Focal adhesion signaling and actin stress fibers are dispensable for progression through the ongoing cell cycle

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Summary

Prevention of cell spreading or disruption of actin filaments inhibits growth factor stimulated cell cycle re-entry from quiescence, mainly because of a failure to induce cyclin D expression. Ectopic cyclin D expression overrules anchorage-dependency, suggesting that cell spreading per se is not required as long as cyclin D is otherwise induced. We investigated whether cyclin D expression in cells exiting mitosis is sufficient to drive morphology-independent cell cycle progression in continuously cycling (i.e. not quiescent) cells. Disruption of post-mitotic actin reorganization did not affect substratum reattachment but abolished the formation of filopodia, lamellipodia and ruffles, as well as stress fiber organization, focal adhesion assembly and cell spreading. Furthermore, integrin-mediated focal adhesion kinase (FAK) autophosphorylation and growth factor stimulated p42/p44 mitogen activated protein kinase (MAPK) activation were inhibited. Despite a progressive loss of cyclin D expression in late G1, cyclin E and cyclin A were normally induced. In addition, cells committed to DNA synthesis and completed their entire cycle. Our results demonstrate that post-mitotic disruption of the actin cytoskeleton allows cell cycle progression independent of focal adhesion signaling, cytoskeletal organization and cell shape, presumably because pre-existing cyclin D levels are sufficient to drive cell cycle progression at the M-G1 border.

Key words: Actin cytoskeleton, Cell cycle progression, Cell spreading, Cyclin D, G1 phase, Integrin signaling

Introduction

G1 phase progression of nontransformed adherent cells depends both on the presence of growth factors and adhesion to the extracellular matrix (ECM). Growth factor-stimulated adherent cells progress into S phase, whereas cells deprived of growth factors or cells maintained in suspension arrest in G1 and enter the quiescent state G0 (Guadagno and Assoian, 1991; Schwartz, 1997). In addition to growth factors and adhesion, organization of the actin cytoskeleton is implicated in G1 phase progression since disruption of actin architecture with pharmacological agents leads to G1 arrest in a variety of cell types (Maness and Walsh, 1982; Ohta et al., 1985; Takasuka et al., 1987; Iwig et al., 1995; Bohmer et al., 1996; Fasshauer et al., 1998; Huang et al., 1998; Tsakiridis et al., 1998; Reshetnikova et al., 2000; Bottazzi et al., 2001; Huang and Ingber, 2002; Lohez et al., 2003). Cytoskeleton-dependent G1 arrest is mainly caused by a failure to induce sustained activity of the p42/p44 MAPKs, expression of cyclin D1 and downregulation of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Bohmer et al., 1996; Fasshauer et al., 1998; Huang et al., 1998; Bottazzi et al., 2001; Huang and Ingber, 2002), events that are required for quiescent cells to resume G1 phase progression upon growth factor stimulation (Baldin et al., 1993; Pages et al., 1993; Brondello et al., 1995; Sherr and Roberts, 1999). These studies have given rise to the concept that growth control in normal mammalian cells is dependent on cell spreading mediated by actin stress fibers. It is however difficult to develop an appropriate system to investigate the contribution of cell spreading and cytoskeletal organization to cell cycle progression, apart from the actions of growth factors. In a growth factor-starved population, adhesion and cell spreading are not varying conditions, since the cells are already attached to and spread on an ECM. To investigate adhesion as a variable factor, the alternative is to trypsinize quiescent cells or to induce quiescence by prolonged incubation in suspension. Results obtained with the latter systems revealed that reattachment alone can induce immediate early gene expression and cell cycle re-entry into G1 even in the absence of growth factors, whereas for subsequent progression into S-phase cell spreading and growth factors were required, thus subtly demonstrating distinct roles for cell adhesion and cell spreading in cell cycle regulation (Dike and Farmer, 1988; Dike and Ingber, 1996). However, the drawback of using quiescent cells (whether rendered quiescent by growth factor starvation or by incubation in suspension) is that these cells have downregulated protein synthesis in general and downregulation of cell cycle regulators, such as c-myc, c-fos, c-jun and cyclins D and E in particular, whereas cell cycle inhibitors such as p21 or p27 are upregulated. Quiescent cells are thus growth-restricted for multiple reasons and may

therefore not represent a suitable system to study adhesion as a single variable factor in otherwise growth-committed cells. The ideal system to address the requirement for attachment and cell spreading apart from growth factor actions in cells entering G1 phase would be a synchronous population of rounded cells that have lost significant if not all contact with the substratum but in the presence of growth factors and expressing cell cycle regulators, thus being competent for growth (i.e. not quiescent). Such a situation occurs naturally in mitosis in continuously cycling cells. Since expression of both D-type cyclins and cdk inhibitors oscillates only moderately in continously cycling cells (Sewing et al., 1993; Sherr, 1993; Hulleman et al., 1999b), cells entering G1 from mitosis do not need to induce cyclin D expression or downregulate p27 levels, in contrast with cells entering G1 from quiescence. This is particularly interesting since (1) cyclin D1 is the main rate limiting step in G1 phase and (2) forced induction of cyclin D1 is sufficient to drive G0 to S-phase progression in rounded cells with disorganized actin filaments. The latter result argues against the absolute requirement of cell spreading and cytoskeletal tension for proliferation (Welsh et al., 2001; Roovers and Assoian, 2003) and suggests that as long as cyclin D1 is induced, cell cycle progression is uncoupled from an organized cytoskeleton and the corresponding spread cell shape (Welsh et al., 2001; Roovers and Assoian, 2003). This model is supported by the observation that overexpression of cyclin D1 rescues proliferation in non-adherent cells, allowing for anchorage-independent growth as observed in many tumors (Schulze et al., 1996; Zhu et al., 1996; Resnitzky, 1997; Hansen and Albrecht, 1999; Bottazzi et al., 2001). In addition, stress fibers are not commonly detected in cells in living tissue, questioning the need of cytoskeletal tension for proliferation in vivo (Herman et al., 1982; White et al., 1983; Wong et al., 1983).

In this study, we hypothesized that pre-existing cyclin D expression in mitosis may allow cells to progress through G1 phase independent of post-mitotic cell spreading and actin reorganization into stress fibers. Therefore, we investigated the role of the actin cytoskeleton and cell spreading in progression through the ongoing cell cycle employing the mitotic shake-off method. Disruption of post-mitotic actin reorganization inhibited cell spreading, focal adhesion assembly and integrin-mediated FAK signaling, as well as growth factor stimulated p42/p44 MAPK activation. Despite reduced expression of cyclin D in late G1 phase, neither expression of cyclins E and A nor S-phase entry were impaired and cells progressed further through G2 and M-phase, thus completing their entire cell cycle. The same cells do not progress through G1 phase when incubated in suspension or when attached to a substratum to which they can only attach and not spread, such as poly-L-lysine (PLL), demonstrating that (1) adhesion but not spreading is required for cell cycle progression and (2) simple attachment to a non-permissive substratum is not sufficient to activate the cell cycle machinery. Taken together, our results present evidence for the first time that post-mitotic disruption of the actin cytoskeleton allows cells to progress through the ongoing cell cycle independent of cytoskeletal organization, focal adhesion signaling and cell shape and identify a subtle distinction between cell adhesion and cell spreading, at least in the particular cell lines studied here.

