

Stable, Detyrosinated Microtubules Function to Localize Vimentin Intermediate Filaments in Fibroblasts

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Abstract. Separate populations of microtubules (MTs) distinguishable by their level of posttranslationally modified tubulin subunits and by their stability *in vivo* have been described. In polarized 3T3 cells at the edge of an *in vitro* wound, we have found a striking preferential coalignment of vimentin intermediate filaments (IFs) with detyrosinated MTs (Glu MTs) rather than with the bulk of the MTs, which were tyrosinated MTs (Tyr MTs). Vimentin IFs were not stabilizing the Glu MTs since collapse of the IF network to a perinuclear location, induced by microinjection of monoclonal anti-IF antibody, had no noticeable effect on the array of Glu MTs. To test whether Glu MTs may affect the organization of IFs we regrew MTs in cells that had been treated with nocodazole to depolymerize all the MTs and to collapse IFs; the reextension of IFs into the lamella lagged behind the rapid regrowth of Tyr MTs, but was correlated with the slower reformation of Glu

MTs. Similar realignment of IFs with newly formed Glu MTs was observed in serum-starved cells treated with either serum or taxol to induce the formation of Glu MTs. Next, we microinjected affinity purified antibodies specific for Glu tubulin (polyclonal SG and monoclonal 4B8) and specific for Tyr tubulin (polyclonal W² and monoclonal YL1/2) into 3T3 cells. Both injected SG and 4B8 antibodies labeled the subset of endogenous Glu MTs; W² and YL1/2 antibodies labeled virtually all of the cytoplasmic MTs. Injection of SG or 4B8 resulted in the collapse of IFs to a perinuclear region. This collapse was comparable to that observed after complete MT depolymerization by nocodazole. Injection of W², YL1/2, or nonspecific control IgGs did not result in collapse of the IFs. Taken together, these results show that Glu MTs localize IFs in migrating 3T3 fibroblasts and suggest that detyrosination of tubulin acts as a signal for the recruitment of vimentin IFs to MTs.

MICROTUBULES (MTs)¹ in numerous cell types and across diverse species are comprised of different levels of posttranslationally modified tubulin subunits. This has been particularly well established for two modifications of tubulin: removal of a tyrosine residue from the COOH terminus of α -tubulin, known as detyrosination (Raybin and Flavin, 1977; Argarana et al., 1978), and the acetylation of Lys 40 of α -tubulin (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987). Both these modifications are known to be rapidly reversible *in vivo*. Using antibodies directed against posttranslationally modified forms of tubulin, both detyrosinated tubulin (known as Glu tubulin after its newly exposed COOH-terminal Glu residue) and acetylated tubulin have been shown to accumulate in the subset of stable MTs in cells (Gundersen et al., 1984, 1987a; Kreis, 1987; Schulze et al., 1987;

Webster et al., 1987; Piperno et al., 1987). Interestingly, the increased levels of Glu tubulin and acetylated tubulin in stable MTs are most pronounced in cells undergoing morphogenetic events such as the directed motility of fibroblasts (Gundersen and Bulinski, 1988; Nagasaki et al., 1992; Gundersen et al., 1994), formation of myotubes (Gundersen et al., 1989), neurite outgrowth (Baas and Black, 1990), epithelial layer formation (Pepperkok et al., 1990), and during the formation of polarized tissues in embryos (Houliston and Maro, 1989; Warn et al., 1990; McRae et al., 1991). This suggests that the stabilization and posttranslational modification of MTs may be important elements in morphogenetic processes in general (reviewed in Kirschner and Mitchison, 1986; Bulinski and Gundersen, 1991).

The correlation between elevated levels of posttranslationally modified tubulin and enhanced MT stability has been well documented in the case of detyrosination. Two *in vivo* studies have demonstrated directly that Glu MTs (MTs enriched in Glu tubulin and detectable with anti-Glu tubulin specific antibodies) are longer lived than MTs comprised primarily of tyrosinated tubulin (Tyr MTs, de-

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1. *Abbreviations used in this paper:* IF, intermediate filament; MT, microtubule.

tectable with anti-Tyr tubulin specific antibodies) (Webster et al., 1987; Schulze et al., 1987). In addition, Glu MTs are relatively more resistant to dilution-induced depolymerization and to depolymerization induced by MT disrupting agents (e.g., nocodazole) (Kreis, 1987; Khawaja et al., 1988). Also, studies have shown that Glu MTs do not incorporate derivatized (Webster et al., 1987) or endogenous tubulin subunits at their distal ends (Gundersen et al., 1987b), indicating that these MTs are not part of the dynamic population of MTs. Finally, the enhanced longevity of Glu MTs is consistent with how they are formed in vivo: Glu MTs arise by the time-dependent postpolymerization detyrosination of MTs which have formed from a pool of Tyr tubulin subunits (Gundersen et al., 1987a).

Although Glu MTs are more stable as judged by their greater longevity and resistance to antagonists, the posttranslational accumulation of Glu tubulin in stable MTs in vivo does not appear to be responsible for generating MT stability (Khawaja et al., 1988; Webster et al., 1990). These in vivo studies are consistent with in vitro studies which have shown that Glu and Tyr tubulin polymerize equivalently (Raybin and Flavin, 1977; Kumar and Flavin, 1982) and have similar dynamic instability parameters (Idriss et al., 1991; Skoufias, D., and L. Wilson, unpublished observations). Indeed, the factors that stabilize Glu MTs may be proteins whose phosphorylation level is important for their stabilizing activity (Gurland and Gundersen, 1993). The factors responsible for this stabilization are currently unknown.

Although a general function for stable, Glu MTs during cellular morphogenesis has been postulated (Kirschner and Mitchison, 1986; Bulinski and Gundersen, 1991), no specific functions for stable, Glu MTs have been documented to date. Stable, Glu MTs are biochemically differentiated from their dynamic counterparts and this may be used by the cell to distinguish different types of MTs (i.e., stable vs. dynamic MTs). Perhaps the posttranslational detyrosination of tubulin is involved in signaling the interaction of stable MTs with other cellular organelles. Candidate organelles that may respond to such a signal would include many of the organelles in the cell: the ER (Terasaki et al., 1986), mitochondria (Heggeness et al., 1978; Summerhayes et al., 1983; Ball and Singer, 1982), lysosomes (Collot et al., 1984; Matteoni and Kreis, 1987), endosomes (Matteoni and Kreis, 1987), Golgi (Thyberg and Moskalewski, 1985; Couchman and Rees, 1982; Ho et al., 1989) and intermediate filaments (IFs) (Goldman and Knipe, 1973; Croop and Holtzer, 1975; Blose and Chako, 1976; Hynes and Destree, 1978; Forry-Schaudies, 1986) have all been shown to be dependent on MTs for their localization in vivo. For example, IFs, mitochondria, and the ER will collapse toward the nucleus in cells treated with MT antagonists whereas the Golgi and lysosomes will disperse under these conditions. In some cases the interaction between MTs and organelles appears to be mediated by motor molecules; kinesin(s) in the cases of mitochondria (Nangaku et al., 1994), ER (Dabora and Sheetz, 1988; Vale and Hotani, 1988), and IFs (Gyoeva and Gelfand, 1991). MT-associated proteins have also been implicated in cross-bridging of MTs and IFs (Hirokawa, 1982; Leterrier et al., 1982; Heiman et al., 1985).

Given the evidence demonstrating that detyrosination is

not involved in stabilizing MTs and the dependence of organelle position on MTs, we have begun to examine whether organelles interact selectively with subsets of MTs in vivo. In this report, we present evidence that vimentin IFs are preferentially localized with the subset of stable, Glu MTs in the leading edge of polarized, migrating fibroblasts. We show directly that the IFs do not cause Glu MT stability. Instead, we have found that Glu MTs appear to be the preferred sites for IF interaction with MTs present in the lamella of migrating fibroblasts. Treatments that resulted in an increase in Glu MTs caused a coincident colocalization of IFs with the newly formed Glu MTs. More directly, we show that microinjection of affinity purified antibodies against Glu tubulin, but not Tyr tubulin, resulted in the redistribution of IFs to a perinuclear location in the cell ("IF collapse"). Taken together, the data presented in this study show that one function of the directed arrays of stable, Glu MTs in the lamella of wound edge, motile fibroblasts is to localize vimentin IFs. We hypothesize that posttranslational detyrosination of tubulin may be important for regulating the interaction of IFs with MTs.

Materials and Methods

Cell Culture and Treatments

NIH-3T3 fibroblasts were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% calf serum as previously described (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). Cells were seeded onto acid-washed sterile glass coverslips for immunofluorescence and grown until confluent (2 d). Monolayers were wounded as described previously (Gundersen and Bulinski, 1988) to generate a homogeneous population of cells at the wound edge containing arrays of Glu MTs oriented toward the wound edge (see Fig. 1) and were then treated as described in the Results. To prepare cells lacking Glu MTs, confluent monolayers were incubated for 48 h in serum-free medium (DME containing 5 mg/ml fatty acid-free BSA [No. 6003; Sigma Chemical Co., St. Louis, MO] and 20 mM Hepes, pH 7.4) as described previously (Gundersen et al., 1994). Serum-free medium-treated monolayers were wounded as described above and readministered medium containing 10% calf serum to induce the formation of oriented Glu MTs (Gundersen et al., 1994). For MT regrowth experiments, wounded monolayers were treated for 5 h in 10 μ M nocodazole (Aldrich Chemical Corp., Milwaukee, WI), washed three times in EBSS and incubated in DME with 10% calf serum for the times indicated. Long nocodazole treatments were used to insure the collapse of vimentin IFs to a juxtannuclear location (see also Forry-Schaudies et al., 1986). After treatments, cells were fixed in -20°C methanol for immunofluorescence as described previously (Gundersen et al., 1984).

Taxol (the generous gift of Dr. Ven L. Narayanan, National Cancer Institute, Bethesda, MD) and nocodazole were prepared as stock solutions in DMSO and diluted into growth medium so that the final concentration of DMSO did not exceed 0.1% (vol/vol). This concentration of DMSO had no effect on the distribution or levels of Glu or Tyr tubulin in these cells.

Indirect Immunofluorescence

For most experiments, we performed triple indirect immunofluorescence with antibodies to Glu tubulin, Tyr tubulin and IFs by incubating the coverslips in primary antibodies diluted in TBS, pH 7.4, containing 10% normal goat serum. A rabbit polyclonal antibody specific for Glu tubulin (SG) (Gundersen et al., 1984) was used at a dilution of 1/400 (of serum); a rat monoclonal antibody specific for Tyr tubulin (YL1/2) (Kilmartin et al., 1982) was used at a dilution of 1/400 (of ascites fluid) and was the generous gift of Dr. J. V. Kilmartin (Medical Research Council, Cambridge, England); a mouse monoclonal IgM (56B5) specific for IF rod domains (Kaplan et al., 1991), was used at a dilution of 1/4 (of culture supernatant) and was generously provided by Dr. R. Liem (Columbia University, New York). In some microinjection experiments, we stained the injected Glu or Tyr tubulin antibodies directly with the appropriate fluorescently la-

beled secondary antibody and simultaneously stained the endogenous Glu or Tyr MTs by indirect immunofluorescence using an anti-Glu tubulin or anti-Tyr tubulin antibody produced in a second species. Secondary antibodies were fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Durham, NC), amino-methyl-coumarin-conjugated donkey anti-rat IgG (minimum cross reaction with mouse and rabbit IgGs) and tetramethyl rhodamine-conjugated goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA). For some experiments, the secondary antibody to mouse IgM was a coumarin conjugate and the secondary antibody to rat IgG was a rhodamine conjugate; these gave similar results as the other combination of secondary antibodies. The secondary antibodies exhibited no cross reaction with the inappropriate primary antibodies as judged by fluorescence microscopy.

