Generation of a Stable, Posttranslationally Modified Microtubule Array Is an Early Event in Myogenic Differentiation

Gregg G. Gundersen,* Sadiqa Khawaja, and Jeannette Choë Bulinski*

Department of Biology, University of California, Los Angeles, California 90024; and *Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York 10032

Abstract. Microtubules (MTs) have been implicated to function in the change of cell shape and intracellular organization that occurs during myogenesis. However, the mechanism by which MTs are involved in these morphogenetic events is unclear. As a first step in elucidating the role of MTs in myogenesis, we have examined the accumulation and subcellular distribution of posttranslationally modified forms of tubulin in differentiating rat L_6 muscle cells, using antibodies specific for tyrosinated (Tyr), detyrosinated (Glu), and acetylated (Ac) tubulin. Both Glu and Ac tubulin are components of stable MTs, whereas Tyr tubulin is the predominant constituent of dynamic MTs. In proliferating L_6 myoblasts, as in other types of proliferating cells, the level of Glu tubulin was very low when compared with the level of Tyr tubulin. However, when we shifted proliferating L_6 cells to differentiation media, we observed a rapid accumulation of Glu tubulin in cellular MTs. By immunofluorescence, the increase in Glu tubulin was first detected in MTs of prefusion myoblasts and was specifically localized to MTs that were associated with elongating portions of the cell. MTs in the multinucleated myotubes observed at later stages of differentiation maintained the elevated level of Glu tubulin that was observed in the prefusion myoblasts. When cells at early stages of differentiation (<1 d after switching the culture medium) were immunostained for Glu tubulin and the muscle-specific marker, muscle myosin, we found that the increase in Glu tubulin preceded the accumulation of muscle myosin. Thus, the elaboration of Glu MTs is one of the very early events in myogenesis. Ac tubulin also increased during L_6 myogenesis; however, the increase in acetylation occurred later in myogenesis, after fusion had already occurred. Because detyrosination was temporally correlated with early events of myogenesis, we examined the mechanism responsible for the accumulation of Glu tubulin in the MTs of prefusion myoblasts. We found that an increase in the stability of L_6 cell MTs occurred at the onset of differentiation, suggesting that the early increase in detyrosination that we observed is a manifestation of a decrease in MT dynamics in elongating myoblasts. We conclude that the establishment of an oriented array of microtubules heightened in its stability and its level of posttranslationally modified subunits may be involved in the subcellular remodeling that occurs during myogenesis.

THE differentiation of myoblasts in tissue culture is a complex process, involving dramatic changes in gene expression, transcription of new muscle-specific messages, and accumulation of myofibrillar proteins. In addition, cultured myoblasts undergo significant morphological changes during myogenesis; they assume an elongated shape, neighboring cells align in an appropriate orientation, and myoblasts eventually fuse with their neighbors to form multinucleated myotubes. Although a wealth of experimental detail is available concerning transcription and translation of muscle-specific gene products (Devlin and Emerson, 1978; Zevin-Sonkin and Yaffe, 1980; Minty et al., 1982) and fusion of myoblasts (Kalderon and Gilula, 1979; Senechal et al., 1982), the cellular factors responsible for morphogenesis during myogenesis are not yet well understood.

Dramatic alterations in the cytoskeleton are commonly thought to evoke morphogenetic events, and there is circumstantial evidence in support of the idea that the cytoskeleton is involved in the morphogenetic events of myogenesis. Early investigators showed microtubules (MTs),[1] along with intermediate filaments and stress fibers, coursing through the cytoplasm parallel to the long axis of both elongating myoblasts and postfusion myotubes (Okazaki and Holtzer, 1965; Fischman, 1970). MTs, specifically, have been implicated in myogenesis by virtue of their close juxtaposition to forming myofibrils (Warren, 1974) and by the effects of MT antagonists upon the differentiation of muscle cells. Both prefusion myoblasts and differentiated myotubes are altered in their

1. *Abbreviations used in this paper*: Ac MT, microtubule enriched in acetylated alpha-tubulin; Ac tubulin; acetylated alpha-tubulin; Glu MT, microtubule enriched in detyrosinated alpha-tubulin; Glu tubulin, detyrosinated alpha-tubulin; MHC, myosin heavy chain; MT, microtubule; TTL, tubulin tyrosine ligase; Tyr MT, microtubule enriched in tyrosinated alpha-tubulin; Tyr tubulin, tyrosinated alpha-tubulin.

morphology and acquire a disorganized array of myofibrils when cytoplasmic MTs are disrupted by MT-depolymerizing agents (Ishikawa et al., 1968; Toyama et al., 1982). Similarly, the MT-stabilizing compound, taxol, disrupts myogenesis in cultured cells, yielding cells with aberrantly formed myofibrils (Antin et al., 1981; Toyama et al., 1982).

While it is clear that the integrity of the MT array is required for normal myogenesis, the precise role played by MTs in effecting myogenesis is unknown. MTs seem to participate in two distinct events in the myogenic program: the elongation and alignment of myoblasts before their fusion into syncytial myotubes and the formation of myofibrils within the myotubes. Perhaps the better accepted of these is the organizing activity that MTs exhibit in the formation and organization of myofibrils. When myogenic cells are treated with taxol, actin-containing thin filaments do not form, while myosin-containing thick filaments align, instead, along the stabilized MTs (Antin et al., 1981; Toyama et al., 1982). This result suggests that within developing myotubes the MTs serve as "templates," which are depolymerized and replaced by thin filaments to generate the characteristic myofibrillar array of mature myotubes.

A role for MTs earlier in the myogenic program, during the cell elongation and alignment phase, has not been demonstrated directly. However, by inference from other morphogenetic systems in which there is pharmacological evidence that MTs are involved in cell shape changes (for a consideration of numerous examples see Dustin, 1984), one would predict a similar involvement of MTs in myoblast elongation and alignment. In fact, during myoblast elongation, the radially based MT array, with MTs centered at the centrosomes, begins to change to the more elongated, bipolar array that is ultimately observed in syncytial myotubes (Warren, 1974; Tassin et al., 1985). In myotubes, the MTs ultimately emanate from multiple sites around the periphery of the nuclei. While this change from a radial, centrosomal array of MTs to a bipolar, nuclear array of MTs is a characteristic feature of myogenesis, it is a postfusion alteration and, therefore, cannot function in the early morphogenetic events of myoblasts.

Of the mechanisms that might be involved in modulating MT function during the earliest stages of myogenesis, we chose to focus on posttranslational modifications of tubulin since, overall, the changes in MT arrays that are effected by posttranslational modification are relatively rapid when compared with other modulators of MT function; e.g., the differential expression of tubulin isoforms (Fulton and Simpson, 1976; Cleveland and Sullivan, 1985; Villesante et al., 1986) or of microtubule-associated proteins (Vallee, 1984; Olmsted, 1986). Two posttranslational modifications of tubulin, tyrosination and acetylation, are known to yield coexisting tubulin species that may be functionally distinct (Gundersen et al., 1984; Piperno et al., 1987). In fact, tyrosination/detyrosination, in which the COOH-terminal tyrosine of alpha-tubulin is reversibly removed (Barra et al., 1974; Argaraña et al., 1978), has been studied during several types of morphogenetic and differentiative events; alterations in the level of the enzyme that carries out tyrosination or in the level of tyrosinated alpha-tubulin (Tyr tubulin) have been temporally correlated with both neural (Rodriguez and Borisy, 1978; Deanin et al., 1977) and muscle (Deanin et al., 1977) development. Localization of the Tyr tubulin and detyrosinated alpha-tubulin (Glu tubulin) with specific antibodies has shown that most MTs in interphase cultured cells contain predominantly Tyr tubulin while a small subset of MTs is enriched in Glu tubulin (Glu MTs) (Gundersen et al., 1984; Gundersen and Bulinski, 1986; Geuens et al., 1986; Kreis, 1987; Wehland and Weber, 1987). Glu MTs arise by a postpolymerization modification of preexisting MTs composed of Tyr tubulin (Tyr MTs) (Gundersen et al., 1987); thus, Glu MTs are those that were polymerized less recently than Tyr MTs. In fact, in one study, Glu MTs were found to persist for at least 16 h, possibly throughout an entire cell cycle (Webster et al., 1987*b*). The dynamics of Glu MTs are in striking contrast with the dynamics of most cellular MTs (hence, Tyr MTs), whose half-time of depolymerization is 5–10 min (Schulze and Kirschner, 1986). While the function of stable, Glu MTs has not been identified, evidence obtained from 3T3 fibroblasts suggests that Glu MTs may be important for the determination of cell polarity; in 3T3 cells induced to undergo directed cell migration, an oriented array of Glu MTs is established during the cell polarization that occurs before the commencement of active cell migration (Gundersen and Bulinski, 1988).