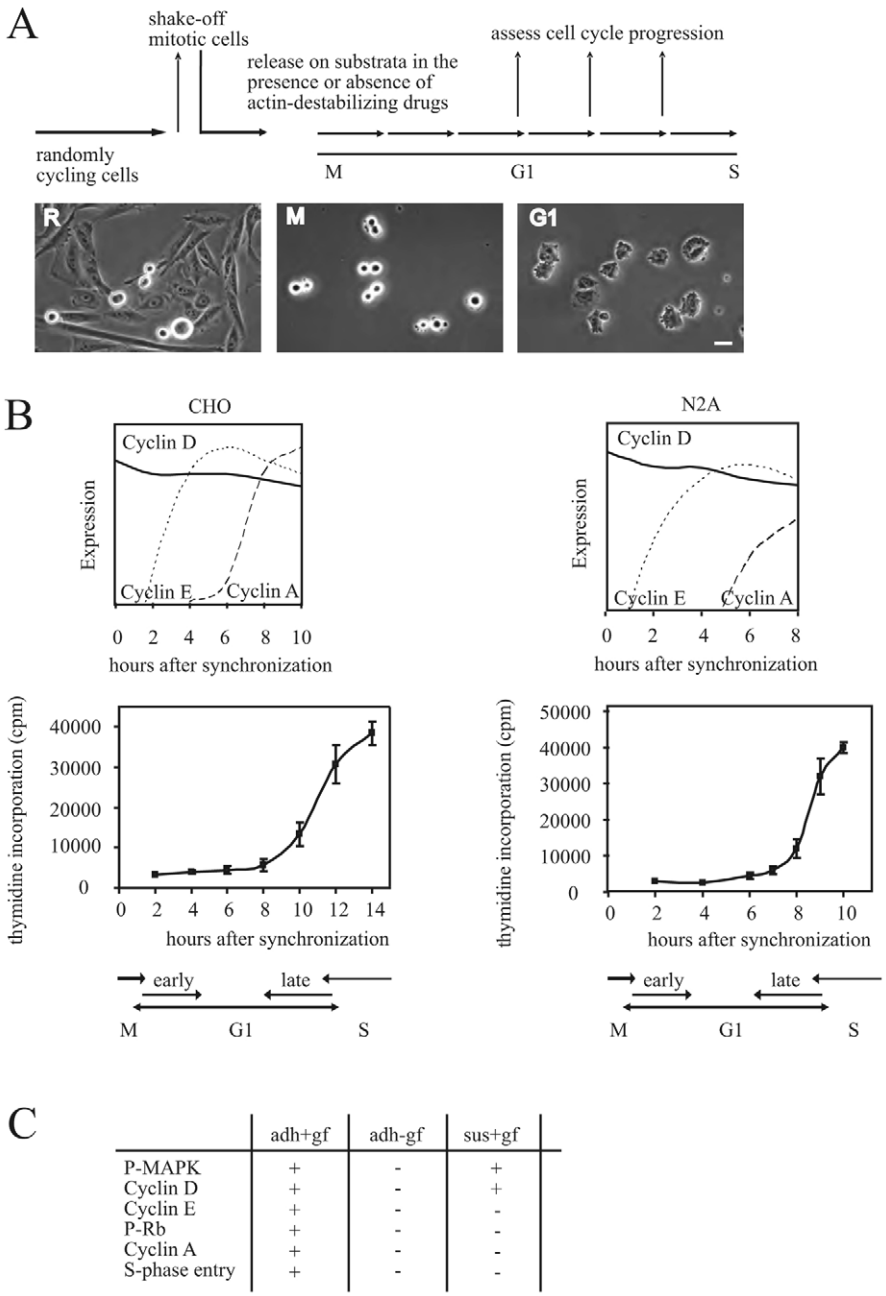
Results

Cell cycle control by growth factors and adhesion during M and early G1 phase

To determine the role of actin cytoskeletal (re-)organization and cell spreading on cell cycle progression in rounded cells exiting mitosis, we used the mitotic shake-off method on N2A and CHO cells. We used these cell types because they yield relatively high numbers of mitotic cells and because we have thoroughly characterized cell cycle regulation from mitosis to S phase in both cell lines in previous studies (Hulleman et al., 1999a; Hulleman et al., 1999b; van Rossum et al., 2002). The experimental design is outlined schematically in Fig. 1A. Randomly cycling cultures (designated R in the phase-contrast images) are shaken firmly by hand for 1 minute, detaching the mitotic cells. Medium containing the mitotic cells is then removed and the cells are released in fresh media under the appropriate experimental conditions. The obtained cells constitute a highly synchronous (~90%) M-phase population (designated M), that reattaches to the substratum as early as 15 minutes after release and divides and spreads within 1 hour after synchronization, thus entering G1 phase (designated G1). Although the cell cycle in N2A is generally somewhat shorter than in CHO, the expression patterns of the G1/S cyclins are comparable in both cell lines (represented diagrammatically in Fig. 1B); cyclin D is expressed in mitotic cells and persists only slightly declining throughout G1 phase, while cyclin E is induced in early G1 phase and cyclin A in mid to late G1 phase. Normal cell cycle progression from mitosis to S phase requires 7-8 hours for N2A cells and 9-11 hours for CHO cells, as measured by thymidine incorporation (Fig. 1B). Although the used cell types are unrelated in origin, both exhibit similar cell cycle controls in the progression from mitosis into S phase, as summarized in Fig. 1C. G1 phase progression in adherent cells is dependent on growth factor stimulation and requires sustained MAPK activity and its nuclear translocation (Hulleman et al., 1999a). Upon growth factor removal, MAPK activation is prevented, cyclin D expression is not maintained and cyclin E expression is not induced in early G1. Alternatively, when post-mitotic cells are released in suspension in the presence of growth factors, MAPK phosphorylation and cyclin D levels are initially maintained but early G1 expression of cyclin E is prevented (Hulleman et al., 1999b). Both conditions lead to insufficient phosphorylation of the retinoblastoma protein and a failure to induce cyclin A, preventing entry into S phase. In summary, we use mitogen- and adhesion-dependent cell lines to study the role of post-mitotic cell spreading and cytoskeletal organization on cell cycle progression.

Effect of inhibition of actin reorganization during G1 phase on cell morphology

Renewed attachment to the substratum and subsequent cell spreading after mitosis are associated with dramatic changes of the actin cytoskeleton. To determine post-mitotic reorganization of actin filaments, cells were fixed for fluorescence microscopy at several time points after synchronization. Phalloidin staining of filamentous actin (F-actin) in mitotic cells showed a distribution of actin filaments in the contractile ring and additional arrangement into short cortical and cytoplasmic filaments (Fig. 2A). Post-mitotic substratum adherence was accompanied by formation of actin-



C

	adh+gf	adh-gf	sus+gf
P-MAPK	+	-	+
Cyclin D	+	-	+
Cyclin E	+	-	-
P-Rb	+	-	-
Cyclin A	+	-	-
S-phase entry	+	-	-

Fig. 1. Cell cycle control by growth factors and adhesion in N2A and CHO cells entering G1 phase from mitosis.
(A) Experimental set-up. Mitotic cells were collected from randomly proliferating cell cultures by shake-off and released in the presence or absence of inhibitors as indicated. Cells were harvested at several time points after synchronization to assess cell cycle progression. Phase-contrast images depict a randomly cycling culture of CHO cells (R), a synchronous population obtained by mitotic shake-off (M) and a population 2 hours after synchronization (G1). Bar, 20 μm.
(B) Kinetics of cell cycle progression in adherent N2A and CHO populations traversing G1 phase from mitosis in the presence of growth factors. Upper panel, representations of expression patterns of G1-S cyclins over time. Lower panel, kinetics of S-phase entry as measured by thymidine incorporation. Representative experiments are shown; data are expressed as means ±s.e.m. (n=3). (C) Summary of the main cell cycle controls in M-G1-S phase regulation by growth factors and the ECM in N2A and CHO cells synchronized by mitotic shake-off. Adherent cells in the presence of growth factors progress into S phase, whereas post-mitotic growth factor depletion or incubation in suspension induce an arrest in G1. Adh, adherent; gf, growth factors; P-Rb, phosphorylated retinoblastoma protein; sus, suspension.

mediated surface protrusions such as microspikes, filopodia, lamellipodia and ruffles (Fig. 2B-E). Reorganization of F-actin into stress fibers was visible within 2 hours after mitosis and corresponded with cell spreading over the substratum (Fig. 2B-E).

