# Rapid Movements of Vimentin on Microtubule Tracks: Kinesin-dependent Assembly of Intermediate Filament Networks

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*Abstract.* The assembly and maintenance of an extended intermediate filament (IF) network in fibroblasts requires microtubule (MT) integrity. Using a green fluorescent protein–vimentin construct, and spreading BHK-21 cells as a model system to study IF–MT interactions, we have discovered a novel mechanism involved in the assembly of the vimentin IF cytoskeleton. This entails the rapid, discontinuous, and MT-dependent movement of IF precursors towards the peripheral regions of the cytoplasm where they appear to assemble into short fibrils. These precursors, or vimentin dots, move at speeds averaging $0.55 \pm 0.24$ μm/s. The vimentin dots colocalize with MT and their motility is inhibited after treatment with nocodazole.

Our studies further implicate a conventional kinesin in the movement of the vimentin dots. The dots colocalize with conventional kinesin as shown by indirect immunofluorescence, and IF preparations from spreading cells are enriched in kinesin. Furthermore, microinjection of kinesin antibodies into spreading cells prevents the assembly of an extended IF network. These studies provide insights into the interactions between the IF and MT systems. They also suggest a role for conventional kinesin in the distribution of non-membranous protein cargo, and the local regulation of IF assembly.

Key words: intermediate filaments • vimentin • microtubule • kinesin • cytoskeleton

CYTOPLASMIC intermediate filaments (IF)[1] form complex networks that extend from the cell periphery to the nucleus. Little is known about the mechanisms underlying the assembly and organization of these networks in vivo. However, a variety of experimental data suggest that interactions with other cytoskeletal elements contribute significantly towards establishing and maintaining networks of IF.

Initial evidence for interactions between IF and other cytoskeletal components came from light and electron microscopic studies of fibroblasts showing the existence of close parallel arrays of IF and microtubules (MT) (Goldman and Knipe, 1973; Heuser and Kirschner, 1980; Ball and Singer, 1981; Geiger and Singer, 1980). Interestingly, the localization of vimentin IF has been correlated with a sub-population of MT, the detyrosinated stable MT in fibroblasts (Gurland and Gundersen, 1995). These structural features of IF and MT support other results that indicate that the normal assembly and maintenance of vimentin IF networks are dependent on an intact MT network. Fibroblasts allowed to spread in the presence of MT-depolymerizing drugs after trypsinization fail to organize extended IF networks (Goldman, 1971). In spread cells the depolymerization of MT, as well as the microinjection of tubulin antibodies, causes the movement of IF away from the cell periphery (Goldman, 1971; Croop and Holtzer, 1975; Blose and Chacko, 1976; Bloom and Vallee, 1983; Wehland and Willingham, 1983; Gurland and Gundersen, 1995).

Little is known regarding the specific mediators of IF and MT interactions. In neurons, it has been suggested that the neurofilament heavy chain (NF-H), one of the subunits of neuronal IF, can bind to MT (Hirokawa, 1982). There is also evidence for other possible crossbridging elements including a fibroblast 95-kD IF-associated protein (Lin and Feramisco, 1981), plectin (Svitkina, 1996), and the MT-associated proteins, MAP-2 and 210-kD MAP (Leterrier et al., 1982; Bloom and Vallee, 1983; Draberova and Draber, 1993). For example, the microinjection of an

1. *Abbreviations used in this paper*: GFP, green fluorescent protein; IF, intermediate filaments; MT, microtubules; TX-100, Triton X-100.

antibody against the 95-kD IF-associated protein causes the apparent retraction of IF away from the cell periphery.

Interestingly, the microinjection of kinesin antibodies into fibroblasts also induces the retrograde movement of IF away from the cell periphery towards the nucleus (Gyoeva and Gelfand, 1991). Furthermore, the expression of truncated kinesin heavy chain cDNA in epithelial cells causes a retraction of vimentin IF (Navone et al., 1992). These observations suggest that this MT-dependent motor is required to actively maintain the IF network in its normal extended configuration. Recently, in vitro binding assays using purified kinesin and vimentin suggest that kinesin heavy and light chains are capable of interacting with purified vimentin (Avsyuk et al., 1995; Liao and Gundersen, 1998).

There is evidence that IF in fibroblasts also interact with the microfilament system (Green et al., 1986). These interactions appear to contribute to the movements of IF towards the juxtanuclear region (Hollenbeck et al., 1989; Tint et al., 1991). Interactions between the IF and microfilament systems also appear to be mediated by associated proteins, such as plectin, and BPAG1n/dystonin (Brown et al., 1995; Yang et al., 1996). These observations imply that the mechanisms governing the distribution of cytoskeletal IF are the result of a complex interplay of IF with MT and microfilaments (Yoon et al., 1998).

In this study we attempt to shed light on the mechanisms underlying IF–MT interactions by examining the process by which the different structural subunits of IF are assembled into extended networks. An ideal model system for this purpose is provided by spreading fibroblasts, after trypsinization and replating. During this process, IF form complex assemblies extending from the nucleus to the cell surface. We describe for the first time, the formation of a large number of protofilamentous aggregates of vimentin, vimentin dots, during the early stages of spreading of BHK-21 cells. Many of these dots are rapidly translocated to the peripheral regions of cells where they appear to assemble into IF. This process is dependent upon MT and a kinesin motor protein. These observations help provide a framework for understanding the interactions between the IF and MT cytoskeletal networks.

## Materials and Methods

### Cell Culture

BHK fibroblasts (BHK-21) were grown in plastic culture dishes (Corning Glass Works, Corning, NY) at 37°C in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% calf serum, 10% tryptose phosphate broth (DIFCO Laboratories, Detroit, MI), 100 units/ml penicillin and streptomycin. In some cases cells were maintained at 4°C for 2 h in DME containing 100 mM Hepes (Sigma Chemical Co., St. Louis, MO). Leibovitz L-15 medium (GIBCO BRL) containing 10% calf serum, 10% tryptose phosphate broth, and 100 units/ml penicillin and streptomycin was used for live cell observations. When required, nocodazole (Sigma Chemical Co.) at a final concentration of 600 nM was added to the culture medium to disassemble MT. For observations on recovery from nocodazole, cells were rinsed and transferred to normal growth medium. For membrane staining, live cells were incubated in one of the following lipophilic vital dyes: $DiOC_6$, rhodamine-6-G, Acridine orange, Neutral red, and rhodamine 123 (Molecular Probes, Eugene, OR). These dyes were added to the culture medium for 15 min at the recommended concentrations (Haugland, 1996; Spector et al., 1997).

### Trypsinization/Replating

Confluent cultures grown on plastic dishes were trypsinized and replated (Goldman and Follet, 1969) onto No. 1 cover glasses (Corning Glass Works), in either of the two culture media described above. Specifically, trypsin (GIBCO BRL), diluted 1:5 in 0.5 mM EDTA and $Ca^{2+}$-, $Mg^{2+}$-free PBS (6 mM $Na^+$-$K^+$ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4), was used for detaching cells from their substratum. Upon detachment, cells were dispersed into culture medium, and plated onto coverslips at densities of $10^4$–$10^5$/cm$^2$. After replating, cells were monitored as they spread into characteristic fibroblastic shapes.

### Transfection

A green fluorescent protein (GFP)–vimentin cDNA construct was used for transfection to observe vimentin dynamics in live cells as described by Yoon et al. (1998). 72 h after transfection, cells were trypsinized and replated onto glass coverslips as described above for live cell observations.

### Antibodies

For studies of vimentin localization in BHK-21 cells, the following antibodies were used: a polyclonal anti-BHK IF (Yang et al., 1985), a polyclonal anti–human vimentin (Chou et al., 1996), and V9, a monoclonal anti-vimentin (Sigma Chemical Co.). For MT localization, a polyclonal antibody against calf brain tubulin (Green and Goldman, 1983) and an mAb against bovine brain tubulin IgM, 5H1 (Wang et al., 1993) were used (a gift of Dr. L. Binder, Northwestern University, Chicago, IL).

