Cytoskeletal Integrity Is Required throughout the Mitogen Stimulation Phase of the Cell Cycle and Mediates the Anchorage-dependent Expression of Cyclin D1

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Submitted July 24, 1995; Accepted October 23, 1995
Monitoring Editor: Timothy J. Mitchison

The proliferation of many nontransformed cells depends on cell adhesion. We report here that disrupting the cytoskeleton in normal human fibroblasts causes the same cell cycle phenotype that is observed after blocking cell adhesion: suspended cells and cytochalasin D-treated monolayers fail to progress through G1 despite normal mitogen-induced expression of c-myc mRNA. Midway between G0 and the beginning of S-phase, cell cycle progression becomes independent of adhesion and the cytoskeleton. At this stage, the cells are also mitogen independent. Molecular analyses showed that Rb hyperphosphorylation and the induction of cyclin D1 occur slightly earlier than the transition to cytoskeleton independence. Moreover, these molecular events are blocked by cytochalasin D. Overall, our data indicate the following: 1) anchorage and cytoskeletal integrity are required throughout the mitogen-dependent part of G1; 2) mitogens and the cytoskeleton jointly regulate the phosphorylation of Rb; and 3) this interdependence is manifest in the regulation of cyclin D1.

INTRODUCTION

The G1 phase of the fibroblast cell cycle is regulated by external signals that come from mitogenic growth factors and the extracellular matrix. Mitogen stimulation results in the activation of several intracellular kinases such as the MAP kinases, protein kinase C, and PI-3 kinase (Cantley *et al.*, 1991; Johnson and Vaillancourt, 1994; Schlessinger, 1994). Cell adhesion to the extracellular matrix stimulates the same kinases as well as focal adhesion kinase (Guan and Shalloway, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992; Vuori and Ruoslahti, 1993; Chen *et al.*, 1994a,b; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995). Ultimately, the signal transduction cascades stimulated by mitogens and the extracellular matrix must regulate the expression or activation of the cyclin-dependent kinases (cdks) because these enzymes trig-

ger the biochemical events that allow cells to progress through distinct transitions of G1 phase (reviewed in Hunter and Pines, 1994 and Sherr, 1993, 1994). Once past G1, completion of the cell cycle requires neither mitogens nor the matrix (Otsuka and Moskowitz, 1975; Matsuhisa and Mori, 1981; Pardee, 1989; Han *et al.*, 1993).

The transition from mitogen-dependent to mitogen-independent cell cycle progression occurs in late G1 and has been termed the restriction point R (Pardee, 1989). It now seems clear that mitogen-stimulated transit through R requires the activation and action of the G1 cdks. Mitogens stimulate the expression of the D type cyclins (D1, D2, and D3) and cyclin E (Lew *et al.*, 1991; Matsushime *et al.*, 1991; Koff *et al.*, 1992). These proteins bind to and activate cdk4/6 and cdk2, respectively; cyclin D1 and cdk4 form the major D/cdk complex in fibroblasts (Dulic *et al.*, 1992; Koff *et al.*, 1992; Matsushime *et al.*, 1992, 1994; Meyerson and Harlow, 1994). Cyclin D1 is induced by mitogens in mid G1, and cyclin D-cdk4 activity is detected coinci-

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dent with Rb phosphorylation in vivo (Matsushime *et al.*, 1994). Rb is also the principal substrate for the cyclin D-cdk4 in vitro (Matsushime *et al.*, 1992, 1994; Meyerson and Harlow, 1994), and Rb-null fibroblasts lose their requirement for cyclin D (Lukas *et al.*, 1994, 1995). Cyclin E/cdk2 is also likely to be involved in the phosphorylation of Rb, but this cdk complex has substrates other than Rb (Dulic *et al.*, 1992; Koff *et al.*, 1992; Ohtsubo *et al.*, 1995). Although the relative contributions of cyclin D-cdk4 and cyclin E-cdk2 in the phosphorylation of Rb are still a matter of investigation, it now seems likely that phosphorylation of Rb by the G1 cyclin-cdks represents both the culmination of mitogen signaling and cell cycle progression through R (Sherr, 1994).

In some fashion, the cell cycle events regulated by mitogens and the extracellular matrix must interact because neither mitogens nor the extracellular matrix alone are sufficient to induce transit through G1 in nontransformed cells. Our recent studies indicate that cell adhesion regulates the G1 cyclin-dependent kinases (Guadagno *et al.*, 1993; Zhu, X., Ohtsubo, M., Böhmer, R.-M., Roberts, J.M., and Assoian, R.K., unpublished data). For example, by comparing cell cycle progression in monolayer and suspended cells, we found that mitogen-treated cells in suspension poorly express cyclin D1 and cyclin A mRNAs. Adhesion to extracellular matrix also has a small repressive effect on the expression of the cdk inhibitor p21.