Fluorescence microscopy was performed on a Nikon Optiphot microscope using a narrow excitation band fluorescein cube (DM510, B1E; Nikon), a rhodamine cube (DM590, 565DR-P, Omega Optical) and a coumarin cube (DM400, UV2A; Nikon) with a 420–490 nm band pass barrier filter substituted for the one supplied by Nikon to avoid overlapping fluorescein fluorescence. Photographs were taken using sensitized Kodak 2415 film as described previously (Gurland and Gundersen, 1993).

Preparation of Antibodies for Microinjection

We used five different antibodies in our microinjection experiments: (a) IFA, a mouse monoclonal (IgG1 class) against IFs (Pruss et al., 1981) (hybridoma cells were from the American Type Culture Collection, Rockville, MD); (b) SG, a rabbit polyclonal antibody against a COOH-terminal peptide corresponding to Glu tubulin (Gundersen et al., 1984); (c) 4B8, a mouse monoclonal antibody (IgG1 class) against the same COOH-terminal peptide corresponding to Glu tubulin (see Gundersen et al., 1984); 4B8 was produced and characterized by M. Y. Chang and J. C. Bulinski and was the gift of Dr. Bulinski (Columbia University, New York); the specificity of 4B8 is shown in the Results; (d) W², a rabbit polyclonal antibody against a COOH-terminal peptide corresponding to Tyr tubulin (Gundersen et al., 1984); and (e) YL1/2, a rat monoclonal antibody against Tyr tubulin (Kilmartin et al., 1982) (affinity purified YL1/2 was the generous gift of Dr. J. Kilmartin, Medical Research Council). An IgG fraction of each antibody was prepared on protein A-agarose (Sigma Chemical Co.) using TBS, pH 8.0, as the loading buffer. Bound IgGs were eluted with 0.2 M glycine, pH 2.8, and fractions containing IgG were immediately adjusted to pH 7.0 with 2 M Tris, pH 9.6, and dialyzed against TBS, pH 8.0. The IgG fractions of antibodies to Glu tubulin (SG and 4B8) were affinity purified on a column of Glu-tubulin-Sepharose (prepared as described below). IgG fractions were applied to the Glu-tubulin-Sepharose column in TBS, pH 8.0, and the flow-through fraction was saved as a control IgG for injection experiments. Bound antibodies were eluted with 0.5 M acetic acid and were immediately neutralized with 2 M Tris-base and dialyzed against injection buffer (10 mM Hepes, pH 7.4; 140 mM KCl). The IgG fractions of antibodies to Tyr tubulin (W²) were affinity purified on a column of brain tubulin-Sepharose (a mixture of Glu and Tyr tubulin; see Gundersen et al., 1987a) as described above for the Glu tubulin antibodies. All purified antibodies were concentrated by vacuum dialysis, dialyzed exhaustively against injection buffer, and stored as aliquots at –80°C. Protein concentrations were determined using the BCA protein assay using BSA as a standard.

Preparation of Tubulin-Sepharose Columns

Tubulin was prepared from fresh calf brains by two or three cycles of assembly-disassembly and was purified by DEAE-Sephadex chromatography as described by Murphy (1982). Pure Glu tubulin was prepared by incubating microtubule protein with pancreatic carboxypeptidase A (10 µg/ml; Sigma Chemical Co.) as described previously (Gundersen et al., 1987a). Glu tubulin (12 mg) was dialyzed against coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.6 M NaCl, 0.1 mM GTP), and then coupled to 3 ml CNBr-activated Sepharose (Sigma Chemical Co.) by overnight end-over-end mixing at 4°C. Remaining active sites on the Sepharose were blocked by overnight incubation with 1 M ethanolamine. The Glu tubulin-Sepharose column was then washed successively in coupling buffer and 0.1 M NaOAc, pH 4.0, 0.6 M NaCl. The column was stored at 4°C in 100 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 0.1 mM GTP, pH 6.9, and 0.1% NaN₃. The column for the purification of Tyr tubulin antibodies was prepared in the same way; however the tubulin used on the affinity column was not carboxypeptidase A treated.

Microinjection

Confluent 3T3 cell monolayers were wounded and incubated in medium for 3 h to insure that the proportion of cells with oriented Glu MTs was maximal. Cells were then pressure microinjected with antibodies using back-loaded glass capillaries and a Narishige micromanipulator (Narishige, Greenvale, NY), as previously described (Mikhailov and Gundersen, 1995). We estimate that approximately 10% of the cell volume was routinely introduced into injected cells. As controls, non-specific mouse IgG (1 mg/ml; Jackson ImmunoResearch Laboratories, Avondale, PA), rabbit IgG (8 mg/ml; Sigma Chemical Co.) or the flow-through IgG fraction of SG IgG from the Glu tubulin-Sepharose column (10 mg/ml) (see below) were injected as described above. Before and after injection, cells were maintained at 37°C in a humidified CO₂ environment until they were fixed in –20°C methanol. In some experiments, to detect clearly the structures labeled by the injected antibodies, cells were detergent extracted by rapidly rinsing twice in 37°C PEM (100 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl₂), permeabilizing in PEM containing 200 µg/ml saponin (Sigma Chemical Co.) for 1 min, and then rinsing twice in PEM prior to fixation in –20°C methanol. This extraction procedure removed the majority of unbound antibody from the cells without disrupting the MTs. Injected cells were detected by first staining the coverslips with fluorescently conjugated secondary antibodies reactive with the injected primary antibody. Depending on the experiment, cells were then stained with other primary and secondary antibodies as described above.

Quantification of Vimentin IF Collapse in Injected Cells

After immunofluorescent staining, injected cells were located by fluorescence microscopy (using a 60 × 1.4 NA oil Planapo objective; Nikon). Images of the injected cells were captured with a SIT camera (model 65; Dage MTI, Wabash, MI) and stored as digitized images using an Image-1 work station (Universal Imaging Corp., West Chester, PA). Quantification of the extent of IF collapse in injected cells was performed using the “measure with calipers” function of the Image-1 program. Using the fluorescent image of the IFs, we measured the distance from the nucleus to the most distal IFs extended toward the leading edge of the cell and, using a phase image, we measured the distance from the nucleus to the cell periphery. From these values, the ratio of IF extension to the extension of the lamella was calculated and converted to percent extension of IFs from the nucleus to the leading edge of the cell. At least 92 cells were measured for each different antibody we microinjected.

Western Blotting

Whole cell extracts and brain tubulin samples for SDS-PAGE and Western blotting were prepared as described previously (Gundersen et al., 1994). Samples were then subjected to SDS-PAGE on 7.5% polyacrylamide gels, transferred to nitrocellulose sheets, and blocked as described in Gundersen et al. (1994). Blots were then reacted with either affinity purified 4B8 IgG (10 µg/ml) or affinity purified SG IgG (25 ng/ml). Alkaline phosphatase-conjugated secondary antibodies against mouse (1:7,500) or rabbit (1:12,000) (Promega Biotec, Madison, WI) were used to detect Glu tubulin antibody reactivity. The blots were developed with nitro-blue-tetrazoleum and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Glu MTs and Vimentin IFs Are Colocalized in Polarized 3T3 Fibroblasts at the Edge of an In Vitro Wound

In examining the relationship of Tyr and Glu MTs with other organelles in the leading edge of polarized fibroblasts migrating into an in vitro wound, we found a striking colocalization between Glu MTs and vimentin IFs. Fig. 1 shows representative distributions of vimentin IFs, Glu MTs and Tyr MTs in cells at the edge of a wound as revealed by triple-indirect immunofluorescence. As described previously (Gundersen and Bulinski, 1988; Nagasaki et al., 1992; Gundersen et al., 1994), Glu MTs in such cells are largely confined to the lamella, i.e., the area between the

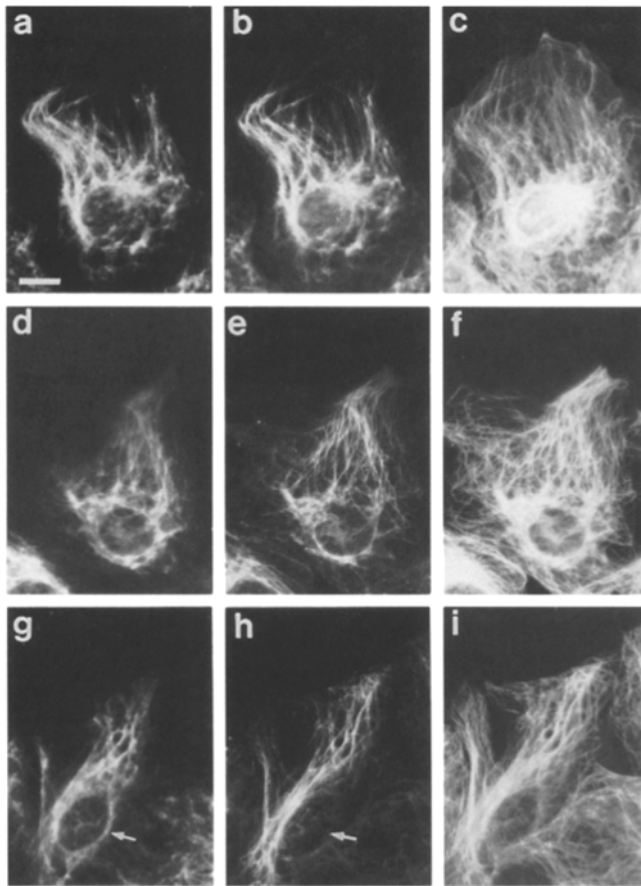


Figure 1. Vimentin IFs are colocalized with Glu MTs in the lamella of 3T3 cells at the edge of an in vitro wound. Wounded monolayers of 3T3 cells were fixed and triple immunostained for the presence of vimentin IFs (a, d, and g), Glu MTs (b, e, and h) and Tyr MTs (c, f, and i). Note that IFs generally extend outward from a juxtannuclear position toward the periphery of the leading lamella in wound edge cells and are preferentially localized with Glu MTs rather than Tyr MTs. Arrows in g and h indicate a region of the cell lacking Glu MTs but containing perinuclear IFs. Bar, 10 μ m.

nucleus and the leading edge of the cell (Fig. 1, b, e, and h), whereas Tyr MTs are more generally distributed throughout the cytoplasm (Fig. 1, c, f, and i). By comparison, the vimentin distribution (Fig. 1, a, d, and g) closely paralleled the distribution of Glu MTs, rather than that of Tyr MTs. The similarity in the distributions of Glu MTs and vimentin IFs was most striking in cells which exhibited an asymmetric distribution of Glu MTs; for example, in the cell shown in Fig. 1 (a–c), the IF and Glu MT distributions are strongly biased to the left side of the lamella, whereas the Tyr MTs are distributed throughout the lamella. A second example of the asymmetric distribution of Glu MTs and IFs is shown in Fig. 1 (d–f). Although there was strong coalignment of vimentin IFs with Glu MTs in the lamella, there was not perfect colocalization throughout the cell. In some cells the IFs did not extend toward the leading edge as far as the Glu MTs (e.g., see Fig. 1, g and h). Also, IFs were frequently found around the nucleus where there were no Glu MTs (e.g., see arrows in Fig. 1, g and h). The juxtannuclear IFs were also observed

in early post-mitotic cells, which have no Glu MTs (not shown) (Gundersen and Bulinski, 1986).