To analyze the role of the actin cytoskeleton in post-mitotic events, normal post-mitotic actin reorganization was disrupted with the actin destabilizing agents CCD and LB. Both drugs favor depolymerization but they act by distinct mechanisms; CCD caps the growing ends of actin polymers, whereas LB inhibits actin polymerization by sequestering actin monomers (Spector et al., 1989). Release of mitotic N2A cells in medium containing either 500 ng ml^{-1} CCD or 100 ng ml^{-1} LB did not affect reattachment but completely inhibited cell spreading (Fig. 2G and 2I, respectively). Both LB and CCD inhibited the formation of adhesion-mediated membrane protrusions and actin reorganization into stress fibers, whereas cortical F-actin persisted (Fig. 2G,I). To investigate whether inhibition of post-mitotic F-actin redistribution and cell spreading is reversible, cells were exposed for 3 hours to either drug, washed and incubated in fresh medium. Within 1 hour after drug release, cells treated with CCD (Fig. 2H) or LB (Fig. 2J) gained the ability to spread over the substratum and spreading was associated with membrane ruffling, lamellipodia formation and stress fiber assembly. DMSO (0.1%) did not affect post-mitotic reattachment and cell spreading, nor did it interfere with stress fiber formation (Fig. 2F). These data show that CCD and LB, at the concentrations used, reversibly inhibit actin rearrangements and associated changes in cell morphology, as occurring normally after mitosis.

Effect of inhibition of actin reorganization during G1 phase on MAPK and FAK phosphorylation

The previous results have shown that early G1 cells exhibit major cytoskeletal and morphological changes related to reattachment and cell spreading. Attachment and cell spreading depend on integrin binding to ECM proteins, which elicits the formation of focal adhesions and tyrosine phosphorylation of several intracellular signaling proteins including FAK (Burridge et al., 1992; Hanks et al., 1992; Yamakita et al., 1999). Furthermore, integrin anchorage to the substratum links the actin cytoskeleton to the extracellular environment. To investigate whether inhibition of normal post-mitotic actin reorganization and concomitant cell spreading interferes with integrin-mediated focal adhesion assembly, mitotic N2A cells incubated with or without CCD were fixed at several timepoints after synchronization and vinculin as well as F-actin distribution were visualized by fluorescence microscopy. In untreated cells, the presence of focal adhesions was evident from punctate vinculin staining at the cell periphery associated with the tips of actin stress fibers. This was not visible in cells treated with CCD, indicating that focal adhesion assembly was inhibited (Fig. 3A). As a parameter for focal adhesion signaling, FAK autophosphorylation on tyrosine residue 397 was investigated from mitosis into G1 phase in the absence and presence of CCD or LB. FAK autophosphorylation was absent in mitosis and increased in reattached cells after division, which was dramatically inhibited by either drug (Fig. 3B). These results indicate that inhibition of actin reorganization in early G1 phase prevents integrin-stimulated focal adhesion assembly and autophosphorylation of FAK.

Another important event early after mitosis is the growth factor induced activation of p42/p44 MAPK, which is required for progression through G1 phase (Hulleman et al., 1999a). Growth factor receptors and many of their downstream substrates are associated with actin filaments (Payrastre et al., 1991; den Hartigh et al., 1992; Diakonova et al., 1995; van der Heyden et al., 1997; Tsakiridis et al., 1998) and disruption of the actin cytoskeleton inhibited activation and nuclear translocation of MAPK in growth factor-stimulated G0 cells (Aplin and Juliano, 1999; Aplin et al., 2001). We therefore investigated whether early growth factor signaling to MAPK is related to post-mitotic actin reorganization by monitoring p42/p44 MAPK phosphorylation in the presence of the actin inhibitors. MAPK phosphorylation was absent or low in mitotic cells but increased significantly thereafter (Fig. 3B), as has been established before (Hulleman et al., 1999a). Both CCD and LB considerably reduced post-mitotic MAPK phosphorylation (Fig. 3B). It was also examined whether the observed effects on signal transduction were reversible. Synchronized cells incubated for 3 hours with CCD or LB were washed and released in fresh medium. Cells showed full rescue of FAK autophosphorylation and MAPK phosphorylation within 1 hour after release (Fig. 3C), corresponding with the morphological recovery observed (Fig. 2H,J; indicated by R). In conclusion, these data suggest that both integrin signaling and growth factor signaling in early G1 phase depend on integrity of the actin cytoskeleton.

Effect of inhibition of actin reorganization during G1 phase on G1 phase progression

The previous results have shown that CCD and LB reversibly inhibit typical early G1 phase events, both morphological (Figs 2, 3) and on the level of signal transduction (Fig. 3). Since several studies have described cell spreading and focal

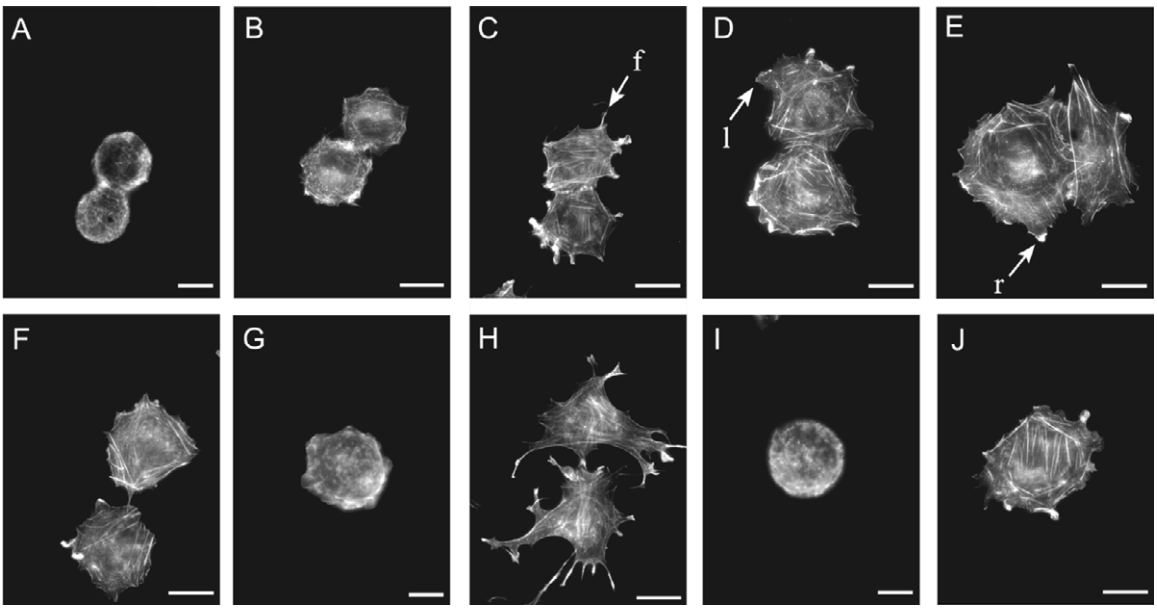


Fig. 2. CCD and LB reversibly inhibit the formation of filopodia, lamellipodia and ruffles, as well as stress fiber assembly and concomitant cell spreading in post-mitotic N2A cells. Untreated N2A cells were fixed in mitosis (A) and 1 hour (B), 2 hours (C), 3 hours (D) and 4 hours (E) thereafter. Alternatively, mitotic cells were incubated with 0.1% DMSO (F), 500 ng ml^{-1} CCD (G), or 100 ng ml^{-1} LB (I) and fixed 3 hours after synchronization. To determine reversibility of drug effects, cells were treated for 3 hours either with CCD (H) or LB (J), washed and released in fresh medium for 1 hour. After fixation, cells were labeled with phalloidin and visualized with fluorescence microscopy. Arrows indicate filopodia (f), lamellipodia (l) and ruffles (r). Bars, 20 μm.

