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Microtubule Dynamic Turnover Is Suppressed During Polarization and Stimulated in Hepatocyte Growth Factor Scattered Madin-Darby Canine Kidney Epithelial Cells

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The dynamic behavior of microtubules has been measured in non-polarized, polarized, and hepatocyte growth factor treated Madin-Darby canine kidney epithelial cells. In a nocodazole disassembly assay, microtubules in polarized cells were more resistant to depolymerization than microtubules in non-polarized cells; microtubules in scattered cells were nearly completely disassembled. Analysis of fluorescent microtubules in living cells further revealed that individual microtubules in polarized cells were kinetically stabilized and microtubules in scattered cells were highly dynamic. Individual microtubule behavior in polarized cells was characterized by a suppression of the average rate of shortening, an increase in the average duration of pause, a decrease in the frequency of catastrophe transitions, and an increase in the frequency of rescue transitions, when compared with microtubules in non-polarized cells. In contrast, microubule behavior in epithelial cells treated with hepatocyte growth factor was characterized by increases in the average rates of microtubule growth and shortening, a decrease in the frequency of rescue transitions, and an increase in the frequency of catastrophe transitions, when compared with polarized cells. Dynamicity, a measure of the gain and loss of subunits from microtubule plus ends, was 2.7 μm/min in polarized cells and 11.1 μm/min in scattered cells. These results demonstrate that individual microtubule dynamic behavior is markedly suppressed in polarized epithelial cells. Our results further demonstrate that in addition to its previously characterized effects on cell locomotion, hepatocyte growth factor stimulates microtubule dynamic turnover in lamellar regions of living cells.

Key words: microtubule dynamics, polarized cells, MDCK epithelial cells

INTRODUCTION

It is now well established that microtubules in interphase and mitotic cells undergo a dynamic assembly/disassembly behavior referred to as dynamic instability [Mitchison and Kirschner, 1984a,b]. Direct observation of microtubules in vitro and in vivo reveals that some microtubules assemble while other microtubules rapidly shorten [Mitchison and Kirschner, 1984a,b; Schulze and Kirschner, 1986; Cassimeris et al., 1988; Samak and Borisy, 1988b; Walker et al., 1988; Shelden and Wadsworth, 1993]. Although the exact mechanism responsible for the regulation of dynamic instability behavior is not known, the presence of a cap of GTP-tubulin subunits at the microtubule end is correlated with continued

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microtubule elongation, while the loss of the terminal GTP- tubulin subunits is thought to contribute to a transition to disassembly [Stewart et al., 1990; Walker et al., 1991; Caplow, 1992; Erickson and O'Brien, 1992]. Microtubule dynamic behavior can be quantified by measuring the rates of microtubule elongation and shortening and the frequencies of switching between phases. Examination of microtubule behavior in interphase and mitotic extracts has demonstrated that microtubule dynamic behavior is regulated throughout the cell cycle. For example, in mitotic *Xenopus* extracts the frequency of catastrophe is approximately 10-fold higher than in interphase extracts [Belmont et al., 1990]. Recently two different proteins have been identified that increase the frequency of catastrophe in cell extracts, and thus might contribute to the cell cycle dependent regulation of microtubule dynamics [Belmont and Mitchison, 1996; Walczak et al., 1996].

In addition to cell cycle dependent regulation, measurements of dynamic instability behavior in various cell types demonstrate that dynamic instability behavior is cell type specific [Shelden and Wadsworth, 1993]. For example, microtubules in epithelial cells both grow and shorten at slower rates and rescue more frequently than microtubules in fibroblasts. Microtubules in epithelial cells are characterized by numerous transitions between growth and shortening and little net change in the microtubule array over time. Taken together these observations clearly demonstrate that microtubule dynamic behavior is regulated in living cells [Cassimeris, 1993]; however, the molecular mechanisms responsible for the control of microtubule dynamic behavior during cell growth and differentiation are largely unknown.

Madin-Darby canine kidney (MDCK) epithelial cells are a useful model system to examine cytoskeletal reorganization during differentiation [Bacallao et al., 1989; Bre et al., 1990]. Following trypsinization, these cells lose their polarized organization and reestablish an epithelial morphology with time in culture. In non-polarized cells, microtubules radiate from a perinuclear microtubule organizing center (MTOC), while in polarized cells bundles of microtubules extend from a dispersed, apical MTOC toward the basal region of the cell. In addition, MDCK cells respond to scatter factor (SF) or hepatocyte growth factor (HGF), becoming fibroblast-like and invasive. HGF was originally described as a mitogenic factor involved in liver regeneration [Nakamura et al., 1987], while SF, first detected in fibroblast conditioned media, was shown to induce dissociation of epithelial cells [Stoker et al., 1987]. Molecular analysis has revealed that HGF and SF are identical molecules [Naldini et al., 1991; Weidner et al., 1991] which bind with high affinity to a transmembrane receptor tyrosine kinase, the c-MET proto-oncogene product [Bottaro et al., 1991]. Recent work has shown that HGF/SF is produced by mesenchymal cells while the receptor is expressed in neighboring epithelial tissues [Sonnenberg et al., 1993]. Additional studies have shown that HGF/SF mediates responses to tissue injury, is involved in hematopoiesis, functions as a morphogen for epithelia, and contributes to neural induction [for review see Montesano et al., 1991; Bronner-Fraser, 1995; Zanegar and Michalopoulos, 1995].