Another posttranslational modification of alpha-tubulin has been described in which the epsilon-amino group of lysine 40 is acetylated (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987). The existing information concerning acetylation argues that this modification is mechanistically distinct from detyrosination, although the two modifications share some features. For example, in most types of interphase cells in culture, a subset of MTs contains segments enriched in acetylated alpha-tubulin (Ac tubulin) (Piperno et al., 1987; Bulinski et al., 1988); in cells in which both acetylation and detyrosination are detectable, the subsets of MTs enriched in Glu and Ac tubulin (called Glu and Ac MTs, respectively), are nearly coincident (Bulinski et al., 1988). Similarly, acetylation of alpha-tubulin has been shown to increase during developmental events such as neurite outgrowth (Black and Keyser, 1987; Sale et al., 1988). Ac tubulin, like Glu tubulin, is enriched in vivo in stabilized MTs (Piperno et al., 1987; Webster and Borisy, 1989).

In this paper, we have characterized the alterations of tubulin posttranslational modification that accompany myogenesis in cultured L_6 cells. We also demonstrate a dramatic change in MT dynamics that occurs early in myogenesis and present evidence suggesting that the stabilization of an array of MTs in myoblasts brings about their increased posttranslational modification. We propose that stable MTs oriented within elongating myoblasts may be involved in the morphological and differentiative changes of myogenesis.

Materials and Methods

Cell Culture

Except as noted, all tissue culture chemicals and solutions were obtained from Gibco Laboratories (Grand Island, NY). The L_6 myogenic line, originally isolated from rat muscle tissue by Yaffe (1968), was obtained from American Type Culture Collection (CRL 1458; Rockville, MD). L_6 cells were grown on 100-mm plastic tissue culture dishes or on glass coverslips. The growth medium used consisted of DME supplemented with 10% FBS (HyClone Laboratories, Logan, UT). In the differentiation medium, the serum was replaced with 3% equine serum (Flow Laboratories, Inc., McLean VA; equine serum used was heat inactivated at 50°C for 30 min). In general, proliferating L_6 cells were fed every other day and passaged (releasing them from the dish with Viokase) or differentiated when subconfluent. Differenti-

ation of L_6 cultures was measured by determining the percentage of nuclei that were found in multinucleated cells; for each time point, a total of 2,000 nuclei per dish were scored from random fields.

We assessed the stability of MTs during myogenesis by treating L_6 cells with the microtubule antagonist, nocodazole (Aldrich Chemical Co., Milwaukee, WI). Cells were exposed to 10 μM nocodazole (added directly to the culture medium from a 5 mM stock solution in DMSO) for 6 or 25 min. Drug-treated cells were then extracted for 1 min with 0.6% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in an MT-stabilizing buffer, essentially as described previously (Khawaja et al., 1988), to reduce the diffuse staining contributed by monomeric tubulin.

Indirect Immunofluorescence

Indirect immunofluorescence of Glu, Tyr, and Ac tubulin was performed on methanol-fixed cells as previously described (Bulinski et al., 1988). Rabbit polyclonal antibodies to Tyr and Glu tubulin (Gundersen et al., 1984) were used as 2 and 1% solutions, respectively, diluted in PBS or directly in Ac tubulin antibody (6-11-B1; a hybridoma supernatant kindly provided by Dr. Gianni Piperno, Rockefeller University, New York). Total cellular tubulin was detected using a 1% solution of an ascites fluid of a mouse monoclonal antibody reactive with beta-tubulin (3F3; the generous gift of Dr. James Lessard, University of Cincinnati, Cincinnati, OH). Muscle-specific myosin heavy chain (MHC) was detected in the fixed L_6 cells using a 1:10 dilution of a mouse hybridoma supernatant (MF20; kindly provided by Dr. D. Fischman, Cornell University Medical College, New York). Fluorescein-conjugated goat anti-mouse antibodies (diluted 1:50) were used to visualize primary antibodies to myosin and to total tubulin; other secondary antibodies were used as described (Bulinski et al., 1988).

Immunoblots

Western blots were performed and analyzed as previously described (Bulinski et al., 1988); blots stained for total beta-tubulin were incubated with a 1:2,000 dilution of 3F3 ascites fluid and processed analogously to the others. Total protein was determined with the modified Lowry assay of Geiger and Bessman (1972) or, for SDS samples, the procedure of Tornqvist and Belfrage (1976).

Assay of Tubulin Tyrosine Ligase (TTL)

TTL activity was determined using the assay procedure of Flavin and Murofushi (1984). Thrice-cycled bovine brain microtubule protein, purified as described by Vallee (1986) was used as the substrate in our assay. Cell extracts to be assayed were prepared as follows, with all steps carried out at 0–4°C: four 100-mm culture dishes (per time point) were washed twice with ligase stabilization buffer (25 mM morpholino ethane sulfonic acid, pH 6.8, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 1 mM DTT) and then once with the same buffer to which 20% glycerol and 0.2 mM PMSF had been added. Next, the cells were collected with a rubber policeman in 0.5–1 ml of the latter buffer and briefly homogenized with a motor-driven teflon and glass homogenizer. The homogenate was clarified by centrifugation (45 min at 40,000 rpm at 4°C in a type 65 rotor [Beckman Instruments, Inc., Fullerton, CA]). The supernatant was assayed immediately, although we found that storage at −80°C for up to 2 wk did not diminish the TTL activity.

Results

The rat L_6 myogenic line is a valuable model for the in vitro study of myogenesis. As shown in Fig. 1, upon changing the medium bathing the L_6 myoblasts from growth medium to differentiation medium, myogenesis ensued rapidly; cells first became elongated and aligned, and fusion commenced on day 1. Striated myofibrils began to appear on day 4 and began to show contractile behavior on day 8. We observed only slight variation in the kinetics of fusion from experiment to experiment (see error bars in Fig. 1). This uniformity and final high level of myogenic differentiation made L_6 cells amenable to the assay of tubulin forms in populations of cells at various stages of differentiation.

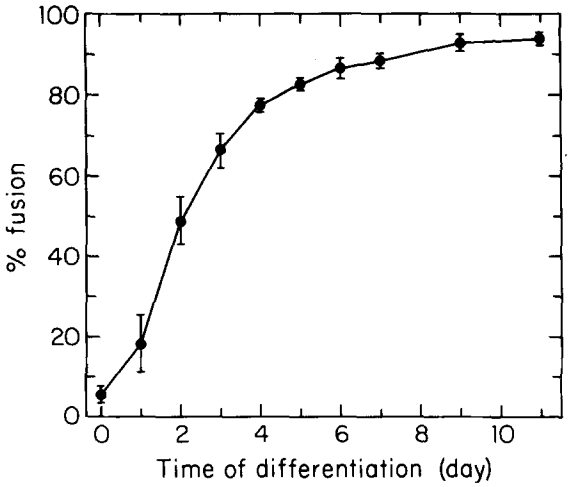

Figure 1. Time course of L_6 cell myogenesis. L_6 cells were switched to differentiation medium at day 0, and the extent of myoblast fusion (*% fusion*) was defined as the percentage of nuclei in the culture that were found in multinucleated myotubes. 2,000 nuclei were counted for each time point; data represent the mean ± SD from three separate experiments.

Alterations in Posttranslational Detyrosination of Tubulin during Myogenesis

To determine whether any change in posttranslational detyrosination occurred during the morphogenetic events of myogenesis, we compared the distribution of Tyr tubulin in L_6 cells (Fig. 2, *b, e, h,* and *k*) with that of total tubulin (data not shown), and Glu tubulin (Fig. 2, *a, d, g,* and *j*) at each stage of L_6 cell differentiation. At all stages, the distribution of Tyr tubulin mimicked that of total tubulin; thus, our results on the distribution of Tyr tubulin during myogenic differentiation resembled results obtained previously by others on the distribution of tubulin during myogenesis (Warren, 1974; Tassin et al., 1985). Before differentiation, the distribution of Tyr MTs in mononucleated myoblasts is similar to that observed in many types of undifferentiated cultured cells. Tyr MTs are focused at the centrosome, which is located near the nucleus, and extend throughout the myoblast cytoplasm to the cell periphery (Fig. 2, *b* and *c*). The relative flatness of the L_6 myoblasts is advantageous for immunofluorescence; some MTs can be traced from the cell margin almost all of the way back to the centrosome. As cells elongate and align for fusion, the Tyr MT array becomes oriented parallel to the long axis of the cell (Fig. 2, *e* and *f*). After fusion, the L_6 cells remain flat, such that distinct MTs can still be visualized by Tyr tubulin antibody staining along the length of the myotubes, parallel to the nascent myofibrils (Fig. 2, *h* and *i*). As previously reported by Tassin et al. (1985), at this stage of myogenesis MTs no longer appear to be organized by the centrosome. At later stages of myogenic development (e.g., at day 11; Fig. 2 *k*), we observed grainy, indistinct immunofluorescent staining with the Tyr tubulin antibody. This grainy pattern of tubulin staining, which was also obtained with other anti-tubulin antibodies, was unchanged in cells that were extracted with detergent to remove monomeric tubulin, suggesting that the grainy staining is attributable to polymeric tubulin within the myotubes (data not shown). Presumably, the density of the myofibrillar proteins in some regions of the cells rendered the immunofluorescent staining of MTs grainy and indistinct.