adhesion signaling as well as sustained MAPK activation throughout G1 phase as essential for cell cycle progression, we analyzed whether our cells had arrested in early G1, by investigating expression of cyclins D and E in the presence of the drugs for up to 3 hours after mitosis. As demonstrated previously in our laboratory, cyclin D levels were readily detectable in mitotic cells and in early G1 phase, whereas cyclin E was absent in mitotic cells but was induced shortly thereafter (Fig. 4A,C) (Hulleman et al., 1999b). Expression of both cyclins seemed not significantly affected by the drugs within this timeframe, suggesting no effect on cell cycle progression (Fig. 4A,C). Several studies have however demonstrated a direct link between organization of the actin cytoskeleton and sustained cyclin D expression. We therefore monitored expression levels of cyclins D and E throughout the entire G1 phase. In contrast to early G1, prolonged incubation with the drugs did induce a progressive decline in cyclin D levels later in G1, however expression of cyclin E remained unaffected (Fig. 4B,C). Cyclin A levels increased steadily from 5 hours after mitosis, which was not inhibited in cells treated with CCD and LB (Fig. 4B,C), suggesting cell cycle progression into S phase. S-phase entry was further determined by incubating synchronized cells overnight with BrdU (10 μM) and measuring incorporation with an ELISA assay. Consistent with cyclin A expression, the majority of the cells treated with either drug had incorporated BrdU (91% and 89%, respectively; Fig. 4D). To establish whether the rate of G1-phase progression in these cells was affected, mitotic cells were incubated with the actin inhibitors and [^{3}H]thymidine and incorporation was analyzed using a scintillation counter every hour after synchronization. No delay or acceleration in the progression to S phase was detected (not shown). Taken together, these results indicate that cytoskeletal integrity is not a prerequisite for G1-phase progression in the ongoing cell cycle of N2A cells.

Effect of inhibition of actin reorganization during G1 phase on cell-cycle progression and cytokinesis

We have shown that in N2A cells, progression from M to S phase is not impaired by inhibition of post-mitotic actin reorganization, despite reduced levels of cyclin D in late G1 phase (Fig. 4). The next question we addressed was whether these cells were also able to progress through G2 and M phase. In M phase, actin filaments assemble with myosin filaments to generate the contractile ring (Fig. 2A), which is required for formation of the cleavage furrow leading to cytokinesis. Suppression of actin polymerization during mitosis leads to cleavage failure, creating bi-nucleated cells. Therefore, we used bi-nucleation as a parameter for cell cycle completion in the presence of CCD and LB. Cells were synchronized in mitosis as described earlier and subsequently incubated with or without the actin inhibitors during the entire cell cycle. After 20 hours of incubation cells were still rounded, indicating that the agents had lost none of their potency (not shown). Cells were then washed once in medium and incubated in fresh medium for 3 hours, allowing the cells to recover. Released cells were able to spread and form ruffles and stress fibers, indicating that even after long-term exposure the drug effects

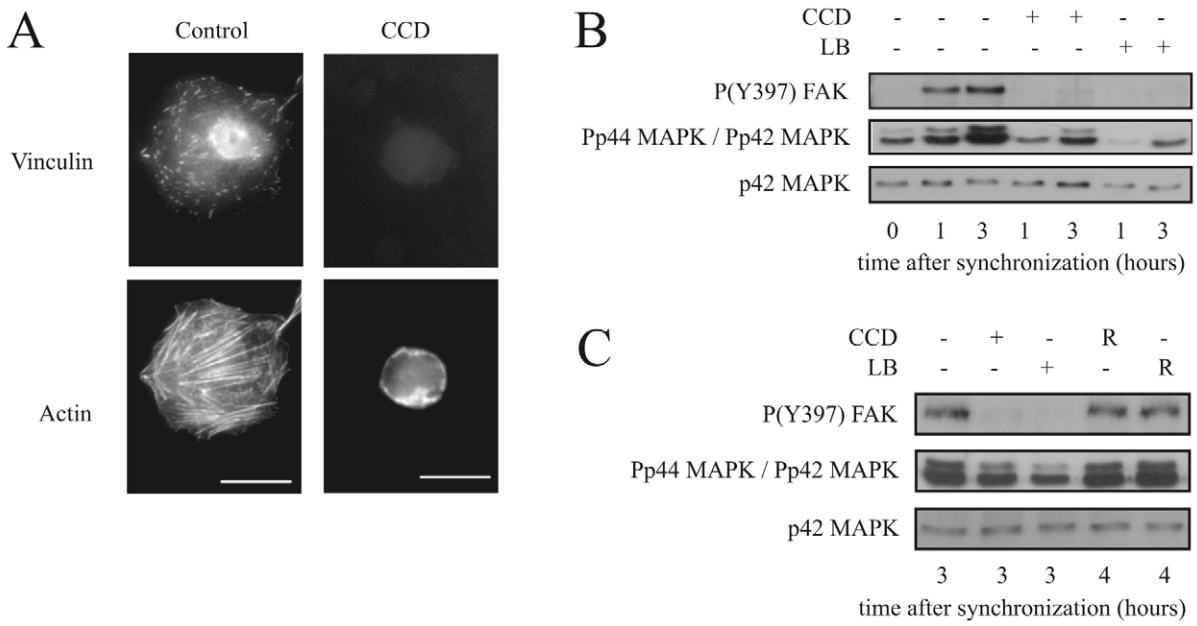


Fig. 3. Disturbed post-mitotic actin reorganization inhibits integrin-mediated focal adhesion assembly, autophosphorylation of FAK and growth factor-stimulated MAPK phosphorylation in early G1 phase. (A) Synchronized N2A cells were incubated with or without CCD and fixed 3 hours thereafter. Vinculin association with actin filaments was determined by fluorescence microscopy. Bars, 20 μm. (B) p42/p44 MAPK phosphorylation and (Y397) FAK autophosphorylation were investigated by western blotting in lysates of mitotic and post-mitotic cells treated for up to 3 hours with CCD or LB (p42 MAPK=loading control). Representative results out of at least three independent experiments are shown. (C) Synchronized cells were incubated for 3 hours with CCD or LB as in (B), after which they were washed once and released for 1 hour in fresh medium. Phosphorylation of FAK and p42/p44 MAPK was then analyzed by western blotting. p42 MAPK, loading control; R, recovery.

are reversible (Fig. 5A; left panel). DAPI staining revealed that the vast majority of CCD and LB treated cells had more than one nucleus (85% and 82%, respectively; Fig. 5A,B). Cells with one nucleus probably reflect slowly cycling cells that have not come to mitosis yet or cells that have arrested at some point in the cycle. This result is consistent with the observed progression through G1 into S phase (Fig. 4) and demonstrates that actin cytoskeleton organization into stress fibers and cell spreading are not required for progression through the ongoing cell cycle, at least in N2A cells.

