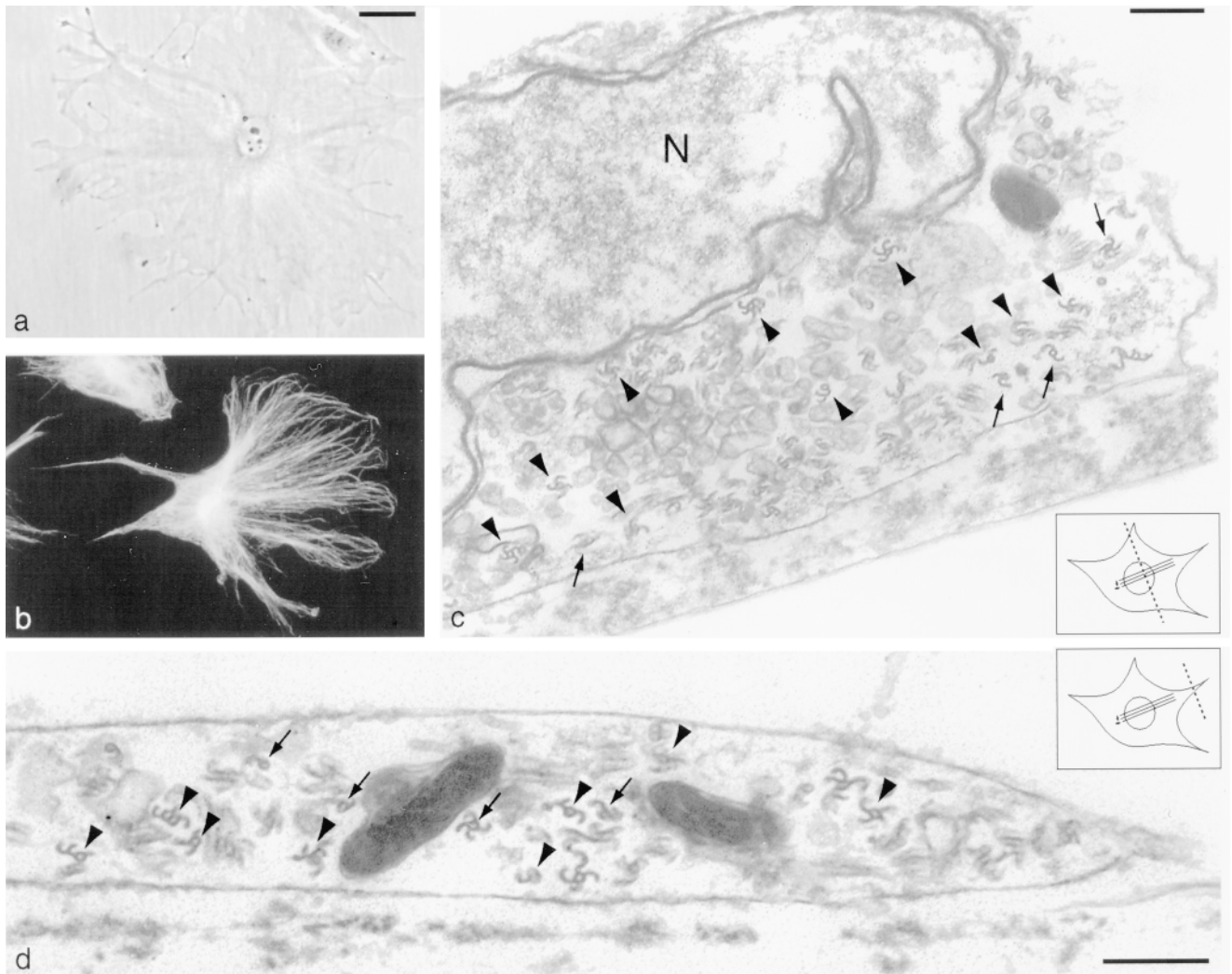
Differentiated podocytes possess prominent cell processes containing MTs that radiate from the perinuclear region into the cell periphery (Fig. 1, *a* and *b*). By applying the hook decoration technique in a series of ultrathin sections, the polarity of MTs in differentiated podocytes was determined. When tracing MTs from their distal end, clockwise hooks show their plus end–distal polarity, whereas counterclockwise hooks indicate their minus end–distal polarity (Heidemann, 1991). The results from two independent sets of ultrathin serial sections are shown in Table I. The analysis of cross-cut MT profiles of 15 sections from the two series revealed that 36% of MTs showed minus end–distal polarity with counterclockwise hooks (Table I). Heidemann (1991) reported that the rate of mismatching between the handedness of hooks and the polarity of MTs is less than 10%, i.e., if all MTs are oriented in a uniform plus end–distal fashion, then the percentage of counterclockwise hooks is less than 10%, e.g., 1–6% in mature neuronal axons where MTs are uniformly oriented (Baas et al., 1989). Therefore, our data show that podocytes contain minus end–distal MTs resulting in the nonuniform (mixed) polarity of MTs. The observed efficiency of ~80% for hook decoration of MTs (the ratio of hook-decorated MTs among all MTs) in the present study was similar to levels of hook decoration observed in previous reports (Baas et al., 1989; Takemura et al., 1995). The frequency of minus end–distal MTs varied not only among different MT bundles but also within individual MT bundles. A given region of an MT bundle could contain up to 50% minus end–distal MTs, while a different region of the same bundle could contain only a few of them. Along the proximal–distal axis of one cell, the percentage of minus end–distal MTs per section was 43, 31, 20, 40, 33, 0, 17, 10, 20, 43, 50, and 20% from the proximal to distal sites. In other words, the percentage of minus end–distal MTs varied randomly along the proximal–distal axis.