In the experiments described here, the behavior of microtubules in living MDCK cells has been measured under three distinct experimental conditions. Cells were examined 1) within 48 h of plating, before the cells acquired a polarized morphology; 2) after culture for 4–5 days, when the cells have differentiated and acquired a polarized morphology; and 3) following scattering in response to the addition of HGF/SF to the cultures. The results demonstrate that as cells differentiate, the rate and extent of microtubule shortening events and the frequency of catastrophe transitions are decreased. The average length of a pause, the percentage of time in pause, and the frequency of rescue transitions are markedly increased. In HGF scattered cells, microtubules are more dynamic than microtubules in either polarized or non-polarized cells. Specifically, both the rate and distance of shortening events are increased and the frequency of rescue is reduced. These results demonstrate that modulation of the behavior of individual microtubules accompanies both cellular differentiation and the response to growth factors.

MATERIALS AND METHODS

Cell Culture and Microinjection

MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5–10% fetal bovine serum and antibiotics. Following trypsinization, cells were plated on glass coverslips for the appropriate time (see Results). For analysis of microtubules in HGF treated cells, 12–25 ng/ml of recombinant HGF was added to the cells after 24 or 72 h in culture; cells were observed after culture for an additional 24 h. The cloning, expression, and purification of recombinant HGF were performed as described previously [Rubin et al., 1991; Cioce et al., 1996]. No quantitative differences in the behavior of individual microtubules could be detected in cells grown for 24 or 72 h before HGF addition. In polarized cells, microtubules were visible near the base of the cell, where they extended into the periphery [Bacallao et al., 1989]; microtubules were imaged in both free edges and in the edges in contact with other cells. In some experiments, only non-edge cells were injected to determine if microtubules in edge cells differed from internal cells. The behavior of microtubules

in internal cells was similar to that observed in edge cells (data not shown). In both locations, only individual microtubules in the periphery were analyzed; more central areas densely packed with microtubules were not suitable for individual microtubule tracking. However, the loss of microtubules seen in nocodazole disassembly experiments (Results) occurs throughout the cytoplasm, suggesting that microtubules in peripheral regions behave in a manner that is similar to microtubules in other regions of the cell.

For microinjection, cells were plated on etched coverslips (Bellco Glass, Inc., Vineland, N.J.) and injected with rhodamine labeled tubulin as described previously [Shelden and Wadsworth, 1993]. Following injection the cells were returned to the 37°C incubator for 3–5 h to allow incorporation of the labeled subunits into microtubules before observation.

Immunofluorescence and Confocal Microscopy

For tubulin immunofluorescence, cells were rinsed briefly in phosphate buffered saline (PBS) at room temperature, and briefly lysed (10 sec) in 80 mM PIPES (pH 6.9), 1 mM $MgSO_4$, 5 mM EGTA, and 0.5% Triton X-100 and immersed in −20°C methanol for 10–15 min. Coverslips were then rehydrated in PBS containing 0.1% Tween-20 and 0.02% azide (PBS-Tw-Az) and incubated with tubulin antibodies in PBS-Tw-Az containing 2% bovine serum albumin (BSA) for 2 h at 37°C in a humid chamber. A monoclonal anti-alpha tubulin antibody (DM1a, Sigma Immunochemicals, St. Louis, MO) or the E7 monoclonal antibody developed by Dr. M. Klymkowsky and obtained from the Developmental Studies Hybridoma Bank was used in these experiments, with identical results. Coverslips were rinsed in PBS-Tw-Az and incubated in affinity purified, fluorescently conjugated goat anti-mouse antibodies (Organon Teknika, Durham, NC) diluted 1:100 in PBS-Tw-Az containing 2% BSA for 45 min at room temperature. For staining of tight junctions, cells were rinsed in PBS and fixed in cold acetone for 10–15 min. Cells were rehydrated in PBS-Tw-Az and incubated in the R26.4C monoclonal antibody developed by Dr. D.A. Goodenough and obtained from the Developmental Studies Hybridoma Bank. Cells were incubated in appropriate secondary antibodies as described above. For confocal observation, cells were imaged using a BioRad MRC-600 confocal microscope mounted on a Nikon Diaphot; a 60× objective lens was used.

Analysis of Individual Microtubule Dynamics

Images of rhodamine labeled microtubules were obtained as previously described with the exception that some image sequences were obtained using a 100× 1.3 NA phase objective lens, rather than a 100× 1.4 NA apochromat [Shelden and Wadsworth, 1993; Dhamodharan and Wadsworth, 1995]. Quantification of microtubule dynamic behavior was performed as described by Dhamodharan and Wadsworth [1995] without modification. Traces of microtubule behavior ("life history" plots) were used to identify the various phases of microtubule dynamics (growth, shortening, and pause) and the transitions between phases. The Student's t-test was used to determine the significance of difference between groups. "Significant" is used in the text for differences significant at greater than or equal to the 99% confidence level; differences significant at the 95% confidence level are noted in the text. Catastrophe frequency was calculated by dividing the number of catastrophes by the sum of the total time spent in growth and pause or the distance of growth. Rescue frequency was calculated by dividing the number of rescues by the total time or distance of shortening. Dynamicity was calculated by dividing the sum of growth and shortening distances by the lifespan of the particular microtubule.

RESULTS

The dynamic behavior of individual microtubules has been measured in polarized and non-polarized MDCK epithelial cells and in MDCK cells treated with the cytokine growth factor, HGF, or SF (HGF/SF) [Stoker et al., 1987; Lieuvin et al., 1994]. The morphology of the MDCK cells used in these experiments is shown in Figure 1. When plated on glass coverslips and examined ≤48 h in culture, the cells are highly flattened and well spread (Fig. 1a). With additional time in culture, the cells differentiate, becoming morphologically and functionally polarized (Fig. 1b) [Balcarova-Stander et al., 1984; Simons and Fuller, 1985; Bacallao et al., 1989; Rodriguez-Boulan and Nelson, 1989; Bre et al., 1990]. When either polarized or non-polarized MDCK cells were incubated with 12–25 ng/ml recombinant HGF/SF for 24 h, the cells scattered across the coverslip, taking on a fibroblast-like morphology (Fig. 1c), and were highly motile (data not shown).