At higher magnification, the codistribution of IFs and Glu MTs can be seen at the level of individual filamentous elements (Fig. 2, a and b, arrows). While individual IFs in the lamella were almost always colocalized with Glu MTs, we observed frequent cases in which Glu MTs were present in the lamella without a colocalized IF (Fig. 2, a and b, arrowhead). At high magnification, it is also clear that IFs are absent in regions of the cell lacking detectable Glu MTs but containing numerous Tyr MTs (Fig. 2 c, asterisk).

Injection of Monoclonal Anti-IF Antibodies Results in the Collapse of Vimentin But Not Glu MTs in 3T3 Cells

The factors responsible for the stability of Glu MTs are not known (see Introduction). In the present study, we observed some Glu MTs that did not colocalize with IFs, suggesting that Glu MTs were not stabilized by interactions with IFs. Nonetheless, we wanted to confirm this experimentally. Accordingly, we microinjected monoclonal anti-IFA antibody (1 mg/ml IgG) into 3T3 cells at the edge of a wounded monolayer. Previously, this has been shown to result in the redistribution of vimentin IFs from an extended array, filling the cytoplasm, to a perinuclear or “collapsed” location (Klymkowsky, 1981). 2 to 6 h after injection, we fixed the cells and determined the distribution of the injected IFA IgG, Glu MTs, and Tyr MTs by triple indirect immunofluorescence. As expected, vimentin IFs collapsed to a juxtannuclear location within 4 h after injection of anti-IFA IgG (Fig. 3 a). In the injected cells with collapsed IFs, Glu MTs were clearly present and extended virtually the entire distance to the leading edge of the cells (Fig. 3 b). We did not detect a significant difference in either the distribution or the number of Glu MTs in the injected cells. In 70 cells in which the IFs were induced to collapse, we observed fewer than 5% which lacked extended Glu MTs; this number reflects the percentage of cells without extended Glu MTs in uninjected controls. Tyr MTs also appeared to be unaffected by the injection of antibody (Fig. 3 c), consistent with earlier anti-IF antibody microinjection studies (Klymkowsky, 1981; Gawlitta et al., 1981). We have obtained similar results in NRK fibroblasts injected with the anti-IFA antibody (not shown). These results directly show that extended vimentin IFs are not required for the maintenance of an array of stable Glu MTs in vivo.

Selective Localization of Extended Vimentin IFs with Newly Formed Glu MTs After Release from Nocodazole Treatment

The above result indicates that IFs are not stabilizing Glu MTs. The colocalization of IFs with Glu MTs in the lamella, but not necessarily near the nucleus (see Fig. 1), suggests that Glu MTs may be important for maintaining an extended array of IFs in the lamella. To test this possibility, we determined whether IFs, induced to collapse to a perinuclear position by nocodazole-induced breakdown of MTs, would reextend into the lamella rapidly or with a lag after the nocodazole was washed out. Since Glu MTs are formed by the postpolymerization detyrosination of Tyr MTs, their reappearance following release from a nocodazole block to polymerization occurs some 30–60 min after

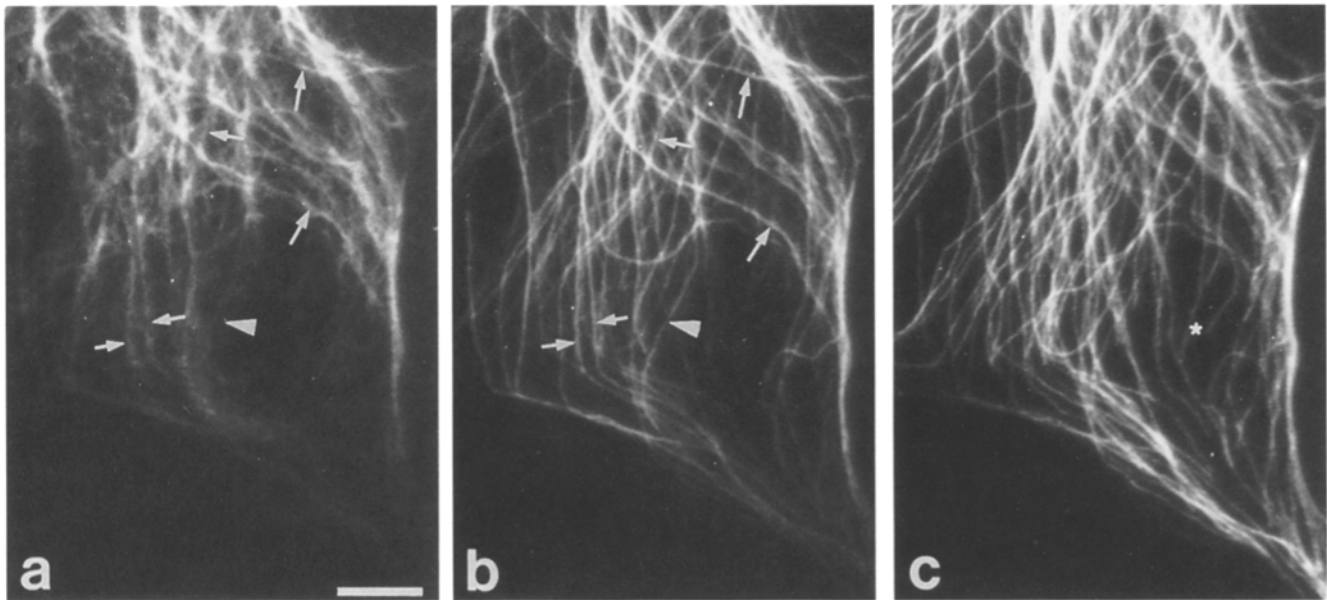


Figure 2. Vimentin IFs and Glu MTs are colocalized at the level of individual filaments in wound edge 3T3 cells. Higher magnification of IFs (*a*), Glu MTs (*b*), and Tyr MTs (*c*) in the leading lamella of a wound edge 3T3 cell. Arrows indicate some of the IFs (*a*) that are coaligned with Glu MTs (*b*). The *arrowhead* indicates a Glu MT (*b*) that is not coaligned with IFs in *a*. The *asterisk* in *c* indicates a region that contains numerous Tyr MTs but is devoid of IFs (*a*) and Glu MTs (*b*). Bar, 5 μm .

Tyr MTs are reformed (Gundersen et al., 1987*a*; Nagasaki et al., 1992). Thus, rapid reextension of IFs after nocodazole wash-out would suggest that IFs are not dependent on Glu MTs, whereas delayed reextension would be consistent with their dependence on Glu MTs. Cells incubated in nocodazole (10 μM for 5 h) contained no cytoplasmic MTs (Fig. 4, *b* and *c*) and exhibited collapsed IF bundles near the nucleus (Fig. 4 *a*), consistent with results of previous studies (see Introduction). The position of the nucleus and the leading edge of the cells can be seen in the Tyr immunofluorescence panel (Fig. 4 *c*) in which the diffuse staining of the monomeric tubulin outlines the cell periphery and the nucleus. Collapsed IFs were observed in all of

the cells at the wound edge. After release from nocodazole, Tyr MTs rapidly repolymerized, filling the cytoplasm completely within 15 min (Fig. 4 *f*) (see Gundersen et al., 1987*a*). The overall appearance of Tyr MTs did not change significantly at later time points (Fig. 4, *i* and *l*). Although some hazy Glu tubulin staining was observed as early as 15 min after nocodazole removal (Fig. 4 *e*), individual Glu MTs were not evident until ~ 30 min after nocodazole removal (not shown) and an oriented array of Glu MTs in the lamella was not formed until 1 h after release (Fig. 4 *h*). These results are similar to those obtained previously (Gundersen et al., 1987*a*; Nagasaki et al., 1992). No change in the IF organization was evident 15 min after removal of

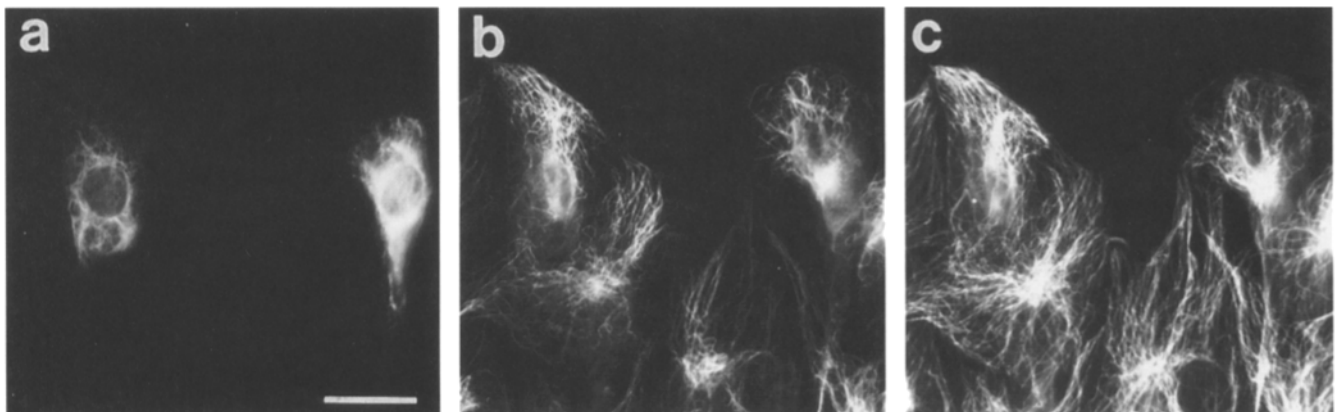


Figure 3. Collapse of vimentin IFs after injection of monoclonal anti-IF antisera does not affect Glu MT distribution. Selected 3T3 cells at the wound margin were injected with anti-IFA IgG (1 mg/ml) and incubated for 4 h prior to methanol fixation and processing for immunofluorescence staining. The injected cells were visualized by staining directly with a fluorescent secondary antibody that reacted with the injected anti-IFA IgG (*a*). The distribution of Glu MTs (*b*) and Tyr MTs (*c*) were visualized by indirect immunofluorescence. Bar, 20 μm .

