Experimental Cell Research **275,** 255–264 (2002)
doi:10.1006/excr.2002.5504, available online at http://www.idealibrary.com on IDEAL®

A Discrete Cell Cycle Checkpoint in Late G_1 That Is Cytoskeleton-Dependent and MAP Kinase (Erk)-Independent

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Cell spreading on extracellular matrix and associated changes in the actin cytoskeleton (CSK) are necessary for progression through G_1 and entry into S phase of the cell cycle. Pharmacological disruption of CSK integrity inhibits early mitogenic signaling to the extracellular signal-regulated kinase (Erk) subfamily of the mitogen-activated protein kinases (MAPKs) and arrests the cell cycle in G_1. Here we show that this block of G_1 progression is not simply a consequence of inhibition of the MAPK/Erk pathway but instead it reveals the existence of a discrete CSK-sensitive checkpoint. Use of PD98059 to inhibit MAPK/Erk and cytochalasin D (Cyto D) to disrupt the actin CSK at progressive time points in G_1 revealed that the requirement for MAPK/Erk activation lasts only to mid-G_1, while the actin CSK must remain intact up to late G_1 restriction point, *R*, in order for capillary endothelial cells to enter S phase. Additional analysis using Cyto D pulses defined a narrow time window of 3 h just prior to *R* in which CSK integrity was shown to be critical for the G_1/S transition. Cyto D treatment led to down-regulation of cyclin D1 protein and accumulation of the cdk inhibitor, $p27^{Kip1}$, independent of cell cycle phase, suggesting that these changes resulted directly from CSK disruption rather than from a general cell cycle block. Together, these data indicate the existence of a distinct time window in late G_1 in which signals elicited by the CSK act independently of early MAPK/Erk signals to drive the cell cycle machinery through the G_1/S boundary and, hence, promote cell growth. © 2002 Elsevier Science (USA)

Key Words: **cell cycle progression; microfilament; cell shape; growth; angiogenesis.**

INTRODUCTION

Cell shape is a critical regulator of cell cycle progression in anchorage-dependent cells [1–3]. This mode of growth control may play an important role in the generation of localized growth differentials that drive tissue patterning during morphogenesis by allowing subsets of cells that can spread in regions of accelerated extracellular matrix (ECM) modeling to proliferate while neighboring compacted cells remain quiescent [4]. Cell spreading is an active process mediated by integrin binding to the ECM and associated tension-dependent rearrangements of actin microfilaments within the cytoskeleton (CSK) [5–8]. Studies using drugs, such as cytochalasin D (Cyto D), that disrupt microfilaments have revealed that cells require an intact CSK in order to pass through the G_1/S restriction point [9–11]. Past work on the role of the CSK in growth control focused on the early events of growth signaling, notably the ras-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) pathway [12–14]. However, our laboratory has shown that prevention of cell spreading by lowering ECM coating densities on otherwise nonadhesive substrates or by culturing cells on micrometer-sized ECM islands that physically prevent cell extension arrests capillary endothelial (CE) cells in late G_1 despite normal activation of MAPK/Erk [3]. Here we explore the possibility that CSK signals which are distinct from those elicited by the early ras-MAPK/Erk cascade act later in G_1 to drive cell cycle progression.

Critical biochemical events that occur downstream of receptor signal pathways and promote cell cycle progression into S phase include the induction of G_1 cyclins (D/E-type cyclins) [15] which activate their catalytic partners, the cyclin-dependent kinases (cdks) and the down-regulation of cdk inhibitors, such as $p21^{Cip}$ and $p27^{Kip1}$ (p27) [16]. These events collectively lead to the progressive phosphorylation of the retinoblastoma protein (pRb) by the cdks [17]. Inactivation of pRb by hyperphosphorylation in late G_1 marks the passage through the critical cell cycle restriction point, *R*, after which cells are committed to enter S phase [18].

Most work on the control of G_1 progression by external signals has focused on the role of soluble mitogens and insoluble ECM in early G_1. These factors activate

