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Induction of stable microtubules in 3T3 fibroblasts by TGF-β and serum

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SUMMARY

Previous studies have shown that fibroblasts induced to migrate into an in vitro wound rapidly generate an array of stable, post-translationally detyrosinated microtubules (Glu MTs) oriented toward the direction of migration. To understand how cells generate a stable array of MTs at a specific location, we have analyzed the contribution of media components to the formation of oriented Glu MTs in wounded monolayers of 3T3 fibroblasts. When confluent monolayers were placed in serum-free medium (SFM) for 2 days before wounding, the cells contained virtually no Glu MTs or nocodazole-resistant MTs and were incapable of generating Glu MTs in response to wounding. Such SFM-treated monolayers were capable of generating oriented Glu MTs within 1 hour of wounding, if calf serum (CS) was added back to the medium. The Glu MTs in the CS refed cells were oriented toward the wound in cells at the wound edge, and were juxtanuclear in cells within the monolayer, demonstrating that CS restored the Glu MT array characteristic of each cell type.

To determine the nature of the 'Glu MT-inducing' factor in CS, we subjected CS to different treatments and found that the CS factor was nondialyzable, resistant to heat, mild acid and trypsin, but inactivated by treatment with dithiothreitol. The factor was not absorbed by charcoal and was present in lipoprotein-deficient serum. These properties are consistent with the properties of a number of polypeptide growth factors, so we screened purified growth factors for their ability to induce Glu MTs in wounded SFM-treated monolayers. Of all the growth factors tested, only TGF-β1 and TGF-β2 induced a significant level (≥ 70% of the CS response) of oriented Glu MTs. The SFM-treated cells were exquisitely sensitive to TGF-β1, with significant induction of Glu MTs observed at 0.01 ng/ml TGF-β1. Induction of Glu MTs observed by immunofluorescence after CS or TGF-β treatments were paralleled by increases in Glu tubulin detected on western blots. The Glu MTs formed after either CS or TGF-β1 treatment showed enhanced resistance to nocodazole, confirming that both treatments increased the level of stable MTs in cells. The TGF-β1 induction of stable MTs was slower than that of CS (2-4 hours onset versus 1 hour onset), but by 24 hours the level of MT stabilization in TGF-β1 was even greater than that in CS. Unlike CS, TGF-β1 did not stimulate the migration of SFM-treated cells into the wound or the entry of SFM-treated cells into the cell cycle, showing that MT stabilization is independent of these events. These results demonstrate that MT stabilization can be regulated by external factors and that TGF-β is a potent inducing factor for stable MTs.

Key words: detyrosination, microtubule, TGF-β, fibroblast

INTRODUCTION

Microtubules (MTs) are conspicuous elements of the cytoskeleton that are found in nearly every eukaryotic cell. In addition to a well-characterized role as the principle structural elements of the mitotic spindle, MTs are thought to contribute in nonmitotic cells to the generation and maintenance of cell shape and polarity. In this capacity, one would expect that MTs would exhibit a certain degree of longevity. Indeed, this seems to be the case. In undifferentiated, proliferating cells, MTs exhibit tremendous dynamics, turning over with a half-life of about 5-10 minutes (Saxton et al., 1984; Schulze and Kirschner, 1986). The rapid dynamics of interphase MTs is paralleled by a dramatic sensitivity of the MTs to antagonists such as nocodazole or colchicine (Kreis, 1987; Khawaja et al., 1988). In contrast, MTs in cells that have exited the cell cycle and have undergone morphogenesis, in general, exhibit decreased dynamics and a lower sensitivity to MT antagonists (reviewed by Bulinski and Gundersen, 1991).

In some cases this stabilization process can be followed in culture, providing a system in which to study the stabilization process. In these examples, MT stabilization is correlated both temporally and spatially with the generation of cellular asymmetry (Gundersen et al., 1989; Pepperkok et al., 1990; Baas and Black, 1990). This correlation and the observation that the basal state of MTs is a dynamic one have led to the idea that the MT involvement in morphogenetic changes of cells occurs by a selective stabilization process, whereby dynamic MTs are stabilized in those areas of the cell that are actively acquiring a novel shape. This results in the formation

of an asymmetric MT array that is subsequently used, perhaps as a scaffold, to rearrange or redirect other components in the cell (Kirschner and Mitchison, 1986; Bulinski and Gundersen, 1991).

The stabilization process itself is likely to be complex and in cases of terminal differentiation is likely to involve new gene expression. In at least one system, namely directed motility of fibroblasts, selective stabilization of MTs appears to occur without the complexity of new gene expression. Experiments with wounded monolayers of fibroblasts have shown that cells at the edge of the wound rapidly develop stable MTs oriented toward the leading edge of the cell (i.e. toward the direction of locomotion) (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). In these experiments, the stability of MTs was assessed by their accumulation of post-translationally detyrosinated (Glu) tubulin and by their increased resistance to nocodazole depolymerization (reviewed by Bulinski and Gundersen, 1991). MTs oriented away from the leading edge were not stabilized by these critera. Thus, fibroblasts appear to be capable of sensing a particular direction for motility and then locally activating machinery to stabilize MTs.

Motility per se does not seem to be the signal for MT stabilization, since cells can generate stable MTs before adopting a motile morphology (Gundersen and Bulinski, 1988), and at least for a period of time, can retain stable MTs after motility has been blocked by cytochalasin (Nagasaki et al., 1992; unpublished observations). While the MTs in the leading edge of motile fibroblasts appear to be stabilized, they are not irreversibly stabilized, since they have been observed to disappear rapidly from the leading edge of motile cells during cell-cell collisions (Nagasaki et al., 1992). These results are consistent with the possibility that MT stabilization is an important mediator of cell locomotion, although this has not been tested directly. In any case, the directed motility of fibroblasts into an in vitro wound provides a unique and readily manipulatable system in which to test the idea that the selective stabilization of MTs contributes to the generation of functional cellular asymmetry.

To test the functional role of stable MTs in locomotion (and other morphogenetic processes), it will be necessary to understand the molecular mechanism involved in generating stable MTs and to develop tools to interfere with their formation. As a step in this direction, we have developed culture conditions that render MT stabilization in wounded fibroblasts dependent on the addition of serum to the media. We have characterized the activity in serum as a polypeptide growth factor-like component and, by testing a number of purified growth factors, have found one, TGF-β, that is capable of stimulating abundant formation of stable MTs in cells under serum-free conditions. Our results show that MT stabilization in cells is critically regulated in response to external factors and additionally suggest that MTs are an important target of TGF-β action.

MATERIALS AND METHODS

Cell culture, serum-free treatments and wounding

NIH-3T3 cells (passage no. 127-137) were cultured in DMEM (GIBCO) supplemented with 10% calf serum (CS) as previously described (Gundersen and Bulinski, 1988). Cells were seeded on acid-washed glass coverslips for immunofluorescence or on tissue culture dishes for preparation of SDS samples and grown until they were just confluent (2-3 days). Cells were then used directly or were treated with serum-free medium (SFM; DMEM containing 5 mg/ml fatty acid-free BSA (no. 6003, Sigma) and 20 mM HEPES, pH 7.3) by washing three times with SFM and then culturing the cells for an additional 48 hours in SFM. We found that adding BSA to the SFM improved the viability of the cells. A number of different commercial BSA preparations were tried and this one gave the greatest viability and the lowest level of stable MTs (see Results).

Wounding of the monolayer was performed by scraping a narrow strip of cells with either a teflon cell scraper or a jeweler's screwdriver (Gundersen and Bulinski, 1988). The medium was immediately removed and the monolayer was washed three times before adding fresh medium with the appropriate additions and incubating the cells at 37°C in a CO_2 incubator.

Serum treatments

To characterize the factor(s) in CS responsible for inducing stable MTs in cells, CS was subjected to a number of treatments before adding it to SFM-treated cells. CS was extensively dialyzed against Dulbecco's PBS for 2 days in 12,000 to 14,000 molecular mass cut-off dialysis tubing. CS was boiled for 5 minutes, cooled on ice and then clarified by centrifugation (30 minutes, 40,000 ***g***, 4°C). The boiling of whole serum caused a massive precipitation of serum proteins, but did not seem to inactivate the MT stabilizing activity (see Results). Similar results were found if CS was first diluted to 20% (in DMEM) prior to boiling. Boiled CS was subjected to two additional treatments. It was treated with TPCK-trypsin (Sigma) at a protein mass ratio of 1:50 for 30 minutes at 37°C. The trypsin was subsequently inactivated with a 2-fold molar excess of soybean trypsin inhibitor (Sigma). By SDS-PAGE, most of the serum proteins had been digested by this trypsin treatment (data not shown). Boiled CS was also absorbed with charcoal (Norit A): 50 mg charcoal/ml boiled CS was added and it was allowed to incubate for ~15 minutes at room temperature; the charcoal was removed by centrifugation (10 minutes at 16,000 ***g***) and the procedure repeated with fresh charcoal. For treatment with reducing agents, CS was incubated with 50 mM DTT for 60 minutes at room temperature. The DTT-treated CS was then dialyzed and clarified as described above for 'dialyzed CS'. CS was treated with acid by adding HCl until the pH reached 3.0, and then neutralizing the acid as described previously for the activation of latent TGF-β (Lawrence, 1991). Lipoprotein-deficient fetal bovine serum (LPDS; d, 1.21 g/ml) was isolated by preparative ultracentrifugation and was obtained from F. Maxfield (Columbia University, NY); matched, untreated fetal bovine serum was used as a control. Following treatments, all sera were filtered through 0.22 μm sterile filters. CS and fetal bovine serum were from Hyclone (Denver, CO); horse serum and horse platelet-poor plasma were from Sigma. Treated sera were stored at –20°C and added to SFM just prior to use.