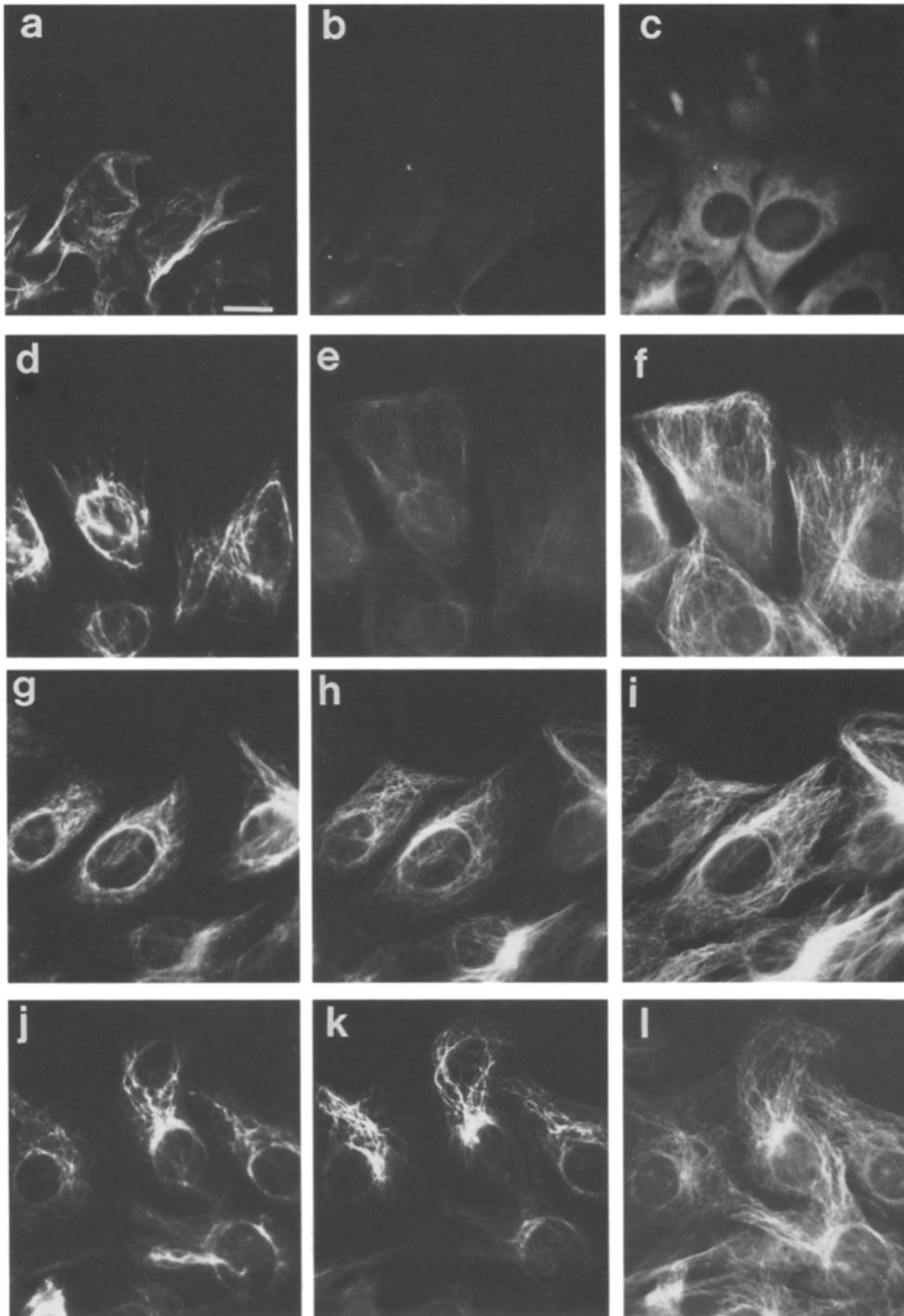


Figure 4. Redistribution of vimentin IFs with newly formed Glu MTs after release from prolonged nocodazole treatment. Wounded 3T3 monolayers (wound edge toward the top of the page) were incubated in 10 μ M nocodazole for 5 h, fixed, and triple-stained for vimentin IFs (a), Glu MTs (b), and Tyr MTs (c). After 5 h, nocodazole was removed and cells were incubated in fresh medium for 15 min (d-f), 1 h (g-i) or 3 h (j-l). Vimentin staining is shown in a, d, g, and j. Glu MTs are shown in b, e, h, and k and Tyr MTs are depicted in c, f, i, and l. Note that IF extension does not begin until Glu MTs have begun to reappear (g-i) and that complete reextension of the IFs does not occur until a complete array of Glu MTs is present (j-l). Bar, 10 μ m.

nocodazole (Fig. 4 d), a time when Tyr MTs were already fully reformed (Fig. 4 f). Vimentin reorganization was first evident after 1 h: at this time, the IF array could clearly be observed to extend greater than half of the distance to the leading edge of most cells (Fig. 3 g). Pinpointing the earliest time of IF reextension in these experiments was difficult since the collapsed array in the nocodazole-treated cells was not always tightly coiled around the nucleus. Nonetheless, it was clear that IFs did not significantly re-extend toward the periphery of the leading edge until brightly labeled Glu MTs had reformed 60 min after nocodazole removal (compare Fig. 4, d with g). By 3 h after

nocodazole removal, the IFs were, again, fully reextended and clearly colocalized with Glu MTs in the lamella (Fig. 4, j, k, and l).

Realignment of Vimentin IFs with Newly Formed Glu MTs after Readdition of Serum to Serum-starved Cells

As a second test of whether the distribution of IFs is influenced by the presence of stable, Glu MTs, we examined the distribution of vimentin IFs in serum-starved 3T3 cells following the induction of Glu MT formation by serum treatment. As described previously (Gundersen et al.,

1994), serum starvation of 3T3 cells resulted in the loss of stable, Glu MTs (Fig. 5 *b*) without substantially reducing the number of Tyr MTs (Fig. 5 *c*). Surprisingly, in serum-starved cells, the array of vimentin IFs extended into most areas of the cell (Fig. 5 *a*). This appears to be inconsistent with our previous results showing that the reextension of IFs after nocodazole release correlated with the reappearance of Glu MTs. However, we found that the IFs in serum-starved cells would not collapse after nocodazole depolymerization of all the MTs (data not shown). This suggests that previously extended IFs (when the cells were in serum) became independent of MTs for their localization upon serum-starvation and that the mechanism involved in collapsing IFs is not active under these conditions. An inhibition of the collapsing mechanism in serum-starved cells would be consistent with previous studies showing that the collapsing activity involves actin-dependent centripetal movement (Hollenbeck et al., 1989; Tint et al., 1991) and that serum-starvation reduces actin assembly in 3T3 cells (Ridley and Hall, 1992). Further evidence that the IFs were not dependent on MTs for their localization in serum-starved cells is shown by the lack of colocalization of the IFs and MTs; while some IFs appeared to codistribute with MTs, many did not (compare Fig. 5, *a* and *c*; arrows indicate IFs that are not colocalized with MTs).

To test whether the IFs in the serum-starved cells could become colocalized with Glu MTs, we incubated the quiescent cells in medium containing 10% calf serum. Calf serum has previously been shown to induce the rapid formation of oriented arrays of stable, Glu MTs (Gundersen et al., 1994). 1 h after cells were refed medium containing serum, IFs were aligned with the newly formed, Glu MTs (see arrows in Fig. 5, *d* and *e*). In fact, whereas before serum addition it was easy to find IFs that were not aligned with MTs, after serum addition it was difficult to find any that were not colocalized with Glu MTs.

Vimentin IFs Rapidly Realign with Taxol-stabilized MTs in Serum-starved 3T3 Cells

In addition to stimulating the formation of stable, Glu MTs, calf serum treatment undoubtedly induces many other processes in the serum-starved cells. To determine whether the induction of MT stability and the attendant increase in Glu tubulin was sufficient for the realignment of IFs with MTs, we treated serum-starved cells with taxol, a drug which rapidly induces MT stability and MT detyrosination in normal and serum-starved cells (Gundersen et al., 1987a; Gundersen et al., 1994). When we added 1 μ M taxol to serum-starved cells, we detected the formation of Glu MTs within 30 min and the complete colocalization of IFs with MTs within 30 min (Fig. 6, *a-c*). This colocalization should be compared with that in serum-starved cells, which exhibited only partial codistribution of IFs and MTs (see Fig. 5, *a-c*). In cells treated briefly with taxol, MTs appeared uniformly distributed and polarized arrays of Glu MTs were not observed in cells at the leading edge of the wound (Fig. 6 *b*). Consistent with the idea that IFs preferentially associate with stable, Glu MTs, vimentin IFs were observed to colocalize with MTs throughout the cytoplasm of taxol-treated cells and were not restricted to the lamella of wound-edge cells (Fig. 6, *a* and *b*).

Characterization of Affinity Purified Anti-Glu Tubulin Antibodies

In the experiments described above, we consistently observed the association of IFs with MTs after the MTs had been stabilized (either by cellular factors or by taxol) and posttranslationally detyrosinated. To test directly whether MT detyrosination is critical for the interaction of IFs with MTs, we developed a strategy to block interactions of Glu MTs with IFs by microinjecting affinity purified antibodies specifically reactive with Glu tubulin into cells. As controls, we microinjected affinity purified antibodies against Tyr tubulin or preparations of non-specific IgGs. With the exception of the monoclonal antibody to Glu tubulin, all of the antisera we used in these experiments have been well characterized previously. The specificity of the anti-Glu tubulin mouse monoclonal 4B8 antibody was determined by Western blot analysis and by immunofluorescence microscopy. The affinity purified 4B8 monoclonal antibody specifically reacted with α -tubulin in a preparation of purified brain tubulin (Fig. 7, lane 1) and a single band corresponding to α -tubulin in whole cell extracts prepared from TC-7 cells (Fig. 7, lane 2). 4B8 reacted strongly with α -tubulin and weakly with a number of other proteins in whole cell extracts from 3T3 cells (Fig. 7, lanes 3 and 4). We do not know whether these minor bands represent significant cross-reactivity of the 4B8 antibody, or are just background due to high protein loading necessary to detect Glu tubulin in the 3T3 cell extract. After microinjection into cells, 4B8 labeled only Glu MTs (see below), suggesting that the weak reactivity of 4B8 with other bands on western blots was not a complicating factor in our study (see below). Additionally, the polyclonal anti-Glu tubulin antibody (SG) did not react with these bands on western blots (Fig. 7, lane 5-8), yet gave similar results as 4B8 antibody when injected into cells (see below), arguing again that the minor reactivity of 4B8 antibody with other proteins was not a significant problem in our study. With both antibodies, the α -tubulin band detected in cell extracts increased after incubation with pancreatic carboxypeptidase A (not shown), consistent with antibody specificity directed toward Glu tubulin. The flow-through IgG fraction from the affinity purification of SG IgG reacted only weakly with brain tubulin when used at 100-fold higher concentrations (not shown). Both the YL1/2 monoclonal antibody and the W² polyclonal antibody have been characterized in detail previously (Wheland and Willingham, 1983; Gundersen et al., 1984) and were shown to react specifically with Tyr tubulin.