The previous results demonstrate that in N2A cells, disturbed actin reorganization after mitosis does not induce cell cycle arrest in the ongoing cell cycle. To exclude that the observed effect is restricted to this cell line, we next analyzed the relation between cell cycle progression and actin integrity in CHO cells, which exhibit very similar cell cycle regulation as demonstrated previously in our laboratory (Hulleman et al., 1999a; Hulleman et al., 1999b; van Rossum et al., 2002). CHO cells were synchronized in mitosis as described above and released in fresh medium or medium supplemented with 500 ng ml^{-1} CCD, which impaired both post-mitotic cell spreading and actin reorganization into stress fibers as well as phosphorylation of p42/p44 MAPK and autophosphorylation of FAK (Fig. 6A and data not shown). Early G1 phase expression of cyclins D1/D2 and induction of cyclin E were not inhibited by CCD, although cyclin D1/D2 levels dropped later in G1 (Fig. 6A and data not shown). To investigate whether progression from M-phase into S was dependent on cytoskeletal reorganization, BrdU incorporation was assessed in cells treated with CCD. Alternatively, cells were incubated with 100 ng ml^{-1} LB. No differences in BrdU incorporation were detected (Fig. 6B). In addition, analysis of [^{3}H]thymidine incorporation revealed that the majority of the cells treated with CCD or LB committed to DNA synthesis with kinetics comparable to untreated cells, suggesting that the rate of G1 phase progression was not affected (Fig. 6C). These results are identical to those obtained in N2A cells and suggest that G1 phase progression in post-mitotic CHO cells is not dependent on cell spreading and cytoskeletal tension. We also determined cell cycle progression to the next M-phase by incubating mitotic cells with the actin inhibitors for at least 25 hours, while normal doubling time for CHO cells is approximately 20

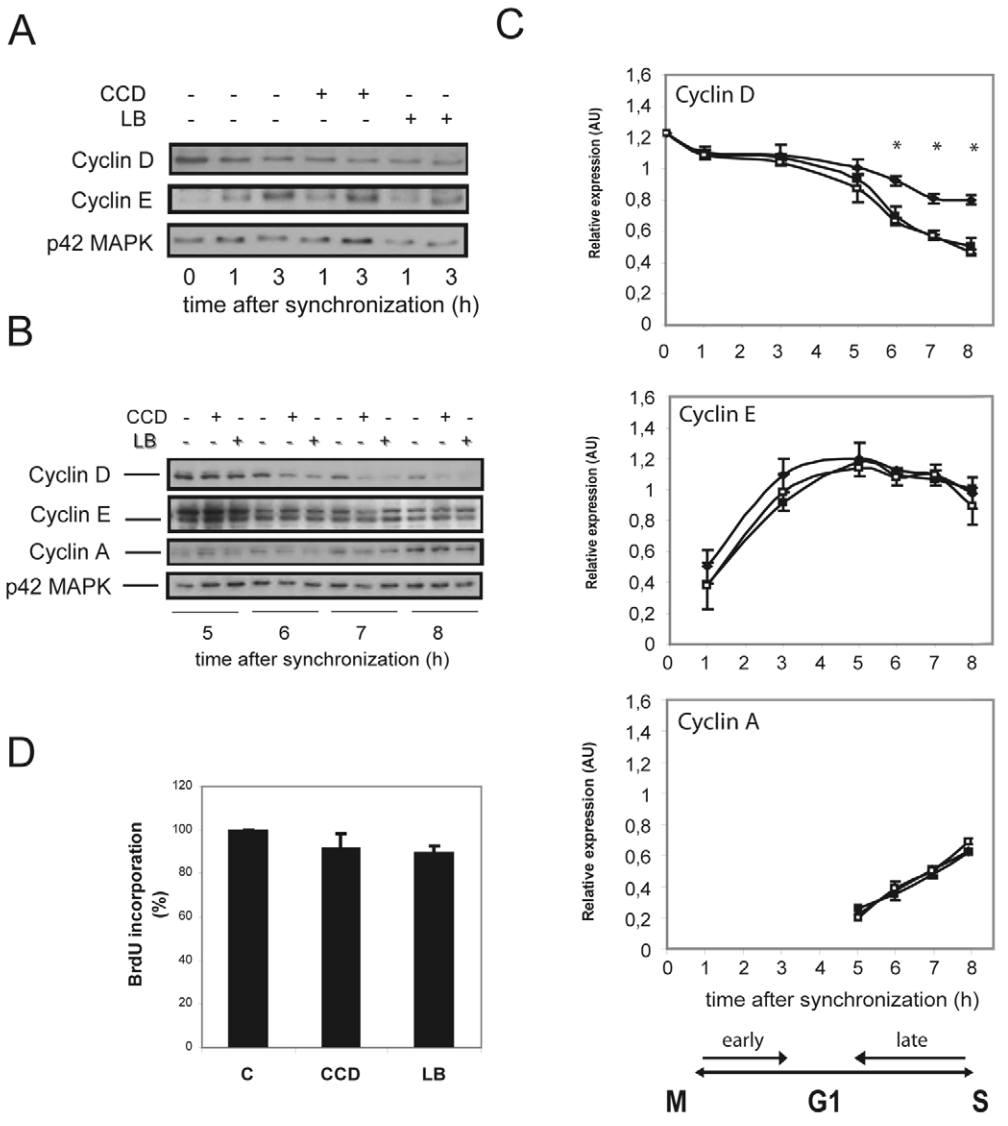


Fig. 4. Actin integrity is not required for G1-phase progression in the ongoing cell cycle of N2A cells. (A) Cyclin D and cyclin E expression levels were monitored by western blotting in lysates of mitotic cells and post-mitotic cells treated for up to 3 hours with either CCD or LB (p42 MAPK=loading control). Each experiment was conducted at least three times with similar results and representative blots are shown. (B) Effect of cytoskeletal disorganization on expression levels of G1/S cyclins in mid- to late G1 phase. Depicted are representative blots out of at least three experiments with identical results (p42 MAPK, loading control). (C) Expression levels of cyclins D, E and A were quantified by densitometry. Values shown are means ±s.e.m. from three independent experiments normalized to expression of p42 MAPK. Statistically significant differences are marked by asterisks (triangles, untreated; open squares, CCD; closed squares, LB). AU, arbitrary units. (D) DNA synthesis was examined in synchronized N2A cells incubated overnight with actin inhibitors and 10 μM BrdU. Values shown represent the average percentages ± s.e.m. obtained from three independent experiments.

hours. Drugs were then washed out and the cells were incubated in fresh medium for another 3 hours. Recovery after drug removal was evident from cell spreading over the substratum and the formation of ruffles and stress fibers (Fig. 6D; left panel). As in N2A cells, long-term incubation of CHO cells with CCD and LB induced bi-nucleated cells (Fig. 6D; right panel), although the numbers of bi-nucleation were slightly lower (68% for CCD treated cells and 79% for LB treated cells; Fig. 6E) than those observed in N2A (Fig. 5B). In conclusion, the results presented here demonstrate that CHO cells can progress through the continuous cell cycle despite aberrant cytoskeletal reorganization and a lack of cell spreading after mitosis.