### **CHO1/MKLP1 Motor Protein Is Found along Microtubules in Podocytes**

Since the nonuniform polarity of MTs in podocytes could be achieved by motor-dependent assembly, we next examined the expression of CHO1/MKLP1. Differentiated podocytes showed CHO1/MKLP1 immunolabeling in a punctated pattern in the cytoplasm (Fig. 2). Double staining with  $\alpha$ -tubulin showed that CHO1/MKLP1 was localized in a punctated pattern on MTs, indicating the association of CHO1/MKLP1 with MTs in podocyte processes (Fig. 2 *c*). To confirm the immunocytochemical results, we analyzed the expression of CHO1/MKLP1 by RT-PCR using RNA from undifferentiated and differentiated podocytes. In both samples, a PCR product with the expected length of 232 bp was amplified (Fig. 2 *d*). These findings corroborate the expression of CHO1/MKLP1 in podocytes during proliferation and after differentiation.

### **Inhibition of CHO1/MKLP1 Expression Abolished Process Formation of Podocytes**

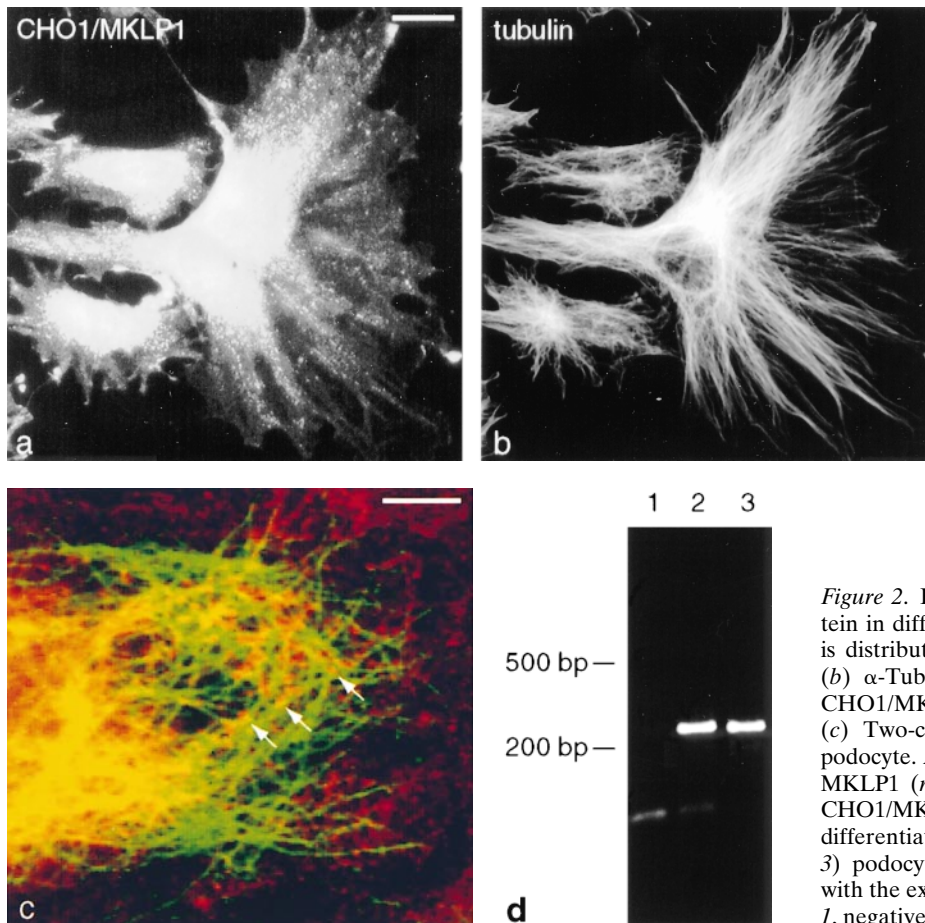
To establish a functional role of CHO1/MKLP1 in podocytes, expression of CHO1/MKLP1 was inhibited by AS oligonucleotide treatment of process-forming podocytes (for details see Materials and Methods). Treatment of dif-



**Figure 1.** MTs of process-bearing podocytes show a nonuniform polarity. (a) Phase contrast micrograph illustrating the morphology of differentiated podocytes. (b) Immunocytochemistry for  $\alpha$ -tubulin visualizing MT bundles in the cell body and processes. (c and d) TEM analysis of hook-decorated MTs viewed from the distal side of the examined podocytes. Both micrographs belong to a series of serial sections including centrosomal profiles. Arrowheads point to MTs with clockwise hooks, representing plus end–distal MTs. Arrows point to MTs with counterclockwise hooks, representing minus end–distal MTs. In the perinuclear region (c) and in the tips of processes (d), podocytes show a nonuniform polarity with minus end–distal MTs (arrows). *N*, nucleus. The dotted lines of the cartoons show the position where the sections are cut. Paired dots indicate centrosomes, and parallel lines show MT bundles. Bars: (a and b) 50  $\mu$ m; (c and d) 0.2  $\mu$ m.