To test the specificity of the affinity purified anti-Glu tubulin IgG *in vivo*, we microinjected 4B8 IgG and SG IgG into TC-7 monkey kidney epithelial cells. The stable, Glu MTs in TC-7 cells have been well characterized and appear as a distinct subset of sinuous MTs (Gundersen et al., 1984, 1987a; Webster et al., 1987). Fig. 8 *a* shows the staining of the injected antibody in a cell injected with 4B8 IgG (2 mg/ml). Only MTs were labeled by the injected antibody and the labeled MTs exhibited the sinuous morphology characteristic of Glu MTs (Fig. 8 *a*). A comparison with the endogenous Tyr MTs (Fig. 8 *c*), detected by indirect immunofluorescence using YL1/2 antibody, clearly illustrates that the injected 4B8 IgG labeled only a subset of

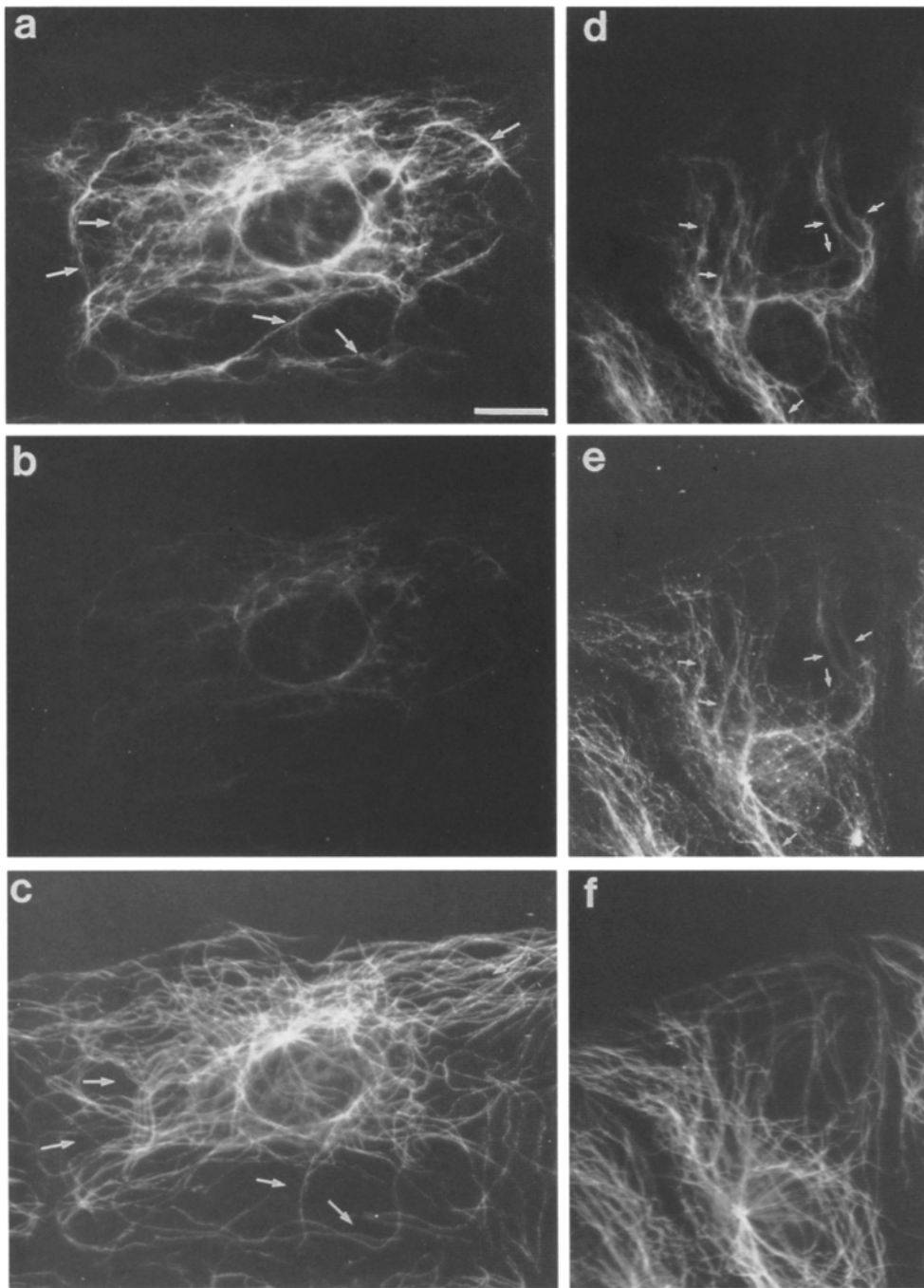


Figure 5. Redistribution of vimentin with newly formed stable, Glu MTs after addition of serum to serum-starved cells. Confluent 3T3 cells were serum-starved for 48 h as described in Materials and Methods. Serum-starved cells were wounded, fixed and triple stained for vimentin IFs (*a* and *d*), Glu MTs (*b* and *e*) or Tyr MTs (*c* and *f*). *a*–*c* are serum-starved cells; arrows indicate IFs (*a*) that are not colocalized with Tyr MTs (*c*). *d*–*f* are serum-starved cells 1 h after addition of 10% calf serum; arrows indicate IFs (*d*) that are colocalized with Glu MTs (*e*). Bar, 10 μ m.

the MTs. Additionally, by comparing the MTs labeled by the injected antibody with the endogenous Glu MTs (detected with a second anti-Glu tubulin antibody raised in a different species [rabbit], Fig. 8 *b*), it is clear that the injected antibody labeled all of the endogenous Glu MTs. We did not observe significant differences in the level or distribution of Glu MTs in cells injected with Glu antibodies (compare the injected cell in Fig. 8 *a* with the surrounding uninjected cells in Fig. 8 *b*). Similar results were observed in TC-7 cells injected with the affinity purified SG IgG (not shown).

As with the TC-7 cells, when 3T3 cells were injected with affinity purified SG or 4B8 IgG, a subset of the MTs corresponding to those that stain with the Glu antibody by

indirect immunofluorescence were labeled by the injected antibody and no dramatic changes in the level of Glu MTs were detected in the injected cells (see below). Additionally, staining of Tyr MTs confirmed that the injected SG antibody labeled only a subset of the total MTs (e.g., see Fig. 9, *e* and *f*). This was also true in 3T3 cells injected with the 4B8 IgG (not shown).

Injected Polyclonal Anti-Glu Tubulin Antibody But Not Anti-Tyr Tubulin Antibody Results in Collapse of Vimentin IFs

Since the injected antibodies faithfully bound to Glu MTs in vivo, we were able to test directly the relationship be-

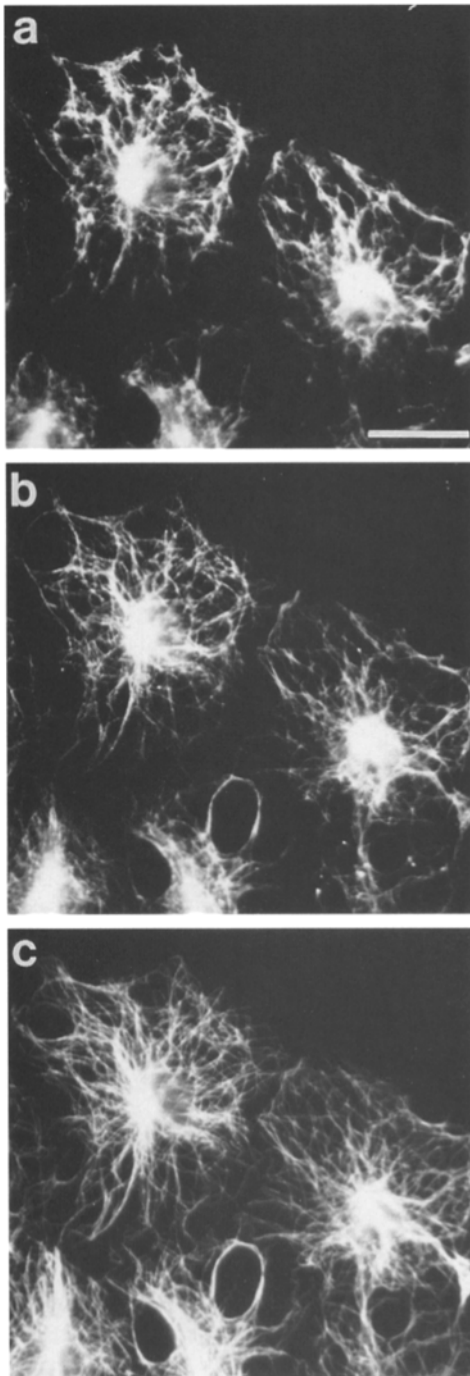


Figure 6. Vimentin IFs rapidly realign with taxol-stabilized MTs in serum-starved 3T3 cells. 3T3 cells were serum starved for 48 h (see Materials and Methods), wounded, and then treated with 1 μ M taxol for 30 min prior to methanol fixation and triple immunofluorescence staining. *a* shows vimentin IF staining; *b* shows Glu MT staining; and *c* shows Tyr MT staining. Bar, 20 μ m.

tween Glu MTs and IFs in vivo. We microinjected wounded 3T3 cells with various concentrations of affinity purified SG IgG or W² IgG, the Tyr tubulin specific rabbit polyclonal antibody, and, after a suitable time, determined the effect of the injected antibody on the distribution of IFs. Microinjection of 10 mg/ml affinity purified W² IgG labeled most of the cytoplasmic MTs (compare Figs. 9, *b*

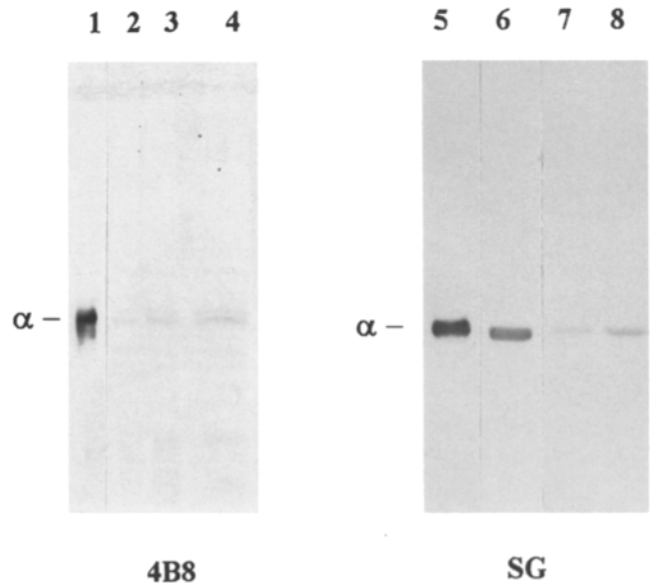


Figure 7. Western blot analysis of affinity purified anti-Glu tubulin antibodies. The specificity of the affinity purified IgGs was determined by Western blot analysis. DEAE-purified tubulin from calf brain (1 μ g, lanes 1 and 5) and whole cell extracts from TC-7 cells (lane 2, 25 μ g; lane 6, 10 μ g), and 3T3 cells (lane 3, 50 μ g; lane 4, 100 μ g; lane 7, 5 μ g; lane 8, 25 μ g) were electrophoresed on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose. Following transfer and blocking, the membranes were incubated with 10 μ g/ml affinity purified 4B8 IgG (lanes 1–4) or 25 ng/ml affinity purified SG IgG (lanes 5–8) followed by alkaline phosphatase conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. Antibodies were detected using BCIP and NBT as substrates. α marks the position of brain α -tubulin detected on Amido black-stained blots.

and *c*) but did not cause collapse of the IF network after a 2-h incubation (Fig. 9 *a*). Additional examples of W² IgG injected cells are shown in Fig. 9, *g* and *h* and 9, *i* and *j*. Qualitatively, vimentin IFs in the W² IgG injected cells appeared to be indistinguishable from those in the neighboring uninjected cells, which extended outward from the nucleus toward the leading edge of the cell.