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immediate early signaling pathways that lead to cyclin D1 induction, a critical event that links external signals to the cell cycle machinery [19–24]. Activation of Ras by growth factor receptors and integrins triggers a signaling cascade that results in the activation of MAPK/Erk, which translocates to the nucleus and induces the expression of growth-related genes, including cyclin D1 (reviewed in [12, 13, 25]). Importantly, while most of the known signaling events activated by mitogens occur transiently and are confined to early G_1, continuous growth factor stimulation is required until the end of G_1 phase several hours later in order for cells to successfully enter S phase [18]. Analysis of requirements for late G_1 signaling has focused on soluble signaling cascades, notably, the Ras and the phosphatidyl-inositol-3-kinase (PI3K) pathways [20, 26–30]. However, studies on cell shape-dependent growth control in fibroblasts, epithelial cells, and endothelial cells note that the actin CSK also must remain intact through late G_1 in order for spread cells to enter S phase [4, 9–11]. This observation suggests that the CSK of a spread cell may convey mitogenic signals in late G_1 that are critical for passage through the restriction point *R*. However, the drugs that disrupt the actin CSK can also interfere with many early signaling pathways, including MAPK/Erk, and thus, this possibility remains controversial [31–34]. Moreover, CSK disruption causes a cell cycle arrest characterized by changes in cyclin levels, cdk activation, and pRb phosphorylation that are similar to those produced by the arrest induced by growth factor withdrawal [4, 11, 35]. This finding raises the question whether the effects of CSK disruption on G_1 cell cycle proteins merely reflect a late consequence of an early block of general mitogenic signaling, or instead result directly from the abrogation of distinct CSK signals that normally act in late G_1. Here we demonstrate that while MAPK/Erk activation is necessary for cell cycle progression through early G_1 in human CE cells, distinct signals from the CSK act in a narrow time window hours later to alter cyclin D1 and p27 protein levels in late G_1 and, thereby, promote passage through the restriction point, *R*, and S-phase entry.

MATERIAL AND METHODS

Cell culture. Primary human pulmonary CE cells were obtained from Clonetics/BioWhittaker (San Diego, CA) and cultured for additional two to four passages. Culture medium was EGM, consisting of EBM medium (Clonetics) supplemented with 10 ng/ml human recombinant EGF, 12 mg/ml bovine brain extract, 1 mg/ml hydrocortisone, and 10% fetal bovine serum (FBS; all from Clonetics/BioWhittaker). Cells were synchronized at G_1/G_0 by treatment with 40 μM lovastatin (Merck, Rahway, NJ) in standard culture medium for 32–36 h [3, 36]. Cells were released into cell cycle by washing the cells free of lovastatin, and replated in or transferred to experimental medium, which is the above culture medium modified in the following ways [3]: reduction of FBS to 2% and addition of 4 mM mevalonate (Sigma, St. Louis, MO) prepared from the lactone form, 5 ng/ml recombinant bFGF (Takeda Chemical Industries Ltd, Osaka, Japan), 10 μg/ml high-density lipoprotein (HDL) (Perimmune, Rockville, MD), and 5 μg/ml transferrin (Collaborative Research, Lexington, MA). In some experiments, EGM-2 medium EGM-2 (BioWhittaker/Clonetics) was used, prepared according to the manufacturer's instructions.

Cyto D (Sigma) was used at a final concentration of 1 μg/ml (from 1 mg/ml stock in DMSO); PD98059 (Calbiochem, LaJolla, CA) was used at 50 μM (from a 20 mM stock in DMSO, stored in small aliquots at −20°C) [37]. Fresh aliquots were used for every other time point to ensure that loss of cell cycle inhibition was not due to loss of drug activity. Calpain inhibitor 1, acetyl-Leu-Leu-nLeu-aldehyde (LLnL) [38] was used at a final concentration of 50 μM (from 100 mM stock in DMSO). Cycloheximide was used at 5 μg/ml (from 5 mg/ml stock); 0.25% DMSO had no significant effects on CE cell morphology and proliferation.

5-Bromo-2′-deoxyuridine (BrdU) labeling and detection. Entry into S phase was measured by quantitating the percentage of cells in 1-cm^2 LabTek chambers (Nunc Niagene, Rochester, NY) that exhibited nuclear incorporation of BrdU after continuous or pulsed (4 h) exposure to 5-bromo-2′-deoxyuridine:5-fluoro-2′-deoxyuridine (1:1000 v/v final dilution of commercial solution, RPN201, Amersham Biosciences). Cells were fixed at the end of the BrdU treatment in (5% acetic acid, 90% ethanol) for 30 min at room temperature and washed with PBS. BrdU-positive cells were identified by incubating cells with anti-BrdU antibody and nuclease (RPN202, Amersham Biosciences) for 90 min, and staining with biotinylated anti-mouse Ig antibodies and Texas red–avidin (Vector Laboratories, Burlingame, CA). BrdU-positive cells were visualized and scored using a Nikon epifluorescence microscope with oil immersion, 25× objectives; all nuclei were counterstained with the DNA-binding dye DAPI (1 μg/ml). Twelve random fields with a total of more than 400 cells were counted in duplicate cultures per data point. Standard deviation was less than 10%, except as shown in Fig. 1B, where error bars are shown. Data shown were from at least three independent experiments.