Discussion

In the present study, we examined the role of the actin cytoskeleton in cell cycle progression in continuously cycling cells. Using the mitotic shake off method on N2A and CHO cells, we obtained highly synchronized rounded cells which are competent for growth in the sense that they do not need to downregulate cdk inhibitors or induce expression of cyclin D, in contrast to quiescent cells. We found that inhibition of post-mitotic cell spreading and actin reorganization into stress fibers by the pharmacological inhibitors CCD and LB did not inhibit progression through G1 phase. In fact, cells completed the entire cell cycle with disorganized actin cytoskeletons. Our results show that these cells can progress through the ongoing cell cycle independent of stress fiber formation and the corresponding spread cell shape. This observation seems to be in contrast with a number of studies demonstrating G1 arrest upon disruption of the actin cytoskeleton in a variety of cells (Maness and Walsh, 1982; Ohta et al., 1985; Takasuka et al., 1987; Iwig et al., 1995; Bohmer et al., 1996; Fasshauer et al., 1998; Huang et al., 1998; Reshetnikova et al., 2000; Bottazzi et al., 2001; Huang and Ingber, 2002; Lohez et al., 2003). The majority of these reports, however, focused on cells entering G1 phase from quiescence, demonstrating that cytoskeletal disorganization blocks events crucial to resume proliferation at the G0-G1 border such as downregulation of $p27^{KIP1}$, activation of the p42/p44 MAPKs and the induction of cyclin D1 (Bohmer et al., 1996; Huang et al., 1998; Bottazzi et al., 2001; Huang and Ingber, 2002). Cyclin D1 induction is considered the main rate-limiting step in G0-G1 transition and its expression is induced by growth factor-induced MAPK activity, which is sustained throughout G1 by organization of the cytoskeleton and cell spreading (reviewed by Assoian and Zhu, 1997; Assoian, 1997). In our system, we identified a similar link between cytoskeletal tension/cell spreading and growth factor signaling, since growth factor-induced MAPK activity was considerably reduced by prevention of post-mitotic cytoskeletal reorganization. Furthermore, we observed a progressive decline in cyclin D levels in late G1 phase, thus confirming the link between cell spreading and cyclin D expression. Cyclin D expression was however unaffected early after mitosis, probably because residual MAPK signaling can rescue expression for some time but fails to sustain it throughout the remainder of G1 phase. Alternatively, de novo synthesis of cyclin D ceases already in this stage but it takes a few hours of degradation of existing protein levels before the loss becomes apparent. Either way, cyclin E expression is

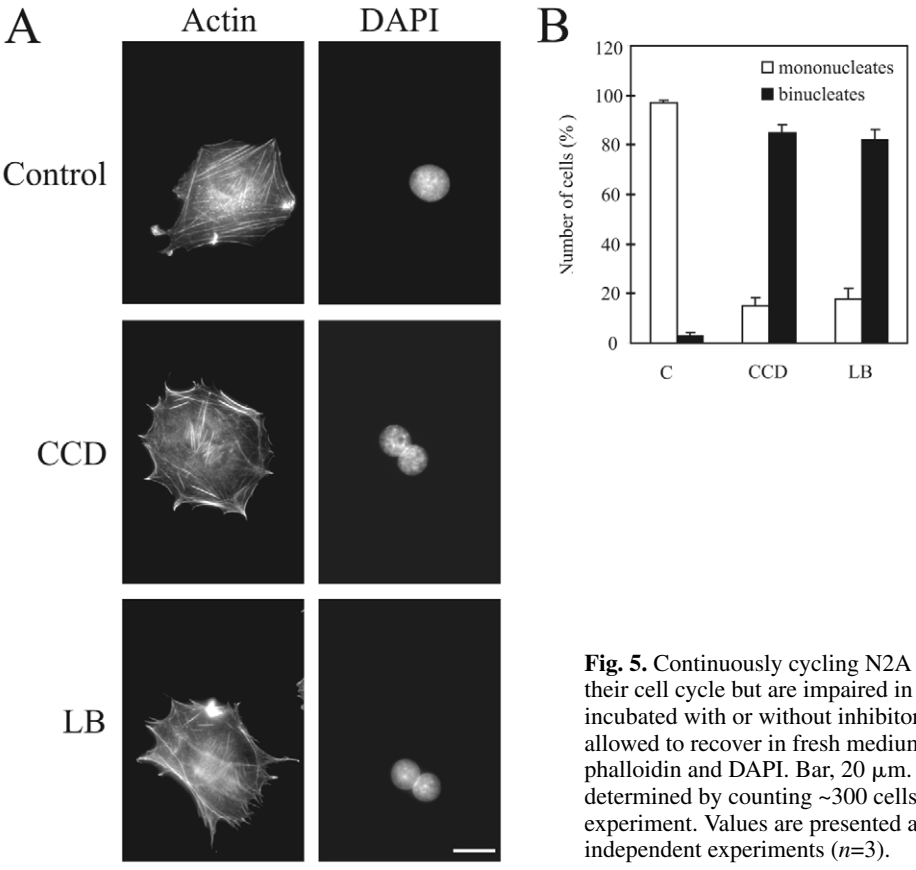


Fig. 5. Continuously cycling N2A cells with a disorganized cytoskeleton complete their cell cycle but are impaired in cytokinesis. (A) Synchronized N2A cells were incubated with or without inhibitors during the length of a cell cycle, washed and allowed to recover in fresh medium for 3 hours, after which they were stained with phalloidin and DAPI. Bar, 20 μm. (B) Mono- and bi-nucleated cells were determined by counting ~300 cells in random fields in each independent experiment. Values are presented as average percentages ± s.e.m. from three independent experiments (n=3).