Two antibodies against conventional kinesin were used in this study. First, a polyclonal antibody against the stalk domain of ubiquitous conventional kinesin (aa 523–775) was prepared and affinity purified as described previously (Niclas et al., 1994). A second polyclonal antibody was raised against a peptide found in the L8/β5 region of conventional kinesin (aa 153–172; acetyl-CLSVHEDKNRVPYVKGVTERF-amide). For affinity purification, the peptide was coupled with sulfolink resin (Quality Controlled Biochemicals, Hopkinton, MA) at a ratio of 1 mg peptide per ml resin. Serum was incubated with the resin for 12 h at 4°C, the resin was washed with 50 mM $NaH_2PO_4$, pH 6.5, and antibodies were eluted with 100 mM glycine-HCl, pH 2.5, followed by rapid neutralization.

FITC- and lissamine-rhodamine–conjugated anti–mouse and anti–rabbit (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies for immunofluorescence studies. 10-nm gold–conjugated secondary antibodies (anti–mouse and anti–rabbit; Amersham Life Sciences, Arlington Heights, IL) were used for electron microscopic studies. Peroxidase-conjugated anti–rabbit secondary antibodies (Jackson ImmunoResearch) were used for immunoblotting.

### Live Cell Observations

For live cell observations, BHK-21 cells were plated on coverslips in L-15 medium (GIBCO BRL) as described above, mounted on glass feet, and then sealed with a mixture of Vaseline, beeswax, and lanolin (1:1:1). The cells were maintained at 37°C with an air stream stage incubator (Model ASI 400; NEVTEK, Burnsville, VA) during the period of observation.

### Indirect Immunofluorescence

BHK-21 cells plated on coverslips were rinsed rapidly in PBS, and fixed at room temperature in 4% formaldehyde (Tousimis Research Corporation, Rockville, MD) in a MT-stabilizing buffer, PEM (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.9, 37°C) (Weatherbee et al., 1978) for 6 min. This buffer was used to preserve MT, which are extremely labile, especially at the cell periphery during the early stages of cell spreading. After fixation, cells were permeabilized in 0.1% NP-40 in PEM buffer for 3 min at room temperature, and then briefly washed in PEM for 3 min. Subsequently, cells were incubated with the appropriate primary antibody for 30 min at 37°C in a moist chamber, washed with PEM, and then incubated with the appropriate secondary antibody for 15 min. The coverslips were then mounted for observation, using Gelvatol (Air Products and Chemicals, Inc., Allentown, PA) containing DABCO (Sigma Chemical Co.) at 100 mg/ml.

### Ultrastructural Immunogold Localization

For the ultrastructural localization of vimentin during spreading, cells were trypsinized and replated (see above), and then processed for immu-

nogold electron microscopy using a modification of the Yang et al. (1985) protocol. Cells were fixed and lysed after 45 min in 0.2% glutaraldehyde (Tousimis Research Corporation) and 0.1% Triton X-100 (TX-100; Sigma Chemical Co.) in MES buffer (0.1 M MES, pH 6.6, 0.5 mM $MgSO_4$, 2 mM EGTA) for 30–45 s. The coverslips were washed in MES buffer for 5 min. Cells were further permeabilized in 0.15% TX-100 in MES buffer for 2 min. This was followed by three washes in MES buffer for 10 min, treatment with 0.5 mg/ml $NaBH_4$ in MES buffer for 20 min, and five washes in MES buffer for 20 min. Subsequently, coverslips were incubated with vimentin mAb (1:5 in MES buffer) for 4–6 h at 37°C, washed three times for 10 min in MES buffer, and then incubated with 10-nm colloidal gold–conjugated secondary antibodies for 4–6 h at 37°C. The cells were then fixed in glutaraldehyde–osmium tetroxide, flat embedded, and then stained for electron microscopy according to the procedure of DeMey et al. (1986).

## Image Acquisition and Analysis

Images of both live and fixed-stained cells were acquired using a Zeiss LSM 410 confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany). Optical sections were set to ~0.7–1.0 μm. GFP–vimentin was visualized using excitation at 488 nm, and emission at 515–545 nm. Images of live cells during spreading were acquired from the same focal plane at 5–10-s intervals for 5–10 min at a resolution of 512 × 512 dpi. Image analysis was conducted using Metamorph (Universal Imaging Corp., West Chester, PA). The rates of movement were calculated using the Measure Distance function on successive frames. Colocalization studies used lissamine-rhodamine/FITC or lissamine-rhodamine/GFP.

A JEOL 1200EX electron microscope (JEOL USA, Peabody, MA) was used at an accelerating potential of 60 kV to examine the immunogold preparations.

## Microinjection

The L8/β5 kinesin antibody (2.4 mg/ml), the stalk domain kinesin antibody (0.25 mg/ml), and control preimmune IgG (5 mg/ml) were dialyzed into PBS, clarified by centrifugation, and then microinjected into cells (Vikstrom et al., 1989) 30 min and 24 h after replating.

## The Preparation of IF-enriched Cytoskeletons, SDS-PAGE, and Immunoblotting

IF-enriched cytoskeletal fractions were prepared from spreading, and fully spread BHK-21 cells maintained at either 37°C or 4°C according to a modified version of the protocol of Starger and Goldman (1977). For immunofluorescence studies, cells grown to 50% confluency on coverslips were lysed for 15 s in IF lysis buffer (PBS containing 0.6 M KCl, 5 mM EDTA, 5 mM EGTA, and protease inhibitors [1 mM PMSF, 1 mM TAME, 1 mg/ml leupeptin, pepstatin, and aprotinin; Sigma Chemical Co.]) containing 0.1% TX-100, and fixed and processed for immunofluorescence as described above.

For biochemical studies, cells grown to 80% confluency in 100-mm plastic dishes were washed with PBS, and then lysed in IF lysis buffer containing 1% TX-100. The insoluble fraction containing IF was pelleted at 15,000 g at 4°C in a Sorvall RC-2B centrifuge. Chromatin and actin in the pellet were removed by treatment with 5 mg/ml DNase for 30 min. After centrifugation at 18,000 g in a Sorvall centrifuge, the pellet, enriched in IF and associated proteins, was used to assay for the presence of kinesin by immunoblotting. Whole cell extracts were prepared by solubilizing live cells in boiling Laemmli buffer containing protease inhibitors (1 mg/ml leupeptin, pepstatin, and aprotinin). The samples were separated on 7.5% polyacrylamide gels according to the method of Laemmli (1970).