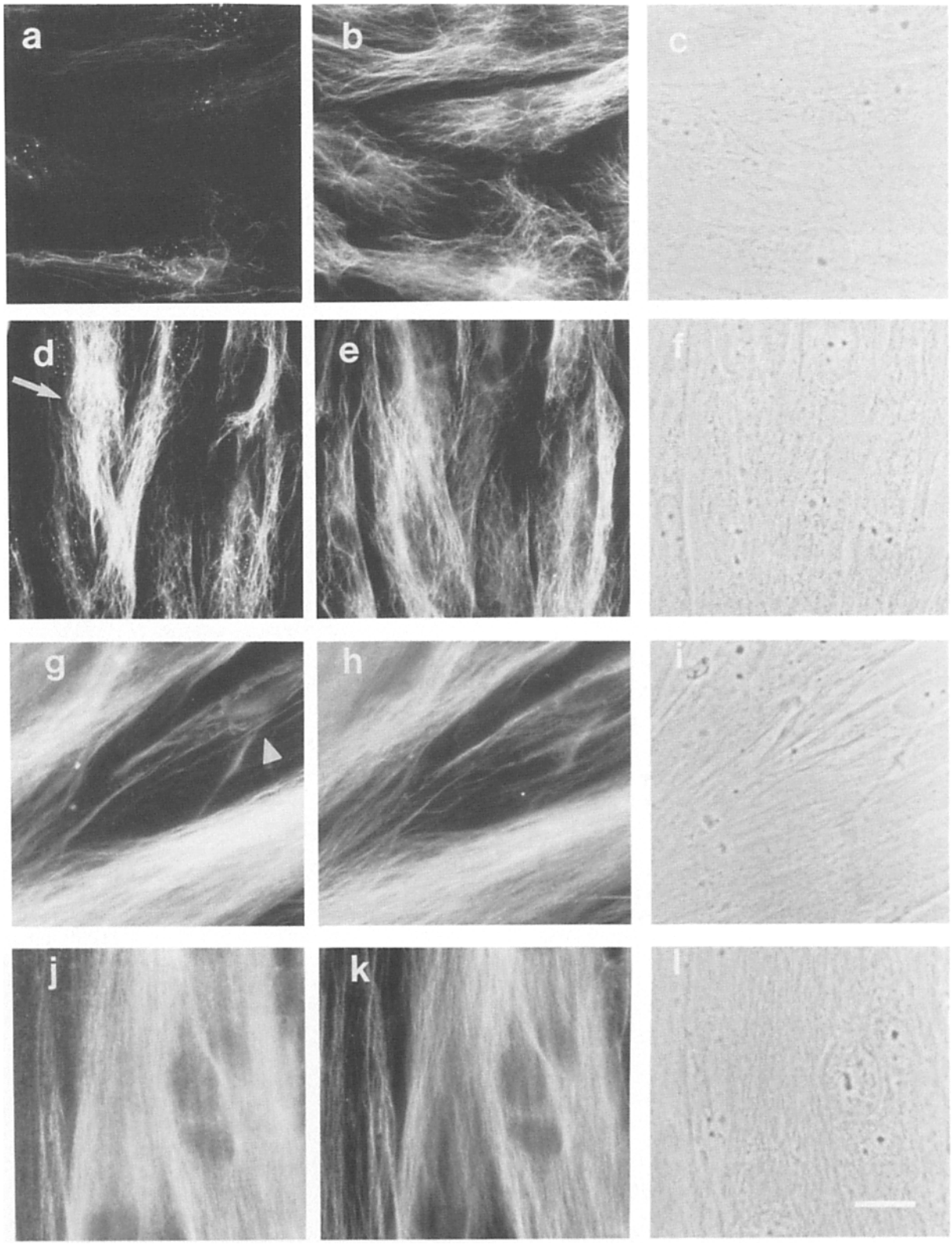

Figure 2. Immunofluorescence distribution of Glu and Tyr tubulin during myogenesis. L_6 cells at days 0 (*a–c*), 1 (*d–f*), 4 (*g–i*), and 11 (*j–l*) of myogenesis are shown; for each time point, the distribution of Glu (*a*, *d*, *g*, and *j*) and Tyr tubulin (*b*, *e*, *h*, and *k*) and the corresponding phase image are shown. Arrow in *d* indicates an elongated myoblast with virtually all MTs stained with the Glu tubulin antibody; arrowhead in *g* indicates an unfused myoblast amongst fused myotubes. Bar, 15 μm.

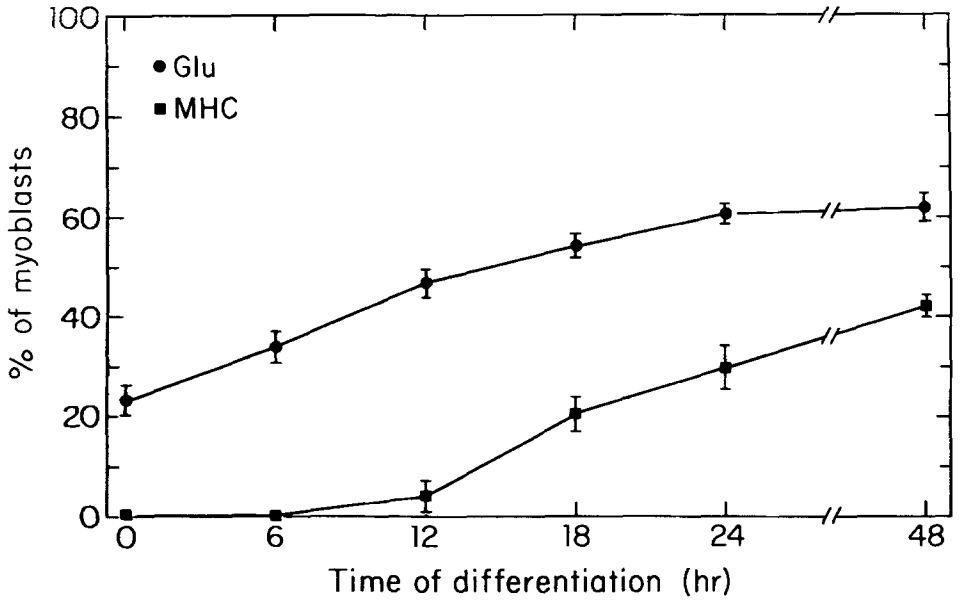

Figure 3. Appearance of muscle-specific MHC and Glu MTs during the early stages of L_6 cell myogenesis. Double immunofluorescent labeling was used to detect muscle-specific MHC (■) and Glu MTs (●) in myoblasts differentiated for the indicated intervals. Data was obtained from 300–500 cells in random fields for each time point. Data are expressed as the average of three separate determinations ± SD.

Glu tubulin appeared from immunofluorescent staining to constitute only a minor species in myoblasts; in only ~20% of the myoblasts were MTs detectably stained with the Glu tubulin antibody, and in these cells only a small number of MTs were brightly labeled (Fig. 2 *a*). Western blotting of L_6 cell extracts and brain tubulin standards with the Tyr and Glu tubulin antibodies confirmed that only a small proportion of the tubulin in myoblasts was detyrosinated (data not shown). Thus, both the distribution and level of Tyr and Glu tubulin in myoblasts are similar to that observed in many other types of proliferating cells (Gundersen et al., 1984; Gundersen and Bulinski, 1986; Geuens et al., 1986; Wehland and Weber, 1987; Kreis, 1987).

In contrast to the situation in proliferating myoblasts, we observed a dramatic increase in the level of Glu tubulin during myogenic differentiation. Even on day 1 after switching to differentiation medium, the L_6 cells showed a significant increase in the number of MTs that stained with the Glu tubulin antibody (Fig. 2, *d–f*). These day-1 cultures were composed predominantly of unfused myoblasts (Fig. 1 and Fig. 2 *c*); thus, it is clear that the increase in the number of Glu MTs occurred before actual myoblast fusion (also see below). In fused myotubes, which were first observed in day-1 cultures, we consistently found high levels of Glu tubulin staining. In fact, as is shown for myotubes in day-4 (Fig. 2, *g–i*) and day-11 (Fig, 2, *j–l*) cultures, virtually all of the MTs were brightly stained with the Glu tubulin antibody. Some graininess in the staining with Glu tubulin antibody, analogous to that observed with the Tyr or total tubulin antibodies, was observed in myotubes from long-term cultures (Fig. 2 *j*).