Growth factor treatments

Growth factors, obtained from the sources listed below, were prepared as stock solutions in PBS containing 1 mg/ml fatty acid-free BSA (except for TGF-β and PDGF, which were prepared in 4 mM HCl containing 1 mg/ml fatty acid-free BSA; Assoian et al., 1983). Growth factors were added to SFM just prior to use. The PDGF, IGF1, IGF2, insulin, EGF and FGF used in this study were all judged to be active as mitogens, since they stimulated DNA synthesis of quiescent NIH 3T3 cells prepared by treatment with 0.2% CS for 2 days. However, none of these same factors was capable of stimulating DNA synthesis when added individually to the SFM-treated cells used in this study (data not shown). Purified porcine TGF-β1 and TGF-β2, and recombinant interleukins 1-α and 6, were obtained from R & D (Minneapolis, MN). Purified forms of human IGF I and IGF II, and bovine pituitary FGF (predominantly basic FGF), were obtained from Collaborative Research (Bedford, MA). Human PDGF was from UBI

(Lake Placid, NY). PDGF purified from human platelets according to Heldin et al. (1981) and obtained from Dr L. Witte (Columbia University, NY), was also tried, but was no more active in stimulating stable MT formation than the commercial preparation of PDGF. EGF, purified from mouse submaxillary gland as described previously (Taylor et al., 1970), was obtained from F. Maxfield (Columbia University, NY). Bovine insulin, fibronectin and collagen were all purchased from Sigma.

Assessment of the induction of oriented Glu MTs

The extent of induction of Glu MTs was assessed microscopically, essentially as previously described (Gundersen and Bulinski, 1988). Briefly, preparations of wounded monolayers that had been immunofluorescently labeled for Glu and Tyr MTs (see below) were examined with a Zeiss ×40 Planapo (1.4 NA) objective on a Nikon Optiphot fluorescence microscope. The extent of the induction of Glu MTs was determined on a cell-to-cell basis by scoring cells at the edge of wounds for the presence of an 'oriented array' of Glu MTs. A cell was judged to have an oriented array of Glu MTs if it contained a significant number of Glu MTs (generally ≥10) and virtually all the Glu MTs extended from the centrosomal area toward the leading edge. Cells without oriented Glu MTs contained either no Glu MTs or a small number of randomly arrayed Glu MTs. None of the conditions that we examined (with the exception of the taxol treatments) induced > ~10% of the cells at the wound edge to contain a large number of Glu MTs randomly disposed in the cell. Thus by scoring cells with oriented Glu MTs, we count as positive only those cells that have generated a significant response.

Assessment of MT stability toward nocodazole treatments

To examine the resistance of the MTs to breakdown by nocodazole, we tested a range of nocodazole concentrations to find one that caused most of the MTs to break down over a period of about 1 hour at 37°C. For the SFM-treated cells used in this study, 2 μM nocodazole was found to be an optimal concentration (not shown). SFM-treated cells that had been refed SFM, SFM+10% CS or SFM+2 ng/ml TGF-β for 7 hours, were treated with 2 μM nocodazole. At different times after adding the nocodazole (from 10-60 minutes), cells were extracted to remove the monomeric tubulin generated by nocodazole and fixed for indirect immunofluorescence. The extraction protocol was essentially as described previously (Gundersen et al., 1987a), except that 200 μg/ml saponin was used as the detergent, since the cells were less prone to come off the coverslips than when Triton X-100 was used.

Assessment of proliferation

To assess the re-entry of cells into the cell cycle in response to growth factors, we used the bromodeoxyuridine (BrdU) technique to measure the capability of the cells to synthesize DNA, essentially as described (Lamb et al., 1990). Briefly, cells were either pulsed with 10 μg/ml BrdU for 2 hours (to examine the onset of DNA synthesis) or were incubated continuously for 24 hours in BrdU (to determine the extent of mitogenic stimulation). At the appropriate times, cells were fixed in methanol as described below. Before indirect immunofluorescence, methanol-fixed cells were treated with 2 M HCl for 15 minutes to render the DNA accessible to the anti-BrdU antibody (Lamb et al., 1990). The HCl treatment only slightly interfered with the preservation of MTs as judged by the immunofluorescence staining of MTs (e.g. see Fig. 8).

Indirect immunofluorescence

Double indirect immunofluorescence with a rabbit peptide antibody specific for Glu tubulin (Gundersen et al., 1984) and rat monoclonal antibody specific for Tyr tubulin (YL 1/2; Kilmartin et al., 1982) was performed as previously described (Gundersen et al., 1987a). YL 1/2 was a generous gift from Dr J. V. Kilmartin (MRC, Cambridge). For some experiments, we double stained cells with the rabbit anti-Glu tubulin antibody and a mouse monoclonal antibody (3F3) reactive with all β-tubulin isoforms as previously described (Khawaja et al., 1988). 3F3 was generously provided by Dr J. Lessard (University of Cincinnati). Secondary antibodies were 1:400 dilution of high F/P fluorescein-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) and 1:100 dilutions of rhodamine conjugates of goat anti-rat and goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA).

To assess the extent of DNA synthesis, cells that had been pulsed with BrdU, and then fixed in methanol and HCl-treated (see above), were double stained with the anti-Glu tubulin antibody (as above) and a 1:25 dilution of a mouse monoclonal antibody specific for BrdU (Becton-Dickinson, Lincoln Park, NJ). Secondary antibodies were the same as those used above. The percentage of cells capable of synthesizing DNA was then determined by counting cells with fluorescently labeled nuclei in random fields along the edge of the wound and in the interior of the wound.

Fluorescence microscopy and photography with hypersensitized 2415 film (Kodak) was as described previously (Nagasaki et al., 1992).

Western blotting

Samples for SDS-PAGE and western blotting were prepared as follows. A 60 mm dish of confluent cells was used for each sample. At the appropriate time, the cells were rinsed three times in 37°C Earle's balanced salt solution followed by two rinses in 37°C D-PBS (the extensive washing removed adherent material (cell debris, etc.) from the monolayers). After carefully removing the last rinse, 0.25 ml (or 0.5 ml for cells not treated with SFM) of SDS sample buffer without reducing agent (2% SDS, 100 mM Tris-HCl, pH 6.8, 20% glycerol and freshly added 0.2 mM phenylmethylsulfonyl fluoride) was added to the dish and rapidly spread over the cells. To reduce the viscosity of the SDS extract and to ensure complete recover of protein from the dish, the sample was triturated several times over the dish before transferring to a microfuge tube, vortexing thoroughly and then boiling for 5 minutes. The boiled samples were stored at −70°C until analyzed by western blotting.

Before SDS-PAGE, the protein concentration was determined with the BCA assay using BSA as the protein standard (Pierce, Rockford, IL). The samples were then adjusted to 50 mM DTT and 0.1% bromophenol blue (by leaving these components out of the sample buffer until after the protein assay, we were able to determine the protein concentrations without resorting to modifications that compromise the reliability of the assay). Samples were then subjected to SDS-PAGE on 7.5% polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose sheets (overnight at 5 V/cm in the buffer of Towbin et al., 1979). After transfer, blots were blocked with gelatin and BSA (Towbin et al., 1979) and reacted with either the rabbit Glu antibody (diluted 1:50,000) or monoclonal 3F3 (ascites fluid, diluted 1:1000). Alkaline phosphatase-conjugated second antibodies (Promega, Madison, WI) were used to detect Glu antibody reactivity and peroxidase conjugated-second antibodies (Cappel) were used to detect 3F3. The phosphatase substrate was NBT/BCIP and the peroxidase substrate was 4-chloro-1-naphthol.

RESULTS

Serum requirement for the formation of Glu MTs in 3T3 cells

During the polarization and subsequent migration of 3T3 cells into the denuded area of a wounded monolayer, cells at the edge of the wound generate an array of Glu MTs that is selectively oriented toward the wound (Gundersen and Bulinski, 1988). The Glu MTs in this array exhibit increased resistance to breakdown by MT antagonists (Gundersen and Bulinski, 1988). In order to examine the role of serum factors in the generation and regulation of these 'stable' MTs during the wound

response, we developed a wounded monolayer system for which the generation of stable MTs was dependent on the addition of serum factors. By focusing our attention on the cells at the edge of a wound, we lessened the problem of assessing the induction of stable MTs, since the response of these cells is so uniform (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). We initially utilized the presence of elevated levels of Glu tubulin as an indicator of MT stability, since immunofluorescence detection of Glu MTs is relatively facile, and the enhanced stability of Glu MTs in vivo has been well established by results demonstrating the resistance of Glu MTs to breakdown by MT antagonists (Kreis, 1987; Khawaja et al., 1988) and by direct estimates of the half-life of Glu MTs (Webster et al., 1987; Schulze et al., 1987). Furthermore, the mechanism by which Glu MTs are generated, i.e. by the post-polymerization detyrosination of pre-existing Tyr MTs, is consistent with the enhance longevity of Glu MTs (Gundersen et al., 1987a).

When a confluent monolayer of 3T3 cells was treated for 48 hours with DMEM containing only buffer and BSA (SFM), the cells of the monolayer became almost completely devoid of MTs immunofluorescently stained for Glu tubulin (Glu MTs) (compare Fig. 1a and c). In fact, about the only structures consistently stained with the Glu antibody in the SFM-treated cells