The extracellular matrix transmits growth information through the activation of integrins, and integrins have effects that occur as a direct response to clustering and as an indirect response to integrin-dependent organization of the cytoskeleton. For example, integrin clustering leads to the activation of protein kinase C, focal adhesion kinase, PI-5 kinase, and MAP kinase (Schwartz *et al.*, 1991; Kornberg *et al.*, 1992; Schwartz and Lechene, 1992; McNamee *et al.*, 1993; Vuori and Ruoslahti, 1993; Chen *et al.*, 1994b; Morino *et al.*, 1995; Zhu and Assoian, 1995). The events mediated by integrin-dependent cytoskeletal organization, and the resulting change in cell shape, are not well defined biochemically, but they seem to map to mid-late G1 in both hepatocytes and endothelial cells (Hansen *et al.*, 1994; Ingber *et al.*, 1996). In this report, we have studied the kinetics of DNA synthesis, the phosphorylation of Rb, and the expression of cyclin D1 to compare the roles of cell adhesion and adhesion-dependent organization of the cytoskeleton in regulating cell cycle progression of normal human fibroblasts.

MATERIALS AND METHODS

Cell Culture

Early passage human skin fibroblasts were obtained from explants of infant foreskins according to standard procedures. The cultures were maintained in DMEM (low glucose; Life Technologies, Gaith-

ersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS). Cultures were grown to density-based arrest and then incubated for 6 days in serum-free DMEM supplemented with a 1:500 dilution of ITS⁺ (a defined medium supplement; Collaborative Research, Bedford, MA). To stimulate entry into the cell cycle, the cultures were trypsinized, suspended in fresh medium with 10% FCS, and 12 ng/ml epidermal growth factor (EGF), and transferred either to normal tissue culture dishes or to dishes coated with 1% agarose as described below. For all experiments, cells were seeded subconfluently at a density of 3000–6000 per cm² (35-mm dishes for flow cytometry and 150-mm dishes for molecular studies). Cytochalasin D, nocodazole, and staurosporine were dissolved in dimethyl sulfoxide (DMSO) as 1000-fold stock solutions and diluted in medium before use. For experiments performed with serum-free medium, type I collagen was used to support cell spreading and purified EGF was used as the mitogen.

Fresh serum-free medium strongly inhibits cell cycle progression of human fibroblasts (Böhmer and Burgess, 1989). Therefore, EGF-stimulation in the absence of serum was performed with conditioned medium. To generate sufficient quantities of conditioned medium, extra serum-free medium (50–100 ml per 150-mm dish) was added to the quiescent cultures 2 days before the experiments. Trypsinized quiescent cultures were briefly washed with DMEM and 1 mg/ml soybean trypsin inhibitor, suspended in conditioned medium with and without EGF, and seeded onto 150-mm dishes coated with agarose or 200 µg of type I collagen (Vitrogen 100, Celtrix, Palo Alto, PA).

NIH-3T3 cells were rendered quiescent similarly to the procedure described by Zhu and Assoian (1995). Normal rat kidney (NRK) fibroblasts (clone 49F) were grown in DMEM with 10% FCS. These cells were rendered quiescent and stimulated as described above for human fibroblasts. Under these conditions, NRK fibroblasts become largely anchorage independent for progression through the first cell cycle. Similar results have been obtained by others (Newman *et al.*, 1986).

To prepare the cultures of nonadherent cells, plastic petri dishes were coated with a layer of 1% agarose in water. A 1% solution of agarose (electrophoresis grade) in water was sterilized by boiling for 20 min. The boiling-hot solution (~15 ml) was used to quickly coat a 150-mm dish, and as much as possible of the solution was removed. The remaining agarose film (~2 ml) was allowed to harden at room temperature, and then the dishes were equilibrated by two overnight incubations at 37°C with 20 ml of DMEM containing the additives that would be used in the subsequent experiment.