differentiating cells with AS oligonucleotides for 7 d abolished process formation of podocytes; cell bodies remained small, and only a few short processes developed (Fig. 3 a, compare with Figs. 3 b and 1 a). The effects of AS oligonucleotides were dose dependent; cells treated with 3  $\mu$ M were smaller in size than those treated with 1  $\mu$ M. In all subsequent experiments, podocytes were treated with 3  $\mu$ M oligonucleotides. In AS-treated cells, cytoplasmic expression of CHO1/MKLP1 was absent. Only a perinuclear, nonspecific background staining was found in these cells (Fig. 3 c). In contrast, S-treated cells showed a normal punctated distribution pattern CHO1/MKLP1 (Fig. 3 d, compare with Fig. 2 a). Hook decoration revealed that in AS-treated podocytes, MT polarity was almost uniform (Fig. 3 e). 83% of MTs showed plus end–distal polarity,

which is significantly higher than those in control cells (Table I). In contrast, S oligonucleotide–treated cells contained MTs with nonuniform polarity in usual frequency (Fig. 3 f), proving that CHO1/MKLP1 is required for the generation of nonuniform MT polarity in podocytes. The antisense treatment was not accompanied by a reduction of polymerized MTs (data not shown). However, the cytoplasm of AS-treated cells contained less membrane-bounded organelles (Fig. 3 e) as compared with S-treated (Fig. 3 f) or nontreated cells (Fig. 1, c and d). In addition, AS-treated cells sometimes contained abnormally clustered organelles in their cell bodies (data not shown). These observations suggest an impaired transport of membrane bounded in AS-treated podocytes. In some experiments, cells were treated with AS oligonucleotides for 7 d



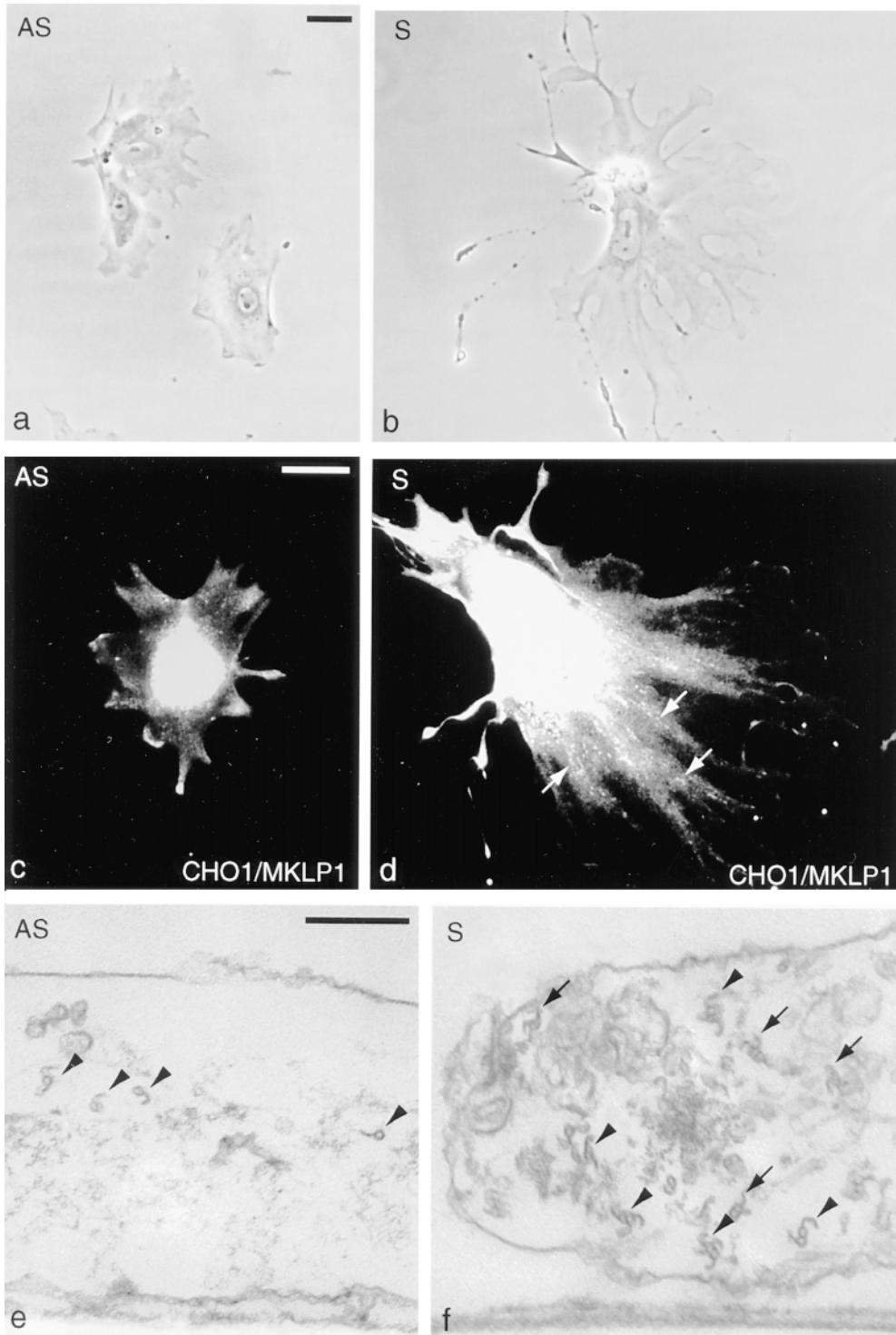
**Figure 2.** Expression of CHO1/MKLP1 motor protein in differentiated podocytes. (a) CHO1/MKLP1 is distributed in podocytes in a punctated pattern. (b)  $\alpha$ -Tubulin staining reveals the association of CHO1/MKLP1 with MTs (double staining in a). (c) Two-color overlay image of a differentiated podocyte. Arrows indicate the association of CHO1/MKLP1 (red) with MTs (green). (d) Detection of CHO1/MKLP1 mRNA expression by RT-PCR in differentiated (lane 2) and nondifferentiated (lane 3) podocytes. In both samples, a DNA fragment with the expected size of 232 bp was amplified. Lane 1, negative control without cDNA. Bars, 0.2  $\mu$ m.