## Results

### The Organization of Vimentin in Spreading Cells

To observe cells in the process of assembling their networks of IF, we studied the localization of vimentin in BHK fibroblasts during cell spreading. For this purpose, trypsinization/replating was used as the experimental system. During trypsinization, IF are reorganized from their previous extended configurations in spread cells. After replating and attachment to the substratum, most of the vi-
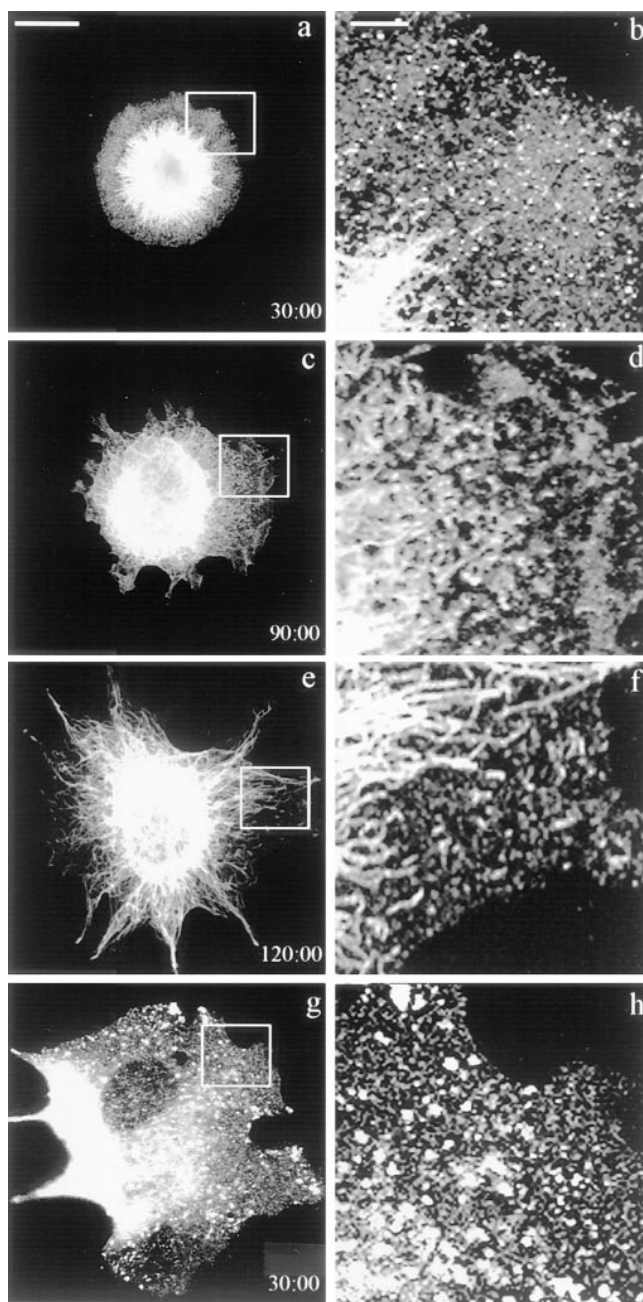


*Figure 1.* Vimentin localization in BHK-21 cells during cell spreading. Cells were fixed (*a–f*) at 0.5 h (*a, b*), 1.5 h (*c, d*), and 3.0 h (*e, f*) after trypsinization/replating. Lower magnification views of spreading cells (*a, c, e*) and higher magnification views from peripheral regions within the same cells (*b, d, f*). The perinuclear region of intense fluorescence corresponds to the IF cap that typifies cells in this stage of the spreading process. Note the abundance of discrete vimentin-containing dots in early spreading (*a, b*), the presence of squiggles as the dots decrease in number at later stages (*c, d*), and longer vimentin fibrils at later stages of cell spreading (*e, f*). The localization of GFP–vimentin in a live cell at low (*g*) and high (*h*) magnification, 30 min after replating. In the more peripheral regions of spreading cytoplasm, GFP–vimentin is present in discrete punctate structures, vimentin dots, similar to those seen in fixed untransfected cells (compare with *a, b*). Time is indicated on the lower right. Bars: (*a, c, e, g*) 10 μm; (*b, d, f, h*) 2.5 μm.

mentin is localized initially in the juxtanuclear region as previously described (Goldman and Follet, 1970). However, with the use of cross-linking fixatives like formaldehyde, discrete dot-like vimentin-rich structures can be distinguished between this juxtanuclear region and the cell surface (Fig. 1). These dots had previously eluded our detection, since the majority of them are not preserved by methanol, the most commonly used fixative for immunolocalization of vimentin IF. Within 30–45 min after replating, most of the vimentin in the peripheral regions of cells was concentrated in dots (Fig. 1, *a* and *b*). By 1–1.5 h after replating, however, short filamentous structures with two free ends, or "squiggles" predominated in the peripheral regions (Fig. 1, *c* and *d*; also see Yoon et al., 1998). Within 2–3 h, the number of dots and squiggles throughout the cell decreased as a function of spreading, accompanied by a concomitant increase in longer fibrils (Fig. 1, *e* and *f*). These observations imply that, in the peripheral regions of the spreading cytoplasm, the dots give rise to squiggles that in turn become incorporated into longer fibrils. Due to the limits of resolution of fluorescence microscopy, we cannot determine whether 10-nm IF are found in each of these structures. This is addressed in a preliminary fashion by electron microscopy (see below). These observations demonstrate the presence of novel vimentin containing structures which appear to be precursors of vimentin IF networks.

## Vimentin Dots Exhibit Rapid Movements

To gain further insights into the interrelationships between the dots, squiggles, and fibrils, BHK-21 fibroblasts were transfected with GFP–vimentin cDNA. These cells were trypsinized and replated 72 h later. The behavior of the expressed GFP–vimentin was monitored by time-lapse confocal microscopy as the cells assumed fibroblastic shapes. Under these conditions, cells appeared healthy as indicated by processes such as intracellular organelle movement, membrane ruffling, and spreading (Yoon et al., 1998).

In live spreading cells we observed dots and squiggles containing GFP–vimentin and these were indistinguishable from those seen in fixed, untransfected spreading cells (Fig. 1, *g* and *h*). We also observed that the GFP–vimentin dots were motile. Two types of movements could be discerned within the time frame of the observations: vibrational movements with no net translocation, and a fast, predominantly unidirectional movement. The former movements, displayed by 50–60% of the dots at any given time interval, appeared to be due to Brownian motion. The latter movements, exhibited by ~40–50% of the vimentin dots, were fast and discontinuous. Similar movements have been described for various organelles by others (Rebhun, 1967; Freed and Lebowitz, 1970). The vimentin-rich dots involved in vibrational movements ap-
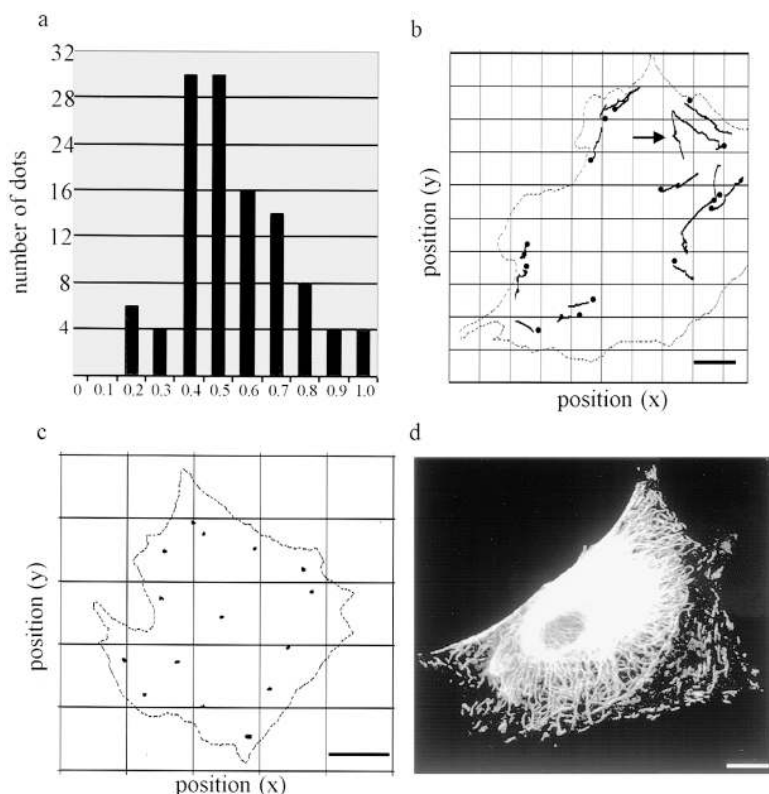
*Figure 2.* (*a*) A cumulative histogram of the number versus speed (μm/s) of GFP-vimentin–containing dots in transfected spreading cells, 30–45 min after trypsinization/replating. 116 dots that displayed net displacements >1 μm/10 s were analyzed. A typical Gaussian distribution was obtained, with most dots displaying movements of about 0.4-0.5 μm/s. The mean rate of speed was calculated to be 0.55 ± 0.24 μm/s, with peak velocities of 1.0 μm/s. (*b*) Vector plots of the movements of 15 dots in another spreading cell over a period of 2.5 min, as determined by confocal time lapse observation. The cell periphery is depicted by the dashed line, and the movements of the dots by solid lines. The ● located at the beginning of the solid lines represents the origin of movements at the time of observations. The arrow singles out a rare example of a directional change in the movement of a single vimentin dot. Note that the net movement of the dots is towards the cell periphery. (*c, d*) The movement of the vimentin dots is dependent on microtubules. When transfected cells are treated with 600 nM nocodazole 15 min after replating, and allowed to continue to spread in the presence of nocodazole for 30 min, no movements of the dots could be detected. This is indicated by the vector plots of GFP–vimentin dot movements in a nocodazole-treated living cell over a period of 5 min (*c*). (*d*) When cells are treated with nocodazole 15 min after replating, and allowed to continue to spread in the presence of nocodazole for 3 h, squiggles can be detected at the edge of the cell. Note the presence of a vimentin cap in the juxtanuclear region. The dashed line (*b, c*) represents the edge of the cell. Bars, 5 μm.