Cell Cycle Kinetics by Flow Cytometry

The proliferative response of G0-synchronized cultures was monitored using two alternative flow cytometric methods as appropriate. Cell cycle transit in suspension and during exposure to cytochalasin D was monitored under conditions where cell division was blocked by incubation with the microtubule-disrupting agent nocodazole (stathmokinesis; Barford and Barford, 1980; Bohmer, 1980; Darzynkiewicz *et al.*, 1981; Dosik *et al.*, 1981). Nocodazole (5 µg/ml) was added to the cultures at the onset of S phase (12 h for NRK and 3T3, 22 h for human fibroblasts). The cells were stained with the DNA-specific dye 33258 Hoechst (2 µg/ml in phosphate-buffered saline with 25% ethanol) and analyzed by flow cytometry. A sequence of single-parameter DNA histograms was used to determine the proportions of cells that had progressed past the middle of S-phase as a function of time after stimulation with mitogens. Discrimination at the mid-S point was chosen as a simple and reliable alternative to the difficult discrimination at the G1/S boundary. Measurements of passage through G1/S and mid-S are equivalent for the purpose of our experiments because cell adhesion does not regulate progression through S phase (Otsuka and Moskowitz, 1975; Matsuhisa and Mori, 1981; Han *et al.*, 1993).

The time course of cell division was quantified using the quenching of Hoechst fluorescence by BUDR incorporation. Briefly, cultures

were exposed to 20 $\mu\text{g/ml}$ BUdR and 5 $\mu\text{g/ml}$ deoxycytidine, beginning at the time of mitogen stimulation. The cells were harvested at selected times and suspended in dye solution (2 $\mu\text{g/ml}$ 33258 Hoechst and 10 $\mu\text{g/ml}$ ethidium bromide in phosphate-buffered saline with 25% ethanol). Fluorescence was excited with a 320 nm UV laser and recorded at 420–440 nm (Hoechst) and >630 nm (ethidium bromide). Incorporation of BUdR prevents the increase in Hoechst-stainable DNA that normally accompanies cell cycle progression through S phase. Therefore, newly divided cells have one-half the Hoechst fluorescence of the G0/G1 cells that had not traversed the cycle. Ethidium bromide fluorescence, which is not affected by BUdR, reveals the distribution of cells within the cell cycle before and after division. For details, see Böhmer and Ellwart, 1980 and Böhmer, 1990.

Western and Northern Blot Analysis

Quiescent human fibroblasts were stimulated with mitogens in monolayer and suspension, and collected at selected times (10^6 cells per time point). For Western blotting, cellular protein was extracted, and 200 μg aliquots from each sample were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies specific for Rb (CIBA-Corning, Alameda, CA) and cyclin D1 (Upstate Biotechnology, Lake Placid, NY); immunoreactive proteins were detected by enhanced chemiluminescence (see Zhu and Assoian, 1995 for all details). For Northern blotting, total RNA was extracted in guanidine isothiocyanate-containing buffer. Equal amounts of the purified RNAs were fractionated on 1% agarose gels containing formaldehyde, transferred electrophoretically to Hybond-N membranes (Amersham, Arlington Heights, IL), and hybridized to random-primed cDNAs: a 1.35-kb *EcoRI*–*PstI* fragment encoding human cyclin D1 cDNA and a 1.1-kb *EcoRI*–*HindIII* fragment encoding c-myc cDNA. Filters were washed with $0.2\times$ SSPE, 0.1% SDS at 68°C and exposed to x-ray film for 1/2 day (cyclin D1) or 2 days (c-myc) at -70°C . See Wager and Assoian (1990) for details of RNA isolation, fractionation, and hybridization.

RESULTS

Cell Cycle Progression of Quiescent Human Fibroblasts Requires Cell Anchorage

Although it is well established that cell adhesion is required for cell cycle progression through the G1 phase of fibroblasts, the points at which adhesion exerts its effects within G1 are less clear. Quiescent NRK cells enter G1 when stimulated with mitogens in suspension (Guadagno and Assoian, 1991) whereas events associated with the G0/G1 transition fail to occur when other fibroblastic cell lines are exposed to mitogens in suspension (Dike and Farmer, 1988; Schwartz *et al.*, 1991). Because these differences could be due to the use of established cell lines, we asked whether normal, early-passage human skin fibroblasts can enter the cell cycle in the absence of substratum. We also used flow cytometry (rather than a selected molecular marker) to quantify actual progression toward mitosis. Quiescent human fibroblasts were incubated with mitogens in suspension for different times (0, [control], 9, and 20 h), reseeded in monolayer (in the continued presence of mitogens), and the subsequent time course of cell division was determined using BUdR/Hoechst flow cytometry (Figure 1A). The kinetics of cell division for the pre-stimulated and