To evaluate polarity, MDCK epithelial cells were stained with antibodies to ZO-1, a component of tight junctions [Stevenson et al., 1986; Bacallao et al., 1989]. In non-polarized cells, ZO-1 staining occurs in the regions of cell-cell contact and lies in a single focal plane in these flattened cells (Fig. 2a). In older cultures, ZO-1 staining is restricted to the apical region of the cells (Fig. 2b); no staining is detected when the plane of focus is shifted to the more basal portion of the cells (Fig. 2c). Thus, by the criteria of cellular morphology and the apical location of tight junctions, these cells have established polarity. Scattered cells did not stain with the ZO-1 antibody, with the exception of small patches of

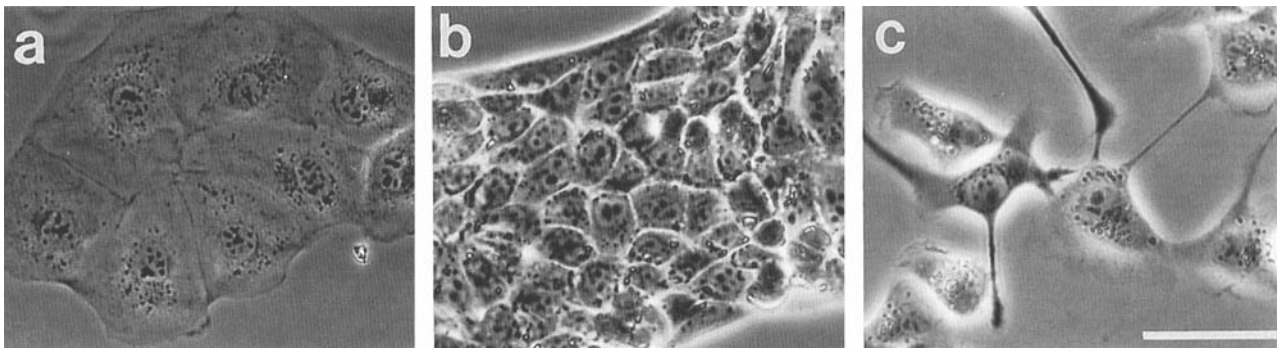

Fig. 1. Morphology of the MDCK epithelial cells used in these experiments. **a:** Non-polarized cells (48 h after plating on glass coverslips). **b:** Polarized cells (96–120 h after plating on glass coverslips). **c:** Cells incubated with HGF/SF. HGF (20–25 ng/ml) was added to the cells after 72 h in culture and incubated for an additional 24 h. Bar = 50 μm.

ZO-1 staining in regions where a cell remained in contact with another cell (data not shown).

To determine the dynamic properties of the population of microtubules in polarized and non-polarized MDCK cells and cells scattered with HGF/SF, a nocodazole disassembly assay was used [Cassimeris et al., 1986; Wadsworth and McGrail, 1990]. In this method, cells are incubated with high concentrations of nocodazole for various times to block microtubule assembly and reveal the intrinsic rate of microtubule disassembly. Following incubation with nocodazole, cells were processed for indirect immunofluorescence localization of microtubules and examined using confocal microscopy. The results demonstrate that a substantial proportion of the microtubules in non-polarized cells disassembled following incubation with 20 μM nocodazole for 20 min (Fig. 3a,b). The nocodazole induced disassembly of microtubules was markedly reduced when polarized cells were treated in the same manner (Fig. 3c,d). Microtubules in HGF/SF scattered cells appeared the most sensitive to disassembly induced by nocodazole (Fig. 3e,f) and little or no polymer remained in the cells after a 20 min incubation with 20 μM nocodazole. These observations demonstrate that microtubules in polarized epithelial cells are relatively resistant to disassembly induced by nocodazole and thus can be considered more stable [Cassimeris et al., 1986; Wadsworth and McGrail, 1990]. This observation is consistent with results from photobleaching and tubulin incorporation experiments [Bre et al., 1990; Pepperkok et al., 1990] which reveal that microtubules in polarized MDCK epithelial cells are more stable than microtubules in fibroblasts or non-confluent epithelial cells [Wadsworth and McGrail, 1990]. The nearly complete disassembly observed in HGF/SF treated cells shows that microtubule stability was modified in response to growth factor treatment. The relatively uniform loss of microtubules throughout the cells (Fig. 3) suggests that changes in microtubule behavior during differentiation and scattering occur throughout the microtubule array. However, analysis of the population of microtubules does not reveal the specific features of individual microtubule dynamic behavior which are modified in the cells following differentiation and in response to growth factors.

Individual Microtubules Are Stabilized During Polarization of Epithelial Cells

To further examine the behavior of microtubules in MDCK epithelial cells, the dynamic behavior of individual microtubules in living cells has been measured. Cells were injected with rhodamine labeled tubulin and incubated for several hours to allow incorporation of the injected tubulin into microtubules [Gliksman et al., 1992; Shelden and Wadsworth, 1993]. Images of fluorescent microtubules were recorded using low-light-level fluorescence microscopy and dynamic behavior was quantified. Video sequences of fluorescent microtubules in non-polarized cells reveal that microtubules in these cells are dynamic, alternating between periods of growth and shortening (data not shown). Life history plots of individual, representative microtubules (Fig. 4A) illustrate the dynamic behavior of these microtubules.

The parameters of microtubule dynamic behavior in non-polarized cells were quantified from the life history plots (Table I). The average rates of growth and shortening (9.2 ± 5.3 and 17.1 ± 11.3 μm/min, respectively) are remarkably similar to values previously obtained in PtK_1 epithelial cells (11.9 ± 6.5 and 19.8 ± 10.8 μm/min for growth and shortening) [Shelden and Wadsworth, 1993]. Overall, microtubule behavior in these cells was qualitatively similar to that observed in other relatively undifferentiated, cultured cells [Cassimeris et al., 1988; Sammak and Borisy, 1988b; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993].