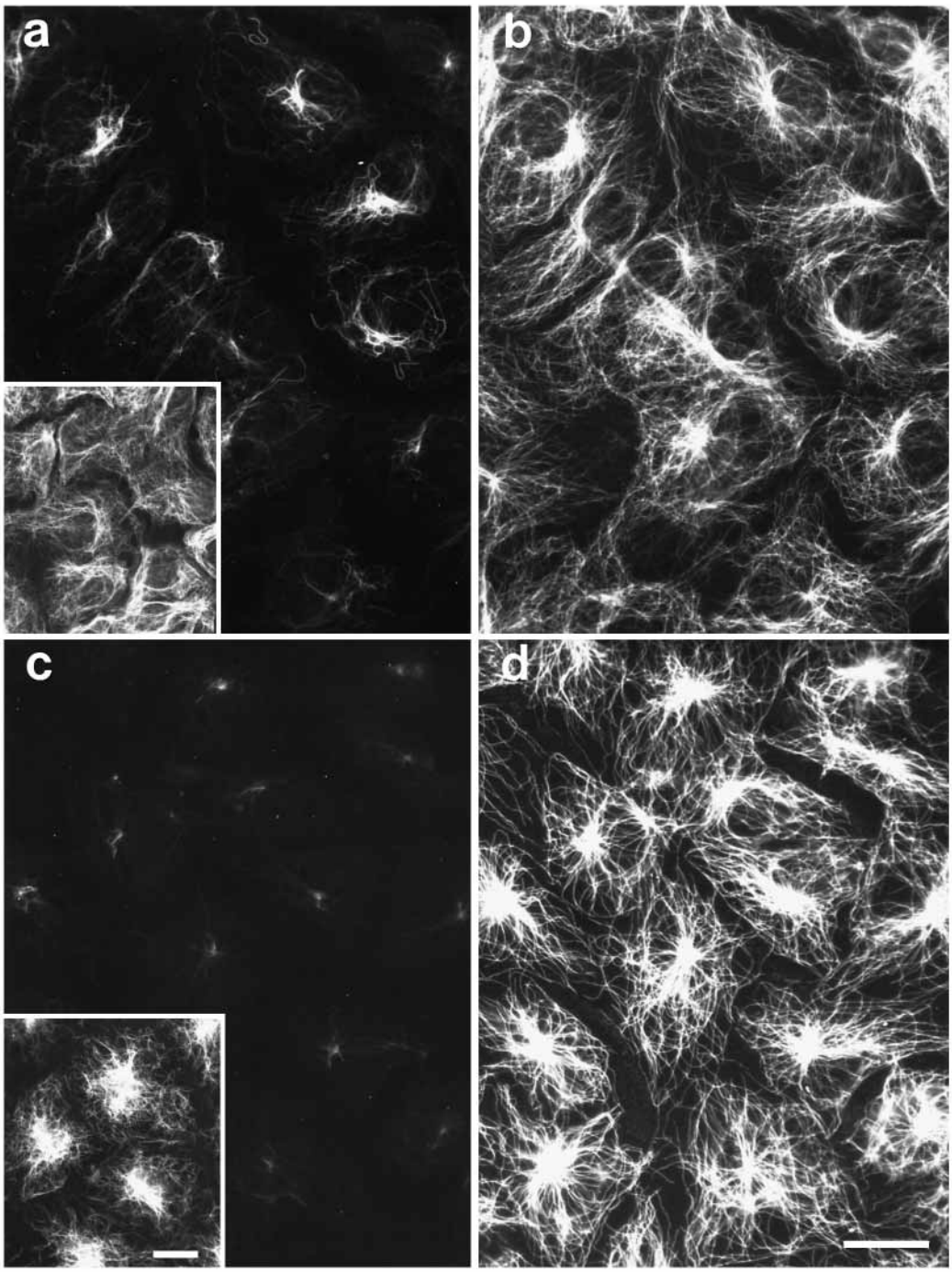

Fig. 1. Distribution of Glu and Tyr MTs in confluent monolayers of 3T3 cells before and after SFM treatment. 3T3 cells were grown to confluency and either fixed directly for immunofluorescence (a,b) or treated with SFM for 48 hours before fixation for immunofluorescence (c,d). The distribution of Glu MTs is shown in (a) and (c), and the distribution of Tyr MTs is shown in (b) and (d). In (c) the comet-like spots of immunofluorescence correspond to the centrosome and the primary cilium. The insets show the Glu MTs observed after treating cells with 5 μM taxol for 30 minutes to stabilize the MTs. Inset a, taxol-treated confluent cells; inset b, taxol-treated confluent cells that had been previously incubated with SFM for 48 hours. Bars, 20 μm.

were the primary cilium and the centrosome (Fig. 1c); both are stable MT structures that have been found previously to label with the Glu antibody (Gundersen and Bulinski, 1986a; Geuens et al., 1986). Western blots of samples prepared from SFM-treated cells showed that the level of Glu tubulin was quantitatively reduced by the SFM treatment (see below), consistent with the reduction in Glu MTs observed by immunofluorescence.

We have not examined the loss of Glu MTs induced by SFM in detail; however, shorter incubations (e.g. ≤ 24 hours) in SFM did not result in such a complete loss of Glu MTs. Also, the complete absence of serum seemed to be necessary to deplete cells of Glu MTs, since cells treated in reduced serum (e.g. 0.2%) still contained abundant Glu MTs after 48 hours (data not shown).

Although the SFM-treated cells did not contain Glu MTs they still contained abundant MTs as shown by Tyr tubulin immunofluorescence (Fig.1d) or β-tubulin immunofluorescence (data not shown). Yet, in comparison to cells in medium containing serum (Fig. 1b), the array of MTs in SFM-treated cells appeared less dense. This probably reflects an actual decrease in the number of MTs in the SFM-treated cells, although it may also be due in part to the highly spread and flattened morphology of the SFM-treated cells. Because of the difficulty in quantifying the numbers of individual MTs in interphase cells, we have not investigated further the apparent decrease in density of MTs in SFM-treated cells.

The lack of immunodetectable Glu MTs in the SFM-treated cells suggests that the cells lack stabilized MTs; however, it is also possible that they contain stable MTs but have inactivated the enzyme responsible for generating Glu tubulin, i.e. tubulin carboxypeptidase (Argarana et al., 1978; Webster et al., 1992). The inset in Fig. 1c shows Glu MT immunofluorescence in quiescent cells that had been treated with taxol (5 μM, 30 minutes). As can be seen, the SFM-treated cells were capable of generating Glu MTs in response to artificial stabilization of the MTs by taxol, indicating that the tubulin carboxypeptidase was indeed active. For comparison, the response of control

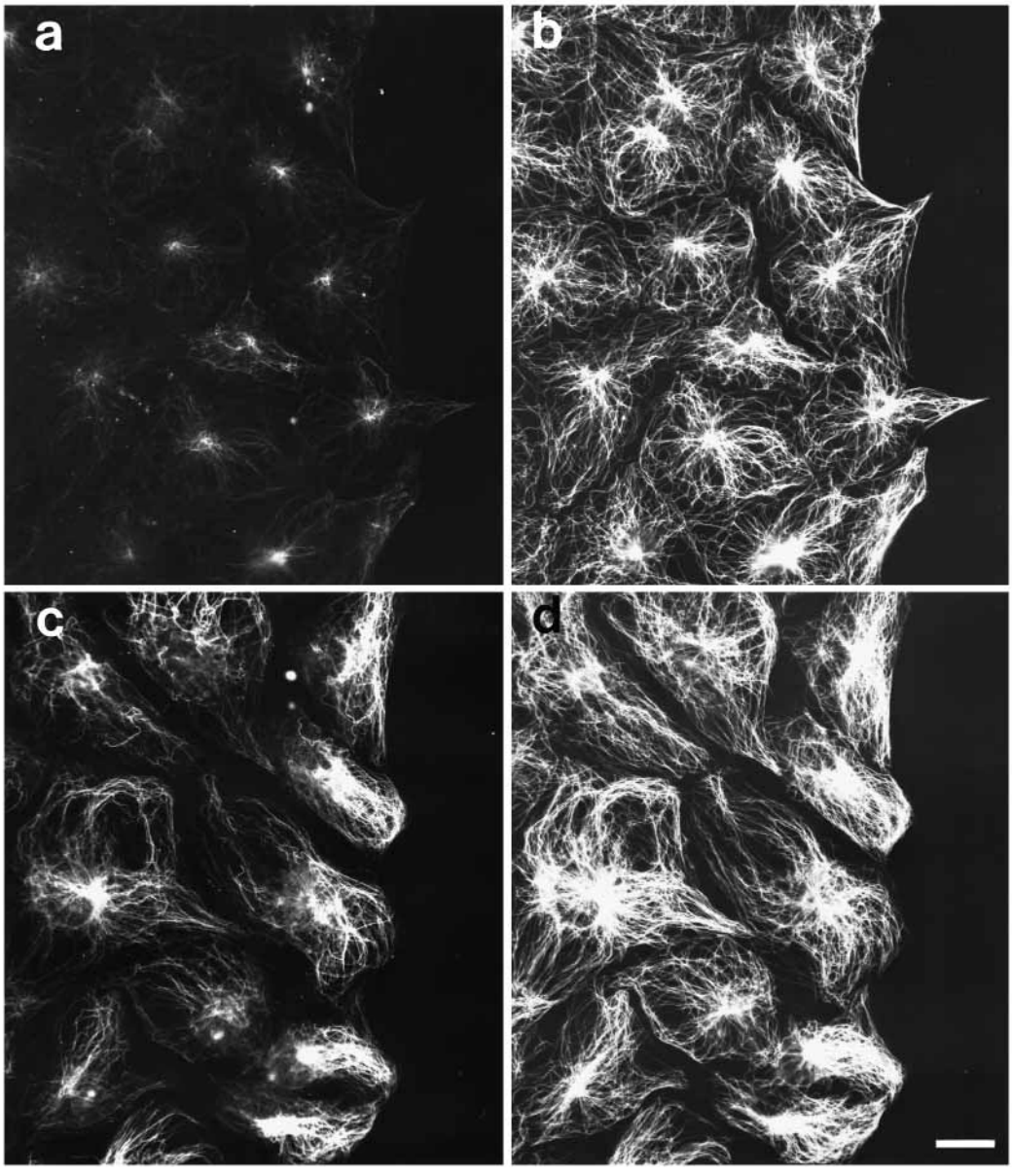

Fig. 2. Distribution of Glu and Tyr MTs in wounded, SFM-treated monolayers after refeeding SFM or SFM containing CS. 3T3 cells were grown to confluency, treated with SFM for 48 hours and then wounded and refed SFM (a,b) or 10% CS (c,d) for 7 hours. The cells were then fixed and processed for immunofluorescence. The distribution of Glu MTs is shown in (a) and (c), and the distribution of Tyr MTs is shown in (b) and (d). Note that CS refeeding results in a large increase in Glu MTs and in the orientation of Glu MTs toward the wound in cells at the wound margin. Bar, 20 μm.

cells (in serum) to the same taxol treatment is shown in the inset in Fig. 1a. In fact, comparisons of the appearance of Glu MT in response to taxol between control (in serum) and SFM-treated cells showed that the first increase in Glu MTs in response to taxol was 30 minutes for both cell populations, suggesting that there was no major quantitative difference in the activity of tubulin carboxypeptidase between the two cell populations. This supports the idea that the lack of Glu MTs in SFM-treated cells was due to the absence of stable MTs. Below, we show that the SFM-treated cells do not contain MTs resistant to nocodazole treatment, consistent with the idea that they do not contain stable MTs.