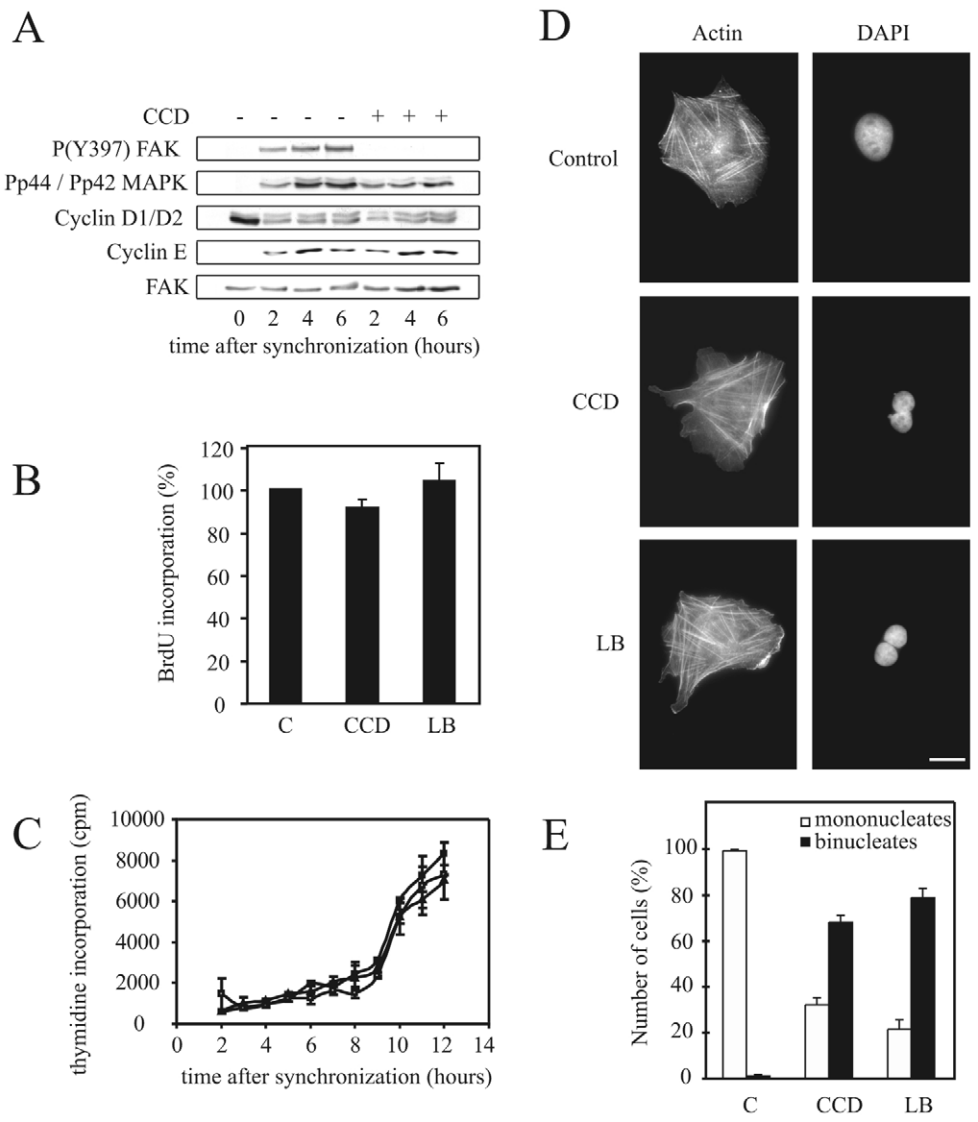


Fig. 6. CHO cells progress through the continuous cell cycle in the absence of actin stress fibers and cell spreading. (A) Phosphorylation of p42/p44 MAPK and (Y397) FAK, as well as expression levels of cyclins D1/D2 and cyclin E were determined by western blotting in mitotic and post-mitotic CHO cells treated with or without CCD (FAK=loading control). (B) DNA synthesis was examined in synchronized CHO cells incubated over night with actin inhibitors and 10 μM BrdU. Values shown represent the average percentages ± s.e.m. obtained from three independent experiments. (C) [^{3}H]thymidine incorporation was assessed in CHO cells progressing from mitosis into S phase in the presence or absence of CCD or LB (untreated=closed squares, LB=open squares, CCD=triangles). A representative experiment is shown; data are expressed as means ± s.e.m. (*n*=3). (D) CHO cells were incubated with the actin inhibitors through one continuous cell cycle (~25 hours), washed and allowed to recover for 3 hours in fresh medium prior to staining with phalloidin and DAPI. Bar, 20 μm. (E) Mono- and bi-nucleated cells were scored in random fields counting ~ 300 cells in each individual experiment. Depicted are the average percentages ± s.e.m. (*n*=3).

normally induced in this timeframe. We assume that pre-existing cyclin D expression in mitotic cells is sufficient to activate the cell cycle machinery at the M-G1 border and to govern progression through G1 phase at least until cyclin E levels are sufficient to take over, thus overriding the need for post-mitotic cell spreading and cytoskeletal organization. In addition, focal adhesion signaling is presumably not required, as concluded from the absence of focal adhesions and FAK phosphorylation. Consistent with this idea is the finding that forced induction of cyclin D1 is sufficient to drive G0-S phase progression in rounded cells with disorganized actin filaments and no focal adhesions (Welsh et al., 2001; Roovers and Assoian, 2003). It was proposed that cell cycle progression can be uncoupled from an organized cytoskeleton and the corresponding spread cell shape, as long as cyclin D1 is induced (Welsh et al., 2001; Roovers and Assoian, 2003). Furthermore, many studies have demonstrated induction of anchorage-independent growth by overexpression of cyclin D1 (Schulze et al., 1996; Zhu et al., 1996; Resnitzky, 1997; Hansen and Albrecht, 1999; Bottazzi et al., 2001), which is an additional indication that D-type cyclins are sufficient to drive proliferation, irrespective of adhesive and cytoskeletal conditions.

Cell cycle progression from M to S phase in our system is summarized in Fig. 7. Normal post-mitotic reattachment and cell spreading in the presence of growth factors are accompanied by stress fiber assembly and focal adhesion formation, which enable full activation of MAPK and FAK, leading to sustained cyclin D expression throughout G1 and the sequential induction of cyclins E and A (condition d). Growth factor withdrawal does not interfere with cell spreading and integrin signaling but induces inhibition of MAPK phosphorylation, the loss of cyclin D and consequent inhibition of cyclin E expression (condition e), whereas transfer of mitotic cells to suspension induces G1 arrest by preventing cyclin E expression (condition a) (Hulleman et al., 1999b). In addition, cells that are transferred to a non-specific substrate such as PLL also arrest in G1 in a similar fashion to cells incubated in suspension (condition b) (Hulleman et al., 1999b). The latter finding demonstrates that simple attachment is not sufficient to initiate proliferation; cells attached to a non-supportive substratum such as PLL fail to

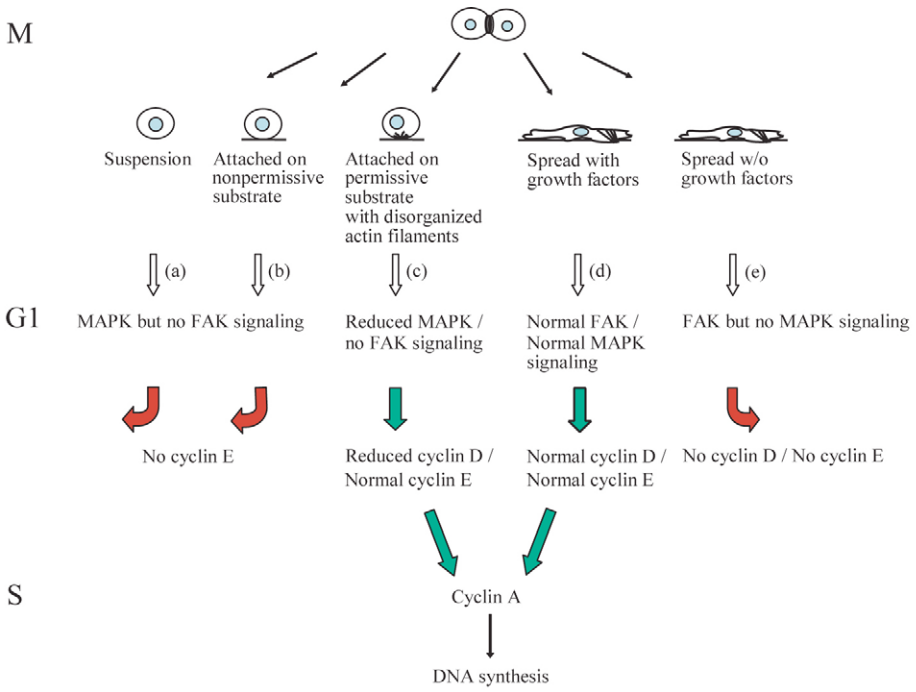


Fig. 7. Model summarizing cell cycle regulation from mitosis to S phase in N2A and CHO cells. Normal cell cycle progression from mitosis into S phase in the presence of growth factors involves cell spreading, stress fiber formation and focal adhesion assembly and is characterized by both FAK and MAPK signaling and the expression of cyclins D and E (condition d). Growth factor depletion after mitosis induces inhibition of MAPK signaling, the loss of cyclin D and the subsequent prevention of cyclin E expression, leading to growth arrest in G1 (condition e). Post-mitotic transfer to suspension or to a non-supportive substratum that allows only non-specific reattachment and no cell spreading also induces cell cycle arrest, via inhibition of FAK signaling and prevention of cyclin E induction (conditions a and b, respectively). However, cells that are released on a permissive substratum (i.e. supportive for integrin signaling and cell spreading) but are not spread because of improper cytoskeletal reorganization do not arrest in G1 despite a progressive loss of cyclin D levels, presumably because pre-existing cell cycle regulators activate the cell cycle machinery sufficiently early after mitosis (condition c).

induce expression of cyclin E and arrest in early G1, whereas cells prevented from cell spreading because of cytoskeletal disorganization on a supportive substratum such as a tissue culture dish do not fail to express cyclin E and cycle through G1 (condition c; this study). Apparently, only a limited contact with the tissue culture dish provides sufficient signals to activate the cell cycle machinery, in contrast to cell adhesion to PLL. The observation that adhesion to a permissive substratum is required for cell cycle progression but cell spreading and cytoskeletal organization are not demonstrates that cell cycle requirements for adhesion and cell spreading are distinct. A similar distinction in cell cycle regulation has been recognized previously in other systems. Reattachment of suspended quiescent fibroblasts was sufficient to induce cell cycle re-entry from G0 into G1, whereas cell spreading and growth factors were required for subsequent progression through G1 into S phase (Dike and Farmer, 1988). Furthermore, quiescent hepatocytes attaching to an (RGD)-peptide which induces integrin activation but not cell spreading exhibited normal activation of junB and ras, but they did not progress through G1 unless cell spreading was allowed (Hansen et al., 1994). In addition, cell shape-dependent effects different from effects of adhesion alone have been demonstrated using ECM-coated adhesive islands; adhesion of quiescent capillary endothelial cells to such micropatterned ECM-islands permitted full activation of MAPKs but not cell cyle progression into S phase (Huang et al., 1998; Mammoto et al., 2004).