Figure 1. Lack of cell cycle progression in suspended fibroblasts. Quiescent human fibroblasts (A) and NIH-3T3 cells (B) were seeded in suspension and incubated with mitogens (FCS/EGF) for selected times (0, 9, and 20 h in panel A and 0, 6, and 12 h in panel B). The cells were then trypsinized and seeded in normal tissue culture dishes in their conditioned media. Under these conditions, the cells adhered within 1 h. Flow cytometry (BUdR/Hoechst/EB method, see MATERIALS AND METHODS) was used to quantify the proportions of divided cells as a function of time after reseeding in monolayer.

control cells were indistinguishable, indicating that the time to cell division is not reduced by exposing nonadherent cells to mitogens. Thus, consistent with results obtained by others (Otsuka and Moskowitz, 1975; Hansen *et al.* 1994; Ingber *et al.*, 1996), significant cell cycle progression does not appear to occur when normal human fibroblasts are exposed to mitogens in the absence of substratum. This experiment was also performed with NIH-3T3 cells, and the same results were obtained (Figure 1B).

Growth Factor Action in the Absence of Substratum

Although mitogens did not appear to induce G1 cell cycle progression in suspended human fibroblasts, they were able to regulate the expression of c-myc

mRNA. In fact, serum and EGF induced similar levels of c-myc mRNA in monolayer and suspension cultures of quiescent human fibroblasts (Figure 2A; the slightly stronger stimulation of c-myc in suspended cells was not reproducible). Moreover, the subsequent decrease in c-myc mRNA levels that accompanies mitogen-stimulated cell cycle transit was also observed in both culture conditions (Figure 2A). Because serum contains vitronectin and small amounts of fibronectin that could potentially activate adhesion-regulated cell cycle events, we asked whether EGF alone would induce c-myc mRNA in suspended human fibroblasts

cultured in serum-free medium. Figure 2B shows that EGF induced c-myc mRNA in suspension to nearly the same extent as the combination of EGF and serum. These data show that the apparent absence of G1 cell cycle progression in suspended human fibroblasts is not due to an inability of these cells to respond to mitogens.

Cytochalasin D as a Probe for Anchorage Dependence

Disruption of the cytoskeleton by cytochalasin D can inhibit cell cycle progression as well as molecular events that are associated with integrin signaling (Lipfert *et al.*, 1992; Chen *et al.*, 1994a,b; Morino *et al.*, 1995; Zhu and Assoian, 1995; Ingber *et al.*, 1996). However, cytochalasin D-treated cells retain a significant degree of attachment and can therefore be used to help distinguish the effects arising from adhesion-based signaling and adhesion-dependent organization of the cytoskeleton.

The concentration-dependent effects of cytochalasin D on mitogen-induced cell cycle progression of human fibroblasts were determined using flow cytometry (Figure 3A). Concentrations greater than 1 $\mu\text{g/ml}$ resulted in a G1 block whereas lower concentrations (0.1–1 $\mu\text{g/ml}$) yielded a G2/M block and an incomplete G1 block. S phase progression was not blocked by cytochalasin D (our unpublished observations). The G2/M block precluded analysis of cell cycle progression by division kinetics. Therefore, cell cycle progression in cytochalasin D-treated cells was determined by measuring the accumulation of cells in G2/M (stathmokinesis; see MATERIALS AND METHODS). In human fibroblasts, cytochalasin D completely blocked mitogen-stimulated progression into S phase (Figure 3B) whereas in anchorage-independent cultures of NRK fibroblasts (see MATERIALS AND METHODS), cytochalasin D caused the same small cell cycle delay that was observed in suspension cultures (Figure 3C). We also found that cytochalasin D did not prevent cell cycle progression of murine T-lymphocytes stimulated with phorbol ester and ionomycin (our unpublished observations). We conclude that incubation with cytochalasin D and incubation in suspension appear to be equivalent with regard to their effects on cell cycle progression into S phase.

Figure 2. Anchorage-independent expression of c-myc mRNA. (A) Quiescent human fibroblasts were exposed to mitogens in monolayer (M) and suspension (S). The cells were stimulated with FCS/EGF and collected at 0, 5, and 24 h after stimulation. (B) Monolayer and suspension cultures of quiescent human fibroblasts were incubated for 5 h as follows: in the absence of mitogens (–); with EGF; or with FCS/EGF. For both experiments, equal amounts of total RNA were analyzed by Northern blot hybridization to quantify the level of c-myc mRNA. RNA loading was assessed in ethidium bromide-stained gels and is shown as 28S rRNA.