As described above, we found that an increase in Glu MT staining occurred before myoblast fusion. To more accurately pinpoint the timing of this change, we examined cultures of L_6 cells that had been switched to differentiation medium for ≤1 d. During the first day of myogenesis (from day 0 until day 1), the number of myoblasts that exhibited distinctly labeled Glu MTs increased from 22 to 60% (Fig. 3). Increased Glu tubulin staining was also manifested as an increase in the number of Glu MTs present in each myoblast; in fact, in the 60% of the myoblasts that exhibited Glu MTs on day 1, the number of Glu MTs in each cell was higher than in any myoblast at day 0 (e.g., compare Fig 2 *a* with *d*). Finally, in a significant proportion of day-1 myoblasts (~15%; Fig. 2 *d*, *arrow*), virtually all of the MTs were stained with the Glu tubulin antibody, a level of staining similar to that detected in fused myotubes. We have confirmed that the increases in Glu tubulin staining apparent from immunofluorescence assays correspond to actual increases in the level of Glu tubulin measured by quantitative immunoblotting (see below).

The results described above show that the increase in Glu MT staining occurs at an early, prefusion stage of myogenesis. To relate the timing of the increase in Glu tubulin to other well-characterized myogenic events, we correlated the timing of increased Glu MT staining with two prefusion markers of myogenic differentiation: the acquisition of an elongated morphology and the accumulation of muscle-specific MHC (Clegg et al., 1987). As rapidly as 6 h after the L_6 cells had been switched to differentiation medium, we observed an increase in the number of cells exhibiting Glu MT staining (Fig. 3). This increase in Glu MT staining was almost always associated with myoblasts that possessed an elongated morphology, and the Glu MTs were frequently localized to the extended areas of the cell (Fig. 4). Cells that maintained an unextended morphology never exhibited the high levels of Glu tubulin staining observed in elongated cells, although some of them did contain a small number of randomly distributed Glu MTs (Fig. 4).

As a second marker of early myogenic differentiation, we examined the accumulation of muscle-specific MHC. Accumulation of immunologically detectable MHC is frequently used as an early marker of myogenic differentiation and is known to occur in advance of myoblast fusion (Clegg et al., 1987). Fig. 5, *a* and *b*, shows the Glu tubulin and MHC immunofluorescent staining patterns of L_6 myoblasts that have been in differentiation medium for 24 h. All of the myoblasts positive for MHC also exhibit brightly labeled Glu MTs. In addition, several cells with labeled Glu MTs, but no detectable MHC staining, can also be seen (Fig. 5, *arrows*). In fact, when we examined cultures that had been switched to differ-

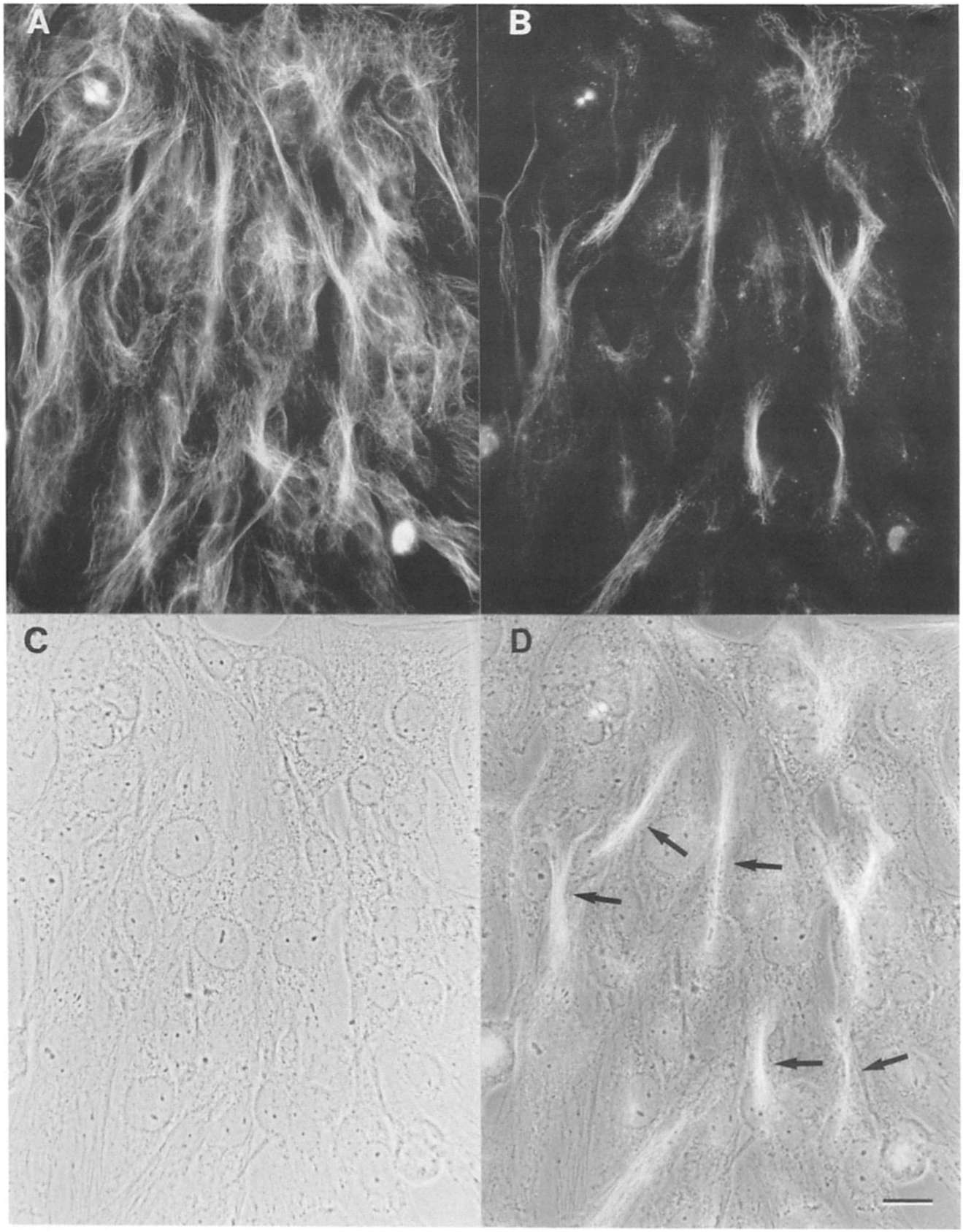

Figure 4. Distribution of Glu MTs in elongating myoblasts. Prefusion L_6 myoblasts were stained for (*a*) total tubulin and (*b*) Glu tubulin. (*c*) Phase image of the myoblasts shown in *a* and *b*; (*d*) Glu tubulin immunofluorescent staining (as in *a*) shown with simultaneous phase illumination to show Glu MTs oriented along the length of elongated myoblasts (*arrows*). The field of cells included in the micrograph contains only myoblasts. Bar, 15 μm.

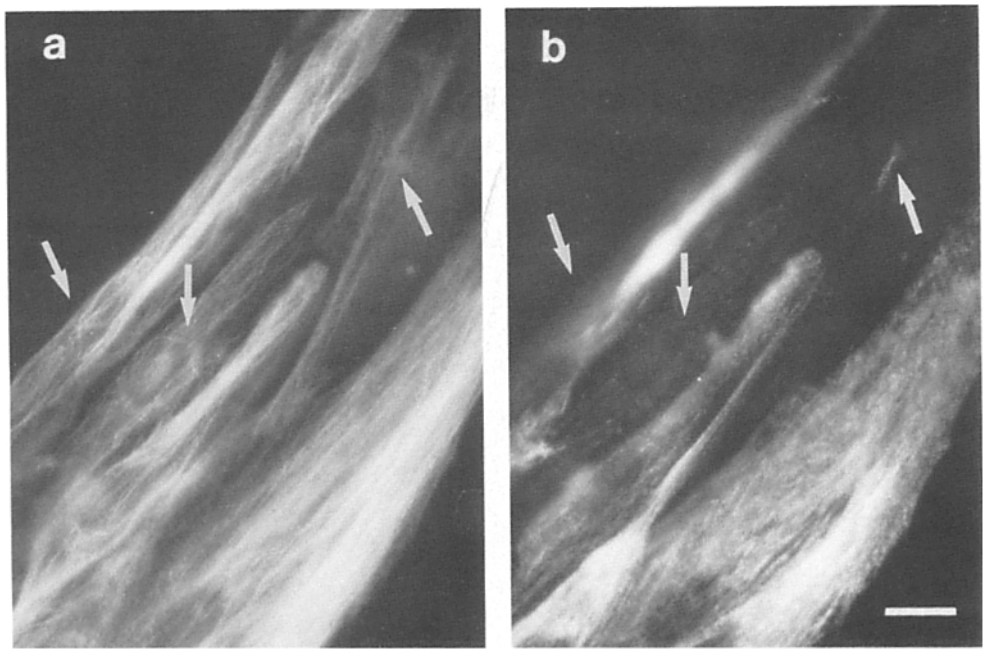

Figure 5. Immunofluorescence detection of (*a*) Glu MTs and (*b*) muscle-specific MHC in differentiating myoblasts. L_6 cells 24 h after the switch to differentiation medium are shown; arrows indicate elongated myoblasts that contain detectable Glu MTs but do not contain detectable MHC staining. Note other myoblasts that contain both Glu tubulin and MHC staining. Bar, 15 μm.

entiation medium for varying intervals, we always found a proportion of myoblasts that exhibited brightly labeled Glu MTs, but did not contain detectable MHC (Fig. 3). One can see from Fig. 3 that the increase in Glu MT staining occurred at least 6 h earlier than the increase in MHC staining. The sensitivity of detection of Glu tubulin and MHC may be different, allowing us to detect the increase in posttranslationally generated Glu tubulin sooner than the newly accumulated MHC; nonetheless, our results clearly demonstrate that the elaboration of Glu MTs is one of the very early events of myogenesis.