To determine whether the cells in the SFM-treated monolayer were still capable of generating Glu MTs and orienting them in response to wounding, we treated confluent monolayers for 48 hours in SFM, wounded the SFM-treated monolayers and then refed them either SFM or SFM containing 10% CS. Cells were fixed 7 hours after wounding to allow them ample time to generate Glu MTs (the percentage of wound-edge cells with Glu MTs oriented toward the wound reaches a maximum by 2 hours in medium containing serum; see Fig. 3, and Gundersen and Bulinski, 1988). Fig. 2 shows typical results from such an experiment. In SFM-treated monolayers that were refed SFM after wounding, only a few of the cells at the wound edge (see Table 1) were capable of generating an array of Glu MTs oriented toward the wound (Fig. 2a). This argues that wounding alone is an insufficient stimulus for the generation of oriented Glu MTs. A slightly larger percentage of the SFM-refed cells (usually ~25%) contained a small number of randomly oriented Glu MTs. However, this did not appear to be a response to wounding, since a similar percentage of cells within the monolayer also contained randomly arrayed Glu MTs. Thus, in wounds allowed to recover in SFM there was almost no increase in the number of cells with oriented Glu MTs and only a slight increase in the total percentage of cells exhibiting any Glu MTs (either random or oriented). Wounding and/or SFM refeeding had little effect on Tyr MTs in the SFM-treated cells (Fig. 2b).

In contrast, when wounded SFM-treated monolayers were refed medium containing 10% CS, virtually all of the cells responded by generating abundant Glu MTs (Fig. 2c). This was true both for cells at the wound edge and for cells in the interior of the monolayer. In $\geq$ 70% of the cells at the wound margin, the Glu MTs were predominately found oriented toward the wound, whereas in cells within the monolayer, the Glu MTs did not show a particular orientation to one side of the cell and were frequently closely coiled arounded the nucleus (Fig. 2c). Thus, re-addition of CS to SFM-treated monolayers induced both the formation of Glu MTs and the characteristic distribution of these MTs in cells at the wound edge and within the monolayer (for comparison, see Gundersen and Bulinski, 1988). In the experiments reported below, we have focused on the development of Glu MTs in cells at the wound edge.

Fig. 3 shows a quantitative comparison of the development of oriented Glu MTs in response to wounding in control (not treated with SFM) and SFM-treated cells that had been refed SFM alone or SFM with 10% CS. The generation of oriented Glu MTs in cells at the wound edge was delayed 30-60 minutes in the SFM-treated cells that were wounded and subsequently incubated in CS as compared to the control cells (never treated with SFM) that were wounded and incubated in CS. Nonetheless, the final extent of the response was very similar for the two treatments, suggesting that removal of CS for 48 hours does not irreversibly inhibit the capacity of the cells to produce Glu MTs. SFM-treated cells incubated in SFM after wounding did not develop significant levels of Glu MTs at any time after wounding.

Table 1. Effects of serum on the level of oriented Glu MTs

	Oriented Glu MTs		
Serum treatment*	% of CS†	*N*	ED_{50} (%)‡
Control (SFM)	11±5	11	–
CS (10%)	(100)	11	1.0
CS, dialyzed	98±1	3	0.45
CS, boiled	98±9	3	–
CS, trypsinized	96±2	3	–
CS, pH3	93±9	3	0.38
CS, charcoal	95±5	3	–
CS, DTT	41±15	2	>10
FBS	101±6	3	–
FBS-LPDS	99±2	3	–
HS	96±11	5	0.90
HP	81±15	5	2.7

*Confluent 3T3 cells were treated with SFM for 48 hours, wounded and then incubated for 7 hours with SFM containing the indicated serum at a 10% concentration. Cells were processed for immunofluorescence and then the level of oriented Glu MTs determined (see Materials and Methods). CS, calf serum; FBS, fetal bovine serum; FBS-LPDS, lipoprotein-deficient fetal bovine serum; HS, horse serum; HP, horse platelet-poor plasma.

†Values were normalized to the level of oriented Glu MTs induced by 10% CS in the same experiment. The mean level of oriented Glu MTs induced by CS in these experiments was 72±10%

‡ED_{50} is the half-maximal effective dose (expressed as percentage of serum added to the medium) determined from separate experiments in which the concentration of the indicated serum was varied from 0.01% to 10%. The results of at least two separate experiments were averaged to obtain each ED_{50}.

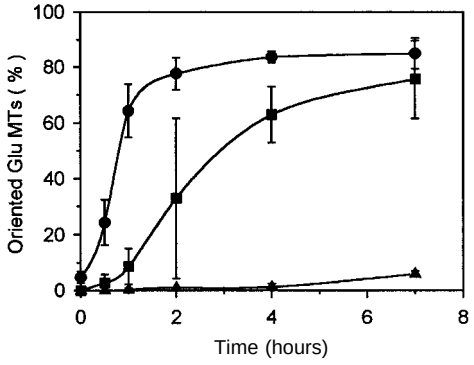

Fig. 3. Comparison of the acquisition of oriented Glu MTs in cells at the wound edge before and after SFM treatment. Confluent 3T3 cells were wounded before (●) or after (▲, ■) 48 hours of SFM treatment. Cells treated with SFM were refed SFM (▲) or 10% CS (■). Cells were fixed at various times after wounding and then processed for immunofluorescence. The wound edge cells were then examined for the formation of oriented Glu MTs (see Materials and Methods). The results shown were obtained by scoring over 100 cells for each time point in each of 4 separate experiments. Error bars are standard deviations.

The array of Tyr MTs in SFM-treated cells that had been refed CS, appeared more crowded than in cells maintained in SFM (compare Fig. 2b and d). As noted above for cells cultured in SFM, the change in the appearance of Tyr MTs may in part reflect differences in cell shape and thickness, since CS appears to reduce cell spreading. To some extent it may also reflect an increase in the number of MTs per cells in response to serum. However, without quantitative measurements it is difficult to be certain which of these two possibilities contributes the most to the apparent change in the Tyr MT array (see Discussion).

Characterization of the serum factor responsible for inducing Glu MT formation

To perform a preliminary characterization of the factor in CS responsible for triggering Glu MT formation, we tested CS that had been subjected to a number of treatments and also examined sera obtained from different sources for their ability to generate oriented Glu MTs in SFM-treated cells after wounding. As shown in Table 1, the 'Glu MT-inducing' activity was not dialyzable (12,000 to 14,000 M_r cutoff), was stable to heat, mild acid and trypsin treatment and was not absorbed by charcoal. In fact, dialysis or mild acid treatment tended to increase the activity of CS (compare ED_{50} values in Table 1). The factor was largely inactivated by DTT treatment as shown by the >10-fold higher ED_{50} of DTT-treated CS. Horse serum worked as well as CS; however, horse platelet-poor plasma was not as effective as whole horse serum (ED_{50} of 2.7% versus 0.9%). Lipoprotein-depleted serum was as active as the untreated control serum. The above characteristics are consistent with the idea that the Glu MT-inducing activity in serum is a polypeptide growth factor, so we proceeded to test the capability of purified growth factors to generate Glu MTs.

Table 2. Effects of growth factors on the level of oriented Glu MTs

		Oriented Glu MTs	
Growth factor*	ng/ml	% of CS†	*N*
Control (SFM)	–	9±4	18
CS	(10%)	(100)	18
TGF-β1	2	75±8	18
TGF-β2	2	79±4	4
PDGF	10	20±6	6
FGF	100	19±6	5
EGF	10	14±8	5
IGF I	100	33±6	4
IGF II	100	30±12	4
Insulin	10000	29±6	6
IL-1α	5	17±2	2
IL-6	100	16±6	2
Fibronectin	20000	15±5	3
Collagen	20000	17±1	2
TGF-β1+CS	2; (10%)	106±14	3
TGF-β1+PDGF	2; 10	60±8	2
TGF-β1+FGF	2; 100	41±15	2
TGF-β1+EGF	2; 10	53±11	3
TGF-β1+IGF I	2, 100	68±22	3
TGF-β1+IGF II	2; 100	78±17	2
TGF-β1+Insulin	2; 10000	75±18	6
Insulin+PDGF	10000; 10	26±6	2
Insulin+FGF	10000; 100	26±8	2
Insulin+EGF	10000; 10	32±16	2

*Growth factor treatments and analysis of oriented Glu MTs were carried out on SFM-treated and wounded 3T3 monolayers as described in Table 1.

†Values were normalized to the level of oriented Glu MTs induced by 10% CS in the same experiment. The mean level of oriented Glu MTs induced by CS in these experiments was 71±8%.

Activity of purified growth factors in stimulating Glu MT formation

We obtained a battery of purified growth factors and tested them, alone and in combination, for their efficacy in eliciting the formation of oriented Glu MT in SFM-treated cells after wounding. The results, shown in Table 2, indicate that of all the growth factors we tested, only TGF-β1 and TGF-β2 were able to induce a significant number of cells to generate oriented Glu MTs. At 2 ng/ml, both TGF-β1 and TGF-β2 induced oriented Glu MTs in about 50% of the cells at the wound edge (see Fig. 4): this is about 75% of the maximal response observed with 10% CS (Table 2). None of the proliferative growth factors (e.g. PDGF, FGF or EGF) was capable of inducing oriented Glu in many cells; for each factor, the response was ≤ 20% of the response induced by 10% CS (Table 2). The interleukins, IL1-α and IL6, both of which are known to stimulate fibroblast proliferation under certain conditions (Schmidt and Tocci, 1990; Hirano and Kishimoto, 1990), were also incapable of stimulating significant numbers of cells to generate oriented Glu MTs. Extracellular matrix components such as fibronectin or collagen were also ineffective when added directly to SFM. Insulin and the insulin-like growth factors, IGF-I and IGF-II, all induced higher percentages (~30% of the CS response) of the cells to respond than did the proliferative growth factors; however, under optimal conditions the response was less than half that observed with TGF-βs. Combinations of TGF-β1 and insulin or IGFs did not increase the level of oriented Glu MT formation above that observed with TGF-β1 alone (Table 2). Similarly, pairwise combinations of TGF-β1 or insulin/IGFs with each of the proliferative growth factors did not increase the percentage of responding cells above that observed with TGF-β1 or insulin/IGFs alone (Table 2). In fact, PDGF, EGF and FGF all appeared to slightly decrease the response to TGF-β1 (Table 2).