In contrast, in cells injected with 2 mg/ml SG IgG and incubated for 2 h, the vimentin IF network collapsed inward from the cell periphery to a perinuclear location (Fig. 9 *d*, compare with uninjected cell in the same image). In this example, the injected SG IgG clearly labeled a subset of the total MTs (compare Fig. 9 *e*, which shows the injected antibody, with Fig. 9 *f*, which shows the distribution of Tyr MTs revealed by indirect immunofluorescence). Another example of collapsed IFs in a cell injected with 2 mg/ml SG IgG is shown in Fig. 9 (*k* and *l*). At higher concentrations of injected SG IgG (10 mg/ml), we observed a similar collapse of the IF network (Fig. 9 *m*) and a more extensive labeling of Glu MTs (Fig. 9 *n*). Quantification of the extent of IF collapse indicated that the collapse was more extensive in cells injected with higher concentrations of Glu tubulin antibodies (see below). As controls, we injected cells with either nonspecific rabbit IgG (8 mg/ml) or the flow-through IgG fraction obtained during the affinity purification of SG IgG (10 mg/ml). Neither of these IgGs induced collapse of the IF network (not shown).

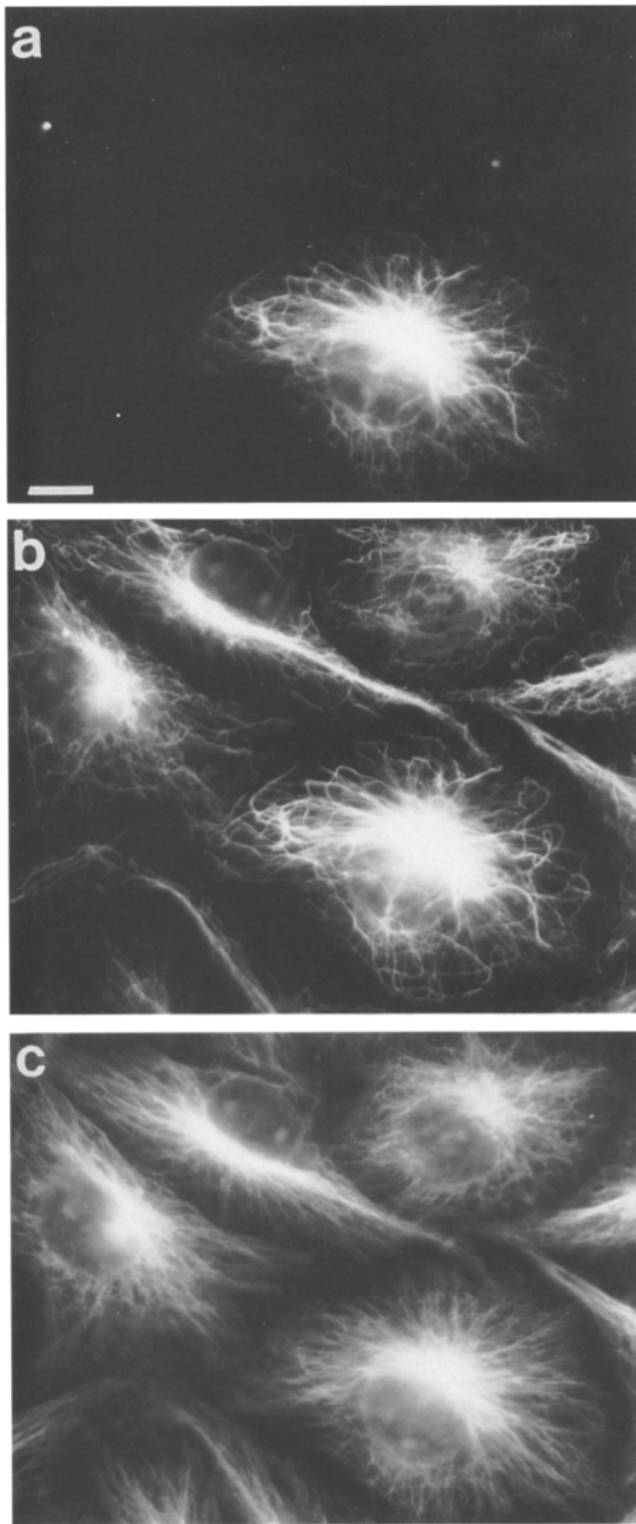


Figure 8. Microinjection of 4B8 IgG into TC-7 cells labels a subset of MTs that correspond to the endogenous Glu MTs. TC-7 cells in a subconfluent monolayer were injected with 2 mg/ml affinity purified 4B8 IgG. 2 h after the injection, the cells were extracted, fixed, and then immunofluorescently stained as described in the Materials and Methods. *a* shows the injected mouse 4B8 IgG detected directly with secondary antibody. *b* shows the MTs labeled by rabbit SG serum by indirect immunofluorescence. *c* shows the MTs labeled by the rat YL1/2 anti-Tyr tubulin antibodies by indirect immunofluorescence. Note that the injected 4B8 IgG labels a subset of the total MT population and this subset corresponds to the Glu MTs. Bar, 10 μ m.

Injected Monoclonal Anti-Glu Tubulin Antibody But Not Monoclonal Anti-Tyr Tubulin Antibody Results in Collapse of Vimentin IFs

As we observed with the polyclonal Glu tubulin antibody, injection of monoclonal 4B8 IgG (10 mg/ml) resulted in extensive collapse of vimentin IFs to a juxtannuclear position within 2 h after injection (Fig. 10 *b*). The injected 4B8 IgG is shown in Fig. 10 *a*. Injection of 4B8 IgG at 2 mg/ml also induced collapse of the IF network (not shown). A previous study reported that microinjected monoclonal anti-Tyr tubulin antibody, YL1/2, did not induce extensive collapse of IFs (Wheland and Willingham, 1983). We observed similar results. At low concentrations (2 mg/ml IgG), injection of YL1/2 antibody labeled many of the cytoplasmic MTs but did not cause the collapse of the IF network (not shown). When YL1/2 antibody was injected at higher concentrations (8 mg/ml) and the cells were incubated for 2 hr, we observed the formation of very thick MT bundles similar to those reported previously (Wheland and Willingham, 1983) but still did not observe collapse of the IF network to a juxtannuclear location (Fig. 10 *d*); the injected cell is shown by direct, secondary antibody labeling of the MTs in Fig. 10 *c*. Thus, the monoclonal antibodies to Glu and Tyr tubulin showed a similar differential capability to induce collapse of the IF network as that exhibited by the polyclonal antibodies.

We have tested the ability of the Glu and Tyr tubulin antibodies to collapse IFs in a second cell line, rat A-10 smooth muscle cells. Microinjection of both the monoclonal and the polyclonal anti-Glu tubulin antibodies into nonconfluent A-10 cells, which contain subsets of Tyr and Glu MTs and have extended vimentin IFs, also resulted in collapse of the IFs to a perinuclear region within 2 h (not shown). Injection of anti-Tyr tubulin antibodies had no effect on the distribution of the IFs in these cells (not shown). Thus, the differential capability of antibodies to Glu and Tyr tubulin to collapse IFs was not restricted to migrating 3T3 fibroblasts.

Quantification of the Degree of Vimentin IF Collapse after Injection of Anti-tubulin Antibodies

To quantify the degree of IF collapse in cells injected with the Glu and Tyr tubulin specific antibodies, we measured the distance that the IFs extended into the lamella compared with the total distance from the nucleus to the leading edge of the cell (see Materials and Methods). The extent of IF extension is represented by the ratio of these two measurements and is expressed as percent extension. The total distance from the nucleus to the leading edge of the cell was measured in over 200 cells and did not change significantly in the injected cells (not shown). Fig. 11 shows frequency plots of the percent extension of IFs after each treatment. In uninjected cells, the mean extension of IFs was $82 \pm 15\%$. In cells injected with 1.5 mg/ml SG IgG, the mean extension was only $55 \pm 20\%$. In cells injected with higher concentrations of SG IgG (10 mg/ml), we observed a more extensive collapse of the IF network to a perinuclear region; the mean extension of IFs was $31 \pm 19\%$. The Glu monoclonal, 4B8, injected at 10 mg/ml IgG was also effective in completely collapsing the IFs; the

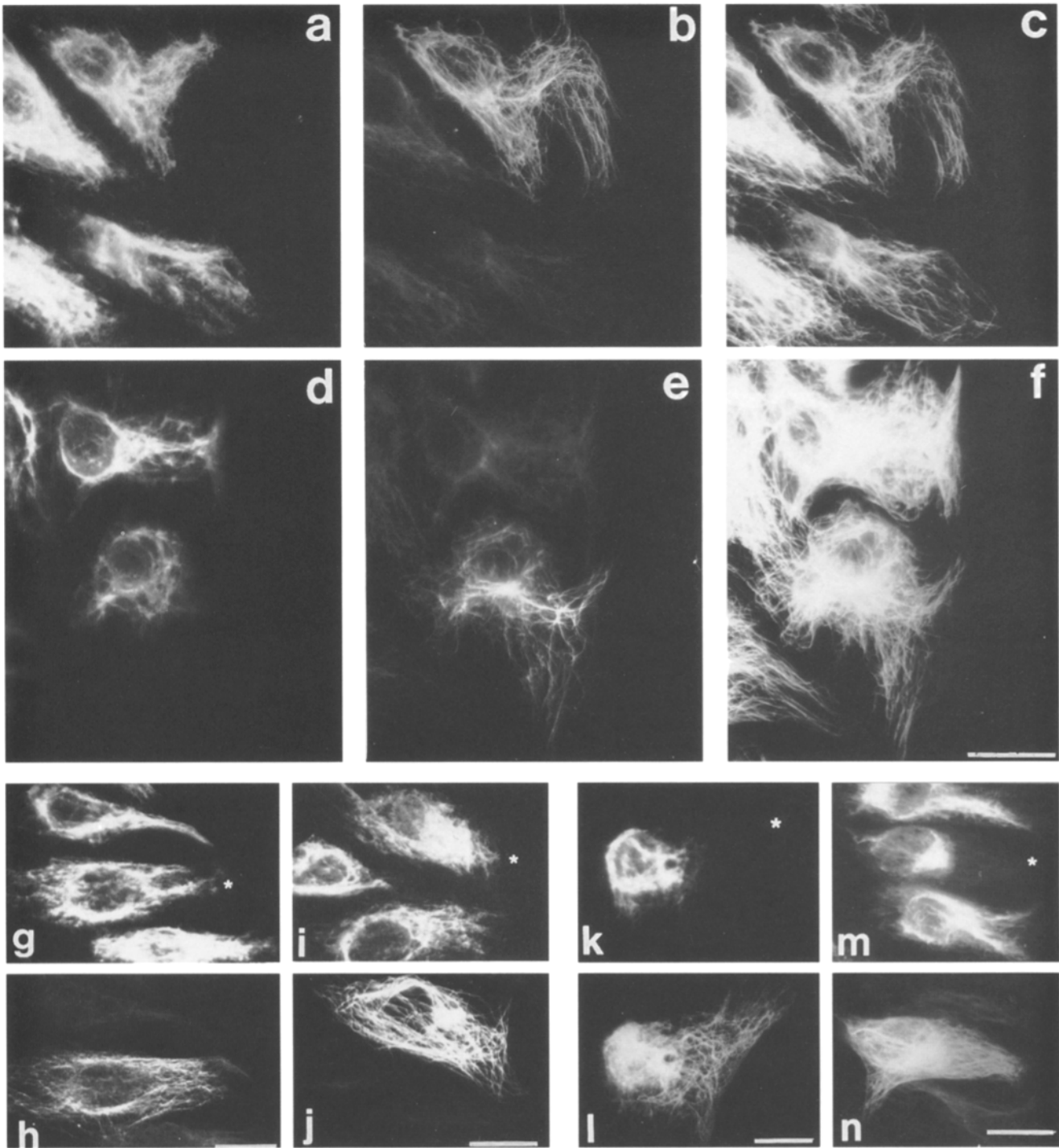


Figure 9. Injection of polyclonal anti-Glu tubulin but not anti-Tyr tubulin IgG causes collapse of vimentin IFs in 3T3 cells. 3T3 cells at the edge of an in vitro wound were injected with 10 mg/ml affinity purified W² IgG (*a-c*, and *g-j*) or with 2 mg/ml (*d-f*, *k*, and *l*) or 10 mg/ml (*m* and *n*) affinity purified SG IgG. Two h after injection, cells were extracted, fixed, and immunostained as described in Materials and Methods. Injected IgG was detected directly with secondary antibodies against rabbit IgG (W², *b*, *h*, and *j*; SG, *e*, *l*, and *n*). Tyr MTs were detected by indirect immunofluorescence with rat YL1/2 antibody (*c* and *f*). Vimentin IFs were detected by indirect immunofluorescence with mouse 56B5 culture supernatant (*a*, *d*, *g*, *i*, *k*, and *m*). Asterisks *g*, *i*, *k*, and *m* indicate the periphery of the injected cells. Bars: (*a-f*) 20 μ m; (*g-n*) 10 μ m.