Alterations in Posttranslational Acetylation of Tubulin during Myogenesis

The close correlation between the patterns of Glu tubulin and a second posttranslationally modified form, Ac tubulin, in fibroblasts and epithelial cells (Bulinski et al., 1988) prompted us to examine the distribution of Ac tubulin during myogenesis. As shown in Fig. 6, the distribution of Ac tubulin did change markedly during L_6 cell myogenesis. In proliferating myoblasts (day 0 of differentiation), ~80% of the cells possessed some Ac tubulin staining. The pattern of Ac tubulin staining resembled that previously observed in other types of proliferating cells (Piperno et al., 1987; Bulinski et al., 1988); that is, segmented staining of a small subset of the MTs, with a few continuously labeled MTs in each cell (Fig. 6 *b*). Most myoblasts at day 1 after switching cells to differentiation media were indistinguishable from proliferating cells in their Ac tubulin staining pattern (compare Fig. 6, *b* with *d*); only a small percentage (~7%) of the small myotubes present on day 1 showed a significant increase in the level of Ac tubulin staining (data not shown). The percentage of myotubes brightly stained with Ac tubulin antibody did increase during later myogenic differentiation; by day 4, 80% of the myotubes were Ac tubulin stained; and, of these, 32% contained distinct Ac MTs, while the remainder contained hazy, indistinct staining (Fig 6 *f*). By day 11, most myotubes (92%) showed bright Ac tubulin antibody staining, most of which appeared hazy or grainy (Fig 6 *h*). The pattern of Ac tubulin staining was clearly distinct from the pattern of Glu tubulin staining, both in prefusion myoblasts and in early myotubes (compare Fig. 6, *a* with *b* and *c* with *d*); however, in more mature myotubes the patterns of the two modified forms of tubulin were nearly indistinguishable (compare Fig. 6, *e* with *f* and *g* with *h*). Notably, the timing of the initial increases in Glu and Ac tubulin were distinctly different; Glu tubulin was already elevated in prefusion myoblasts (Fig 6, *c* and *d*), while Ac tubulin increased later, in the more mature myotubes (Fig. 6, *e–h*).

Quantitative Changes in the Levels of Glu and Ac Tubulin during Myogenesis

To determine quantitatively the changes in Glu and Ac tubulin levels during myogenesis, we performed a densitometric analysis of immunoblots prepared from samples made at various intervals after the cells were switched to differentiation medium (Fig. 7, *a* and *b*). We found that both total tubulin and Tyr tubulin showed a modest increase (~8–10%) during the course of the differentiation. The level of Glu tubulin, however, showed a greater proportional increase; by day 4, when ~80% of the myoblasts had undergone fusion, the level of Glu tubulin was approximately five times the level in proliferating myoblasts (Fig 7 *b*). The level of Glu tubulin actually decreased slightly during the later stages of myogenesis, during maturation of the myotubes. Despite the large proportional increase we observed in the level of Glu tubulin, Tyr tubulin remained the predominant form of alpha-tubulin throughout myogenesis (data not shown). The quantitative immunoblots also showed that the Ac tubulin level increased dramatically during myogenesis; in agreement with the immunofluorescence data, this increase occurred later in myogenesis, when fusion was largely complete (Fig 7, *a* and *b*). Unlike Glu tubulin, the level of Ac tubulin continued to increase during myotube maturation (Fig 7; day 11). Thus,

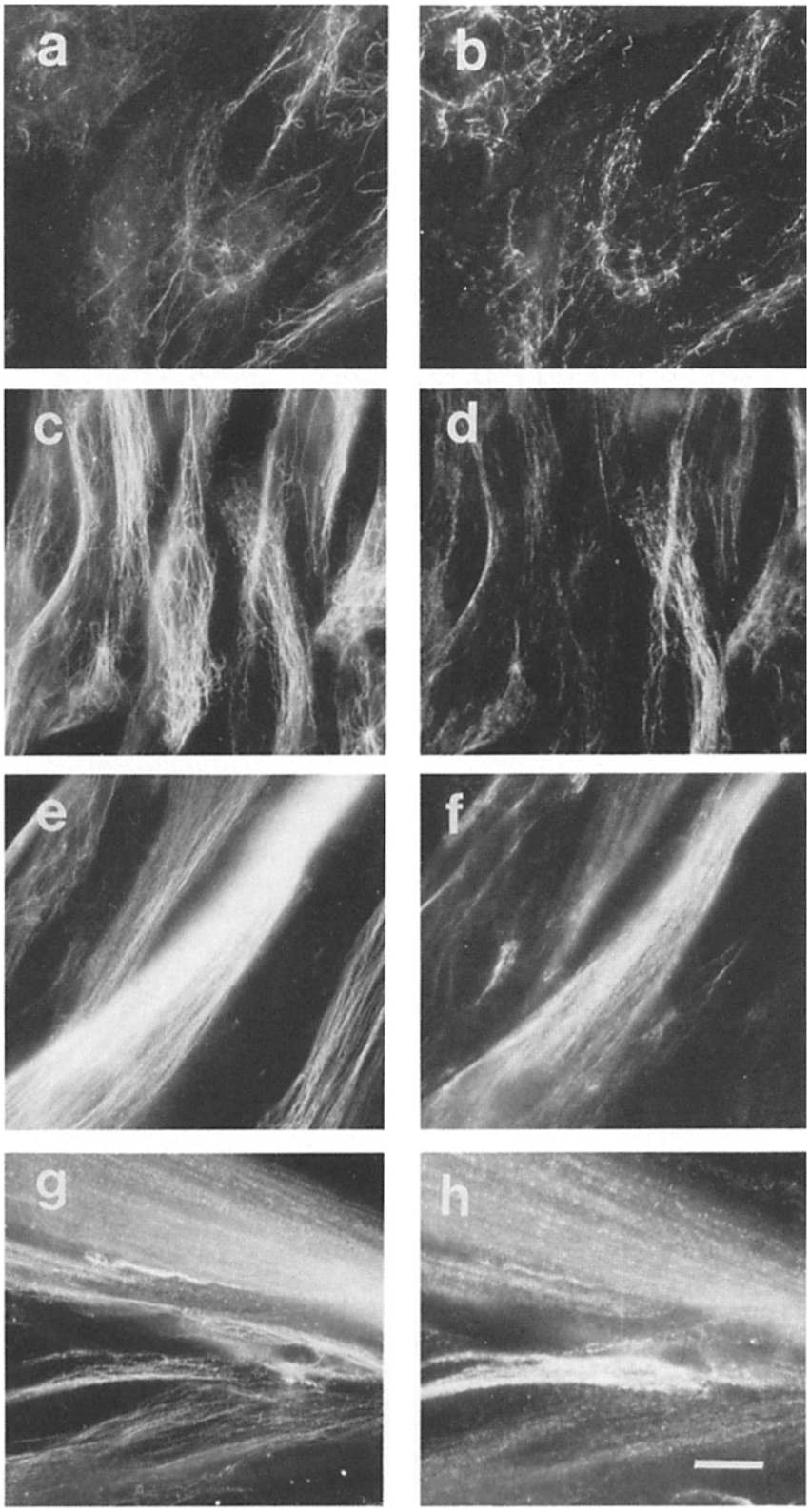

Figure 6. Distribution of Glu and Ac tubulin during L_6 myogenesis. Immunofluorescence patterns of Glu (*a*, *c*, *e*, and *g*) and Ac (*b*, *d*, *f*, and *h*) tubulin are shown in L_6 cells at days 0 (*a* and *b*), 1 (*c* and *d*), 4 (*e* and *f*), and 11 (*g* and *h*) of differentiation. Bar, 15 μm.

for both Glu and Ac tubulin, the quantitative immunoblot data corroborated the immunofluorescence observations and showed that the level of each modified form of alpha-tubulin underwent distinct temporal changes during myogenesis.