peared interchangeable with the rapidly moving ones, with a single dot often displaying vibrational movements for a short period of time, followed by a burst of rapid unidirectional movement. Time-lapse measurements of dots displaying displacements >1 μm/10 s, indicated an average speed of 0.55 ± 0.24 μm/s ($n$ = 116 dots) with peak velocities of 1.0 μm/s (Fig. 2 $a$).

The net movement of the dots was towards the peripheral regions of the cell. This directionality can be seen in the vector plots of the movements of 15 GFP–vimentin dots in a single cell plotted over a period of 2.5 min (Fig. 2 $b$). In some instances, neighboring vimentin-rich dots would move in opposite directions, and orthogonal to the direction of cell spreading. This added to our confidence that the movements of the vimentin-rich dots were not passively induced by cell spreading. The vector plots also depict a rare instance of a directional change in the movement of a single dot (indicated by an arrow). The fast moving dots often followed straight tracks, as seen in the example of a transfected cell 30 min after plating, where two dots follow one another at different rates; one travelling with a peak rate of 1.0 μm/s, and the other 0.5 μm/s (Fig. 3, $a$–$f$). This example also illustrates the variability in the rates of the movements of individual dots as one slows down, and later catches up with the faster moving dot. As the dots move towards the edges of cells, they frequently appear to elaborate a squiggle of increasing length (Fig. 3, $g$–$i$). Invariably the squiggle was elaborated in a direction opposite to the movement of the dots. The motile properties of the squiggles, once formed, are documented in Yoon et al. (1998).

These observations demonstrate that vimentin-containing structures can exhibit rapid and directed motility in actively spreading regions of the cell. The behavior of the vimentin-rich dots also indicates that the squiggles are formed from vimentin dots. These movements appear to be involved in the targeting and elaboration of the IF network to the peripheral cytoplasm of fibroblasts.

### The Motility of Vimentin-containing Dots Depends on MT Integrity

The rates and discontinuous nature of the dot movements suggested that they were MT dependent. We tested this by disassembling MT using nocodazole at a final concentration of 600 nM added to the culture medium 15 min after replating. Within 15 min after treatment with nocodazole, no MT could be detected in these cells (data not shown). Cells were allowed to continue spreading in the presence of nocodazole for an additional 30 min, and then observed. As previously described, BHK-21 cells continue to spread in the absence of MT (Goldman and Follet, 1970).

In nocodazole-treated cells, most of the polymerized IF are present predominantly in a juxtanuclear cap (Fig. 2 $d$; also see Goldman, 1971). As in untreated cells, vimentin-rich dots were present during the early stages of cell spreading. However, these dots did not exhibit movements towards the periphery after the disassembly of MT. Instead they displayed only vibrational movements with no significant net displacements. Even after 5 min, all the detectable dots remained stationary, and vector plots of 15 vimentin dots in a nocodazole-treated cell depict the lack
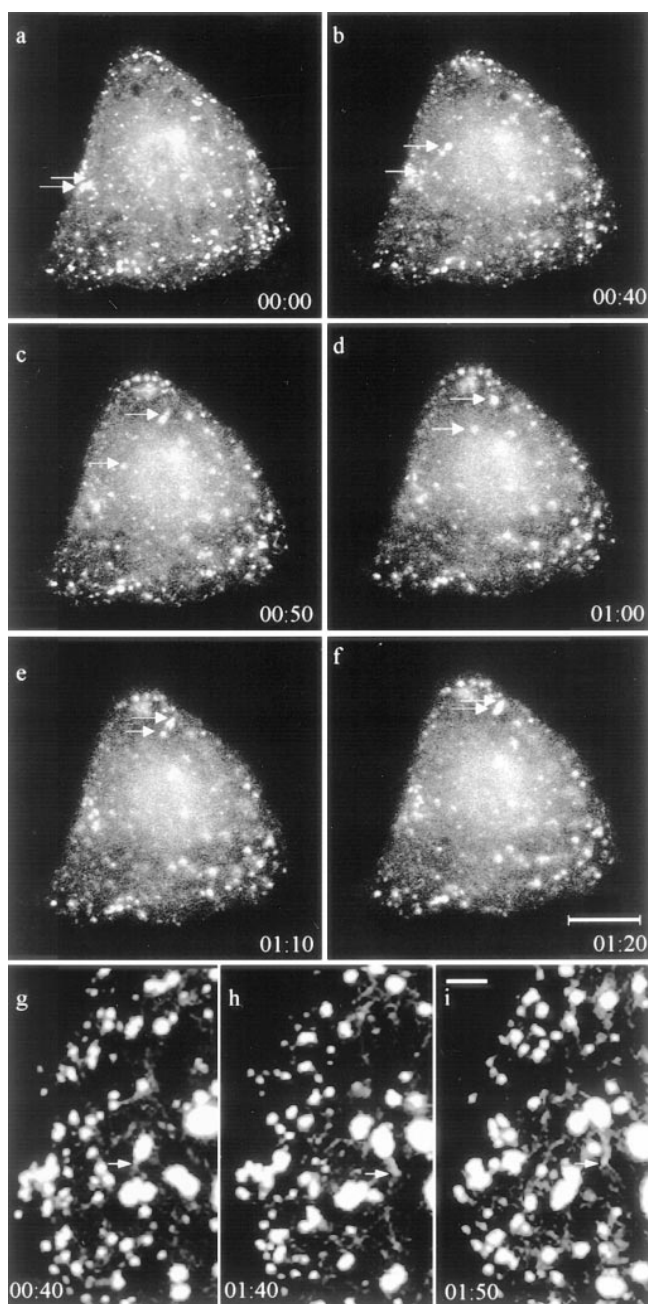


*Figure 3.* The movements of vimentin dots in a live spreading cell. ($a$–$f$) Confocal images of dot movements 30–45 min after replating. Arrows show two GFP–vimentin dots that display rapid movements. The two dots follow one another along a straight track, as they move across the cell. One dot moves across the cell displaying a peak rate of movement of 1 μm/s ($b$, $c$), and then slows down, while the second dot, moving at a peak rate of 0.5 μm/s catches up with it ($d$–$f$). ($g$–$i$) As some vimentin dots move, they appear to elaborate squiggles as seen in the confocal micrographs of the peripheral region of a live cell 1 h after replating. A dot appears to elaborate a squiggle of increasing length, as indicated by the arrow. Time is indicated in min and s at lower right ($a$–$f$), and at lower left ($g$–$i$). Bars: ($a$–$f$) 10 μm; ($g$–$i$), 1 μm.
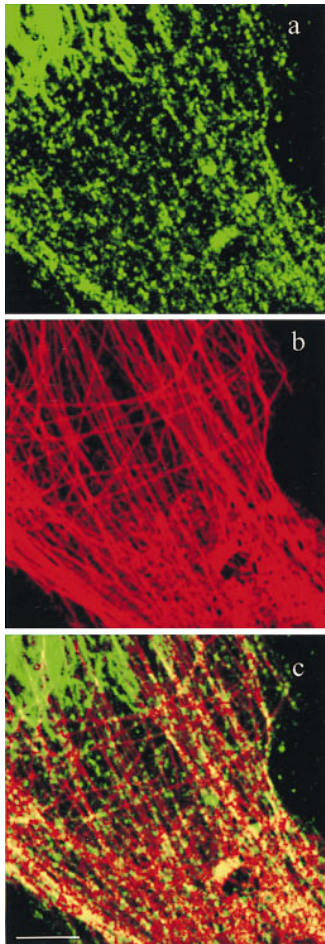
*Figure 4.* The colocalization of vimentin dots and microtubules at the edges of spreading cells. (*a*) Confocal immunofluorescence localization of vimentin at the edge of a fixed cell 30–45 min after replating. (*b*) Microtubule localization in the same cell. (*c*) An overlay of images *a* and *b*. *Yellow*, the regions of colocalization between vimentin dots and microtubules. Bar, 2.5 μm.

immunofluorescence. This was true of both GFP-vimentin–containing dots (data not shown) and dots in untransfected cells (Fig. 4). These studies provide further evidence that the movements of the dots occur along MT tracks.