Characterization of the TGF-β induction of Glu MT formation

Because TGF-β1 and TGF-β2 were the only polypeptide growth factors we tested that were capable of inducing the formation of Glu MTs in a significant number of cells and at a level approaching that of CS, we chose to characterize the TGF-β response further. The induction of Glu MTs by TGF-β1 and β2 appeared qualitatively and quantitatively similar, so we chose to characterize in detail only the response to TGF-β1 (throughout the rest of the paper we will use TGF-β to refer to TGF-β1, unless otherwise indicated). The level of Glu MTs, on a per cell basis, in cells treated with 2 ng/ml TGF-β for 7 hours was similar or perhaps slightly lower than that in cells treated with 10% CS for 7 hours (Fig. 4a and c). However, by 24 hours of treatment with 2 ng/ml TGF-β, the number of Glu MTs was as high or higher than that in cells treated with 10% CS for 24 hours (Fig. 4b and d). In addition to these apparent

quantitative differences in Glu MT levels, which were confirmed by western blot analysis (see below), we also found that the distribution of Glu MTs in cells at the wound edge of cultures treated with TGF-β was frequently parallel to the wound edge rather than perpendicular as is usually observed in control cells (this difference is more evident in the cells shown in Fig. 8).

To further characterize the TGF-β response, we performed a series of experiments to determine the time and concentration dependence of the TGF-β induction of oriented Glu MTs in wounded cells. At an optimal concentration of TGF-β (2 ng/ml), the increase in the number of serum-starved cells that exhibited oriented Glu MTs was first detectable 2-4 hours after wounding (Fig. 5a). The number of cells exhibiting oriented Glu MTs increased over the next couple of hours and reached a plateau at 7-10 hours. This plateau was maintained for at least 24 hours if TGF-β was kept in the medium. Higher concentrations of TGF-β did not induce a more rapid increase in oriented Glu MTs. In contrast to TGF-β, CS was capable of inducing oriented Glu MTs in a significant number of cells by 60 minutes after wounding (Fig. 5a). Thus, the TGF-β response lagged behind that of CS. It is worth noting that many of the known cellular responses to TGF-β exhibit a similar course of onset (see Discussion).

Fig. 5b shows the concentration dependence of the TGF-β induction of oriented Glu MTs. As little as 0.01 ng/ml TGF-β

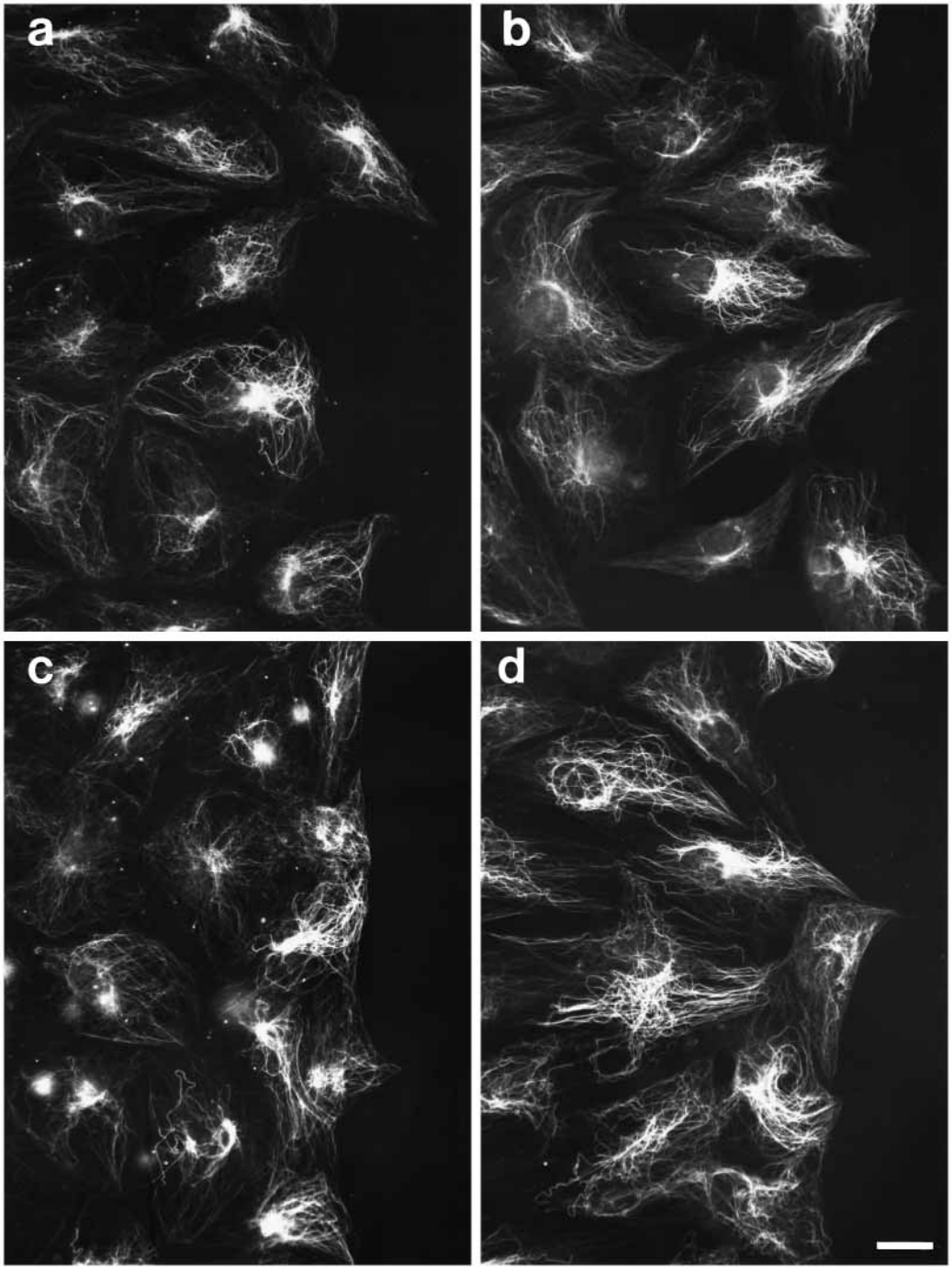

Fig. 4. Distribution of Glu MTs in SFM-treated monolayers after refeeding SFM containing CS or TGF-β. SFM-treated, confluent 3T3 cells were wounded and refed SFM containing 10% CS (a,b) or 2 ng/ml TGF-β (c,d). The cells were fixed at either 7 hours (a,c) or 24 hours (b,d) and processed for immunofluorescence. Glu MTs are shown in (a-d). Tyr MTs did not show significance differences between the different treatments and are not shown. Bar, 20 μm.

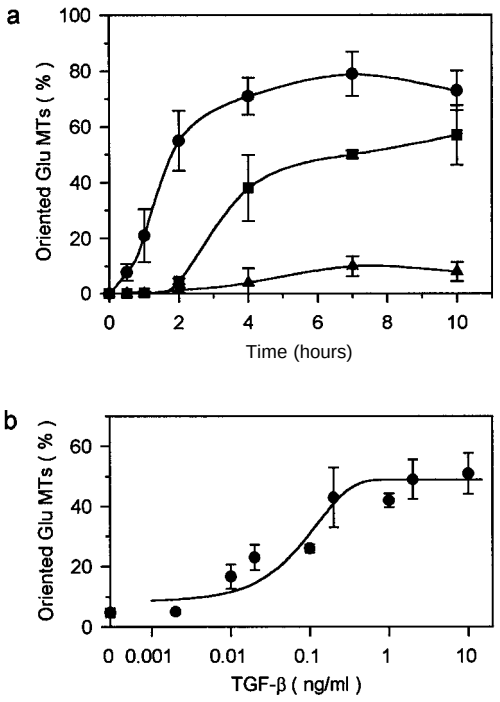

Fig. 5. Time dependence (a) and concentration dependence (b) of TGF-β induction of oriented Glu MTs. In (a) confluent 3T3 cells were treated with SFM for 48 hours, wounded and then refed SFM (▲), 10% CS (●) or 2 ng/ml TGF-β (■). The cells were then fixed at various times, processed for immunofluorescence and the level of oriented Glu MTs assessed (see Materials and Methods). In (b) wounded, SFM-treated monolayers were refed TGF-β at the indicated concentrations, fixed after 7 hours and then processed to assess the levels of oriented Glu MTs. In (a) and (b) the results shown were obtained by scoring over 100 cells for each point in each of 4 separate experiments. Error bars are standard deviations.

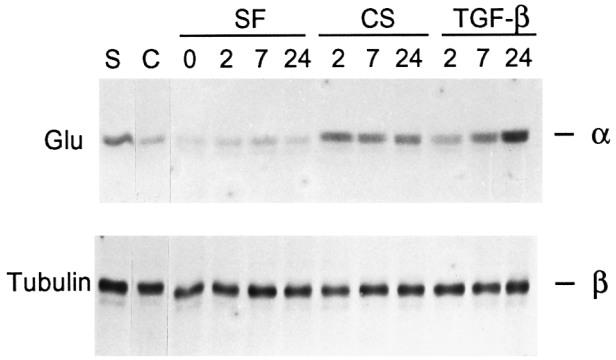

Fig. 6. Western blot analysis of Glu tubulin and total tubulin levels in SFM-treated 3T3 cells after refeeding SFM, CS or TGF-β. SDS samples were prepared from 3T3 cells after the treatments indicated below and subjected to western blot analysis (see Materials and Methods). Samples for determination of Glu tubulin levels were loaded at 20 μg of extract protein per lane and those for total tubulin levels were loaded at 5 μg extract protein per lane. The top panel (Glu) shows a blot probed with Glu tubulin antibody and the bottom panel shows a blot probed with β-tubulin antibody. Only the relevant portions of the blots are shown; α indicates the position of α-tubulin and β indicates the position of β-tubulin. The individual lanes are as follows: S, sparse cells; C, confluent cells; SF (0,2,7,24), SFM-treated confluent cells refed SFM for 0, 2, 7 or 24 hours; CS (2,7,24), SFM-treated confluent cells refed 10% CS for 2, 7, 24 hours; TGF-β (2,7,24), SFM-treated confluent cells refed 2 ng/ml TGF-β for 2, 7, 24 hours. Similar results were obtained in three separate experiments.

(0.4 pM) induced a detectable level of oriented Glu MTs. The half-maximal response was observed at ~0.1 ng/ml TGF-β and the maximal response occurred at concentrations of TGF-β ≥ 1 ng/ml. These data show that the TGF-β effect on stable MTs occurs at pM concentrations of TGF-β, suggesting that the effect of TGF-β on MTs is mediated by specific interaction of TGF-β with its high-affinity receptor/binding proteins (see Discussion).