We noted that Western blots immunostained with the Glu tubulin antibody exhibited a second, weaker band of immunoreactivity (Fig. 7 *a*). Because this band migrated as a 200-kD protein and increased in level during myogenesis, we suspected that our Glu tubulin antiserum was cross-reactive with muscle-specific MHC. This suspicion was confirmed by

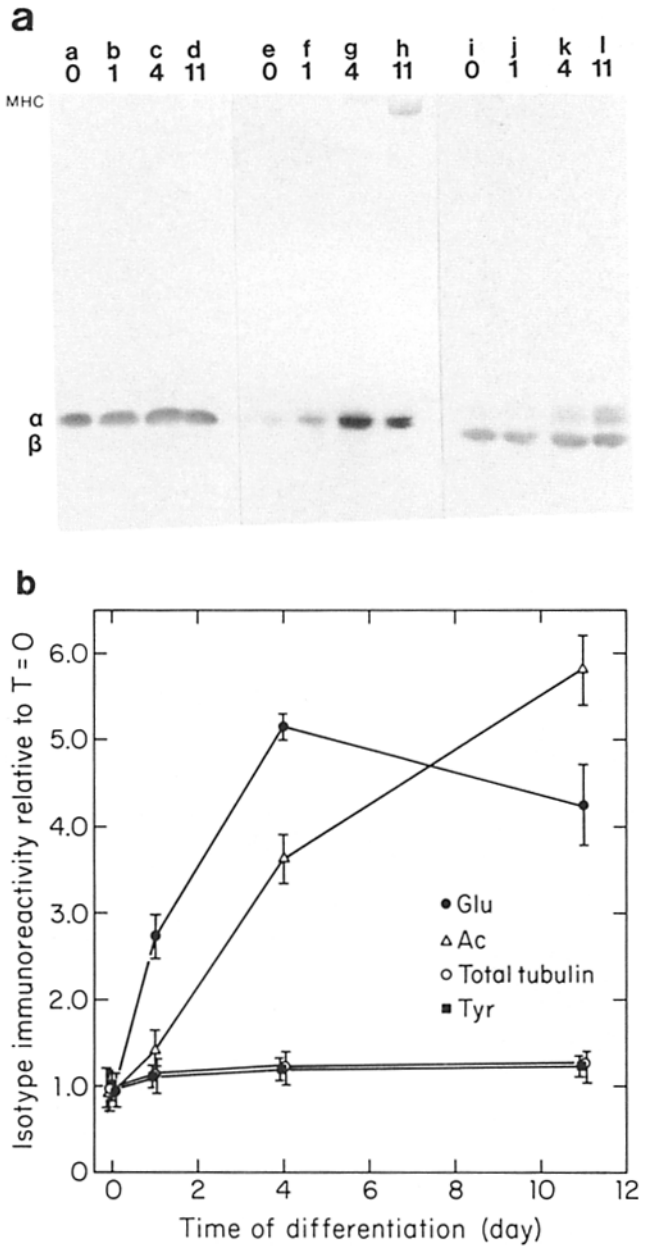

Figure 7. Quantification of Glu and Ac tubulin during myogenesis. (*a*) Western blots of L_6 cell extracts. Extracts were prepared from L_6 cells at the indicated stages of differentiation (expressed as the number of days [0, 1, 4, 11] after switching the cells to differentiation medium). Extract protein (48 μg of protein per lane) was electrophoresed and the gels were transferred to nitrocellulose. Multiple replicas of these blots were stained for Tyr (lanes *a–d*), Glu (lanes *e–h*), Ac (lanes *i–l*), and total tubulin (lanes *i–l*). Migration of alpha- (α) and beta-tubulin (β) and of MHC (*MHC*) are shown. The blot on the right (lanes *i–l*) was sequentially stained for Ac tubulin and total beta-tubulin, which can be seen at the alpha- and beta-tubulin positions, respectively. (*b*) Relative levels of tubulin isotypes during myogenic differentiation. Densitometric scans of western blots were used to calculate the level of Tyr, Glu, Ac, and total tubulin in differentiating L_6 cells, relative to the level in undifferentiated myoblasts (at day 0). Data represent mean ± SD from three experiments.

blots of MHC standards (data not shown); presumably a sequence within muscle MHC is homologous to the peptide with which our Glu tubulin antibody was elicited. What is important for the current study, however, is whether this additional reactivity of the Glu tubulin antibody interfered with our analysis of Glu tubulin during myogenesis. Several lines of evidence demonstrate that the cross-reactivity of Glu tubulin antibody with MHC did not interfere with any of our results. First, we have used greater than saturating concentrations of Glu tubulin antibody to stain blots, such that we have always had enough antibody to saturate the Glu tubulin sites (as well as any myosin sites). Second, we have looked critically at cells double stained for Glu tubulin and total tubulin and confirmed that all Glu tubulin antibody staining represents a subset of the staining with total tubulin antibody. Since the total tubulin antibody does not cross react with any other cellular proteins we can, thus, be sure that the Glu tubulin antibody yields immunofluorescent images of Glu tubulin and not of myosin. In particular, note that the grainy staining of Glu tubulin in mature myotubes (see Fig. 2 *j*) can not be attributed to MHC cross-reactivity of the Glu tubulin antibody since we observed identical grainy staining in mature myotubes with a variety of other tubulin antibodies that do not react with MHC (our Tyr, Ac, and total tubulin antibodies) and Lewis and Cowan (1988) observed similar patterns with other tubulin antibodies. In fact, immunofluorescent staining of MHC was observed only in a few very well-developed myotubes (data not shown) in which sarcomeric staining was apparent with the Glu tubulin antibody and not with the other tubulin antibodies.

Changes in MT Stability during Myogenic Differentiation

In proliferating fibroblasts and epithelial cells in culture, Glu MTs have been shown to be generated by the postpolymerization detyrosination of MTs polymerized from a pool of Tyr tubulin monomers (Gundersen et al., 1987). In these cells then, the level of Glu tubulin in an MT serves as an indicator of the longevity of the MT. We have performed experiments with L_6 cells similar to those that established the postpolymerization detyrosination mechanism for fibroblasts and epithelial cells and have found that the same postpolymerization mechanism is responsible for generating Glu MTs in L_6 cells (data not shown). The postpolymerization model predicts that stabilization of dynamic MTs would result in an increased level of detyrosination. Therefore, we hypothesized that the relative increase we observed in the level of Glu MTs might result from a stabilization of myoblast MTs during myogenesis.

To test this hypothesis, we treated myoblasts and myotubes with the MT antagonist, nocodazole. Relative resistance to nocodazole-induced depolymerization provides an assessment of MT stability and has been used with other cell types to establish the enhanced stability of both Glu and Ac MTs (Piperno et al., 1987; Wehland and Weber, 1987; Khawaja et al., 1988). Fig. 8 shows that MTs in undifferentiated myoblasts (in day-0 cultures) were exquisitely sensitive to nocodazole-induced depolymerization; most MTs were disassembled by a 6-min treatment (Fig. 8, *a* and *b*). With a 25-min treatment, MTs were almost entirely disassembled (data not shown). We observed similar nocodazole sensitivity for the