### The Association of Conventional Kinesin with the Vimentin-containing Dots

Since the net movement of the dots was towards the cell periphery, it was plausible that a plus-end–directed MT-based motor, such as kinesin (Vale et al., 1985), powered their movement. To determine whether or not this was the case, we used two anti-kinesin antibodies to stain spreading cells. The antibodies were generated against the stalk region (aa 523–775) and the L8/β5 region (aa 153–172) of
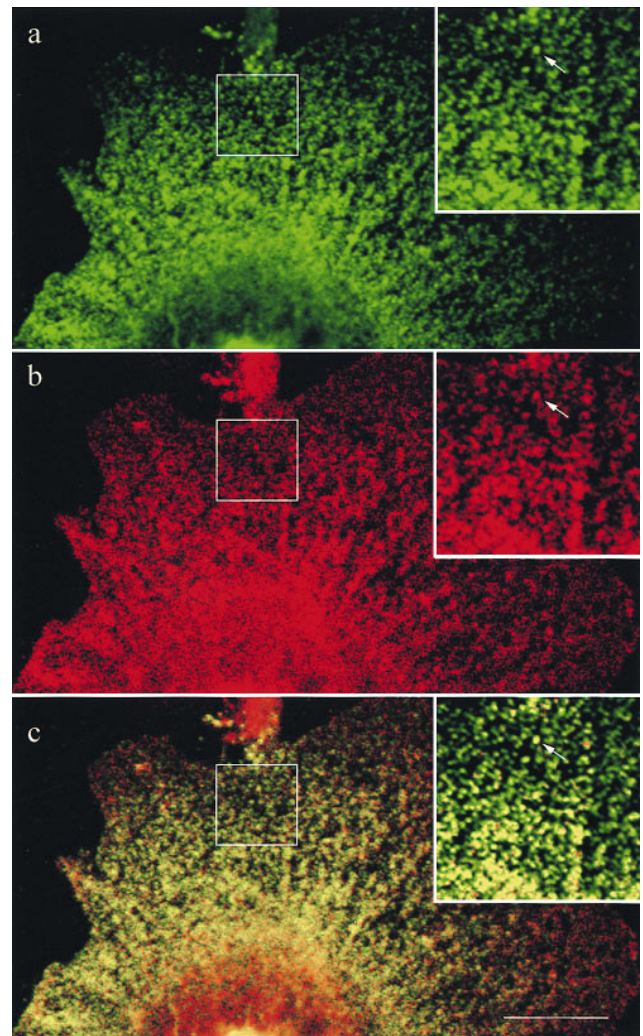


*Figure 5.* The colocalization of vimentin dots with conventional kinesin at the edges of fixed and stained cells, as determined by double label confocal immunofluorescence microscopy. (*a*) The localization of vimentin at the edge of a cell, 45 min after replating. (*b*) Localization of kinesin in the same focal plane, using anti-L8/β5. (*c*) An overlay of images *a* and *b*. Yellow indicates that a majority of the vimentin dots colocalize with kinesin. The insets depict higher magnification views of the boxed regions. Bar, 5 μm.

of significant translocation (Fig. 2 *c*). This arrest of movements in the presence of nocodazole was reversible, and the vimentin dots recovered their directed movements after the nocodazole was removed and MT were allowed to reassemble (data not shown).

When untransfected and transfected cells were allowed to continue to spread in the presence of nocodazole for 3–4 h, the vast majority of IF were arrested around the nucleus. Further, the number of dots in the peripheral regions of the cytoplasm decreased significantly and some squiggles could be detected (Fig. 2 *d*). The distribution of squiggles appeared random, and they remained disconnected from the central mass of filaments even after cells had spread for 7–8 h in the absence of MT. During this time frame, the nocodazole-treated cells failed to organize normal extended IF network. These studies demonstrate that the rapid movements of the vimentin-rich dots are MT dependent. However, the vimentin rich dots that presumably move towards the cell periphery before the disassembly of MT appear to be capable of eventually transforming into squiggles even in the absence of MT (Fig. 2 *d*).

Since dot motility requires an intact MT network, we examined whether the dots colocalized with MT in spreading cells 30–45 min after replating. The majority of the vimentin dots coaligned with MT, as determined by double-label
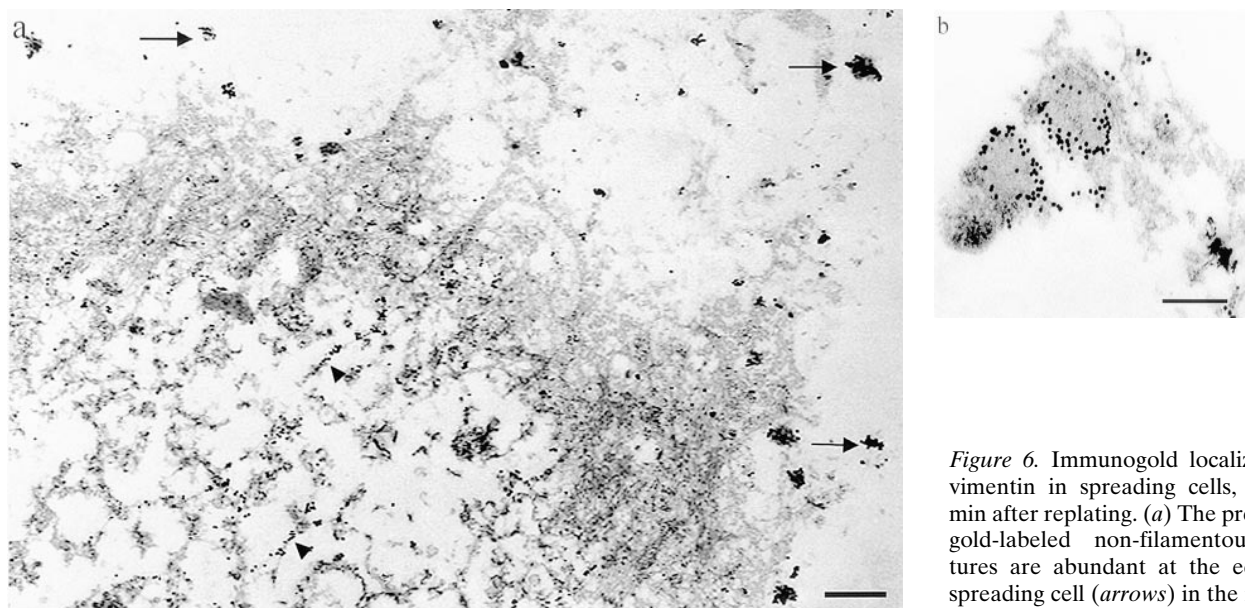
*Figure 6.* Immunogold localization of vimentin in spreading cells, fixed 45 min after replating. (*a*) The presence of gold-labeled non-filamentous structures are abundant at the edge of a spreading cell (*arrows*) in the region of a lamellipodium. In the more proximal regions, linear arrays of gold particles indicate the presence of short IF (*arrowheads*). Longer IF are not apparent in these regions. (*b*) A higher magnification view of two of the non-filamentous structures seen in another cell. Bars: (*a*) 0.5 μm; (*b*) 0.2 μm.

the catalytic core of conventional kinesin. Both antibodies recognized a 120-kD band in BHK-21 whole cell protein preparations as determined by immunoblotting (see Fig. 9 *e*). A 45-kD band could occasionally be detected by the anti-L8/β5 antibody (data not shown), corresponding to a common proteolytic fragment of kinesin (Kuznetzov et al., 1989). Immunofluorescence observations with these antibodies showed that they colocalized with a subset of the vimentin-rich dots especially apparent in the peripheral regions of the cell (Fig. 5). Vimentin dots could often be observed to form straight tracks and in these cases, a majority of them stained with the kinesin antibodies. This was consistent with the fact that only a sub-population of the vimentin-rich dots moved during a given period of time. The colocalization of kinesin with vimentin dots was also observed for GFP-vimentin–containing dots (data not shown) as seen in untransfected cells. These studies suggest that kinesin provides the motive force for the rapid MT-dependent movements of the vimentin-rich dots.