To confirm the immunofluorescence data, we performed a western blot analysis of samples prepared from wounded, serum-starved cells treated with SFM alone, or SFM supplemented with 10% CS or 2 ng/ml TGF-β. A representative example of such an experiment is shown in Fig. 6. In general the western blot results directly corroborated the immunofluorescence results. For example, a reduction in Glu tubulin levels was observed in samples prepared from confluent cells treated with SFM for 48 hours compared to confluent cells that were harvested before SFM treatment (see Fig. 6, lane SF 0 versus lane C). The confluent cells, in turn, had a lower level of Glu tubulin than that of their sparse counterparts (see Fig. 6, lane C versus lane S), consistent with previous results showing that cell-cell contact reduced the level of Glu MTs in fibroblasts (Nakasaki et al., 1992). Despite these differences in Glu tubulin levels, no significant difference in the overall levels of tubulin (as shown by their β-tubulin levels, Fig. 6, Tubulin) could be detected between these two samples. In fact, we did not observe significant differences in the overall tubulin level between any of the different treatments in this study (see Fig. 6, Tubulin).

In serum-starved cells treated with 10% CS, we detected an increase in Glu tubulin levels as early as 2 hours after treating serum-starved cells with 10% CS (Fig. 6, lane CS 2); this increased level of Glu tubulin was maintained up to 24 hours (Fig. 6, CS 24). The level of Glu tubulin in the CS-refed cells mirrored closely that in sparse cells that had never been serum-starved. The lag in the TGF-β response observed by immunofluorescence (relative to that of CS) was also found by western blotting. After a 2 hour treatment with 2 ng/ml TGF-β, virtually no increase in Glu tubulin was observed, whereas at 7 hours the level of Glu tubulin was nearly the same as that of CS (see Fig. 6). Also, as expected from the immunofluorescence data, the level of Glu tubulin after 24 hours of treatment with TGF-β was higher than that found for CS-treated cells. Only slight increases in Glu tubulin levels were observed at any of the time points in cells refed fresh SFM. The immunoblot data thus confirm the immunofluorescence data and suggest that the changes in Glu MT levels can be explained by quantitative differences in the Glu tubulin levels in the various samples.

TGF-β and CS induce the formation of nocodazole-resistant MTs in serum-starved cells

As noted in the Introduction, the presence of elevated levels of Glu tubulin (as detected by immunofluorescence) in MTs has

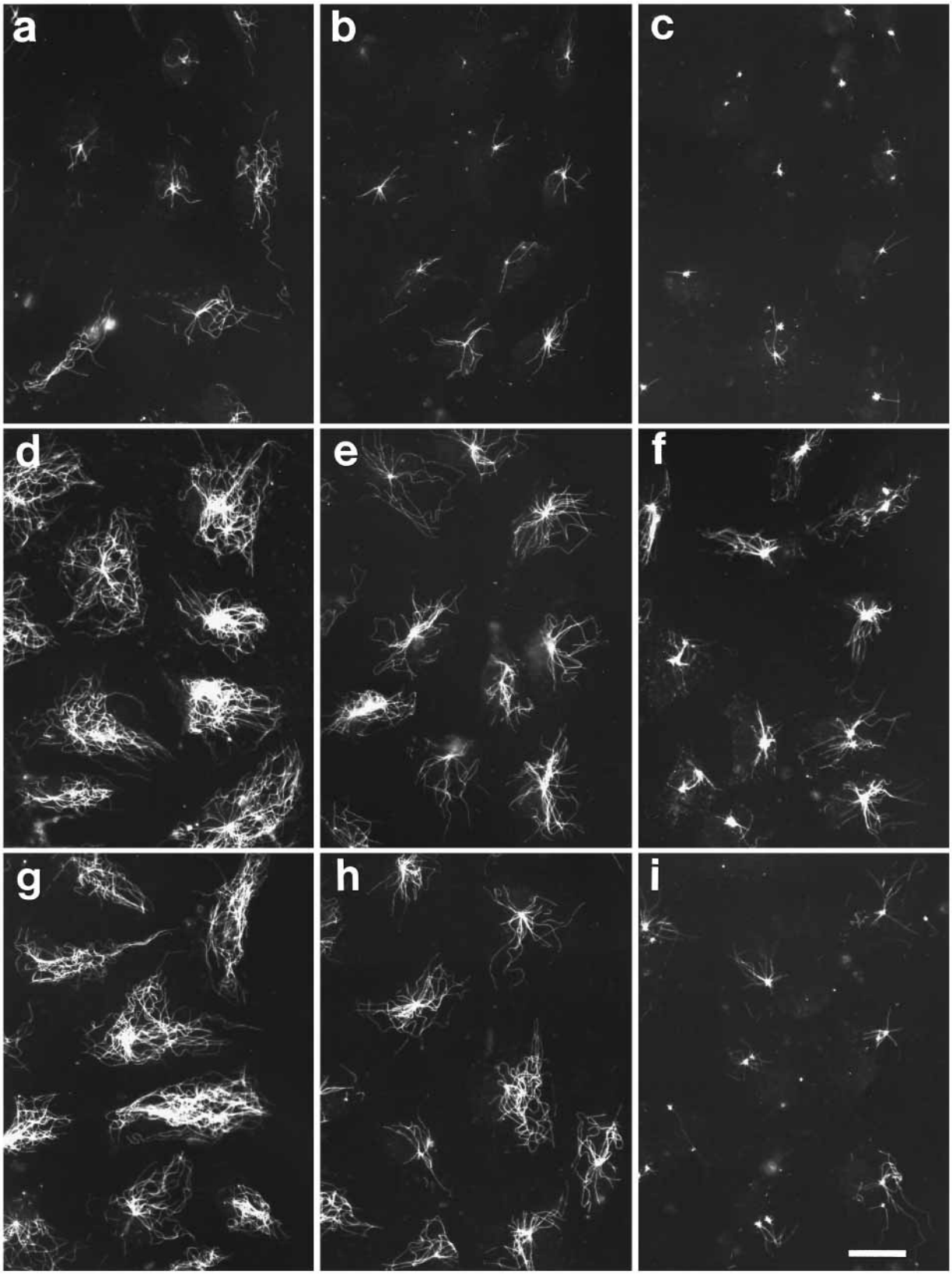

Fig. 7. Nocodazole resistance of MTs in wounded, SFM-treated monolayers refed SFM, CS or TGF-β. Confluent monolayers of 3T3 cells were treated with SFM for 48 hours, wounded and then refed SFM (a-c), 10% CS (d-f) or 2 ng/ml TGF-β (g-i) for 7 hours. Individual cultures were then treated with 2 μM nocodazole for various times before extraction and fixation for immunofluorescence. Shown are MTs revealed by β-tubulin immunofluorescence after nocodazole treatments of 10 minutes (a,d,g), 20 minutes (b,e,h) and 60 minutes (c,f,i). Bar, 20 μm.

been a reliable marker for the enhanced stability of MTs in cells. Nonetheless, to provide an independent test that the induction of Glu MTs by TGF-β and CS reflected the generation of stable MTs, we examined the sensitivity of MTs in TGF-β-, CS- or SFM-refed cells to nocodazole treatments of different lengths. To detect MTs in these experiments, we used a general β-tubulin-specific antibody, so that all of the MTs would be revealed. As previous studies have found (Khawaja et al., 1988), when we double-stained nocodazole-treated cells with the β-tubulin antibody and the Glu tubulin antibody, we found that virtually all of the nocodazole-resistant MTs (i.e. those detected with β-tubulin antibody) were also brightly labeled with antibody to Glu tubulin (data not shown).

In serum-starved cells refed fresh SFM for 7 hours, most of the MTs were depolymerized by 2 μM nocodazole within 10 minutes and after 20 minutes ≤10 MTs were observed in each cell (Fig. 7a and b). There were virtually no MTs in these SFM-refed cells that were resistant to nocodazole for 60 minutes (Fig. 7c). In contrast, cells refed CS for 7 hours exhibited numerous MTs resistant to nocodazole at all time points (Fig. 7d-f). Cells refed TGF-β for 7 hours contained a level of nocodazole-resistant MTs intermediate between that of SFM- and CS-refed cells (Fig. 7g-i). This can be seen most clearly by comparing the number of MTs remaining in the cells after 20 minutes of nocodazole treatment. Also note that there were a significant number of nocodazole-resistant MTs in the CS- and TGF-β-treated cells even after 60 minutes of treatment (Fig. 7f and i), suggesting that at least some MTs in these cells were substantially resistant to nocodazole. In cells treated with CS for 24 hours, the number of MTs resistant to nocodazole was similar to that in cells after 7 hours of CS. In comparison, in cells treated with TGF-β for 24 hours, the number of MTs resistant to nocodzole was higher than that observed in cells after 7 hours of TGF-β and instead was approximately the same as that in CS-treated cells (data not shown).