In contrast to the behavior of microtubules in non-polarized cells, however, microtubules in polarized cells

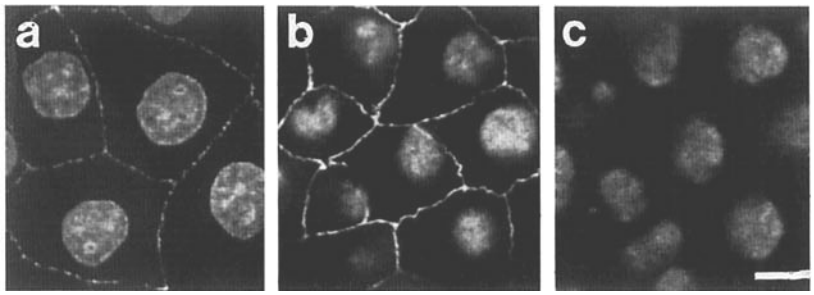

Fig. 2. Confocal immunofluorescence microscopy of MDCK cells stained with an antibody for ZO-1, a marker for tight junctions. **a:** In flattened, non-polarized cells, ZO-1 staining is present at regions of cell-cell contact. An extended focus series is shown. In polarized cells, ZO-1 staining is present only in the apical region (**b**) and not when the plane of focus is shifted toward the basal region of the cells (**c**). Kalman averages of apical and basal optical sections from a complete Z-series are shown. Nuclear fluorescence is non-specific. Bar = 10 μm.

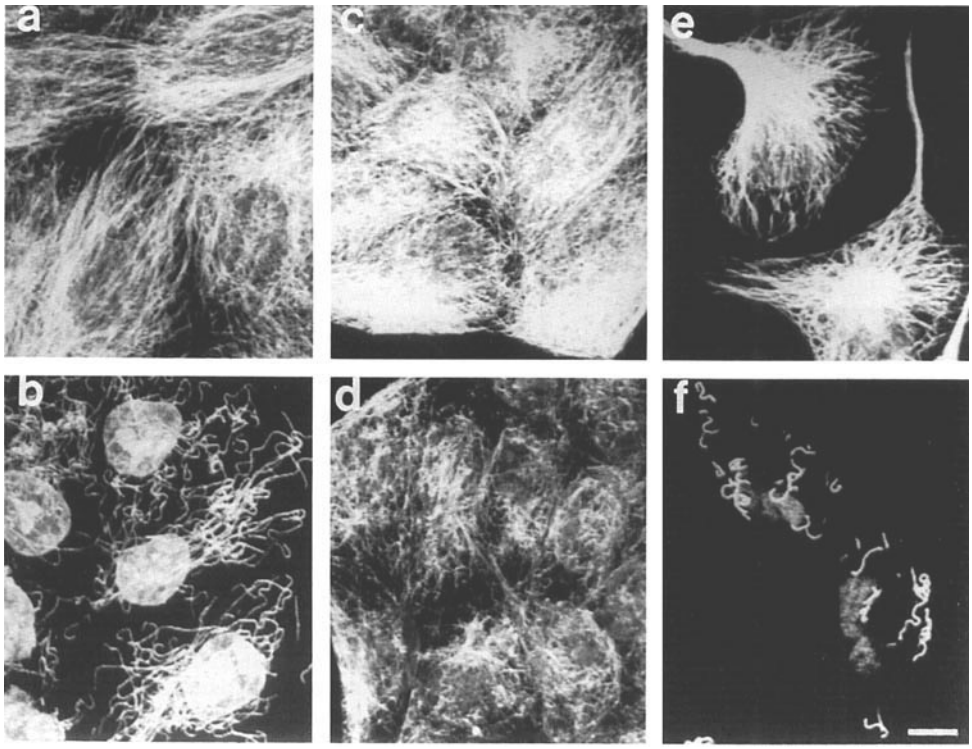

Fig. 3. Differential sensitivity to nocodazole induced microtubule disassembly in polarized, non-polarized, and HGF scattered MDCK cells. Confocal immunofluorescence micrographs of microtubules in MDCK cells. Extended focus series of the cells are shown; each slice in the series was collected as a Kalman average. MDCK cells were cultured for 48 h (**a,b**), 96 h (**c,d**), and 24 h followed by 24 h with 25 ng/ml MGF (**e,f**) and stained with an antibody to tubulin. Cells in b, d, and f were incubated with 20 μM nocodazole for 20 min before fixation. Nuclear staining in b is non-specific staining that is collected by the fluorescein filter set used for confocal microscopy. Bar = 5 μm.

are kinetically stabilized. A sequence of images showing fluorescent microtubules in the peripheral region of a polarized MDCK cell is shown in Figure 5. In this sequence, one microtubule did not detectably change length over the entire observation interval of 88 sec. Another microtubule in the same field of view paused for approximately 40 sec and then disassembled (Fig. 5).

Representative life history plots of microtubules in polarized cells also show the reduction in microtubule dynamic activity in polarized cells (Fig. 4B). Quantitative analysis reveals that the average rate and distance, but not duration, of shortening excursions are significantly reduced in polarized cells (Table I). Thus, fewer dimers are lost from the microtubule plus end during a