### Further Insights into the Nature of the Vimentin Dots

Kinesin has been implicated in the movement of predominantly membrane-bound organelles within cells. We therefore investigated whether the vimentin dots were associated with membranes. Our current evidence suggests that this is not the case. The vimentin dots, short filamentous squiggles, and networks of IF all exhibited similar detergent insolubility suggesting that the vimentin dots are retained along with the IF pellet after extraction with high salt/TX-100. This was determined by Western blot analyses of high speed pellets, and vimentin localization studies of cells before and after extraction (data not shown). As described in greater detail below, kinesin colocalization

with the vimentin dots is also retained after this treatment (see Fig. 9, *a* and *b*). Furthermore, the vimentin dots did not stain with the lipophilic vital dyes DiOC6, neutral red, or Rhodamine 123 (data not shown), which have been used widely as generic markers for membrane-bound compartments (Haughland, 1996; Spector et al., 1997).

Transmission electron microscopy of immunogold-labeled BHK-21 cells fixed at 45 min after replating was performed to characterize the nature of the vimentin-containing dots at higher resolution. In the more interior regions of the cell, the gold localized in linear arrays along IF (Fig. 6 *a*). Immediately subjacent to the edges of these cells, large numbers of discrete electron dense bodies lacking IF were labeled at their perimeters with gold particles (Fig. 6, *a* and *b*). These vimentin-containing aggregates varied in size between 0.15–0.6 μm, and appeared similar to, albeit smaller than, the IF aggregates formed during mitosis (Franke et al., 1982; Rosevear et al., 1990). The gold-labeled aggregates can readily account for the vimentin dots seen in vivo and by immunofluorescence. The fixation conditions required for the optimal labeling of the vimentin-containing dots did not preserve MT or the membranes surrounding cell organelles, such as mitochondria.

It appears, therefore, that the dots contain a non-filamentous form of vimentin. The lack of penetration of the gold-tagged antibodies to the interior of the electron-dense aggregates is very similar to the distribution of gold particles seen surrounding the aggregates of vimentin as well as keratin in mitotic cells (Franke et al., 1982; Rosevear et al., 1990). Taken together with the finding that conventional kinesin colocalizes with the vimentin-containing dots, these observations suggest that this motor may function to transport non–membrane-bound vimentin precursors to the cell periphery.
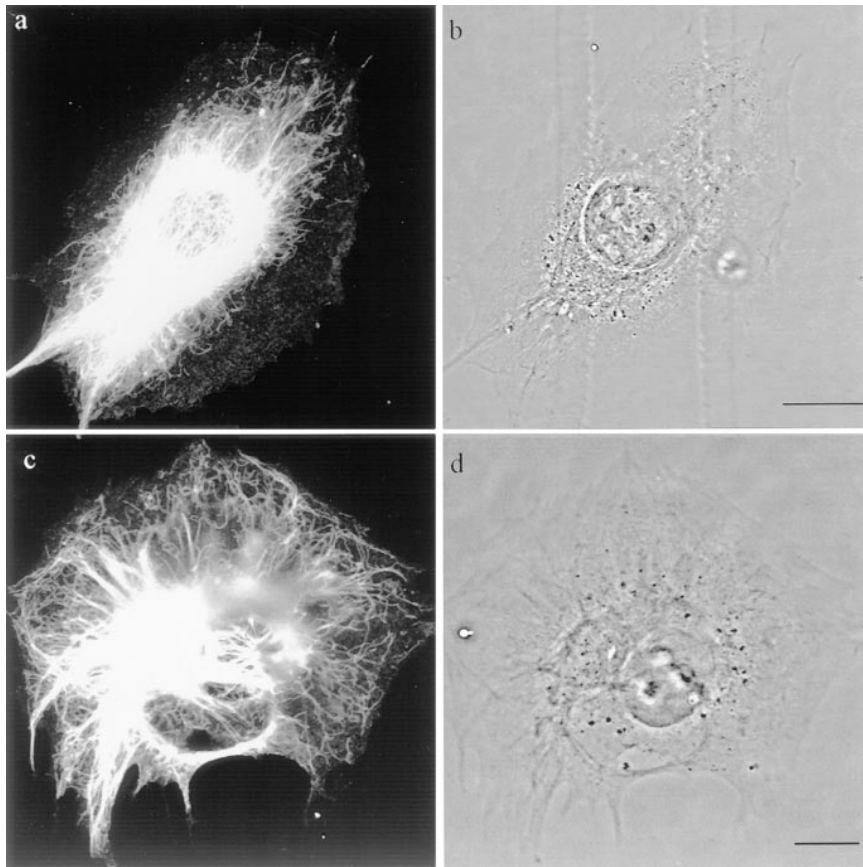
*Figure 7.* The effects of the microinjection of anti-L8/β5 into cells during (*a*, *b*), or after (*c*, *d*), the assembly of an IF network. (*a*) Vimentin localization as determined by immunofluorescence, in a cell microinjected with anti-L8/β5 15 min after replating, and then allowed to continue to spread for 4 h. Notice the absence of an extended IF network into the peripheral regions of the cell. (*b*) Phase-contrast image of the same cell. (*c*) Vimentin localization, as determined by immunofluorescence, in a cell microinjected with anti-L8/β5 24 h after replating. Notice that the majority of the vimentin IF have retracted from the cell periphery by 3 h after injection. (*d*) Phase-contrast of *c*. Bars, 10 μm.

## Kinesin Is Required for the Assembly and Maintenance of IF Networks

To further assess the role of kinesin in the assembly of IF networks, we microinjected kinesin antibodies into spreading BHK-21 cells (see Materials and Methods). If kinesin is responsible for targeting the vimentin dots towards the peripheral regions of spreading cells, we predicted that these cells would not be capable of assembling typical IF networks after the injection of kinesin antibodies. This was indeed shown to be the case. BHK-21 cells were trypsinized, and upon attachment for 15–30 min to the substratum, were microinjected with the affinity-purified antibody (2.4 mg/ml) directed against the L8/β5 region of the kinesin head. After injection, cells were allowed to continue to spread for 3–4 h, and then fixed and stained for vimentin. Injected cells failed to assemble an extended IF network (Fig. 7, *a* and *b*). Similar results were obtained after the injection of the kinesin antibody directed against the stalk domain (not shown). In control injections using nonspecific IgG, no effects on IF network formation were detected (data not shown). These observations indicate that kinesin-based motility is responsible for the targeting of the non-filamentous vimentin dots towards the cell surface, as well as for the assembly of an extended IF network.