and then further incubated for 7 d in the absence of AS oligonucleotides. In these cells, the effects of AS oligonucleotides were fully reversible; after 4 d without AS oligonucleotides, process formation and cytoplasmic expression of CHO1/MKLP1 were completely restored, and cells were indistinguishable from S-treated or untreated cells (data not shown). Treatment with S oligonucleotides did not interfere with process formation or normal expression of CHO1/MKLP1, with this clearly ruling out possible toxicity of oligonucleotides per se. These observations demonstrate that cytoplasmic expression of CHO1/MKLP1 along MTs is required for normal process formation of podocytes. When proliferating podocytes were treated with AS oligonucleotides, their proliferation rate was markedly reduced. In AS-treated cells, no mitotic spindles were found, while in S-treated or untreated preparations dividing cells with spindle formation were frequently seen (data not shown). This inhibition of podocyte cell division is consistent with previous reports describing CHO1/MKLP1 as a mitotic motor (Selitto and Kuriyama, 1988; Nislow et al., 1990, 1992).

#### ***Taxol-stabilized Microtubules Are Redistributed from the Perinuclear Region into the Cell Periphery***

To study the course of MT rearrangement in podocytes, MT arrays were disrupted and then allowed to recover.

The application of taxol to podocytes before process formation induced strong perinuclear clustering of MTs and inhibition of process formation (Fig. 4 a). After removal of taxol, cell bodies increased in size and processes developed. During the recovery phase, strongly labeled MT fragments (probably representing taxol-stabilized remnants) were detected along weakly labeled MTs in the cell body and in processes (Fig. 4 b). The amount of MTs continuously running from the centrosomal region to the cell periphery was much lower in cells recovering from taxol treatment (Fig. 4 b) than in normal podocytes (Fig. 1 b). Treatment of these cells with 10  $\mu$ M nocodazole for 2 h allowed us to distinguish two populations of MTs: weakly stained MTs susceptible to nocodazole treatment and strongly labeled MT fragments resistant to nocodazole treatment (Fig. 4 c). Such strongly stained MT fragments were preferentially observed after taxol treatment and subsequent nocodazole treatment. These findings indicate that taxol-stabilized MT fragments became resistant to nocodazole treatment (de Brabander et al., 1981), while MTs assembled de novo after taxol treatment were sensitive to nocodazole. Furthermore, during recovery of normal podocyte morphogenesis after taxol removal, MT fragments were redistributed from perinuclear MT clusters to the cell periphery in a punctated pattern along newly assembled MTs. The punctated distribution pattern of CHO1/MKLP1 in these cells was quite similar to that of