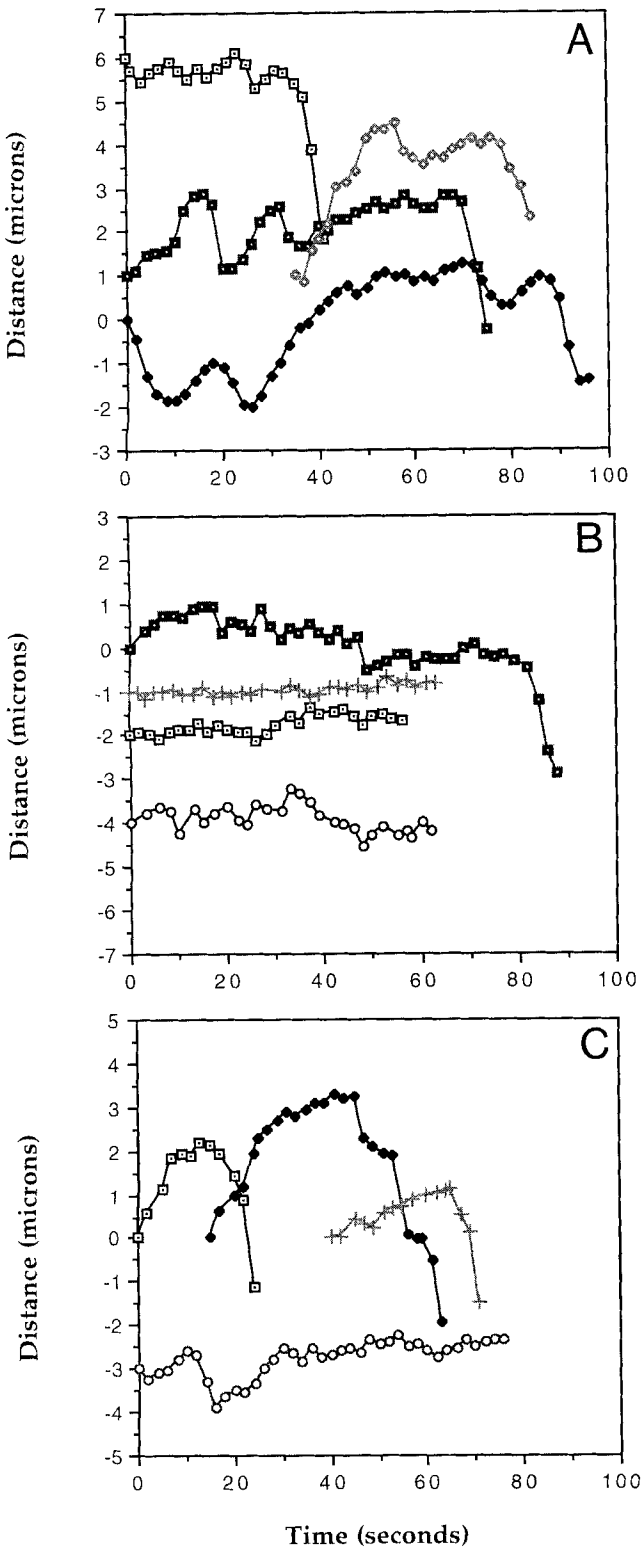

Fig. 4. Distance vs. time plots of individual microtubules. Microtubules in (**A**) non-polarized MDCK cells, (**B**) polarized MDCK cells, and (**C**) MDCK cells treated with HGF are shown. In each panel, traces of 4 representative microtubules are shown; the traces have been offset on the X- and Y-axes for clarity.

TABLE I. Parameters of Microtubule Dynamic Instability in Non-Polarized, Polarized, and Scattered MDCK Epithelial Cells*

Parameter	Non-polarized	Polarized	HGF
Growth			
Duration (sec)	8.9 ± 5.3	8.9 ± 3.9	9.0 ± 6.4
Distance (μm)	1.2 ± 0.6	1.0 ± 0.66	1.5 ± 1.0
Rate (μm/min)	9.2 ± 5.3	7.0 ± 3.5	11.0 ± 5.5
Shortening			
Duration (sec)	6.4 ± 2.4	6.2 ± 3.7	6.3 ± 3.1
Distance (μm)	1.8 ± 1.1	0.9 ± 0.6	2.6 ± 1.5
Rate (μm/min)	17.1 ± 11.3	10.7 ± 7.8	26.6 ± 14.6
Pause (sec)	6.7 ± 4.8	17.6 ± 16.4	10.4 ± 9.2
Dynamicity (μm/min)	8.3 ± 5.0	2.7 ± 2.4	11.1 ± 6.5
No. MTs	26	19	39
Cells	7	10	10

*Values are mean ± standard deviation.

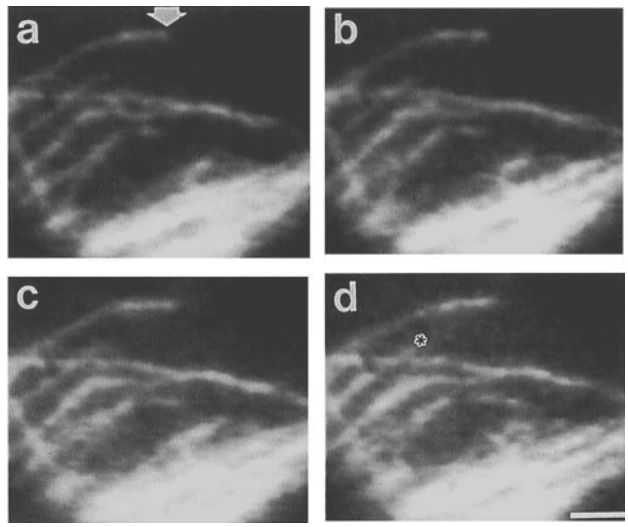

Fig. 5. **a–d:** A kinetically stabilized microtubule in a polarized MDCK epithelial cell. The microtubule marked with an arrow (a) is paused for the entire sequence, of which 40 sec are shown here; the microtubule marked with an asterisk (d) shortens at a later time in the sequence. The interval between displayed images is 10 sec. Bar = 2 μm.

shortening excursion. The rate of microtubule growth also decreased (significant at the 95% confidence level) in polarized cells; the growth distance and duration were not significantly altered (Table I). In previous experiments, no difference in growth rate was observed between single and confluent MDCK cells, when measured from the incorporation of exogenous tubulin in fixed cells (Bre et al., 1990).