mean extension of IFs after 4B8 IgG injection was $23 \pm 17\%$. For comparison, we measured the extension of IFs in cells treated with nocodazole to completely break down all the MTs; in these cells the IFs extended only $41 \pm 44\%$ of

the distance to the leading edge. Thus, injection of anti-Glu tubulin antibodies was as effective in collapsing the IFs as breaking down all the MTs. The differences in extension of the IFs in uninjected controls and in cells in-

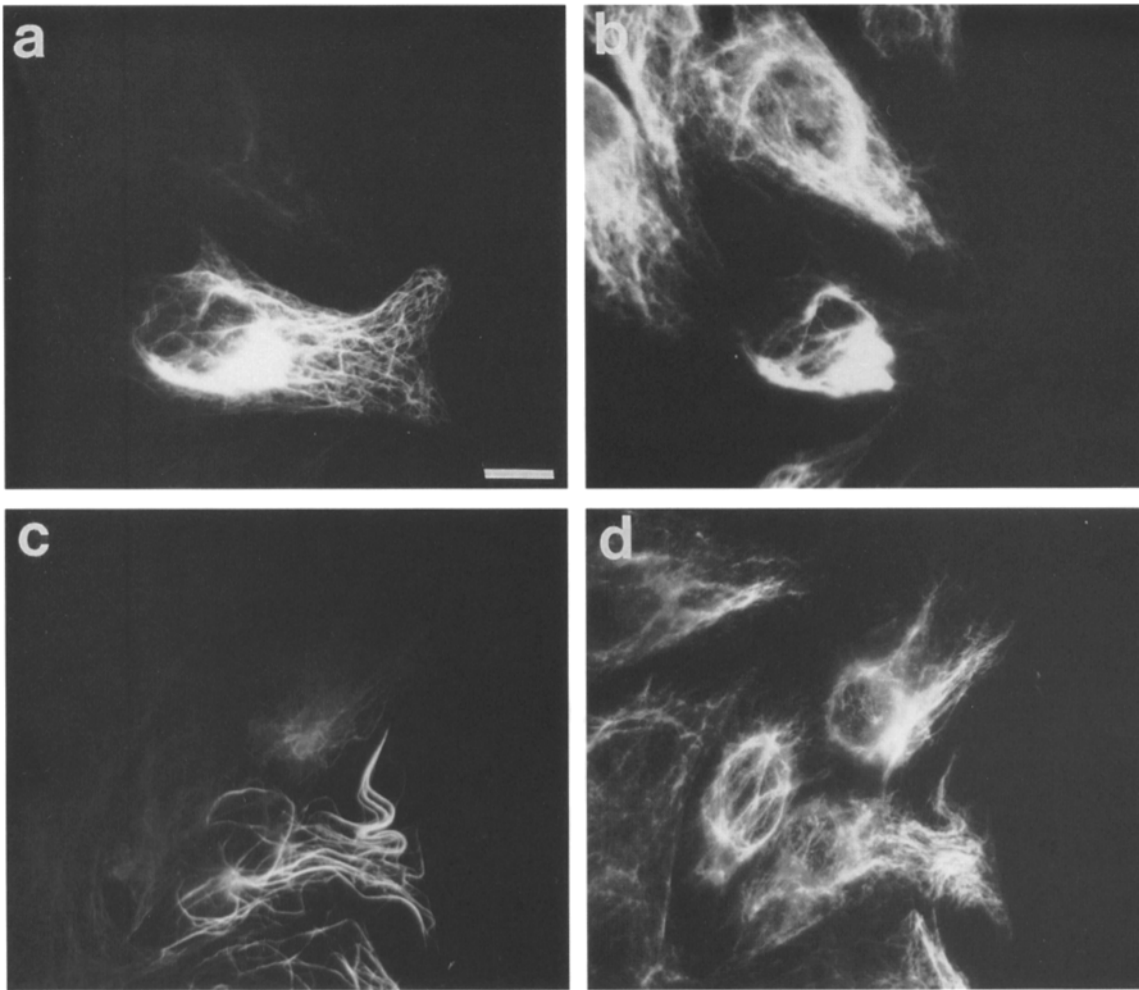


Figure 10. Injection of monoclonal anti-Glu tubulin but not monoclonal anti-Tyr tubulin IgG causes collapse of vimentin IFs in 3T3 cells. 3T3 cells at the edge of an *in vitro* wound were injected with 10 mg/ml 4B8 IgG (*a* and *b*) or 8 mg/ml YL1/2 IgG (*c* and *d*). 2 h after injection, cells were extracted, fixed, and immunostained as described in Materials and Methods. Injected mouse 4B8 IgG detected directly with secondary antibody (*a*). Injected rat YL1/2 IgG detected directly with secondary antibody (*c*). Vimentin IFs detected by indirect immunofluorescence with mouse 56B5 culture supernatant (*b* and *d*). Bar, 10 μ m.

nected with the anti-Glu tubulin antibodies or treated with nocodazole were statistically significant ($P < 0.001$).

Quantification of the IF extension in cells injected with either the polyclonal or the monoclonal Tyr tubulin specific IgGs showed that these antibodies did not significantly collapse the IFs relative to controls (Fig. 11). In cells injected with W² IgG (10 mg/ml), IFs extended $76 \pm 24\%$ of the distance to the leading edge of the cell. After YL1/2 injection (8 mg/ml), the IF array extended, on average, 63% of the distance between the nucleus and the cell periphery. The slight retraction of the IFs in YL1/2 antibody injected cells can be attributed to the formation of extensive MT bundles, which were somewhat retracted from the leading edge of the cell, themselves. When we corrected for the retraction of the MTs within these cells by using the distance from the nucleus to the most peripherally observed MTs as a reference for our measurements (rather than the cell edge), we found that the mean IF extension was $81 \pm 16\%$. This is similar to the 82% extension of the IFs that we measured in non-injected cells in which the MTs were completely extended to the cell periphery.

The differences in the extension of the IFs in uninjected controls and cells injected with either W² or YL1/2 anti-Tyr tubulin antibodies were not statistically significant ($P = 0.042$).

As additional controls, we injected cells with nonspecific rabbit and mouse IgGs and the flow-through IgG fraction from the Glu-tubulin column used to affinity purify SG IgG. Microinjection of the flow-through IgG fraction from the affinity purification of SG antibody did not result in the collapse or other detectable alterations in the IF network; the IFs extended an average of $83 \pm 17\%$ of the distance from the nucleus to the leading edge of the cell (Fig. 11). Injection of nonspecific mouse IgG or nonspecific rabbit IgG also did not cause the collapse of the IFs in these cells (not shown).

Discussion

In this study we have shown that there is a relationship between stable, Glu MTs and vimentin IFs in the lamella of wound-edge 3T3 fibroblasts. In our early experiments, we