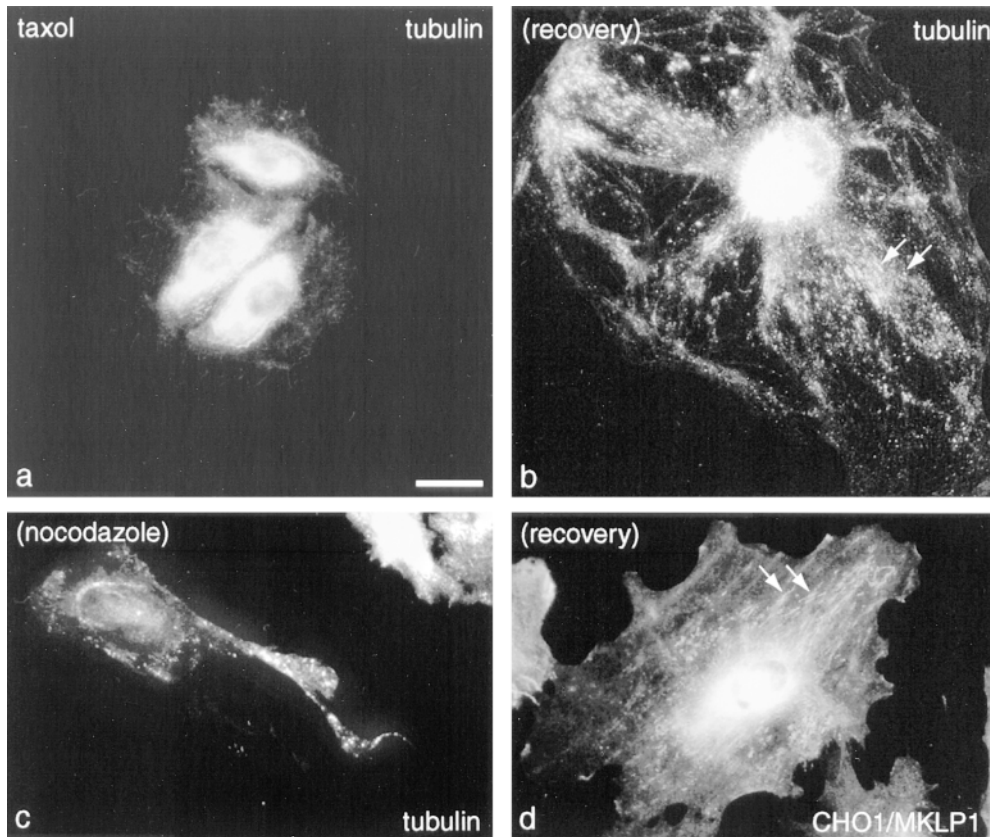
**Figure 3.** Treatment with CHO1/MKLP1 AS oligonucleotides inhibits process formation of podocytes. Process-forming podocytes were treated with CHO1/MKLP1 AS or S oligonucleotides (3  $\mu$ M) for 7 d. (a, c, and e) Treatment with AS2 oligonucleotide. (b, d, and f) Treatment with S2 oligonucleotide as a negative control. (a and b) Phase contrast micrography. (c and d) Immunocytochemistry for CHO1/MKLP1. (e and f) TEM after hook-decoration. Arrowheads point to MTs with clockwise hooks indicating plus end-distal polarity, whereas arrows point to MTs with counterclockwise hooks indicating minus end-distal polarity. AS-treated podocytes lack processes (a) and cytoplasmic expression of CHO1/MKLP1 (c). The perinuclear staining in AS-treated cells is nonspecific. S-treated cells show a normal morphology (b, compare with Fig. 1 a) and dotted localization of CHO1/MKLP1 along MTs (arrows in d, compare with Fig. 2 a). In AS-treated cells, MTs are uniformly arranged with plus end-distal polarity (e), whereas in S-treated cells, MTs are non-uniformly oriented (f) as in nontreated podocytes (see Fig. 1, c and d). Bars: (a–d) 25  $\mu$ m; (e and f) 0.2  $\mu$ m.

nocodazole-resistant MT fragments (Fig. 4, b and d). The association of CHO1/MKLP1 with MTs was confirmed in double labeling experiments (data not shown).

#### ***The Centrosome Is the $\gamma$ -Tubulin-expressing MTOC in Podocytes***

To determine whether MT nucleation also plays a role in the arrangement of minus end-distal MTs, MT nucleation

sites were visualized in podocytes. Treatment with nocodazole for 2 h caused complete disassembly of MTs in differentiated podocytes (Fig. 5, a and b). In nocodazole-treated cells, tubulin staining was diffuse around nuclei or almost absent (Fig. 5 a, compare with Fig. 1 b) since free tubulin subunits had been extracted by detergent during fixation (for details see Materials and Methods). After complete depolymerization of MTs with nocodazole, recovery of MT arrays started immediately after



**Figure 4.** Redistribuition of MTs after taxol treatment. (a–c)  $\alpha$ -Tubulin staining. (a) Treatment of differentiating podocytes with 1  $\mu$ M taxol for 3 d abolishes process formation. MTs cluster around nuclei, leaving only a few MTs in the cell periphery. Three podocytes are seen in this field. (b) After removal of taxol, podocytes were further cultured for 3 d to allow process formation. In addition to perinuclear MT clusters, intensely stained fragments of MTs are found in the enlarging cell body and elongating processes (arrows). These MT fragments are linearly arranged in a punctated pattern. Behind the MTs fragments (arrows), weakly stained long MTs are also observed. (c) After recovering from taxol treatment, podocytes were incubated with 10  $\mu$ M nocodazole to depolymerize newly assembled MTs. Nocodazole-resistant MTs are found around the nucleus as well as in the cell periphery and in

the processes. (d) In cells recovering from taxol treatment CHO1/MKLP1 is localized in a punctated pattern along MTs (arrows), reminiscent of the distribution pattern of the intensely labeled short MT fragments shown in b. Bar, 25  $\mu$ m.