Microtubule Turnover Is Stimulated in Scattered MDCK Epithelial Cells

Individual microtubule dynamic behavior has also been measured in HGF/SF treated MDCK cells. As seen in the video sequence (Fig. 6), microtubules are highly

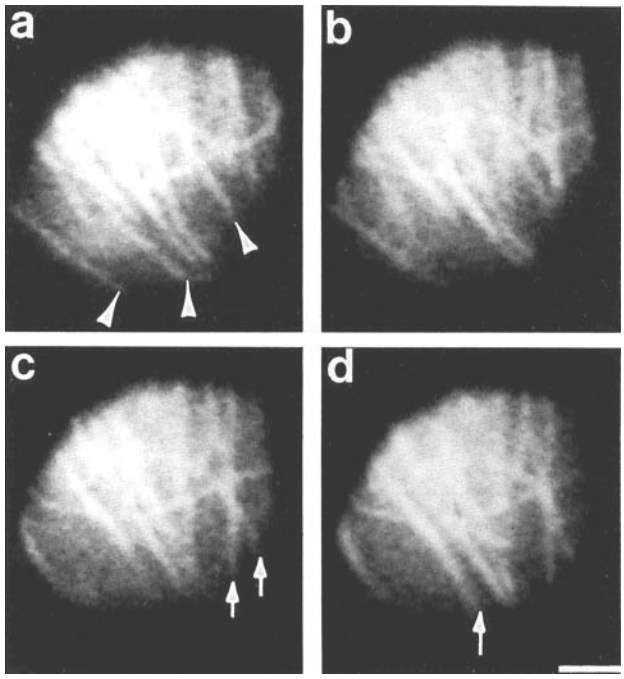

Fig. 6. **a–d:** Individual microtubule dynamic behavior in HGF/SF treated MDCK cells. Images were obtained at 2 sec intervals; the interval between displayed images is 10 sec. Note the changes in the microtubule array over the 40 sec shown. Microtubules that disassemble in a subsequent image are marked with arrowheads (a); microtubules that have grown are marked with arrows (c,d). Bar = 2 μm.

dynamic in lamellar regions of scattered cells. In this sequence, several microtubules grow into, and depolymerize from, the field of view (Fig. 6). Representative life history plots of individual microtubules from HGF/SF treated cells also illustrate this dynamic behavior (Fig. 4C). Many microtubules in scattered cells are characterized by a growth phase, followed by a catastrophe and rapid shortening out of the field of view (Fig. 4C). This behavior is similar to that previously observed in unstimulated Chinese hamster ovary (CHO) fibroblasts [Shelden and Wadsworth, 1993]. Although most microtubules in the scattered cells were dynamic, some microtubules in scattered cells paused for relatively long periods (Fig. 4C, lower trace; see Discussion). Finally, curved and looped microtubules, similar to those observed in motile growth cones, were seen in many scattered cells [Tanaka and Kirschner, 1991; Mikhailov and Gundersen, 1995; Tanaka et al., 1995] (data not shown).

The behavior of microtubules in HGF/SF treated MDCK cells has been quantified and can be compared with microtubule behavior in non-polarized and polarized MDCK cells (Table I). The data reveal significant increases in the average rate and distance of shortening excursions in HGF/SF treated cells when compared with either polarized or non-polarized cells (Table I). Histograms of shortening rates show a narrow distribution of rates in polarized cells while in non-polarized and especially scattered cells, a broad distribution is observed (data not shown). Similarly, a broad distribution of shortening distances is observed in scattered cells (data not shown). The average rate of growth was significantly increased in HGF treated cells when compared with polarized cells. When compared with non-polarized cells, the increase in growth rate in HGF treated cells was significant at the 95% level of confidence (Table I). The growth distance in HGF treated cells was significantly greater than in polarized and non-polarized cells at the 95% confidence level (Table I). The average duration of both growing and shortening events is not changed when HGF treated cells are compared with either polarized or non-polarized cells.

To assess the overall dynamic turnover of microtubules, the total gain and loss of subunits from microtubule plus ends, a parameter known as dynamicity [Toso et al., 1993], has been measured. In polarized cells, dynamicity was significantly less than measured in either non-polarized or polarized cells. In HGF treated cells, dynamicity was significantly increased when compared with polarized cells and increased when compared with non-polarized cells (significant at the 95% level of confidence).

Pause, or Attenuation, Increases During Polarization

In addition to the phases of growth and shortening, microtubules can exist in a state of pause, or attenuation, where no change in microtubule length can be detected using video microscopy [Walker et al., 1988; Gildersleeve et al., 1992; Shelden and Wadsworth, 1993; Toso et al., 1993]. In polarized cells, the average duration of a pause event is longer (Table I) when compared with either non-polarized or HGF treated cells. Analysis of histograms of pause duration (data not shown) reveals an increase in the number of extremely long pauses in polarized cells. In some cases, a microtubule remained paused for the entire sequence of images, so the reported average pause duration underestimates the actual duration of pause. In scattered cells, the average duration of pause (10.4 ± 9.2) was significantly less than polarized cells (17.6 ± 16.4 sec). However, the average pause duration in scattered cells was significantly greater than measured in non-polarized cells (6.7 ± 4.8). This result may reflect the broad distribution of pause durations observed in scattered cells (data not shown).

The percentages of total observation time in the phases of growth, shortening, and pause, or attenuation, have been determined (Table II). Polarization induced a marked increase in the percentage of total observation time spent in pause, from 33% in non-polarized to 72%

TABLE II. Percentage of Time in a Phase for Microtubules in MDCK Epithelial Cells

	Non-polarized	Polarized	HGF
% Time in growth	45.6	15.8	35.7
% Time in shortening	21.4	12.1	21.9
% Time in pause	33.0	72.1	42.5

in polarized cells (Table II). The marked increase in time spent in pause was accompanied by reductions in the percentage of time spent in growth and shortening. The percentages of total observation time that scattered cells spent in the phases of growth, shortening, and pause were similar to those measured in non-polarized cells.