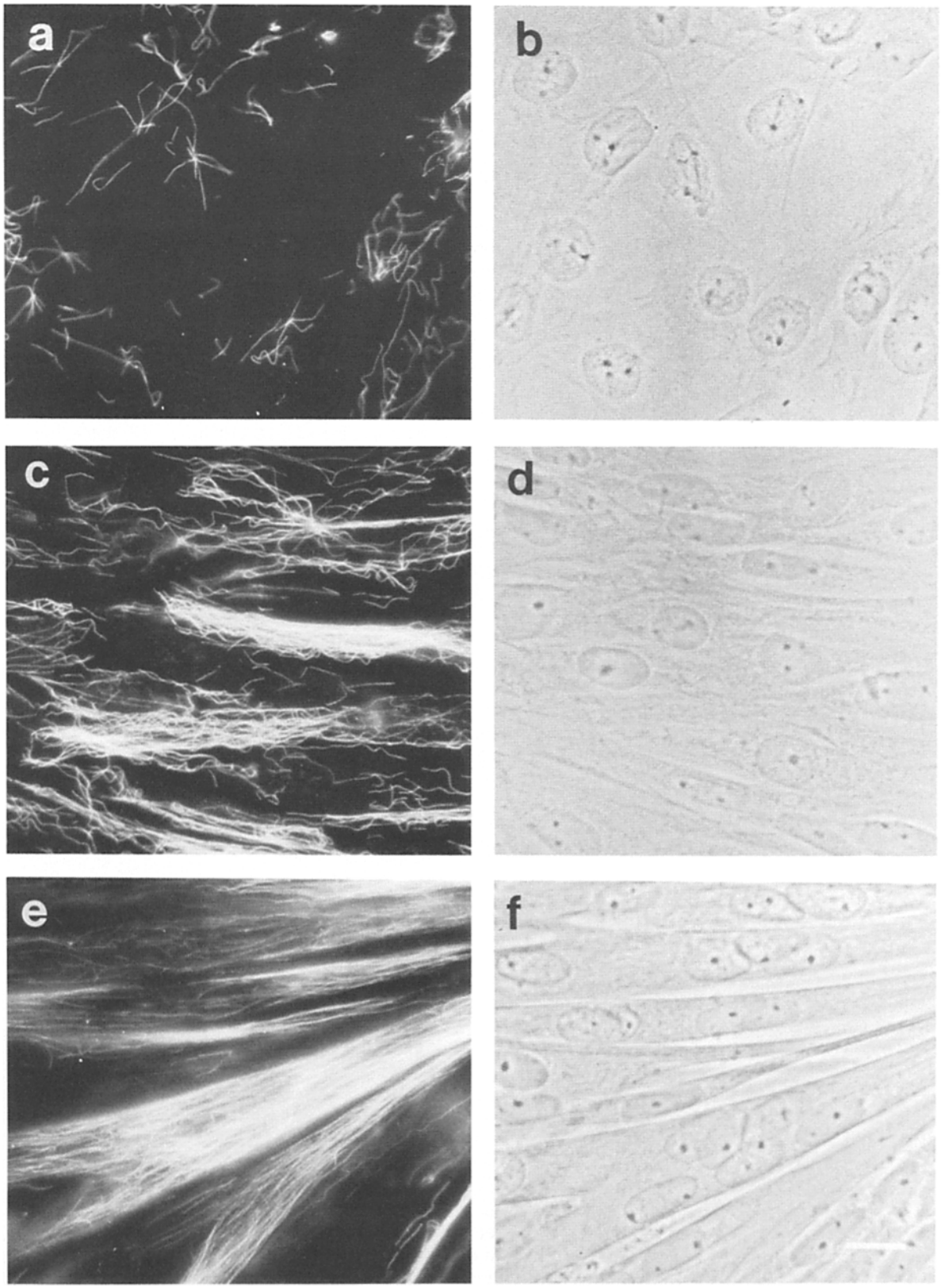

Figure 8. Drug-resistance of MTs in differentiating L_6 cells. Total tubulin distribution (*a*, *c*, and *e*) and phase images (*b*, *d*, and *f*) of differentiating cells (on days 0 [*a* and *b*], 1 [*c* and *d*] and 4 [*e* and *f*]) treated with 10 μM nocodazole for 6 min before processing for immunofluorescence (see Materials and Methods for details). Bar, 15 μm.

MTs in most of the myoblasts in day-1 cultures; however, a subset of the day-1 myoblasts exhibited a significant number of MTs that resisted depolymerization during a 6-min nocodazole treatment (Fig. 8, *c* and *d*). This subset of myoblasts was typically elongated, and the stable MTs in them were, for the most part, oriented along the cell's long axis (Fig. 8 *c*). Longer treatments with nocodazole (25 min) did not change the pattern of stable MTs in elongated day-1 myoblasts; in contrast, myoblasts that did not show an elongated morphology were almost completely devoid of MTs after a 25-min drug treatment (data not shown). The nocodazole stability of MTs in myotubes was similar to that observed in elongated myoblasts; numerous MTs were stable to a 6-min nocodazole treatment (Fig. 8, *e* and *f*), and increasing the treatment time to 25 min had little effect on the array.

While the cells shown in Fig. 8 were stained with an antibody to total tubulin (so that all of the stable MTs were identified), in other experiments we double stained nocodazole-treated cells with the Glu tubulin antibody and the total tubulin antibody. As expected from results obtained in other cell types (e.g., Khawaja et al., 1988), nearly all of the MTs stable to a long (25 min) treatment with nocodazole were stained brightly with the Glu tubulin antibody. These data demonstrate that posttranslationally modified MTs in differentiating prefusion myoblasts and in myotubes are dramatically more stable to the action of nocodazole than MTs in proliferating myoblasts and support the notion that the increased modification of a subset of MTs during myogenesis is the result of their enhanced stability.

TTL Activity during Myogenesis

While the results described above strongly argue that the increased detyrosination of tubulin during myogenesis is due to increased MT stability, the possibility remained that changes in the level of the modifying enzymes might contribute to a change in the cellular level of the Glu tubulin. Two cytoplasmic enzymes, TTL (Raybin and Flavin, 1977; Flavin and Murofushi, 1984) and tubulin carboxypeptidase (Argaraña et al., 1978, 1980), are involved in tyrosinating and detyrosinating cellular tubulin, respectively. Because of insufficient characterization of the tubulin carboxypeptidase and the presence of lysosomal enzymes that mimic its activity in in vitro assays, we were unable to definitively assay the carboxypeptidase during myogenesis. However, to obtain a qualitative measure of the tubulin carboxypeptidase activity in vivo, we took advantage of the observation that when cells are treated with the MT-stabilizing drug, taxol (Gundersen et al., 1987), or when cytoskeletons are prepared by extraction with the mild detergent, Brij 58 (our unpublished observations), all cellular MTs are rapidly detyrosinated by the endogenous tubulin carboxypeptidase. When L_6 cells at each stage of myogenesis were treated with taxol or were extracted with Brij detergent, we observed similar time courses for the appearance of Glu tubulin in cellular MTs (data not shown). This suggests that the activity of the tubulin carboxypeptidase did not change dramatically during myogenesis.

In contrast to the tubulin carboxypeptidase, the TTL has a unique enzyme activity that can be measured easily and accurately in crude cell extracts. When we examined TTL activity during myogenesis, we found that the enzyme activity increased significantly (~50%) by day 4 of differentiation and remained at this level throughout the remainder of myogenesis (Fig 9). Accordingly, the increase we observed in the level of Glu tubulin during myogenesis cannot be attributed to alterations in TTL activity since an elevation in Glu tubulin level could be caused by a decrease, but not by an increase, in TTL activity.

Discussion

In this study, we have documented increases in the level of two alpha-tubulin posttranslational modifications during myogenesis of cultured rat L_6 cells. The level of tubulin detyrosinated by the enzymatic removal of its COOH-terminal tyrosine increased dramatically during early myogenesis, before myoblast fusion. This was manifested as an increase in the number of MTs enriched in Glu tubulin in both prefusion myoblasts and multinucleated myotubes. Significantly, the increase was detectable at even earlier myogenic stages than was muscle MHC, a standard marker of early myogenic differentiation. Thus, the increase in Glu MT levels represents one of the earliest detectable cytoskeletal alterations during myogenesis. It will be interesting to determine whether the change in Glu tubulin level we have observed requires new genetic input or is triggered solely by external factors—e.g., a change in the social interaction of the myoblasts.

We also found that the levels of Ac tubulin, assayed either by immunoblots or immunofluorescence, increased dramatically during myogenesis. However, the increase in Ac tubulin differed from that of Glu tubulin, both in timing and pattern. Ac tubulin did not increase until after myoblast fusion had occurred, in contrast to the early increase of Glu tubulin we observed in myoblasts. Differences in timing of Ac and Glu tubulin increases have been reported previously; in fibroblasts, in which the level of posttranslational modification increased after recovery from drug treatment or cell division, it was Ac tubulin that reached its steady-state level more rapidly than did Glu tubulin (Bulinski et al., 1988). Although both modifications were frequently present on the same MTs in myotubes, Ac tubulin was usually enriched in MT segments, while Glu tubulin was more uniformly distributed along the length of the MTs. Therefore, acetylation and detyrosination of alpha-tubulin appear to be regulated separately

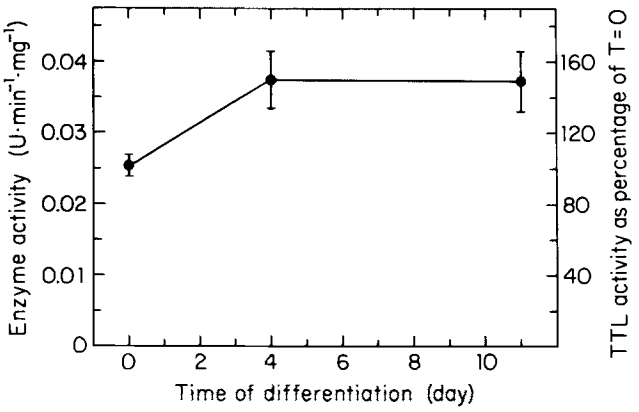

Figure 9. TTL activity during L_6 myogenesis. TTL activity, expressed as $U \cdot min^{-1} \cdot mg^{-1}$ extract protein and as a percentage of the activity in undifferentiated myoblasts (day 0). See Materials and Methods for details of the assay; activities plotted are the mean ± SD from three experiments.

during myogenesis, and each is likely to play a different role in differentiation.