The microinjection of anti-kinesin into fully spread fibroblasts containing an extended IF network has been shown previously to induce the retraction of the vimentin IF network away from the periphery of the cell (Gyoeva and Gelfand, 1991). We determined whether our antibodies were also effective in inducing the retraction of IF. When injected into fully spread BHK-21 cells 24 h after replating, the L8/β5 antibody (2.4 mg/ml in injection buffer) caused a retraction of the majority of the fibrils comprising the vimentin IF network (Fig. 7, *c* and *d*). This retraction of vimentin IF from the peripheral regions took ∼3–4 h after the injection of kinesin antibodies. Similar results were achieved by the injection of the polyclonal antibody directed against the stalk domain (not shown). These results imply that a continuous kinesin-based anterograde movement of vimentin is required for both the assembly and maintenance of extended IF networks in BHK-21 cells.

## Further Insights into the Association Between Vimentin IF and Kinesin

Since the data described above and elsewhere (Gyoeva and Gelfand, 1991) implicate kinesin in the maintenance of IF networks, we looked for conditions under which kinesin might be induced to colocalize with vimentin IF in spread cells. In well-spread BHK-21 cells in culture, kinesin is typically localized throughout the cytoplasm as punctate structures with a diffuse background (Fig. 8 *a*) (Neighbors et al., 1988; Hollenbeck, 1989) and therefore does not obviously colocalize with vimentin IF (Fig. 8 *b*). However, we thought that it might be possible to "freeze" a subset of the kinesin onto its vimentin cargo by lowering the temperature to 4°C for 2 h. Under these conditions, the majority of MT in BHK-21 appear to be stable (data not shown).

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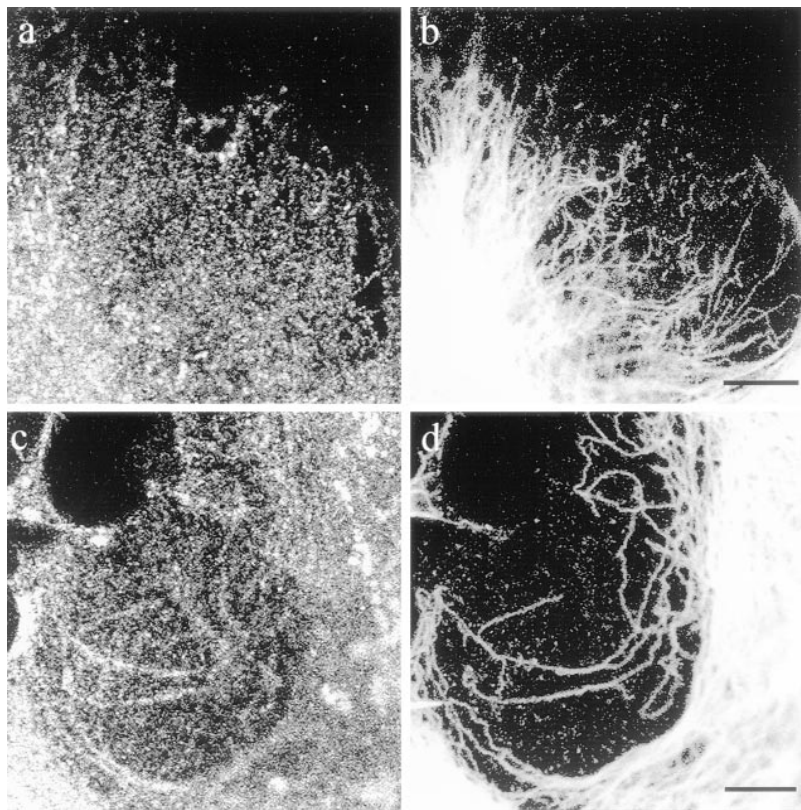
*Figure 8.* The colocalization of kinesin (anti-L8/β4) and vimentin IF in the peripheral regions of spread cells after incubation at 4°C as visualized by double-label immunofluorescence. (*a*) Confocal micrograph showing the presence of kinesin in punctate structures located in the peripheral regions of control, spread cells grown at 37°C. Note that there is no apparent colocalization of kinesin with vimentin (*b*). (*c*) Kinesin localization at the periphery of a spread cell after incubation at 4°C for 2 h. The kinesin now stains filamentous structures at the cell periphery which colocalize with vimentin (*d*). Bars, 5 μm.

When these cold-treated cells were fixed and stained, kinesin colocalization with vimentin IF was detectable in the thin peripheral regions of cells (Fig. 8, *c* and *d*).

Based on the above kinesin/vimentin association, we determined whether kinesin was associated with vimentin in IF-enriched cytoskeletal preparations (see Materials and Methods). These preparations preserved the vimentin-containing dots in spreading cells (see Fig. 9 *a*) and vimentin filaments in cells maintained at 4°C (Fig. 9 *c*). However, this extraction procedure does not preserve MT. In the case of both spreading cells (Fig. 9, *a* and *b*) and cold-treated fully spread cells (Fig. 9, *c* and *d*), colocalization of kinesin with vimentin dots and fibrils was retained after extraction. In support of this observation, the IF preparations from spreading cells and cells maintained for 2 h at 4°C (see Materials and Methods) were reproducibly enriched in kinesin relative to those from spread cells maintained at 37°C (Fig. 9, *f* and *g*). These data provide further support for the interaction between IF and kinesin.
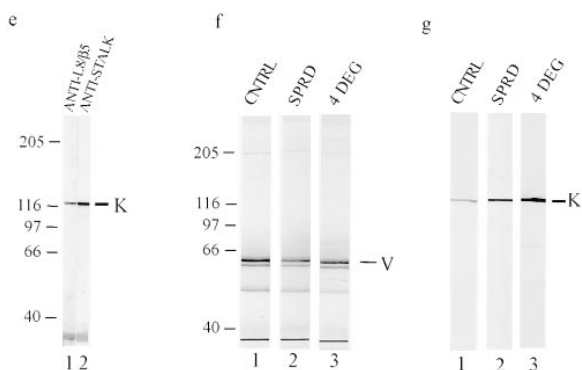
## Discussion

In this study, we describe novel vimentin containing structures that exhibit motility during cell spreading. These vimentin dots contain non-filamentous vimentin and appear to be precursors of vimentin IF networks in BHK-21 cells. Several interesting properties regarding vimentin IF network assembly have emerged from this study. The first is the MT-dependent targeting of vimentin IF precursors to regions of the spreading cytoplasm. Previous studies have documented interactions between MT and IF in fully spread cells, predominantly by showing the retraction of vimentin IF after depolymerization of MT (Goldman, 1971; Wehland and Willingham, 1983; Blose et al., 1984; Gurland and Gundersen, 1995) or disruption of MT-associated proteins (Leterrier et al., 1982; Bloom and Vallee, 1983; Draberova and Draber, 1993). The retractions are thought to be the result of the disruption of IF-MT cross-bridging proteins required to maintain IF networks in extended configurations (Bloom and Vallee, 1983; Draberova and Draber, 1993). However, our results suggest that the reorganization of IF after MT disruption could also reflect an underlying dependence of vimentin IF networks on the continuous MT-based anterograde transport of vimentin precursors; an exaggerated version of which is seen in cells after trypsinization/replating. Similar non-filamentous vimentin dots have been described during cell division (Franke et al., 1982; Rosevear et al., 1990). These dots appear to display similar rapid movements (unpublished observations).