Effect of Microinjected Tubulin Antibodies on IF Distribution

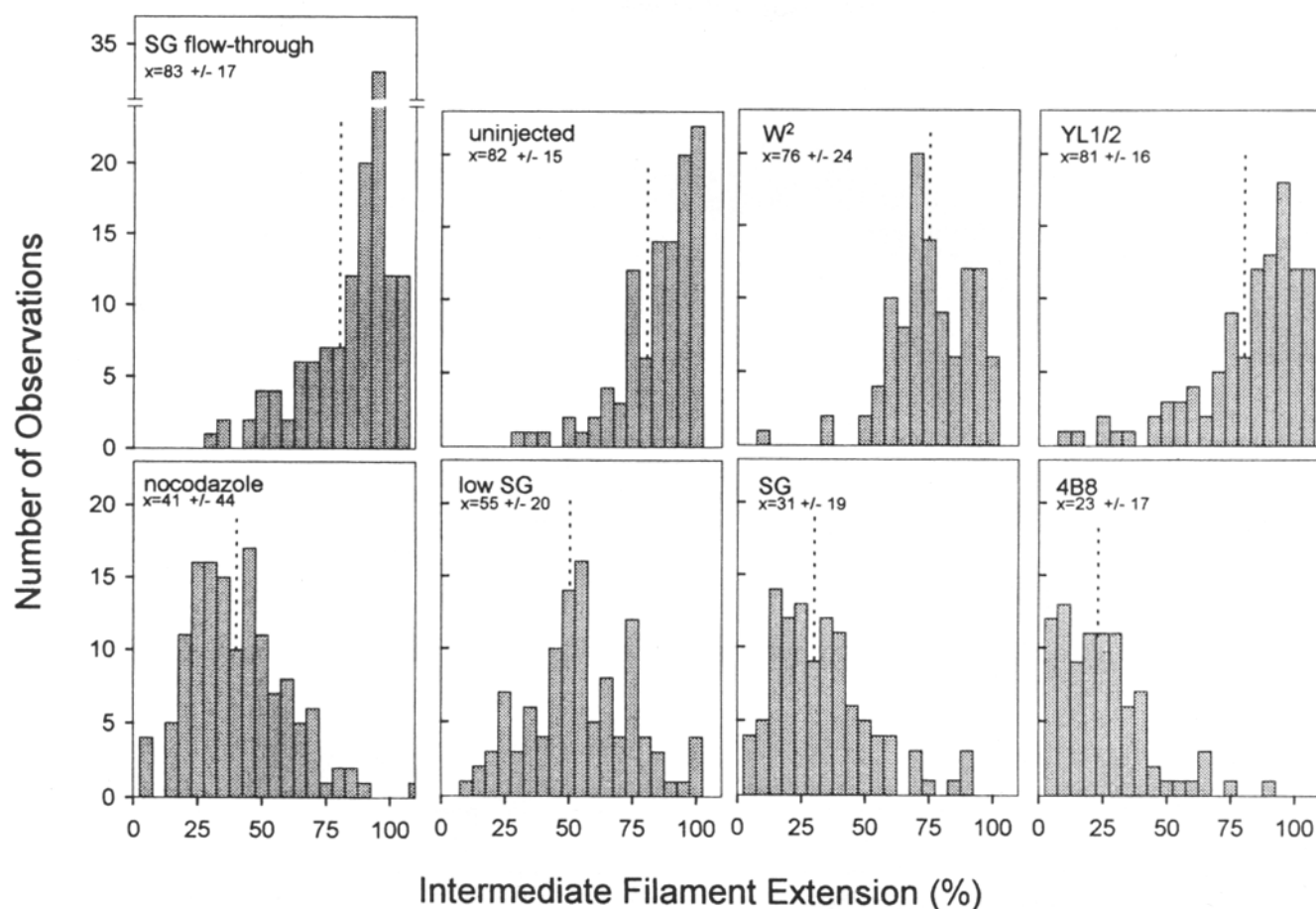


Figure 11. Frequency plots of the percent extension of IFs into the lamella of wound edge 3T3 cells after microinjection with different antibodies. 3T3 cells at the edge of an *in vitro* wound were injected with different antibodies, incubated for 2 h, and then permeabilized, fixed, and immunostained for the injected antibody and IFs as described in the Materials and Methods. IF extension into the lamella of injected cells was measured from digitized images of the immunofluorescently stained IFs and graphed as a frequency plot. The IF extension is expressed as a percent of the distance from the nucleus to the leading lamella (see Materials and Methods). For comparison, the IF extension in wound edge cells treated with 10 μ M nocodazole for 5 h is shown. Dotted lines indicate the mean extension of IFs for each population. $n = 107$ for SG IgG injected cells (both 1.5 mg/ml, *low SG*, and 10 mg/ml, *SG*), 4B8 IgG-injected cells (10 mg/ml), YL1/2 antibody injected cells (8 mg/ml), and the flow-through IgG from the Glu tubulin column (10 mg/ml). The data for the YL1/2 injected cells were corrected by normalizing for the extension of Tyr MT bundles (see text). $n = 129$ for nocodazole treated cells. $n = 92$ for W² IgG injected cells. Data are pooled from five experiments.

were struck by the colocalization of Glu MTs and IFs in the lamella of untreated cells and by the coincident alterations in the IF array when Glu MTs were generated in cells previously lacking Glu MTs. However, to extend our analysis beyond these correlative observations, we needed an approach that would specifically interfere with Glu MT function *in vivo*. The microinjection of affinity purified anti-Glu tubulin and anti-Tyr tubulin antibodies, fulfills several requirements for such a specific approach. First, the rationale for the approach is straightforward; if stable, Glu MTs are specifically interacting with other organelles (like IFs), then introduction of Glu tubulin specific antibodies into the cell might sterically block this interaction and trigger an alteration in the distribution of the organelle. Second, our results show that the injected anti-Glu and anti-Tyr tubulin antibodies labeled the expected

subsets of MTs in the cells, confirming that they can access native MTs and that they have the same specificity on native MTs as they have on fixed MTs. Finally, the fact that both monoclonal and polyclonal anti-Glu tubulin antibodies were effective in collapsing the IFs, whereas neither the monoclonal nor the polyclonal anti-Tyr tubulin antibodies were effective, shows the specificity of the approach and strongly suggests that the detyrosinated tubulin epitope is essential for the effectiveness of the injected antibodies. Furthermore, we showed that the effect of injected anti-Glu tubulin antibodies on the IF distribution was not due to contaminating antibody specificities (e.g., against IFs), since the flow-through IgG fraction from the tubulin affinity column, which would be expected to have a higher concentration of such a “contaminating” antibody specificity, did not induce IF collapse. Thus, we conclude that the in-

jection of anti-Glu tubulin antibodies into cells is a specific approach that is capable of revealing interactions between Glu MTs and other cellular organelles. A similar approach may be useful for examining other post-translational modifications of tubulin for which the function is not known.

The steady-state colocalization of Glu MTs and IFs in the lamella of wound-edge cells, as well as the correlative changes in the distribution of IFs upon the regeneration of Glu MTs in cells lacking these modified MTs, and the effectiveness of anti-Glu tubulin but not anti-Tyr tubulin antibodies in disrupting the IF array all support the idea that vimentin IFs prefer Glu MTs over Tyr MTs *in vivo*. In previous studies (Gundersen and Bulinski, 1988; Gurland and Gundersen, 1993; Gundersen et al., 1994), we have shown that the Glu MTs in 3T3 cells are more stable than Tyr MTs. Thus, a question that arises from the current study is whether the preferred localization of IFs with stable, Glu MTs is due to the increased level of Glu tubulin in the MTs or to the increased stability of the MTs. We favor the former explanation for several reasons. First, if the colocalization with Glu MTs arose simply from the fact that these MTs exhibit increased stability, it is not clear why injected antibodies to Tyr tubulin are almost completely without effect in collapsing the IF array. In a previous study Geuens et al. (1986) estimated that stable, Glu MTs may contain up to 50% Tyr tubulin, so an injected Tyr antibody would be expected to bind to stable MTs and alter the IF distribution to some extent if MT stability was the important factor. Second, it is difficult to explain how the injected anti-Glu tubulin antibodies (which bind only to a small subset of the MTs) are as effective in collapsing the IFs as completely breaking down the MTs with nocodazole. If IFs were simply adopting the position of the most stable MTs, even if the Glu MTs were sterically blocked with antibody, abundant Tyr MTs would still be available to interact with IFs. Finally, it is unlikely that simply decorating stable MTs with any antibody is sufficient to collapse IFs. Blose et al. (1984) microinjected a number of antibodies to tubulin and found that while many of the antibodies labeled all the MTs in the injected cells (including, presumably, the stable MTs), most of them had no effect on the IF distribution. Thus, we think that for the antibodies to effectively disrupt the IF array, they need to be directed to the appropriate epitope on the MTs. Since anti-Glu tubulin antibodies but not anti-Tyr tubulin antibodies are effective in collapsing IFs, this argues that the appropriate epitope is near the COOH-terminus of Glu tubulin, but not Tyr tubulin.

Two previous studies used tubulin antibody microinjections in attempts to interfere with MT-IF interactions (Wheland and Willingham, 1983; Blose et al., 1984). The first study showed that the YL1/2 monoclonal antibody to Tyr tubulin was unable to collapse IFs unless the MTs were themselves bundled and collapsed around the nucleus (this only occurred at antibody concentrations ≥ 12 mg/ml). Even at these high antibody concentrations, the IFs remained mostly colocalized with the bundled MTs, suggesting that YL1/2 antibody did not disrupt the MT-IF interaction. We also observed bundling of MTs with the YL1/2 antibody, but in our study the MT bundles remained extended and the IFs remained codistributed with the MTs and did not collapse. Thus, our results with the

YL1/2 antibody are consistent with the earlier study, but more clearly show that YL1/2 antibody does not disrupt the MT-IF interaction. In the second study, Blose et al. (1984), injected five monoclonal antibodies to tubulin (both α - and β -subunit specific antibodies were tried) and found only two of them to be effective in inducing the collapse of IFs. Coupled with our results and those of Wheland and Willingham (1983), this again points to the limited epitope on MTs that is apparently involved in the interaction between MTs and IFs and lends further support to our argument that the effectiveness of the Glu tubulin antibodies is not due to nonspecific steric effects. Interestingly, one of the two antibodies that was effective in collapsing the IFs in the study by Blose et al. (1984) (DM1A), is directed to a site very close to the COOH-terminus of α -tubulin (the site recognized by the other antibody that induced IF collapse is unknown). The DM1A epitope has been mapped to residues 426–450 of α -tubulin (the COOH-terminus of Glu tubulin is residue 450) and is known to bind to both Glu and Tyr tubulin (Breitling and Little, 1986). Thus, it is entirely possible that DM1A binding to MTs would occlude the same site blocked by antibodies to Glu tubulin thus explaining the ability of DM1A to collapse the IFs.

How do vimentin IFs interact with Glu MTs? The possibilities range from a direct interaction between the two filaments to accessory cross-bridging molecules that would link the two structures. In cultured fibroblasts, a number of studies have shown that IFs and MTs are in close proximity (Hauser and Kirschner, 1980; Schliwa and Van Blerkom, 1981), yet to date, no study has shown evidence for structural cross-bridges. There is, however, abundant evidence for the existence of molecules which cross-bridge MTs and neuronal IFs. By electron microscopy, distinct cross-bridges have been observed between MTs and neuronal IFs (Hirokawa, 1982; Hirokawa et al., 1988). In the latter study, microtubule-associated protein-2 was identified as a component of the cross-bridges in neuronal cells. Microtubule-associated proteins have also been implicated in MT-IF cross-bridging in *in vitro* studies (Leterrier et al., 1982; Heiman et al., 1985). Intermediate filament associated proteins, like the 300-kD protein described by Leiska et al. (1985), may also be candidates for the cross-bridging protein. Functional evidence implicating specific proteins in cross-bridging MTs and IFs in fibroblasts has been obtained in two studies. Lin and Feramisco (1981) found that microinjection of an antibody to a 95-kD protein rapidly induced IF collapse in gerbil fibroma cells without altering the MT distribution. Similarly, Gyoeva and Gelfand (1991) found that microinjection of function blocking antibodies to kinesin heavy chain caused collapse of IFs in 3T3 fibroblasts. Thus, MT motor proteins may also be important for distributing IFs in cells. It will be important to determine whether any of these candidate cross-bridging molecules interact selectively with Glu MTs or whether there is a previously unidentified factor that is responsible for cross-bridging IFs and MTs.

The possibility that detyrosination of tubulin functions to stimulate or signal interactions of MTs with IFs, and perhaps other organelles, needs to be tested with additional experiments. However, it is worth considering the possible advantages that such a regulatory mechanism

might convey to the cell. We have already pointed out that Glu MTs are formed asymmetrically in a number of cases in which cells are undergoing morphogenetic events (see Introduction). Thus, by making organelle location dependent on the deetyrosination of MTs, the cell can build intracellular asymmetry of its organelles. However, the same intracellular asymmetry could conceivably be achieved if organelles bound to MTs solely by a time-dependent mechanism. With such a mechanism, MTs would accumulate organelles as they aged and thus organelle position would be controlled by regulating MT stability. Since a MT stability based system could in theory generate the same asymmetric organelle distributions as one dependent on deetyrosination of tubulin, it is possible that the more fundamental role of regulating organelle interaction by deetyrosination is to separate the two activities of MTs, namely, MT polymerization-depolymerization and MT-organelle interactions. By separating MT dynamics from MT-organelle interactions, the cell could prevent organelles from interacting with MTs while they are in a highly dynamic state. Premature organelle interaction would be inefficient and would slow the ability of the cell to rapidly polarize itself in response to external stimuli. More importantly, premature organelle interaction may interfere with the dynamics of the MTs themselves, leading to mistakes in MT stabilization and consequently cellular polarization. By adding the additional regulatory step of tubulin posttranslational modification, the cell could avoid such problems and increase the efficiency of polarizing cellular organelles. In the future, it will be interesting to determine whether inhibiting deetyrosination, perhaps with antibody microinjections, has an effect on cellular activities that are dependent on cell polarity, e.g., motility.

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