Coincident Requirements for Adhesion, Mitogens, and Cytoskeletal Integrity

Experiments were performed to identify and compare the portions of G1 phase that are dependent upon cell adhesion, mitogens, and cytoskeletal integrity. To map the adhesion requirement within G1, we stimulated quiescent human fibroblasts with mitogens in monolayer, transferred them to suspension at selected

Figure 3. Cytochalasin D as a probe for anchorage-dependence. (A) Quiescent human fibroblasts were trypsinized and seeded with mitogens (FCS/EGF) in the presence of various concentrations of cytochalasin D. The BudR/Hoechst/EB method was used to determine the percent of cells that had divided (DIV) or were blocked in G2/M or G1. To this end, cultures were exposed to BudR from the beginning of mitogenic stimulation and analyzed by flow cytometry 36 h after stimulation. (B) Quiescent human fibroblasts were trypsinized and seeded with mitogens in monolayer (MON), in suspension (SUS), or in monolayer with 2 μ g/ml cytochalasin D (CCD). After 24 h (i.e., before the first cycling cells had

times throughout G1 phase, and determined the proportions of cells that were able to continue through the cell cycle. Figure 4A shows that deprivation of anchorage completely blocked cell cycle progression even when the transfer to suspension was performed 12 h after stimulation with mitogens. However, after longer stimulation times in monolayer, an increasing number of cells were able to progress through the cell cycle after transfer to suspension. After 22 h of mitogen stimulation in monolayer, almost all of the cells had reached the adhesion-independent part of the cell cycle, yet the very large majority (>90%) of the cells were still in G1 phase (DNA histograms not shown). Thus, the transition to adhesion-independent cell cycle progression occurs in late G1 phase.

To examine the requirement for cytoskeletal integrity, parallel cultures were exposed to cytochalasin D rather than being transferred to suspension. At all times throughout G1 the proportion of cells that had become adhesion-independent was equal to the proportion that had become cytoskeleton-independent (Figure 4A). A similar conclusion has been reached with capillary endothelial cells (Ingber *et al.*, 1996).

The experiments in Figure 4A define anchorage- and cytoskeleton-independent growth as the ability of cells to reach G2/M by a fixed time, which was chosen so that all cells that could enter S phase would have completed their progression into G2/M. This assay does not exclude the possibility that the *rate* of cell cycle progression might still be affected by anchorage or the cytoskeleton. To address this question, we used flow cytometry to analyze the speed of cell cycle progression in cells that had already passed the point of adhesion/cytoskeleton independence. We found that these cells traversed the second half of G1 and S phase with the normal speed (our unpublished observations). Thus, the transition to adhesion- and cytoskeleton-independent cell cycle progression is discrete and complete.

When quiescent fibroblasts in monolayer are stimulated with mitogens, they progress through the G1 phase until they reach a point of mitogen independence that has been termed the restriction point R (Pardee, 1989). To investigate the relative positions of R and the point of adhesion-independence, we repeated the experiment shown in Figure 4A except that the incubation in suspension was performed in the absence and presence of mitogens. Figure 4B shows that the proportions of cells that were able to proceed

(Figure 3 cont.) reached mitosis), the metaphase blocker nocodazole was added to the cultures to prevent re-entry of proliferating cells into the G0/G1 phase. Cells were harvested and DNA histograms were recorded to determine the percent of cells that had progressed past mid-S as function of time after stimulation with mitogens. (C) The experiment in panel B was repeated with anchorage-independent NRK cells.

Figure 4. Coincident requirements for adhesion, mitogens, and cytoskeletal integrity. Using the experimental design shown at the top of the figure, we monitored the transition to anchorage-, cytoskeleton-, and mitogen-independent cell cycle progression within the G1 phase of the cell cycle. (A) Quiescent human fibroblasts were incubated with mitogens (FCS/EGF) in monolayer for the times shown. The cells were then either trypsinized and transferred to suspension (SUS) in their conditioned media or directly treated with cytochalasin D (CCD, 2 μ g/ml). (B and C) Quiescent human fibroblasts (B) and NIH-3T3 cells (C) were incubated with mitogens (FCS/EGF) in monolayer for the times shown, trypsinized, washed, and then transferred to suspension either in the absence or the continued presence of mitogens (GF). In all cases, the metaphase blocker nocodazole was added to the cultures (at 24 h for human fibroblasts and 12 h for NIH-3T3 cells) to prevent reentry into G1 phase. Cells were harvested at a time when all cells that were not blocked in G1 had reached G2/M (36 h for human fibroblasts and 28 h for NIH-3T3). The percent of cells that had accumulated in G2/M was determined using DNA histograms. The arrowheads in A–C indicate the time when the fastest cells reached S phase.

through the cell cycle in suspension were not affected by the lack of mitogens. Thus, cells that had lost their adhesion requirement had also lost their mitogen requirement for further cell cycle progression. The same result was obtained with NIH-3T3 cells (Figure 4C) despite their different mitogen requirements and shorter G1 phase (12–14 h). These results indicate that the transition to mitogen independence occurs at the same time or before the transition to adhesion independence.