TGF-β treatment induces Glu MT formation in the absence of stimulation of either cell proliferation or cell locomotion

To determine whether the induction of oriented Glu MTs required re-entry of cells into the cell cycle, we monitored DNA synthesis using the BrdU technique to detect nuclei that had synthesized DNA during a particular interval (see Materials and Methods). In preliminary experiments, we found that refeeding the serum-starved 3T3 cells 10% CS resulted in the initiation of DNA synthesis by 12-14 hours and routinely

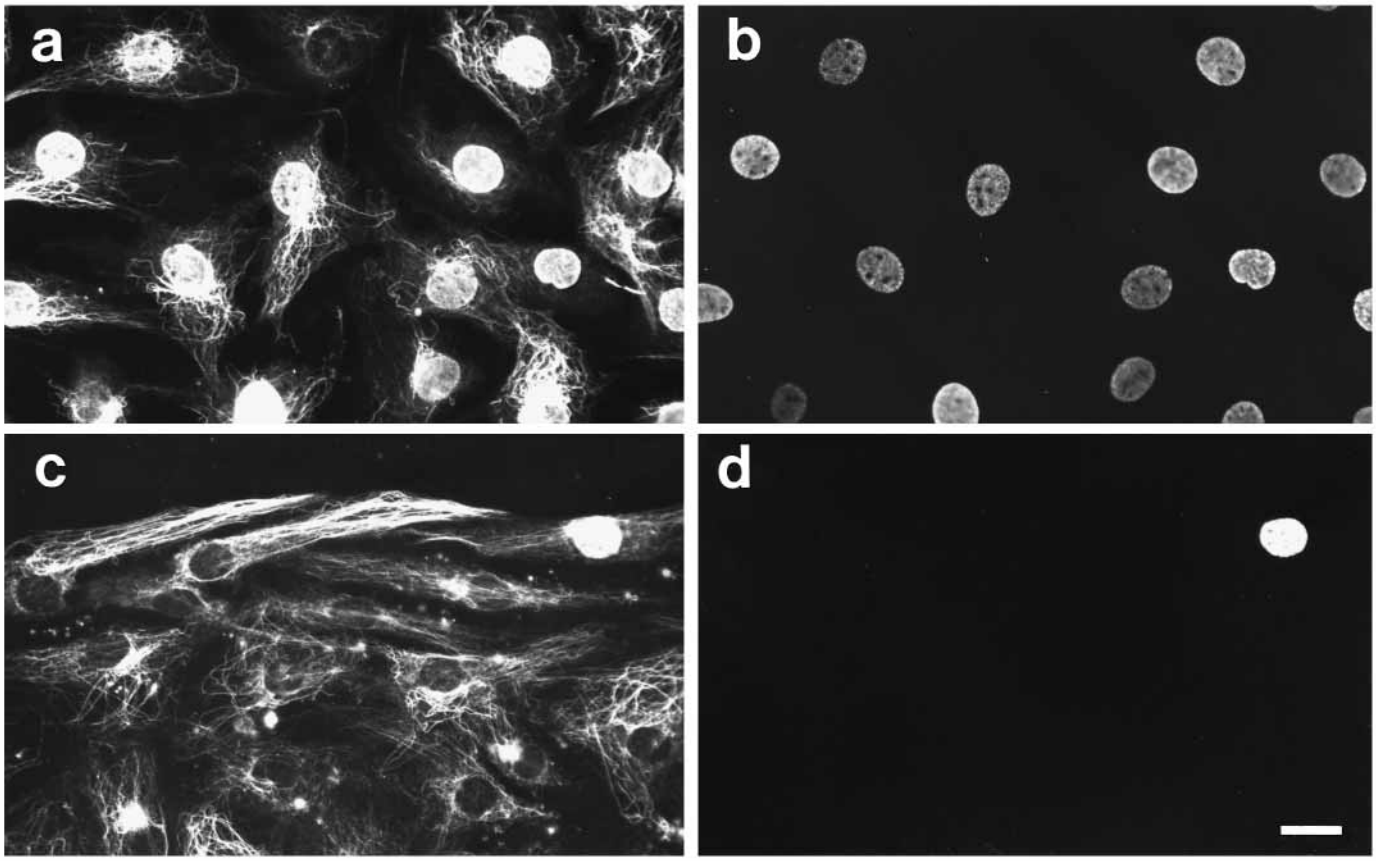

Fig. 8. Distribution of Glu MTs and extent of proliferation of wounded, SFM-treated monolayers refed CS or TGF-β. Confluent monolayers of 3T3 cells were treated with SFM for 48 hours, wounded, and then refed 10% CS+10 μg/ml BrdU (a,b) or 2 ng/ml TGF-β+10 μg/ml BrdU (c,d) for 24 hours. Cultures were processed for double immunofluorescence of Glu tubulin and BrdU (see Materials and Methods). The distribution of Glu MTs is shown in (a) and (c) and the corresponding distribution of nuclei labeled with BrdU antibody is shown in (b) and (d). The Glu MT images were taken using relatively broad fluorescein excitation and emission filters to allow simultaneous visualization of the rhodamine fluorescence of the labeled nuclei. Bar, 20 μm.

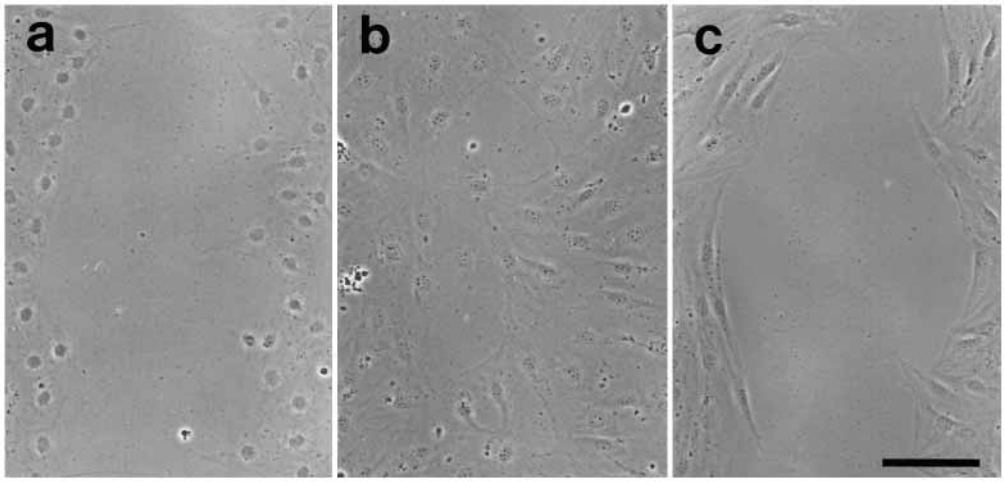

Fig. 9. Phase-contrast images of wounded, SFM-treated cultures refed SFM, CS or TGF-β. Confluent 3T3 cells were treated with SFM for 48 hours, wounded, and then refed SFM (a), 10% CS (b), or 2 ng/ml TGF-β (c) for 24 hours. Phase-contrast images of representative fields of methanol-fixed cultures are shown. Note that whereas distinct wounds are visible in the SFM- or TGF-β-treated monolayers, there is no wound apparent in the CS-treated monolayer. Bar, 100 μm.

stimulated over 80% of the cells to initiate DNA synthesis within a 24 hour period. In the absence of added CS, ≤1% of the cells re-entered the cell cycle by this criterion. Thus, our regime of serum starvation rendered the cells quiescent but fully capable of re-entering the cell cycle if refed appropriate growth factors. In comparison to CS, TGF-β only marginally stimulated DNA synthesis in the serum-starved cells; at the wound edge the percentage of labeled nuclei was generally about 10% after 24 hours of treatment with 2 ng/ml TGF-β. Even fewer of the cells within the monolayer contained labeled nuclei after TGF-β treatment. A longer incubation (48 hours) or higher concentrations of TGF-β (10 ng/ml) did not significantly increase the percentage of cells with labeled nuclei. These results suggests that TGF-β, by itself, was incapable of stimulating 3T3 cell proliferation under our conditions, consistent with numerous other studies demonstrating that TGF-β is generally not mitogenic by itself (see Roberts and Sporn, 1990).

Given that 2 ng/ml TGF-β consistently stimulated oriented Glu MT formation in ~50% of the cells (see Table 2), a purely statistical comparison indicates that re-entry into the cell cycle was not necessary for the generation of Glu MTs. However, since the BrdU method of assessing re-entry of cells into the S phase of the cell cycle is compatible with immunolocalization of MTs, we were able to demonstrate this point on an individual cell basis. Fig. 8 shows wounded monolayers that had been incubated with BrdU throughout a 24 hour incubation and then stained with antibodies to Glu tubulin and BrdU. Virtually all of the cells that exhibited oriented Glu MTs after CS refeeding also had BrdU-labeled nuclei (Fig. 8a and b), whereas many of the cells that contained oriented Glu MTs after TGF-β treatment, did not have BrdU-labeled nuclei (Fig. 8c and d). (Note that the CS treatment stimulated cell migration (see below), so that by 24 hours the wounded monolayer has largely healed; thus, in Fig. 8a and b the wound appears filled in with cells from the two edges of the wound rather than cells at the edge of a distinct wound.) The ~10% of cells that possessed BrdU-labeled nuclei after TGF-β treatment had about the same percentage of oriented Glu MT-positive cells as did the cells without labeled nuclei. The lack of nuclear BrdU staining in some TGF-β-treated cells that showed abundant oriented Glu MTs clearly demonstrates that re-entry of the cells into S phase of the cell cycle (and thus probably the entire cell cycle) is not a prerequisite for the generation of oriented Glu MTs.

The initiation of cell locomotion also did not seem to be a necessary prerequisite for the formation of oriented Glu MTs by TGF-β-treated cells. Fig. 9 shows a low magnification phase-contrast image of wounded monolayers 24 hours after wounding. Serum-starved cells that have been wounded and refed SFM do not migrate into the wound, whereas those refed SFM containing 10% CS have migrated into the wound and have come into contact with cells on the other side (compare Fig. 9a and b). In contrast, if serum-starved cells were wounded and refed TGF-β, virtually no migration of cells into the wound occurred (Fig. 9c). In fact, many of the wound edge cells from TGF-β-treated cultures appeared to align parallel, rather than perpendicular, to the wound edge as was observed with CS-treated cells. Thus, TGF-β is capable of stimulating the formation of oriented Glu MTs without activating cell migration.

DISCUSSION

In this paper, we have shown that the stability of MTs in fibroblasts is acutely dependent upon external factors in serum. Furthermore, we have shown that a single polypeptide growth factor, TGF-β, is capable of inducing the formation of stable MTs. To our knowledge this is the first demonstration that the stability of MTs in cycling cells in culture is subjected to external controls by growth factors. There have been reports of changes in MTs during the mitogenic response; however, these studies have primarily been concerned with the distribution of MTs (Bockus and Stiles, 1984) or with biochemical changes in microtubule-associated proteins (MAPs) (Shaw et al., 1988; Sato et al., 1988). Our results show that the generation of stable MTs is an early response to mitogenic stimulation (CS stimulated both oriented Glu MT formation and cell cycle re-entry) and raise the possibility that changes in MT stability are important for progression of the cell cycle.