Since stress-fiber dependent cell cycle progression is abrogated by transformation (Kurimura and Hirano, 1980; Maness and Walsh, Jr, 1982; Lohez et al., 2003), an important question that arises when interpreting our results is whether the observed cell cycle progression described here does not simply reflect anchorage-independent growth as in many transformed cells. In this respect, it is important to note that post-mitotic serum depletion as well as incubation in suspension abolishes S-phase entry, demonstrating dependency for both mitogens and adhesion as in most nontransformed mammalian cells. However, as anchorage-dependence is progressively lost during transformation (Wittelsberger et al., 1981), the used cell lines may represent an early stage of the transformation process, still requiring mitogen stimulation and adhesion but not extensive spreading. Ultimately, different cell types may differ in their requirements for cytoskeletal tension and cell shape, which may be related to their function in vivo. For example, stress fibers and fibronexus junctions (the in vivo equivalents of focal adhesions) are not commonly detected in living tissues, except in specialized cells including wound fibroblasts and vascular endothelial cells that have to withstand great mechanical stress such as wound contraction and haemodynamic flow (Herman et al., 1982; White et al., 1983; Wong et al., 1983). The lack of these structures in other tissues

questions to what extent cell shape and cytoskeletal tension control proliferation in vivo.

Materials and Methods

Cell culture, synchronization and treatment

Neuroblastoma 2A (N2A) and Chinese hamster ovary (CHO; strain K1) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES (Gibco, Paisley, UK), supplemented with 7.5% fetal calf serum (Gibco) at 37°C under low CO_2 conditions. Asynchronously growing cell cultures were synchronized by mitotic shake off. Two days prior to synchronization, cells were replated at a density of 1.5×10^4 cells per cm^2. Mitotic cells were obtained by shaking the cell cultures firmly by hand for 1 minute at 37°C and collecting the medium as described previously (Boonstra et al., 1981). After shake off, mitotic cells were replated on tissue culture dishes (Nalge Nunc International, Denmark) or in tissue culture plates (Corning, NY) at a density of 1.5×10^4 cells per cm^2 unless otherwise indicated. Cells were incubated with cytochalasin D (CCD; Sigma, Steinheim, Germany), latrunculin B (LB; Calbiochem, San Diego, CA), or dimethylsulfoxide (DMSO; Sigma). CCD and LB were prepared from 1 mg ml^{-1} stock solutions in DMSO. Drugs were further diluted in culture medium, with the final concentration of DMSO not exceeding 0.05%.

Cell extraction and western blotting

Cells were washed once with ice-cold phosphate-buffered saline (PBS) and subsequently lysed on ice in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% sodiumdodecylsulfate (SDS), 1 mM EDTA, 100 mM NaF, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride and 1 mM sodium orthovanadate). Cells that were not allowed to attach to the substratum such as mitotic cells were spun down by centrifugation for 7 minutes at 1400 ***g*** prior to lysis. Collected lysates were cleared for 2 minutes at 8000 ***g*** and the amount of protein was measured using the Bradford assay using a Bio-Rad novapath™ microplate reader. Equal amounts of protein (10 μg) were fractionated on 12% gels and proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Boehringer-Mannheim, Indianapolis, IN) according to standard procedures. Western blots were probed using anti-phosphorylated p42/p44 MAPK (1:1000; New England Biolabs, Beverly, MA), anti-phosphorylated Y397-FAK (1:1000; Biosource International, USA), anti-cyclin E (1:1000; Santa Cruz), anti-cyclin A (1:25; Calbiochem), anti-p34/p36 cyclin D1/2 (1:500) and anti-p42 MAPK (1:1000; both from Upstate Biology, Lake Placid, NY). Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies (1:5000; Jackson Immunoresearch laboratories, West Grove, PA) and enhanced chemiluminescence reagents (Dupont, Wilmington, DE).

For quantification of protein expression levels, western blots were subjected to densitometric analysis using Advanced Image Data Analyzer 340 (version 3.40.029) software. Band intensity of proteins of interest was normalized to band intensity of p42 MAPK and means were calculated from three separate experiments.

Fluorescence microscopy and phase-contrast microscopy

Cells in six-well plates were fixed in 4% (w/v) paraformaldehyde in PBS, washed with PBS and permeabilized with 0.2% Triton X-100. After washing with PBS, cells were blocked with 50 mM glycin in PBS for 10 minutes and subsequently incubated with a monoclonal antibody directed against vinculin (diluted 1:100; Sigma). After several washings with PBS containing 0.2% gelatin, cells were incubated with a FITC-conjugated secondary antibody (1:200; Jackson Immunoresearch laboratories) and TRITC-conjugated phalloidin (1 μg ml^{-1}; Sigma) and washed again as described above. Finally, cells were mounted in mowiol under coverslips (Ø 18 mm; Menzel, Germany) and fluorescence was visualized with a Leitz microscope (Orthoplan Flu 043944) equipped with Leitz objective lenses (40×/1.3 numerical aperture and 63×/1.4 numerical aperture). Images were acquired with a cooled Leica CCD camera (model DC350F) using Leica Image Manager 50 software. Pictures were processed with Adobe Photoshop® 7.0.

For phase-contrast microscopy, cells were fixed as described above and studied on a Zeiss microscope (Axiovert 25) at 10× (numerical aperture 0.25) and 20× (numerical aperture 0.3) magnification. Images were captured on a Zeiss CCD camera (Axiocam MRC) using Zeiss Mr. Grab 1.0 software and processed with Adobe Photoshop® 7.0.

BrdU labeling and quantification

Synchronized cells were plated in 96-well plates at a density of 1×10^4 cells per well and incubated over night with 10 μM 5-bromo-2′-deoxy-uridine (BrdU; Boehringer-Mannheim) at 37°C. BrdU incorporation was determined using the Cell proliferation, Enzyme-linked immunosorbent assay (ELISA) kit (Boehringer-Mannheim), according to the manufacturer's instructions. Absorbance was measured on a Bio Rad novapath™ microplate reader 5 minutes after substrate addition. In each experiment, both medium containing BrdU but without cells as well as cells supplemented with BrdU but fixed before S phase were used as a negative control. Independent experiments were performed with six samples for each condition and each independent experiment was repeated at least three times.

[^{3}H]thymidine incorporation

Mitotic cells were plated in 24-well plates and incubated with or without the inhibitors and 5 μCi ml^{-1} [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 37°C. At the indicated times after plating, cells were washed twice with PBS and 10% trichloroacetic acid and subsequently dissolved in 0.1 M NaOH. The incorporated [^{3}H]thymidine was quantified on a scintillation counter (LS 6000 SE, Beckman instruments, Fullerton, CA).

Cell cycle analysis

Synchronized cells were plated in six-well plates and incubated under the appropriate conditions. Cells were then washed and released in fresh medium for 3 hours, fixed and labeled with phalloidin and 4′,6′-diamidino-2-phenylindole (DAPI; Boehringer-Mannheim). Cells were visualized and images were acquired as described above. In each independent experiment, cell numbers and nuclei were determined for ~300 cells in multiple fields and experiments were performed at least three times.

Statistical analysis

Statistical comparison of means was performed using unpaired two-tailed Student's *t*-tests. Significant differences compared with untreated cells are indicated by asterisks ($P<0.05$).

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