Transition Frequencies Are Altered During Polarization and Scattering

Modulation of the frequency of transitions between the various phases of dynamic instability is likely to contribute to the regulation of microtubule dynamic behavior in cells [Kirschner and Mitchinson, 1986]. To measure the frequency of catastrophe, we have counted transitions to rapid shortening (from growth or pause to shortening); to measure the frequency of rescue we have counted transitions that result in the cessation of rapid shortening (transitions from rapid shortening to growth or pause). Transition frequencies have been determined per unit time and per unit distance [Gliksman et al., 1992; Kowalski and Williams, 1993].

The frequency of catastrophe transitions was significantly reduced in polarized compared with non-polarized cells, when measured per unit time (0.063 ± 0.076 and 0.025 ± 0.025 sec^{-1}, respectively; Table III). The catastrophe frequency in scattered cells was indistinguishable from that measured in non-polarized cells and significantly greater than in polarized cells (Table III). When measured per unit distance, the frequency of catastrophe was not statistically different between any two groups. The frequency of rescue transitions, measured as events per unit time, was not significantly changed during polarization or following scattering with HGF. When determined as a function of distance shortened, however, a significant increase in the frequency of rescue is measured in polarized compared with non-polarized cells (0.712 ± 0.938 μm^{-1} in polarized cells, 0.307 ± 0.393 μm^{-1} in non-polarized cells; Table III). Conversely, when polarized cells are compared with scattered cells, a significant decrease in the frequency of rescue transitions is observed (from 0.712 ± 0.938 to 0.191 ± 0.370 μm^{-1}). Thus, in scattered cells there are fewer rescue events per micron of microtubule disassembled, resulting in a greater loss of subunits from the microtubule. The frequency of rescue was similar in scattered and non-polarized cells.

TABLE III. Catastrophe and Rescue Transition Frequencies in MDCK Epithelial Cells*

Parameter frequency	Non-polarized	Polarized	HGF
Catastrophe, sec^{-1}	$.063 \pm .015$	$.025 \pm .006$	$.072 \pm .014$
Rescue, sec^{-1}	$.071 \pm .015$	$.093 \pm .031$	$.059 \pm .017$
Catastrophe, μm^{-1}	$.733 \pm .177$	$.943 \pm .333$	$.531 \pm .106$
Rescue, μm^{-1}	$.307 \pm .077$	$.712 \pm .215$	$.191 \pm .059$

*Values are mean ± standard error of the mean.

DISCUSSION

Our results demonstrate that microtubule dynamic turnover is regulated in MDCK epithelial cells during differentiation and in response to the growth factor HGF. First, we show that microtubules are less dynamic in polarized compared with non-polarized epithelial cells. Our work extends previous results by measuring the behavior of individual microtubules and thus revealing the specific aspects of microtubule dynamic behavior which are modified in polarized cells [Bre et al., 1990]. We show that the average rates of growth and shortening and frequency of catastrophe (sec^{-1}) decrease and the average duration of pause and frequency of rescue (μm^{-1}) increase in polarized compared with non-polarized cells.

In previous experiments a subset of microtubules, referred to as stable microtubules, was identified that did not incorporate exogenous tubulin within a 1 h incubation period [Schulze and Kirschner, 1986; Schulze et al., 1987; Webster et al., 1987]. Because we observed living cells for short periods (1–2 min), we cannot determine if any of the microtubules we observed correspond to this subclass of microtubules. In non-polarized cells, most microtubules we observed showed some dynamic activity and thus are unlikely to represent examples of the highly stable, minor subclass of microtubules described previously. In polarized cells, some microtubules did not detectably grow or shorten during our observation interval and thus could represent examples of the previously described stable subclass. Perhaps more importantly, however, is the fact that the high temporal and spatial resolution of our experiments permit us to describe the kinetic behavior of microtubules in living cells under various conditions. The kinetic behavior of microtubules in polarized cells is clearly different from that observed in non-polarized or HGF treated cells. Thus we refer to the stabilization that we observe in polarized cells as kinetic stabilization.

Microtubule associated proteins (MAPs), which bind along the length of the polymer, may contribute to the kinetic stabilization of microtubules in polarized epithelial cells. For example, microinjection of brain MAP-2 into non-neuronal cells suppresses individual microtubule dynamic behavior in a manner that is similar to what we observed in polarized epithelial cells: in both

cases, the average rates of microtubule growing and shortening and the frequency of catastrophe (sec^{-1}) were decreased and the rescue frequency (μm^{-1}) was increased [Dhamodharan and Wadsworth, 1995]. Analysis of microtubule dynamics in vitro in the presence of MAP-2 or tau also reveals a decrease in the frequency of catastrophe and an increase in the frequency of rescue, and a marked reduction in the rate of rapid shortening [Drechsel et al., 1992; Pryer et al., 1992; Kowalski and Williams, 1993; Ookata et al., 1995]. Interestingly, in polarized MDCK cells the average duration of pause and the percent of time in pause increased, a change not seen in MAP-2 injected cells. However, low concentrations of vinblastine, which binds to microtubule ends, have been shown to increase pause duration and percentage of time paused, suggesting that molecules which bind to microtubule ends also contribute to microtubule stabilization in polarized epithelial cells [Toso et al., 1993; Wilson and Jordan, 1994; Dhamodharan et al., 1995].