Motile cells continuously send out new cytoplasmic extensions, which can be considered a form of regional spreading. Indeed, in preliminary studies, we have observed motile vimentin dots in some of these extensions in spread cells (unpublished observations). Thus it is possible that IF precursors are constantly moving towards the peripheral regions of the cell where they assemble and connect with the remainder of the IF network. The first step in this assembly process appears to be a conversion of the dots to squiggles. The latter structures also move primarily towards the cell periphery and predominantly in a MT-dependent fashion (Yoon et al., 1998).
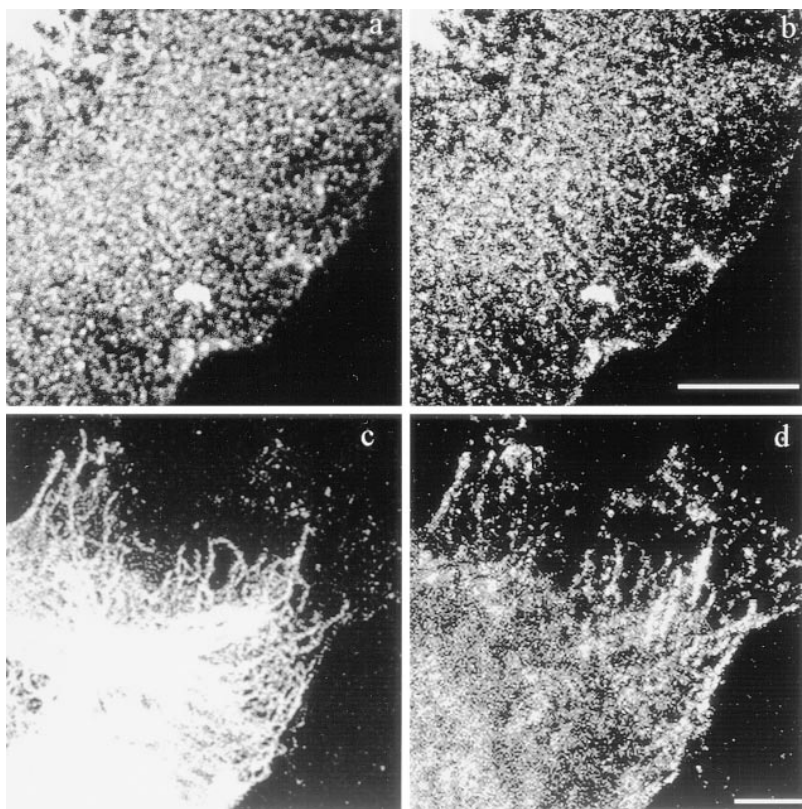
*Figure 9.* Kinesin is retained along with vimentin in IF-enriched cytoskeletal preparations. Double-label immunofluorescence images of (*a*) the peripheral region of a cell extracted 45 min after replating showing that vimentin dots are retained; and that (*b*) many colocalize with anti-L8/β5. (*c*) Vimentin staining at the periphery of a cell incubated at 4°C for 2 h followed by extraction, and kinesin co-localization using anti-L8/β5 (*d*). Note that kinesin colocalizes along many of the vimentin fibrils. (*e–g*) The presence of conventional kinesin in BHK-21 cells as determined by Western blot analyses. (*e*) Total BHK-21 cell protein extracts were probed with anti-L8/β5 (lane *1*), and the anti-stalk region of conventional kinesin (lane *2*). Molecular weight markers are indicated on the left. Note that the kinesin antibodies react with a band of ~120 kD. (*f, g*) Kinesin is found in IF cytoskeletal preparations. (*f*) Coomassie blue–stained SDS-PAGE gels of IF preparations from control spread cells (24 h after replating and maintained at 37°C; lane *1*), spreading cells (45 min after replating; lane *2*) and 4°C-treated cells (24 h after replating at 37°C, and then switched to 4°C for 2 h; lane *3*). Approximately equal amounts of vimentin were loaded in each lane. (*g*) Western blots, using anti-L8/β5, of identical samples as in *f*. Notice the apparent increase in kinesin in lanes *2* and *3*, when compared with lane *1*. This suggests that the amount of kinesin is enriched in IF preparations of spreading cells, and spread cells maintained at 4°C. Molecular weight markers are indicated on the left; *V*, indicates the position of vimentin; and *K*, kinesin. Bars, 5 μm.

Although our studies have focused on vimentin and MT, it is feasible that similar processes occur during the assembly of other types of IF networks, especially those that have been shown to be dependent on MT. For example, although the bulk of neurofilament triplet proteins are generally regarded as being transported by the slow component of axoplasmic flow, there exists some evidence that packets of neurofilament proteins are capable of rapid directed movements within axons (Hollenbeck and Bray, 1987; Prahlad et al., 1998; unpublished observations).

The factors that maintain vimentin in its non-filamentous precursor state (dots), or promote its polymerization into IF are unknown. Previous studies on mitotic cells have shown that the formation of protofilamentous vimentin aggregates is correlated with the phosphorylation of vimentin by p34$^{cdc2}$ (Chou et al., 1990, 1996). Therefore, the state of phosphorylation may also regulate the conversion of the non-filamentous vimentin dots into 10-nm IF during cell spreading.

Our studies using nocodazole indicate that the conversion of vimentin dots into squiggles occurs even in the absence of MT, suggesting that the dots can polymerize independent of their motile behavior. IF assembly can therefore be divided into the MT-dependent targeting of IF precursors, and the MT-independent conversion of the vimentin dots into squiggles. These events must occur in concert for the assembly of "normal" IF networks. A better understanding of the factors regulating each of these processes should ultimately allow us to dissect the mechanisms underlying the formation of abnormal IF aggregates found in fibroblasts and neurons in Giant axonal neuropathy, Lewy bodies of neurons in Parkinson's disease, hepatocyte Mallory bodies in cirrhosis, and neurofilament aggregates in amyotrophic lateral sclerosis (Sim et al., 1978; Goldman et al., 1983; Hirano et al., 1984; Bousquet et al., 1996).

Another observation regarding IF assembly revealed by these studies is its requirement for a plus end–directed MT

motor. The two kinesin antibodies used in our study are directed against the poorly conserved L8/β5 region of the motor catalytic core, and the stalk region specific for the conventional kinesin class (Kull et al., 1996; Sablin et al., 1996). Based upon the nature of the sequences recognized by these antibodies (Navone et al., 1992; Vale and Fletterick, 1997), and their reactivity with a single 120-kD polypeptide, it is likely that the transport of vimentin dots is mediated by a conventional kinesin. In addition, preliminary results with an antibody raised against a peptide corresponding to the highly class-specific conventional kinesin neck coiled-coil (aa 335–356) produced identical results to those shown in this paper (unpublished observations).

The results indicate that conventional kinesin moves non-membranous protein cargo within cells. Conventional kinesin appears unusual among cytoskeletal motors in terms of its high degree of processivity, as it can take >100 steps along a MT without dissociation. Other motors that transport membranes, such as the kinesin-like motor unc104 (Yale, R.D., unpublished observation) and myosin I (Ostap and Pollard, 1996), do not appear to be processive. Therefore, the highly processive motion of conventional kinesin may be an adaptation of this motor for efficiently transporting protein particles. The transport of IF protein that we have described provides an excellent system for dissecting the mechanism by which kinesin docks onto a cargo. A further ramification of these studies lies in the possibility that a greater understanding of IF–kinesin interactions may also lead to an understanding of the translocation of MT described by Vorobjev et al. (1997) and Waterman-Storer and Salmon (1997). In these cases it is conceivable that stationary vimentin IF with associated kinesin could cause MT to "walk" along their surface. Future work will be directed towards identifying the protein components in vimentin-rich dots that are specifically recognized by the kinesin motor. In support of this it has been shown that kinesin light chains as well as the tail portion of the heavy chain bind to vimentin in vitro (Avsyuk et al., 1995; Liao and Gundersen, 1998). The enrichment of kinesin in IF preparations of spreading and cold-treated cells will provide a useful tool for furthering such studies.

Given the novel findings of rapid, and MT-dependent transport of vimentin-containing dots, and the nature of vimentin assembly described in our study on spreading cells, it is interesting to speculate on why such a mechanism may be an important aspect of cell physiology. The regional assembly of IF suggests that cells need to regulate, in a precise manner, the local amounts of vimentin, particularly in rapidly spreading regions of the cytoplasm. The functional significance of such an assembly mechanism may be related to the purported role of IF as mechanical stabilizers of cell shape and mediators of cytoskeletal interactions (Goldman et al., 1996; Fuchs and Cleveland, 1998). Future studies on the composition of the vimentin-rich dots, as well as the factors regulating their delivery to peripheral regions and their local assembly into polymers, may help to define and elucidate the functional significance of IF assembly pathways in living cells.

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