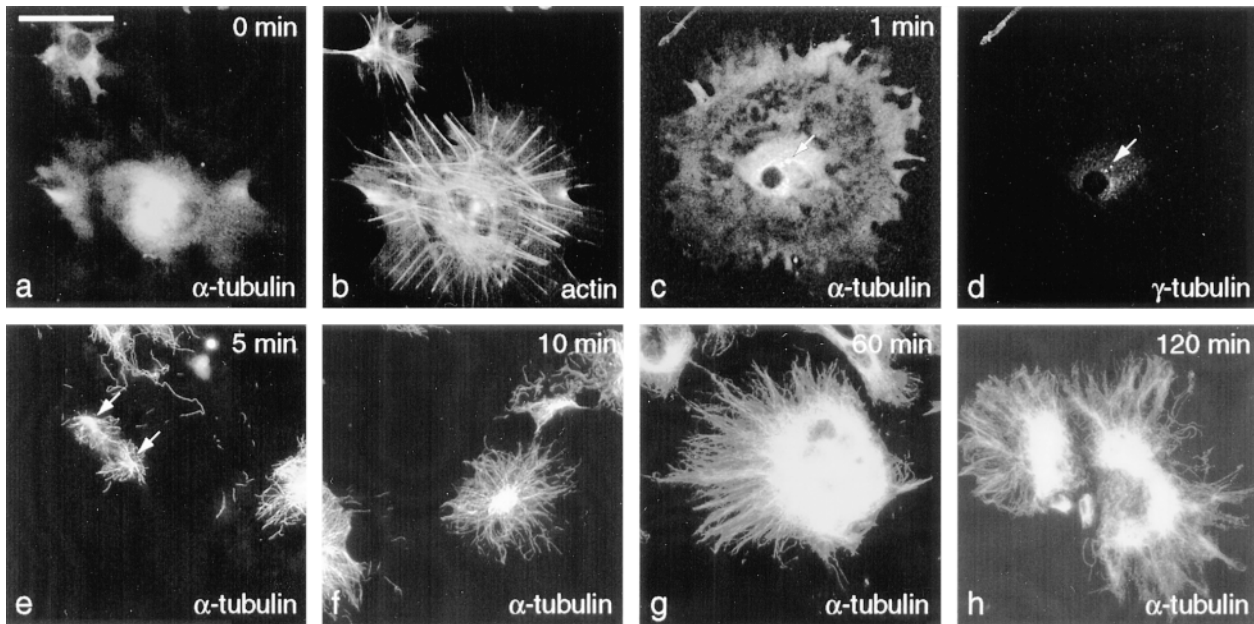
removal of nocodazole. Nucleation of MTs was first observed at  $\gamma$ -tubulin-containing perinuclear foci (Fig. 5, c–e) representing centrosomes (Stearns et al., 1991; Baas and Joshi, 1992). These results show that the  $\gamma$ -tubulin-expressing centrosome is the major MTOC in podocytes. Within 5 min after removal of nocodazole, MTs nucleated in an asterisk-like pattern from centrosomes (Fig. 5, c–e). MTs continued to elongate into the periphery over the next 30 min, and after 60 min almost the entire cytoplasm was filled with MTs (Fig. 5, f and g). At 1–2 h of recovery, MTs were aggregated into discrete bundles, leaving in between regions largely devoid of MTs (Fig. 5 h). Such regions of MT-depleted cytoplasm were also found in nontreated control cells (Fig. 1 b). During the early stages of recovery, in addition to MTs emerging from centrosomes, short MT fragments were observed in cell bodies and processes (Fig. 5 e). During the later stages of recovery, all MTs emerged from the centrosome (Fig. 5, g and h).

To determine the contribution of other cytoskeletal elements to MT organization, we examined the distribution of AFs and IFs during elongation of MT arrays. The distribution of AFs was not affected by nocodazole treatment. During recovery from nocodazole treatment, no spatial relationship between MT nucleation sites and AF foci was found (data not shown). In contrast, vimentin IFs lost their fibrillar distribution and collapsed in the perinuclear region after nocodazole treatment. The reassembly of MTs

was accompanied by a restoration of the normal IF distribution pattern (data not shown). These findings suggest an interaction between MTs and IFs as reported for other cells (Virtanen et al., 1980; Celis et al., 1984). Moreover, time course analysis revealed that the recovery of MT arrays preceded that of IFs. Therefore, we conclude that neither AFs nor IFs are important for the recovery of MT arrays after nocodazole treatment.

### Discussion

The present study shows that podocytes exhibit a nonuniform MT polarity. Since direct formation of minus end-distal MTs is not possible at centrosomes (Pereira and Schiebel, 1997), two hypotheses were proposed for establishing the nonuniform MT-polarity (Baas, 1997; Kobayashi and Mundel, 1998): (a) short plus end-distal MTs are released from the centrosome and transported in antiparallel orientation into the cell periphery along preexisting plus end-distal MTs; or (b) minus end-distal MTs are assembled at MTOCs other than the centrosome. The data from the present study support the first hypothesis. Suppression of CHO1/MKLP1 expression with AS oligonucleotides showed that CHO1/MKLP1 is necessary for the development of nonuniform polarity in podocytes (Fig. 3). CHO1/MKLP1 is located in a punctated pattern along MTs in podocytes (Fig. 2). This pattern is similar to the



**Figure 5.** Visualization of the MTOC during recovery of MT arrays from nocodazole treatment. (a, c, and e–h) Recovering MT arrays are visualized by  $\alpha$ -tubulin-staining. (b) Phalloidin staining to label AFs. (d)  $\gamma$ -Tubulin staining to detect centrosomes. (a, b double staining) No recovery of MTs after exposure to 10  $\mu$ M nocodazole for 2 h. Nocodazole-treated podocytes contained no polymerized MTs (a) but show a normal distribution of AFs (b). (c and d) Double staining of podocytes 1 min after removal of nocodazole. The reassembly of MTs starts from perinuclear foci (arrow in c). These  $\gamma$ -tubulin-expressing foci represent centrosomes (arrow in d). (e–h) Reassembly of MTs 5, 10, 60, and 120 min after removal of nocodazole. MTs elongate from perinuclear foci (arrows in e) into the cell periphery. Short MT fragments not related to these foci can only be observed during the early stages of recovery (e). During the later stages, all MTs extend from centrosomes (g and h). Bar, 50  $\mu$ m.