That stable MTs in cells are under some form of regulation has been suggested by observations that stable MTs are not present in cells just after mitosis but are re-formed within several hours (Gundersen and Bulinski, 1986b; Bulinski et al., 1988) and that treatment with okadaic acid or calyculin A

(Ser/Thr-protein phosphatase inhibitors) results in the selective breakdown of stable MTs in cultured cells (Gurland and Gundersen, 1993). Also, we have observed the rapid depletion of stable MTs from the leading edge of colliding fibroblasts (Nagasaki et al., 1992). Our current results show that the *formation* of stable Glu MTs is regulated during the CS-stimulated re-entry of cells into the cell cycle from a quiescent state. Presumably, this reflects the stimulation of intracellular signalling molecules, due to binding of mitogens to their receptors. With CS, this intracellular signalling probably involves post-translational alterations, since the response is so rapid. We first detect Glu MTs 30-60 minutes after refeeding CS to serum-starved cells (see Figs 3, 5). Yet, the formation of immunodetectable Glu MTs may lag ~30 minutes behind the actual event(s) that stabilize the MTs (e.g. note the 30 minutes lag before detecting Glu MTs after taxol addition, see Fig. 1, insets). This suggests that the actual stabilization event after CS refeding may occur within minutes. We have also found that protein synthesis inhibitors do not block the onset or extent of the CS-stimulated increase in stable MTs (data not shown). These data suggest that the machinery to produce stable MTs is present in quiescent cells, but in an inactive state, and that serum post-translationally activates factor(s) that stabilize a subset of the MTs.

Of the purified growth factors we tested in this study, only TGF-β1 and β2 induced a significant level of stable MTs in the serum-starved cells. This suggests on the one hand that receptor Tyr kinase pathways, which would be stimulated by PDGF, EGF, FGF or insulin, are not sufficient by themselves to induce stable MTs, whereas pathways stimulated by the TGF-β receptor(s) would be possible candidates. Although relatively little is known about the signal transduction events triggered by TGF-β, the recent cloning of one of the TGF-β receptors has shown that the receptor contains homology to Ser/Thr protein kinases (Lin et al., 1992). TGF-β-induced alterations in phospho-proteins would be generally consistent with the idea that stable MTs are regulated by protein phosphorylation of stabilizing proteins (Gurland and Gundersen, 1993). Nevertheless, it is worth noting that the induction of stable MTs by TGF-β lagged behind that of CS by several hours, so it is possible that the CS and TGF-β responses are transduced by different intracellular pathways. Because of technical reasons, we have not been able to determine whether the TGF-β response requires protein synthesis. Thus, at this point, we are unable to conclude whether TGF-β stimulated the formation of stable MTs through a post-translational mechanism or through the increased synthesis of some factor involved in stabilizing MTs. Given the slow induction of stable MTs by TGF-β (2-4 hours onset) and the similar time course for the induction of fibronectin and collagen synthesis by TGF-β (Ignotz and Massague, 1986; Fine and Goldstein, 1987; Roberts et al., 1988), it seems possible that TGF-β results in the increased synthesis of some factor that is limiting for stable MT formation. Presumably, as this factor(s) accumulates in the cell, it is able to overcome the post-translational mechanisms that prevent the formation of stable MTs. Consistent with this hypothesis is the superinduction of stable MTs in cultures treated with TGF-β for 24 hours (Fig. 4c and d; Fig. 6).

While CS stimulated cell locomotion and entry of cells into the cell cycle, TGF-β did not. This result is interesting, since it shows that neither cell locomotion nor cell proliferation is necessary for the formation of stable MTs. We had previously found that oriented stable MTs were formed in cells at the edge of a wounded monolayer prior to the onset of cell migration (Gundersen and Bulinski, 1988) and our current results provide another example of the uncoupling of these processes. We did not test in this study whether the formation of stable MTs is a prerequisite for locomotion of these cells and this remains an open question awaiting the development of specific inhibitors of stable MT formation. In a separate study, we have found that at least some MTs are required for the migration of fibroblasts into a wound (B. Prairie, unpublished observations).

That TGF-β may regulate fibroblast motility, perhaps through the formation of stable MTs, is generally consistent with the known effects of TGF-β on cell motility. TGF-β has previously been shown to be chemotactic for fibroblasts (Postlethwaite et al., 1987) as well as other cells (Wahl et al., 1987; Koyama et al., 1990). Unlike these results, in our studies we did not find that TGF-β alone was sufficient for enhancing cell migration. This may be partially due to the fact that we optimized the TGF-β concentration for formation of Glu MT rather than cell locomotion. In the earlier studies, a sharp TGF-β concentration optimum for chemotaxis was found in each case (peak stimulation occurred at ~10 pg/ml; virtually no stimulation occurred > 100 pg/ml). We have done most of our studies at a significantly higher concentration of TGF-β and this may not stimulate locomotion. Nonetheless, in preliminary studies with lower concentrations of TGF-β, we still failed to detect significant stimulation of locomotion. Thus, other factors (e.g. differences in cell type, state of the cells, assay conditions, etc.) may contribute to the inability of TGF-β to stimulate the locomotion of the serum-starved 3T3 cells used in our study.

With respect to cell proliferation, our results with TGF-β clearly show that re-entry into the cell cycle is not a necessary event for the formation of stable MTs. In fact, if anything, re-entry into the cell cycle results in a lower level of stable MTs (compare 24 hour cultures treated with CS and TGF-β; Fig. 6). Also, the proliferative growth factors, PDGF, EGF and FGF, all tended to lower the level of stable MTs induced by TGF-β (see Table 2). This observation may be related to the finding that the induction of cellular differentiation and the concurrent withdrawl from the cell cycle, leads to the generation of stable, post-translationally modified MTs in a number of systems (reviewed by Bulinski and Gundersen, 1991).

Related to the question of whether CS and TGF-β induce stable MTs through different or similar pathways is the question of whether TGF-β is the factor in serum responsible for the serum effect. Several properties of the serum activity are consistent with the factor being TGF-β. In common with the properties of TGF-β (Childs et al., 1982; Assoian et al., 1983), the serum activity was non-dialyzable, heat-stable, resistant to protease and acid treatments and inactivated by DTT. The ED_{50} for CS was 1.0% (Table 1), which would be consistent with the reported amounts of TGF-β in serum (5-20 ng/ml; Childs et al., 1982; O'Conner-McCourt and Wakefield, 1987; Huang et al., 1988) and the ED_{50} of TGF-β of 0.1 ng/ml (Fig. 5b). The lower activity of platelet-poor plasma (ED_{50} of 2.7%, Table 1), also supports the notion that TGF-β is responsible for the serum/plasma effect, since plasma would be expected to contain less TGF-β (Childs et al., 1982; Assoian

et al., 1983). Despite this evidence, we have been unable to consistently block the CS response with neutralizing antibodies to TGF-β1 and TGF-β2 (unpublished observations; antibodies were kindly provided by Dr M. Sporn and have been shown to block the response to TGF-β in other systems; Danielpour et al., 1989). The the lack of antibody blocking may simply be a technical problem. On the other hand, it may indicate that some other factor in CS is capable of inducing stable MTs or that the TGF-β in CS is in a latent form and is not inhibitable with antibodies (Lawrence, 1991), but is still available to the cells.

Differences in the CS response and the TGF-β response also raise the possibility that there is a second factor in serum that influences the generation of stable MTs. The two principle differences in the responses were in the extent (TGF-β was only able to generate ~75% of the level of oriented stable MTs as that stimulated by CS) and in the time of onset (the TGF-β response lagged behind that of CS by ~2 hours). The former may simply be a scoring artifact. In CS, the cells develop a polarized, fan-shaped morphology and begin migrating into the wound; in TGF-β, the cells do not adopt this migratory morphology and do not locomote into the wound. Instead, the TGF-β-treated cells remain at the wound edge and many of them align parallel to this edge (see Figs 8, 9). When we analyzed these parallel-aligned cells, even if they contained abundant Glu MTs, which was frequently the case, we categorized them as not containing Glu MTs oriented toward the wound. Thus, our method of analyzing the data may underestimate the TGF-β effect on stable MTs. In support of this idea, it is worth noting that both CS and TGF-β induced the formation of Glu MTs in 80-90% of the cells if the orientation of the Glu MTs was ignored in the analysis. Also, by immunofluorescence (Fig. 4) and by western blotting (Fig. 6) the 24 hour cultures of TGF-β-treated cells contained levels of Glu MTs even higher than those of calf serum-treated cells.

In contrast, the difference in onset times between serum and TGF-β is unlikely to be due to a scoring artifact. When orientation of the Glu MTs was ignored in the analysis, or when just the level of Glu tubulin was considered (Fig. 6), TGF-β-treated cells were found to lag behind the CS-treated cells. Thus, either the added, purified TGF-β is not as readily accessible to the cells as the TGF-β in serum or there is some additional factor present in serum that can shorten the TGF-β lag time or can trigger the response itself. That added TGF-β would not be as active as the latent serum TGF-β seems unlikely, since the latent serum TGF-β is known to be inactive on a number of different cell types (Lawrence, 1991). At this time we cannot rule out the latter possibility; however, we note that none of the other candidate polypeptide growth factors that we tried were able to generate a response themslves and, at least, neither insulin nor PDGF was able to decrease the TGF-β lag (data not shown). In fact, a number of these growth factors (PDGF, EGF or FGF) partially inhibited the TGF-β response (Table 2).

Whether the CS factor turns out to be TGF-β, our data clearly show that TGF-β1 and β2 are potent stimulators of MT stability. TGF-β stimulation of MT stabilization in fibroblasts may be important during wound healing responses in vivo. In rats, application of TGF-β to wounds has been shown to increase the response of fibroblasts at the wound site (Roberts et al., 1986; Mustoe et al., 1987). Another important site of TGF-β action appears to be in the developing embryo. Studies that have localized TGF-β in developing mouse embryos have demonstrated a very clear correlation between the tissues with the highest relative concentration of TGF-β and those that are undergoing morphogenesis (Heine et al., 1987). The majority of these examples concern the mesenchymally derived connective tissue, which contains fibroblasts as its predominant cell type. Other studies have shown that cultured cells will undergo distinct morphological changes when treated with TGF-β (Loef et al., 1986; Muller et al., 1987; Koyasu et al., 1988; Coomber, 1991). In this study, we have observed that TGF-β treatment results in the parallel alignment of some 3T3 fibroblasts with the wound. Since this cell shape alteration is probably related to alterations in MTs, perhaps even to their stabilization, this suggests that one of the important targets of TGF-β action on cells is the MT cytoskeleton. It will be interesting to determine whether the TGF-β induction of MT stability is a direct effect on the MTs and the proteins that comprise them or whether the induction is due to indirect effects of TGF-β on, for example, the actin cytoskeleton (Loef et al., 1986; Coomber, 1991) or the extracellular matrix (Ignotz and Massague, 1986; Fine and Goldstein, 1987; Roberts et al., 1988).

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