Hyperphosphorylation of the Retinoblastoma Protein Requires an Organized Cytoskeleton

Cell cycle progression through R is thought to coincide with the mid-late G1 hyperphosphorylation of the retinoblastoma protein (see INTRODUCTION). We therefore asked whether progression into the cytoskeleton-independent portion of G1 would coincide with the hyperphosphorylation of Rb in human fibro-

blasts. Quiescent cells were stimulated with mitogens in monolayer and collected at selected times for an analysis of Rb phosphorylation. Hyperphosphorylation of Rb (detected as reduced electrophoretic mobility by immunoblotting) began $\approx$ 10 h after stimulation and this effect was blocked by cytochalasin D (Figure 5A). Thus, the onset of Rb hyperphosphorylation appears somewhat earlier ($\approx$ 2 h) than the time at which the first cells enter the anchorage/cytoskeleton-independent portion of G1 (Figures 4 and 5).

Staurosporine is a relatively nonspecific inhibitor of protein kinases involved in mitogenic signaling, and it blocks cell cycle progression in G1 phase (Tamaoki *et al.*, 1988; Matsumoto and Sasaki, 1989; Elliott *et al.*, 1990; Fallon, 1990; Badwey *et al.*, 1991; Yanagihara *et al.*, 1991). Cells become resistant to staurosporine in late G1 (Matsumoto and Sasaki, 1989). In initial experiments, we found that incubation with staurosporine from the beginning of mitogenic stimulation inhibited

G1 cell cycle progression in human fibroblasts without having any visible effect on cellular adhesion and spreading (our unpublished observations). Figure 5B shows that the transition to staurosporine-resistant cell cycle progression preceded the transition to cytoskeleton-independent cell cycle progression by ≈ 2 h. Thus, the onset of staurosporine resistance closely matched the onset of Rb hyperphosphorylation (Fig-

Figure 5. Cytoskeleton-dependent Rb phosphorylation and cyclin D1 expression. (A) Quiescent human fibroblasts were incubated with mitogens (FCS/EGF) for selected times from G0 to S phase. The hyperphosphorylation of Rb and the expression of cyclin D1 were determined by SDS-polyacrylamide gel electrophoresis and immunoblotting as described in MATERIALS AND METHODS. The analysis at 21 h was performed in the presence and absence of cytochalasin D (CCD; 2 μ g/ml). The upper and lower arrows show the positions of hyper and hypophosphorylated Rb, respectively. (B) The point in G1 where human fibroblasts become refractory to the action of the kinase inhibitor staurosporine (STP) was determined using the protocol described for Figure 4. Quiescent human fibroblasts were incubated with mitogens (FCS/EGF) in monolayer for the times shown. STP (5 ng/ml) or CCD (2 μ g/ml; as reference) was then added to the cultures and the percent of cells that could enter S-phase and accumulate in G2/M was determined (36 h after the beginning of mitogen stimulation). Note that all data shown in this figure were derived from the same experiment.

ure 5A). This result indicates the following: 1) the phase of adhesion/cytoskeleton-dependent cell cycle progression downstream of Rb phosphorylation does not involve any staurosporine-inhibitable kinases, and 2) the transition to staurosporine-resistance can be considered a marker for R.

Induction of Cyclin D1 mRNA by Mitogens Requires an Organized Cytoskeleton

The expression of cyclin D1 is stimulated by mitogens, and cyclin D-cdk4/6 complexes are known to phosphorylate the retinoblastoma protein (see INTRODUCTION). We therefore asked whether the cytoskeleton requirement for Rb hyperphosphorylation might be linked to the regulation of cyclin D1. The filter used for the Rb immunoblot was reprobed with an antibody to cyclin D1 (Figure 5A, bottom). Densitometric scanning showed the following: 1) cyclin D1 protein was near maximally induced ≈ 11 h after mitogen stimulation of quiescent human fibroblasts, 2) the induction persisted for at least 21 h, and 3) the expression of cyclin D1 was reduced 10-fold by exposure to cytochalasin D (Figure 5A).