The major MAP in non-neuronal cells, MAP-4, may play a role in regulating microtubule dynamics in MDCK cells. Addition of native MAP-4 to microtubules in vitro results in a dramatic increase in the frequency of rescue, compared with control, MAP-free microtubules [Ookata et al., 1995]. MAP-4, which had been phosphorylated in vitro, was much less effective in promoting rescue [Ookata et al., 1995]. Interestingly, however, when the level of MAP-4 is experimentally increased or decreased in living cells, no change in microtubule stability or polymer level is detected [Barlow et al., 1994; Wang et al., 1996]. These observations suggest that changes in the phosphorylation state of MAP-4 in differentiated and scattered cells could contribute to the changes in microtubule dynamics observed here.

Our data further demonstrate that microtubule dynamic turnover is stimulated in MDCK cells scattered with HGF. Previous experiments have shown that during HGF/SF induced scattering, cells initially flatten, and later disperse, across the substrate. Treatment of cells with taxol or colchicine prevents cellular dispersion, but not initial flattening [Prescott et al., 1992; Dugina et al., 1995]. Cell migration is characterized by the continued formation of lamellae at the leading edge, the establishment of contacts with the substratum, and the subsequent retraction of the trailing portion of the cell [Abercrombie, 1988; Vasiliev, 1991]. Microtubules are known to be required for the maintenance of a leading edge in locomoting cells [Vasiliev, 1991]. Microtubules are also required for the selective stabilization of appropriately oriented pseudopodia in chemotaxing amoeba [Ueda and Ogihara, 1994] and may contribute to focal contact formation in locomoting fibroblasts [Rinnerthaler et al., 1988]. Recently it has been shown that the dynamic turnover of microtubules contributes to directed cell locomotion. Suppression of microtubule dynamics, with low concentrations of vinblastine or nocodazole, markedly reduces both directed locomotion of nerve growth cones and the rate of migration of cells into a wound in the monolayer [Liao et al., 1995; Tanaka et al., 1995]. Our observations demonstrate that microtubule dynamic behavior is enhanced in HGF treated, motile cells. Microtubules which are highly dynamic would be best able to extend rapidly into newly forming protrusions, where they can exert their stabilizing effect and maintain the direction of migration.

Our data and previous experiments demonstrate that microtubule dynamic turnover varies in different regions of a cell. Previous experiments have shown that in the thin retraction fibers formed during scattering of PtK_1 cells microtubules are relatively stable [Prescott et al., 1992], in contrast to the predominantly dynamic microtubules we observe in lamellar regions. However, because microtubules in the retraction fibers are bundled, we could not examine individual microtubule behavior in this region of the cell. In addition to regional variation in microtubule dynamic behavior, we observed a high level of heterogeneity in microtubule behavior within lamellar regions of scattered cells. Heterogeneity could result from differences in the amount or kind of MAPs bound along the length of individual microtubules and is consistent with the cooperative binding of MAPs to microtubules in vitro [Wallis et al., 1993]. Interactions of the ends of microtubules with particular cellular structures, e.g., molecules or complexes in the plasma membrane, could potentially alter microtubule dynamic behavior [Liao et al., 1995]. In any case, microtubules must be sufficiently dynamic to extend into rapidly forming lamellae; subsequent stabilization of a fraction of microtubules in a given lamellum may serve to modulate the rate or direction of cell locomotion.

The mechanism by which HGF/SF stimulates microtubule turnover is not known. HGF/SF binds a transmembrane receptor tyrosine kinase called $p190^{MET}$, encoded by the MET proto-oncogene [Bottaro et al., 1991] and cellular responses to HGF/SF are transduced by this receptor [Weidner et al., 1993]. HGF/SF binding to $p190^{MET}$ is followed by receptor autophosphorylation on tyrosine residues and activation of ras [Naldini et al., 1991; Graziani et al., 1993]. Although the downstream events that eventually lead to changes in microtubule dynamics are not known, inhibitors of protein kinase C potentiate scattering, while activation of adenylate cyclase and modulators of calcium/calmodulin have little effect [Rosen et al., 1990].

The changes in microtubule dynamic instability measured in HGF/SF treated cells are similar to those observed when cells or cell extracts are treated with okadaic acid, an inhibitor of phosphoprotein phos-

phatases [Gliksman et al., 1992; Shelden and Wadsworth, 1996]. Okadaic acid treatment also induces the disappearance of post-translationally modified, stable microtubules [Gurland and Gundersen, 1993]. In other experiments, however, addition of serum or transforming growth factor beta to serum starved cells, conditions that might be expected to increase phosphorylation levels, induced the formation of stable microtubules [Gundersen et al., 1994]. Interestingly, addition of XMAP, a Mr 215,000 MAP from *Xenopus* eggs, to brain microtubules in vitro stimulated dynamic turnover of microtubules [Vasquez et al., 1994]. Both the addition of XMAP to microtubules in vitro and treatment of cells with HGF increased microtubule dynamicity, while addition of MAP-2 decreased this parameter. These observations suggest that the activity of XMAP-like factors might be stimulated in scattered cells, increasing the dynamic turnover of microtubules. These data are consistent with a model in which HGF treatment activates MAPs that increase dynamicity and/or inactivates MAPs that stabilize microtubules. Additional experiments are necessary to identify the downstream targets of HGF that modulate microtubule behavior.

SUMMARY

The data presented here document the changes in individual microtubule behavior in a single cell type which occur as the cells differentiate and respond to the growth factor HGF. Polarization of MDCK cells in culture is accompanied by a dramatic stabilization of individual microtubules. Conversely, treatment of cells with HGF/SF markedly stimulates microtubule dynamic turnover. However, because HGF/SF functions primarily to stimulate motility rather than proliferation in MDCK cells [Gherardi et al., 1989], it is not known if mitogens alone are dufficient to enhance microtubule turnover. However, given the dramatic changes in the actin cytoskeleton in response to mitogens [Ridley and Hall, 1992; Ridley et al., 1992], it is likely, but yet untested, that the microtubule cytoskeleton will also be affected.

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