While our data support the idea that MT stabilization during myogenesis results in increased levels of Glu MTs, it is worthwhile to consider the three possible mechanisms that could lead to an increase in Glu MTs: (*a*) a change in the activity of one of the enzymes that carries out the reversible modification; (*b*) an induction of a tubulin gene whose protein product lacks a COOH-terminal tyrosine residue and possesses the COOH-terminal sequence of Glu tubulin; or (*c*) an alteration in MT dynamics. Alterations in the level of the enzymes involved in the tyrosination/detyrosination cycle might be expected to contribute to the elevation in level of Glu tubulin that we have observed in myogenic cells. Tubulin carboxypeptidase cannot be readily assayed in cell extracts since its activity is mimicked by several other carboxypeptidases and exopeptidases. However, when we tested the "in vivo activity" of tubulin carboxypeptidase by treating cells with taxol or with Brij detergent, we did not see a dramatic change in tubulin carboxypeptidase activity during myogenesis. The tubulin carboxypeptidase assays we performed are qualitative, and it is still possible that a quantitative change in the activity of tubulin carboxypeptidase contributes to the increased level of Glu tubulin that we observed in L_6 cells undergoing myogenesis. Yet, even a quantitative change in carboxypeptidase activity could not explain our observation that only certain MTs in elongating myoblasts were detyrosinated (e.g., Fig. 4). When we measured the activity of the tyrosinating enzyme, TTL, we found that the activity increased during myogenesis. This was somewhat surprising, since the steady-state level of TTL's substrate (i.e., Glu tubulin) also increased during differentiation. However, we have also found a parallel increase in TTL activity and Glu tubulin during neurite outgrowth from PC-12 cells (Modesti, N., and J. C. Bulinski, unpublished observations). Naturally, an increase in the activity of TTL during myogenesis could not cause an increase in Glu tubulin. That TTL activity increases concomitantly with Glu tubulin suggests that cells carefully regulate their level of Glu tubulin. Webster et al. (1987*a*) microinjected Glu tubulin into human fibroblasts and found that the injected Glu tubulin was tyrosinated rapidly. One could speculate that the apparent ability of cells to "buffer" their levels of Glu and Tyr tubulin may enable them to avoid some deleterious effect of excess Glu tubulin, especially in the cellular pool of protomer, which is maintained as almost 100% Tyr tubulin (Gundersen et al., 1987).

We have not examined the second possibility directly; however, available evidence suggests that increased synthesis of a tubulin isoform that lacks a COOH-terminal tyrosine residue and terminates, instead, in a glutamic acid residue does not contribute greatly to the increased level of Glu MTs that we have observed. Only one of the six known mammalian genes for alpha-tubulin ends in a COOH-terminal glutamic acid rather than a tyrosine residue (Villasante et al., 1986). Differentiating mouse myogenic cells do express the alpha-tubulin isoform, called M alpha 4, that possesses a COOH-terminal glutamate residue, along with several other tubulin isotypes that possess a COOH-terminal tyrosine residue (Lewis and Cowan, 1988). Expression of a gene analogous to M alpha 4–tubulin in the rat L_6 cells during myogenesis could contribute to the increase in Glu tubulin that we have observed. However, Lewis and Cowan found that, although the M alpha 4–tubulin mRNA is reasonably abundant, its protein product is not. M alpha 4–tubulin was not detectable on immunoblots of extracts of mouse muscle cells at any stage of differentiation and was only weakly detectable in a subset of mouse myotubes examined in immunofluorescence experiments (Lewis and Cowan, 1988). In another study, Gu et al. (1988) demonstrated that, in HeLa cells transfected with the M alpha 4–tubulin gene, the M alpha 4–tubulin protein product was subject to posttranslational tyrosination. Thus, in our study of L_6 cells, it is unlikely that the protein product of the rat analogue of the M alpha 4–tubulin gene, which would not be an abundant tubulin form and which would be subject to posttranslational tyrosination, contributes significantly to the increase in Glu tubulin we have observed.

The third possibility, that increases in Glu tubulin result from a stabilization of some of the myoblast MTs, seems the most plausible. This notion was borne out by a direct demonstration of the enhanced stability of MTs in myotubes and differentiating myoblasts toward nocodazole-induced depolymerization. Although the Glu MTs in prefusion myoblasts and in myotubes represent a stabilized subset of the total array, we do not yet know to what degree these MTs are stabilized. At a minimum, the Glu MTs must have persisted long enough to accumulate levels of Glu tubulin detectable with our Glu tubulin antibody. In fibroblasts and epithelial cells this minimum time is 20 min (Gundersen et al., 1987), and we have found a similar minimum time for Glu MT formation in L_6 cells (our unpublished observations). Nonetheless, it is likely that the Glu MTs in prefusion myoblasts and myotubes persist much longer. In fibroblasts and epithelial cells, most Glu MTs persist for at least 1 h, and a significant number persist even longer (Webster et al., 1987*b*). This is in contrast to the majority of cellular MTs (i.e., Tyr MTs), whose half-life has been measured to be 5–10 min (Schulze and Kirschner, 1986). An upper limit on the type of stabilization event we envision in L_6 cells is invoked by the experiments reported by Toyama et al. (1982). They showed that taxol treatment of myoblasts and myotubes, which presumably halted MT turnover completely, resulted in abnormal myogenesis and dissolution of mature myofibrils. Thus, at some point during myogenesis, it is likely that even the stable Glu MTs must be broken down. Independent measurements of the longevity of Glu MTs with the biotinylated tubulin technique (e.g., Webster et al., 1987*b*) should provide an answer to this interesting question.

Although we have not determined the actual half-life of the stable MTs in differentiating muscle cells, it is interesting to note that this population of MTs appears to accumulate relatively slowly during the course of myogenesis. When we first detect stable MTs in prefusion myoblasts, they represent a small subset of the total MT array (Figs. 2 and 4); later, after fusion has occurred, the array of stable MTs is virtually indistinguishable from the total MT array (Fig. 2). This gradual accumulation of stable MTs may be mechanistically important for the cell to remodel its MT array. In fact, if all of the dynamic MTs in the radial array present in myoblasts were rapidly converted to stable MTs, it is difficult to imagine how the bipolar array characteristic of myotubes could be generated.

What is the function of the stabilized MTs in myogenesis? At least during the early, prefusion course of myogenesis the stable MTs seem to be involved in the alteration of myoblast cell shape. We found increasing numbers of Glu and, hence

stable, MTs in elongating myoblasts, and these MTs were usually distributed parallel to the extended axis of the cell. The location of these stable MTs and the timing of their formation are consistent with a role in the elongation of the cells. MTs might not be limited to this putative role in myoblast elongation; they might act in a different fashion during a second, later phase of myogenesis. Several studies have shown that MTs are necessary for the proper assembly and alignment of myofibrils (e.g., Toyama et al., 1982). Myofibrillogenesis occurs in myotubes, so it is possible that the postfusion increase in Ac MTs is involved in this later MT function.

In a completely different system, namely, the initiation of fibroblast migration in experimentally wounded monolayers, we found that stable, Glu MTs were generated specifically in the direction of impending cell migration (Gundersen and Bulinski, 1988). Thus, for both myogenesis and cell migration, a similar stabilization of MTs occurs during the time that the cells are altering their morphology. In fact, both results support a general model of MT-based morphogenesis recently proposed by Kirschner and Mitchison (1986). In this model, the "ground state" of MTs is taken to be a dynamic one in which MTs turn over rapidly. To initiate a morphogenetic event, MTs are selectively stabilized in a distinct area (or multiple areas) of the cell. Because of the rapid turnover of the remaining dynamic MTs, this generates an asymmetry in the MT array that can, theoretically, be used to effect a morphological change. While details of this model remain to be established, such as the nature of the stabilization process and the mechanism by which some MTs are selected while others are neglected, it appears that the general features of the model can be used successfully to describe the behavior of MTs in two divergent morphogenetic processes, only one of which constitutes a terminal differentiation.

A significant question that remains is how the MTs, specifically the stable MTs, actually contribute to cellular morphogenesis. Previous work points to two possibilities. First, a large body of pharmacological evidence has accumulated demonstrating that MTs lend structural integrity to cells (for review see Dustin, 1984). This structural reinforcement of the cells is presumably due to the limited deformability of the MT fiber itself or to secondary interactions of the MT with other cytoskeletal elements. According to this view, the role played by MT stabilization would be to enhance the structural rigidity of the cell along the particular axis adopted by the stabilized MTs. The second possibility of note is that MTs play a more active, albeit indirect, role by serving as tracks supporting the movement of organelles or vesicles. In particular, if a significant proportion of the membrane precursors necessary for membrane expansion traveled along MTs, then polarized membrane addition leading to cell elongation would occur when MTs were stabilized in a particular area of the cell. An attractive feature of this model is that it suggests a function for the posttranslational modifications of tubulin that accumulate on stable MTs: the modifications, by biochemically differentiating stable MTs from dynamic MTs, could serve as signals for the enhanced transport of membrane vesicles on stable MTs. While direct support for this MT-based membrane growth model has not yet been obtained, evidence has been obtained for both localized stabilization of MTs (Gundersen and Bulinski, 1988) and vectorial membrane insertion (Bergmann et al., 1983) in the case of directed fibroblast motility. That the membrane precursors are actually traveling along MTs and that the selective stabilization of MTs results in vectorial insertion of membrane precursors both remain to be established. The data we have presented here indicate that myogenesis will be a useful system for further study of the role of MTs in cellular morphogenesis.

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