We next asked whether the cytoskeleton dependency of cyclin D1 expression was reflected in the regulation of its mRNA. Consistent with the expression of cyclin D1 protein, the induction of cyclin D1 mRNA was near-maximal after ≈ 12 h of stimulation with mitogens, and this induction persisted for at least 22 h (Figure 6A). We also detected an anchorage- and cytoskeleton-independent induction of cyclin D1 mRNA occurring 3–6 h after stimulation with mitogens and persisting for at least 16 h (Figure 6, B and C). However, this effect was small in comparison to the induction seen in monolayer. Thus, cell anchorage is required for the full induction of cyclin D1 mRNA, and this requirement is based on the need for an organized cytoskeleton rather than adhesion per se. Consistent with the results in Figure 5B, we also found that staurosporine completely inhibited the induction of cyclin D1 mRNA when added together with mitogens (our unpublished observations).

To address the possibility that adhesion and cytoskeletal organization might be sufficient to induce cyclin D1 mRNA, quiescent human fibroblasts were seeded on collagen-coated plates in mitogen-free medium. These cells were fully spread within 2 h, but the induction of cyclin D1 mRNA was very small even after 22 h (Figure 7). Addition of EGF stimulated the expression of cyclin D1 mRNA in the cells spread on collagen, but it was without effect when added to the cells in suspension (Figure 7). We conclude that neither EGF nor adhesion/spreading are sufficient to stimulate the expression of cyclin D1 mRNA.

Figure 6. Cytoskeleton-dependent induction of cyclin D1 mRNA. Quiescent human fibroblasts were incubated with mitogens (FCS/EGF) in monolayer (MON), in suspension (SUS), or in monolayer with cytochalasin D (CCD; 2 μ g/ml). Cells were harvested at selected times from G0 to S phase and total RNA was extracted. Similar amounts of RNA (refer to 28 S) were analyzed by Northern blot hybridization to quantify the level of cyclin D1 mRNA. Cytochalasin D was added 2 h before addition of mitogens.

DISCUSSION

The studies described here address the relative contributions of mitogens, cell adhesion, and cytoskeletal integrity in cell cycle progression of fibroblasts. We found that progression from quiescence into the cell cycle is adhesion dependent in both human fibroblasts and NIH-3T3 cells. This result differs from the results we reported previously in the NRK fibroblast cell line (Guadagno and Assoian, 1991). In light of the present data with normal diploid cells, it now seems likely that NRK cells have lost the controls that render the G0/G1 transition dependent upon cell adhesion. This conclusion is supported by our recent findings that NRK cells, but not NIH-3T3 or human fibroblasts, can express cyclin D1 and phosphorylate Rb in suspension (Zhu, X., Ohtsubo, M., Böhmer, R.-M., Roberts, J.M., and Assoian, R.K., unpublished data). Our data with NIH-3T3 cells and human fibroblasts are also consistent with studies by McNamee *et al.* (1993) who showed that mitogens fail to stimulate the turnover of

phosphatidyl inositol biphosphate (a G0/G1 event) when 10T1/2 fibroblasts are cultured in the absence of substratum.

However, we found that the inhibition of cell cycle progression in suspension depended on the conditions of quiescence for human fibroblasts. When confluent human fibroblasts were serum starved for only 3 days (rather than our standard 6 days), they underwent a slow but readily detectable progression through G1 during exposure to mitogens in suspension (our unpublished observations). It is presently unclear why this prolonged serum starvation is required because cells that are serum starved for 2–3 days appear fully quiescent as judged by complete lack of mitotic figures, >98% of cells with 2n DNA content, and cell cycle re-entry with their characteristic degree of synchrony. In contrast, mitogen-induced cell cycle progression of NIH-3T3 cells was completely anchorage dependent even after only 20 h of serum starvation.

Our studies with human fibroblasts showed that expression of the c-myc gene is anchorage independent, even when the cells are cultured under conditions that optimally revealed the adhesion requirement for G0/G1 cell cycle progression. Both the induction and subsequent decay of c-myc mRNA were similar in adherent and nonadherent cells. Consistent with the anchorage-independent expression of c-myc mRNA, cytochalasin D did not block mitogen-stimulated c-myc expression in monolayer (our unpublished observations). Others have noted that mitogens induce c-myc expression in suspended 3T3 cells (refer to the discussion in Dhawan and Farmer, 1990). We conclude that neither cell adhesion nor cytoskeletal integrity are required for the mitogen-dependent induction of c-myc mRNA in fibroblasts. Recent reports have linked the expression of c-myc and cyclin D1 (Daksis *et al.*, 1994; Philipp *et al.*, 1994), but our data indicate that mitogen-induced expression of c-myc mRNA is not sufficient to ensure the subsequent expression of cyclin D1 mRNA.