distribution of CHO1/MKLP1 in neuronal dendrites, where transport of minus end–distal MTs was also described (Sharp et al., 1997). These data suggest that minus end–distal MTs are transported by CHO1/MKLP1 motor proteins along plus end–distal MTs in podocytes. The alternative hypothesis, i.e., the presence of MTOCs other than the centrosome, which plays a crucial role in the assembly of MTs (Joshi et al., 1992; Moritz et al., 1995), does not receive any support from the present study since  $\gamma$ -tubulin was exclusively found at centrosomes (Fig. 5). Apart from a transient centrosome-independent reassembly of MTs during the early stages of recovery from nocodazole treatment, reassembly of MTs exclusively occurred at centrosomes. (Fig. 5). The transient reassembly of MTs probably represented polymerization without any MTOC activity. As suggested by previous works, after complete depolymerization of MTs, the concentration of free tubulin subunits may be higher than the critical assembly concentration, allowing MTs to spontaneously assemble in the absence of MTOCs (Stearns et al., 1991). Therefore, it is reasonable to conclude that the  $\gamma$ -tubulin-expressing centrosome represents the only regular MT assembly site in podocytes. In neuronal cells, the centrosome is the major MTOC, as shown by the same experimental approach used in the present study (Baas and Joshi, 1992; Yu et al., 1993). Although it has recently become clear that MTs can be polymerized by centrosome-free nucleation (Hyman and Karsenti, 1998), the generation of minus end–distal MTs in podocytes by centrosome-free nucleation was ruled out by the AS experiments. These experiments clearly

showed that the mixed polarity of MTs, which is independent of MT nucleation (Baas, 1997), depends on the expression of CHO1/MKLP1 (Fig. 3).

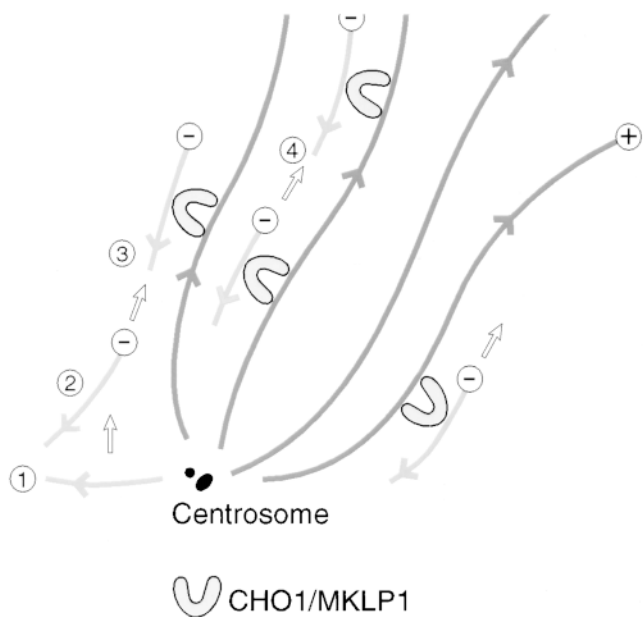
CHO1/MKLP1 is a member of the kinesin superfamily of motor proteins as shown by sequence analysis and *in vitro* motility analysis (Nislow et al., 1992). When over-expressed, CHO1/MKLP1 induces bundling of MTs in a nonuniform orientation, indicating that CHO1/MKLP1 is associated with MTs rather than with membranous organelles (Kuriyama et al., 1994; Sharp et al., 1996). During mitosis, CHO1/MKLP1 causes antiparallel sliding between nonuniformly arranged MTs, accounting for the elongation of mitotic spindles (Nislow et al., 1992). Actually, AS oligonucleotides for CHO1/MKLP1 inhibited cell division of proliferating podocytes (the present study) and neuroblastoma cells (Yu et al., 1997). In addition, reduced expression of CHO1/MKLP1 is accompanied by a uniform plus end–distal microtubular polarity in neuronal cells (Sharp et al., 1997; Yu et al., 1997). These findings suggest that CHO1/MKLP1 acts as a motor protein transporting minus end–distal MTs in dendrites. Our data in podocytes provide further evidence for a role of CHO1/MKLP1 as a transporter of minus end–distal MTs. These minus end–distal MTs are short and colocalize with CHO1/MKLP1 in a punctated pattern along MTs. The MT transport hypothesis is further supported by the similar distribution of taxol-stabilized MT fragments and CHO1/MKLP1 during recovery from taxol treatment (Fig. 4), strongly suggesting that taxol-stabilized MT fragments are transported along MTs by CHO1/MKLP1.



The present study clearly demonstrated the redistribution of taxol-stabilized MT fragments into the cell periphery. Interestingly, process formation of podocytes was restored after removal of taxol, although it had previously been reported that taxol remained in the cytoplasm even after removal from the culture medium (Jordan et al., 1996). Thus, taxol retained on MTs may continue to stabilize MT fragments in taxol-treated podocytes. The inability to demonstrate short MT fragments without taxol treatment (Figs. 1 *b* and 5, *g* and *h*) may simply be due to the fact that immunofluorescence microscopy is not sensitive enough to discriminate between short transported MT fragments and long stationary MTs.

The hypothesis that MTs are transported as preformed segments was controversial with many investigators who claimed that only the transport of tubulin subunits contributes to axonal flow of MTs (see for example Baas and Brown, 1997; Hirokawa et al., 1997). In axons, MTs are uniformly oriented in a plus end–distal fashion (Heide-mann et al., 1981, 1984; Baas et al., 1988), indicating that axons lack a mechanism to arrange MTs in minus end–distal fashion and consistent with the observation that axons do not contain CHO1/MKLP1 (Sharp et al., 1997*b*). Therefore, organization of the microtubular system in axons is fundamentally different from that in podocytes and dendrites. Interestingly, however, Ahmad et al. (1998) recently reported that motor-driven transport of MTs is necessary for axonal elongation.

The present study suggests a mechanism for the generation of the nonuniform MT polarity in podocytes (summarized in Fig. 6). First, plus end–distal MTs, which serve as rails for MT transport, nucleate at the centrosome. Second, short MT fragments are assembled at the centrosome and then released from the centrosome (Fig. 6, 1 and 2). Baas (1997) proposed the MT-severing protein katanin (McNally et al. 1996) as a candidate to release MT fragments from the centrosome. If the released MT fragments are very short, their orientation may be reversed by thermodynamic movements. Third, minus end–distal MT fragments are connected to plus end–distal MTs via CHO1/MKLP1 and then transported into developing processes (Fig. 6, 3 and 4). The results from the AS experiments clearly show that the cytoplasmic expression of CHO1/MKLP1 is crucial for process formation of podocytes (Fig. 3). Similar effects of antisense treatment were recently described in neuronal dendrites (Sharp et al., 1997; Yu et al., 1997). These findings strongly suggest that minus end–distal MTs play a crucial role in process formation. Generally, process formation is dependent on a continuous flow supplying growing processes with cytoskeletal elements as well as membrane precursors. Minus end–distal MTs may support the transport of elements that are carried only by minus end–directed motors into the cell periphery, e.g., components of the Golgi apparatus in dendrites (Baas et al., 1989), since distinct motor proteins selectively carry specific cargos (Hirokawa, 1997). The problem arising from this concept is that minus end–distal MTs do not form continuous rails but are arranged in a discontinuous pattern along plus end–distal MTs. Therefore, an alternative model would be that transported minus end–distal MT fragments are connected with other cytoskeletal and/or membranous elements without the interposition of an-



**Figure 6.** Schematic drawing of the suggested mechanism leading to the nonuniform MT polarity in podocytes. “+” and “–” indicate plus and minus ends of MTs. Arrows show the polarity of MTs. Long lines (dark gray) represent plus end–distal MTs, while short lines (light gray) depict minus end–distal MT fragments. Minus end–distal MTs are assembled at the centrosome (1). After release from the centrosome (2), they are connected to plus end–distal MTs in an antiparallel fashion by CHO1/MKLP1 motor protein (3) and transported into the cell periphery (4).

other motor protein. The transport of these elements into elongating processes may then be achieved together with minus end–distal MT fragments transported by CHO1/MKLP1.

Several transport studies clearly showed that membrane traffic in neuronal dendrites, but not in axons, is driven by a machinery similar to the “basolateral” membrane traffic in polarized epithelia (de Hoop and Dotti, 1993). The present study revealed the role of nonuniform MT polarity established by CHO1/MKLP1 in process formation of podocytes, similar to that reported for dendrites (Sharp et al., 1997). It is tempting to speculate that the development of podocyte processes and neuronal dendrites is driven by the “basolateral” transport machinery depending on nonuniformly oriented MTs. Membrane traffic of neuronal dendrites and of epithelial basolateral membranes is regulated by the small GTPase Rab8 (Huber et al., 1993*a,b*). Transfection of a hyperactive mutant of Rab8 induces process formation in fibroblasts (Peränen et al., 1996), suggesting that Rab8 promotes process formation via regulation of membrane traffic in process-bearing cells. Rab6, another small GTPase, can interact with a kinesin-like motor protein with a high sequence homology to CHO1/MKLP1 (Echard et al., 1998). Therefore, small GTPases may regulate process formation via their interaction with CHO1/MKLP1, although the underlying mechanism remains to be elucidated.

In summary, we have shown that expression of CHO1/MKLP1 motor and nonuniform polarity of MTs are neces-

sary for process formation of podocytes, indicating that podocytes and dendrites share the same mechanism of process formation. In contrast, the uniform MT polarity in axons is achieved by dynein and dynactin-driven transport of plus end-distal MTs (Ahmad et al., 1998). The present study emphasizes that the transport of MT fragments by motor proteins is a general mechanism for process formation in diverse cell types. Since MTs disassemble at minus ends without protection by capping proteins (Keating et al., 1997; Pereira and Schiebel, 1997), the identification of factors preventing disassembly of MT fragments during their transport awaits clarification.

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