Despite their lack of involvement in the regulation of c-myc mRNA, cell adhesion and cytoskeletal organization are required for cell cycle progression of human fibroblasts. These requirements persist throughout the mitogen-dependent portion of G1 phase, which ends with the hyperphosphorylation of Rb. Rb hyperphosphorylation is generally thought to reflect transit through the restriction point R. Thus, our data indicate that progression through R actually requires the coordinated signaling of mitogens and the cytoskeleton.

We note that the transitions to staurosporine-resistant cell cycle progression and hyperphosphorylated Rb slightly precede the transition to adhesion/cytoskeleton-independent cell cycle progression. Thus, an organized cytoskeleton may be required somewhat longer than mitogenic stimulation. This result may

Figure 7. Spreading on collagen or exposure to EGF does not stimulate cyclin D1 mRNA. Quiescent human fibroblasts were trypsinized, suspended in conditioned medium (see MATERIALS AND METHODS), and seeded in the presence and absence of EGF on dishes coated with type I collagen or agarose. At selected times between G0 and the onset of S phase, cells were harvested and the levels of cyclin D1 mRNA were determined by Northern blot hybridization.

reflect the fact that cyclin A expression (which is induced subsequent to cyclin D kinase activity) is also positively regulated by cell adhesion (Guadagno and Assoian, 1993; Zhu, X., Ohtsubo, M., Böhmer, R.-M., Roberts, J.M., and Assoian, R.K., unpublished data) and suggests that an Rb-independent mechanism may contribute to the adhesion-dependent expression of cyclin A. However, we cannot exclude the possibility that the shift in the onset of staurosporine- versus cytochalasin-resistant cell cycle progression is related to technical factors, such as differences in the speed by which these two agents cause their respective biochemical effects.

How does cytoskeletal integrity regulate cell cycle progression? It is known that integrins must cluster to induce the adhesion-dependent cell cycle effects in early G1 (see INTRODUCTION), and it is possible that organization of the cytoskeleton is required to maintain these clusters at sites of focal contacts as cells progress into late G1. Alternatively, as organization of the cytoskeleton results in cell spreading, it may be the spread cell shape (rather than integrin clustering) that allows cell cycle progression through G1. This latter possibility is consistent with studies by others showing that a shape-dependent cell cycle transition can be identified in the G1 phase of hepatocytes and endothelial cells (Hansen *et al.*, 1994; Ingber *et al.* 1996). Moreover, recent reports have shown that the cytoskeletal protein paxillin is phosphorylated by FAK during focal contact formation, and that this phosphorylation creates a binding site for crk, csk, and src (Birge *et al.*, 1993; Hildebrand *et al.*, 1995; Schaller and Parsons, 1995). These data raise the possibility that the cascade of signals initiated by the binding of cells to the extracellular matrix lead to downstream events that depend upon an organized cytoskeleton. Regardless of the specific mechanism, our results show that organization of the cytoskeleton, rather than cell at-

tachment per se, underlies the anchorage dependency of Rb phosphorylation in G1 phase and cell cycle progression through R.

Cyclin D-cdk4/6 complexes are necessary for Rb phosphorylation, and cyclin D1 is the predominant D type cyclin in fibroblasts. Our results indicate that the expression of cyclin D1 is greatly reduced in the absence of an organized cytoskeleton. This result leads to a working model for explaining cytoskeleton-dependent cell cycle progression: G1 phase cdk4 activity is inhibited, Rb fails to hyperphosphorylate, and cell cycle progression through R is blocked. Additionally, the absence of cyclin D1-cdk4 complexes in cytochalasin D-treated cells will likely lead to a redistribution of cdk inhibitors such as p21 and p27 (reviewed in Sherr and Roberts, 1995). This redistribution could block the function of cyclin E-dependent kinase, the other cdk complex thought to be involved in Rb phosphorylation in G1 phase. Thus, the cytoskeleton dependency of cyclin D1 expression may account for the fact that Rb hyperphosphorylation and cell cycle progression through R requires both mitogens and an organized cytoskeleton.

ACKNOWLEDGMENTS

We thank Jerry Hudson, Coulter Corporation, for access to an Elite cell sorter/flow cytometer, Rosendo Morena (University of Miami Tissue Procurement Facility, Department of Pathology) for assistance in obtaining the human fibroblasts, and Donald Ingber for sharing results before publication. These studies were supported by National Institutes of Health grants GM-48224 and GM-51878.

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