pRb hyperphosphorylation. pRb hyperphosphorylation was determined *in situ* as described [3]. In brief, CE cells were washed in PBS at various time points after release into the cell cycle and incubated in nuclear extraction buffer (10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol) for 15 min at room temperature to solubilize the hyperphosphorylated pRb. Cells were then fixed for 20 min in 4% paraformaldehyde/PBS and washed with 0.1 bovine serum albumin/PBS. pRb was visualized by indirect immunostaining using anti-human pRb antibody LM95.1 (2 μg/ml; Calbiochem). The percentage of pRb-negative nuclei was determined using the methodology described above for nuclear BrdU incorporation.

Western blot analysis. For analysis of cell cycle-associated proteins, adherent CE cells in 60-mm culture dishes were lysed with 0.4 ml of boiling lysis buffer (1% SDS, 50 mM Tris–HCl, pH 7.4), scraped, and collected. Homogenized total cell lysates (10 μg protein) were subjected to SDS–PAGE in 1.5-mm-thick minigels, transferred to TransBlot (Bio-Rad, Hercules, CA) nitrocellulose membrane, and immunoblotted with specific primary antibodies which were detected using horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) and a chemiluminescence substrate (Pierce, Rockford, IL). Equal protein loading was confirmed by staining the membranes for total protein with India ink (1:1000) and by probing with antibodies to cdk2, which in these CE cells was not affect by cell cycle position [3], and with antibodies to β-actin. Monoclonal antibodies used were anti-retinoblastoma protein (pRb) (clone LM95.1, Calbiochem), anti-cyclin D1 (clone 14561A, Pharmingen, San Diego, CA), and anti-$p27^{Kip1}$ (clone 57) and anti-cdk2 (clone 55,

both from Transduction Laboratories, Lexington, KY). Rabbit polyclonal antibody to p44/p42 MAPK/Erk (No. 9101, New England Biolabs, Beverly, MA) and monoclonal antibody to activated (phosphorylated) MAPK/Erk (No. 9106, New England Biolabs) were used for analysis of MAPK/Erk activation.

RESULTS

G_1 Progression and S-Phase Entry in Human CE Cells

The length of G_1 phase in CE cells was first established by arresting the cells at the G_0/G_1 interface by treatment with lovastatin and then synchronously activating the cells to reenter the cell cycle by addition of mevalonate [3, 36]. Passage through the restriction point *R* was measured by quantitation of nuclei that expressed hyperphosphorylated pRb. These studies revealed that CE cells passed through the restriction point *R* at approximately 12 to 16 h after cell cycle entry (Fig. 1A). These cells then entered S phase about 6–8 h later, at 18–20 h in G_1, as evidenced by incorporation of BrdU in DNA (Fig. 1A). The fraction of reentry into S phase in these cells was typically 30–50%.

To determine the relative importance of MAPK/Erk signals versus CSK signals in control of this late G_1 checkpoint, we measured the effects of inhibiting MAPK/Erk activation by Mek1 with PD98059 (50 μM) [37] and of disrupting the actin CSK with Cyto D (1 μg/ml) at different time points during G_1. Cyto D completely disrupts the actin network structure and produces an arborized cell morphology within 30 min to 1 h after addition [3]. PD98059 had a similar time delay to onset of its pharmacological effect, as MAPK/Erk phosphorylation became undetectable in anti-phospho-MAPK/Erk immunoblots within 1 h after addition of drug, and began to recover after another 6 h (data not shown). Monitoring S-phase entry by measuring cumulative BrdU incorporation after PD98059 addition revealed that while MAPK/Erk activation was critical in early G_1, blocking MAPK/Erk activation after 8 h of release into G_1 failed to prevent subsequent entry into S phase (Fig. 1B). In contrast, disruption of the actin CSK by Cyto D still inhibited progression to S phase when the drug was added up to 12 h (Fig. 1B) which corresponds to the restriction point *R* (Fig. 1A). This effect appeared to be specific to actin CSK disruption in that Latrunculin B, which disrupts the actin microfilaments by another mechanism [39], produced an almost identical temporal profile in terms of G_1 inhibition (not shown). Thus, growth signals from the actin CSK that act in late G_1 can be temporally dissociated from early MAPK/Erk-dependent mitogenic signals.

To further delineate the time window in which an intact actin CSK is critical for cell cycle progression, cells were treated with short (3 h) pulses of Cyto D at different time points in G_1 in order to measure the

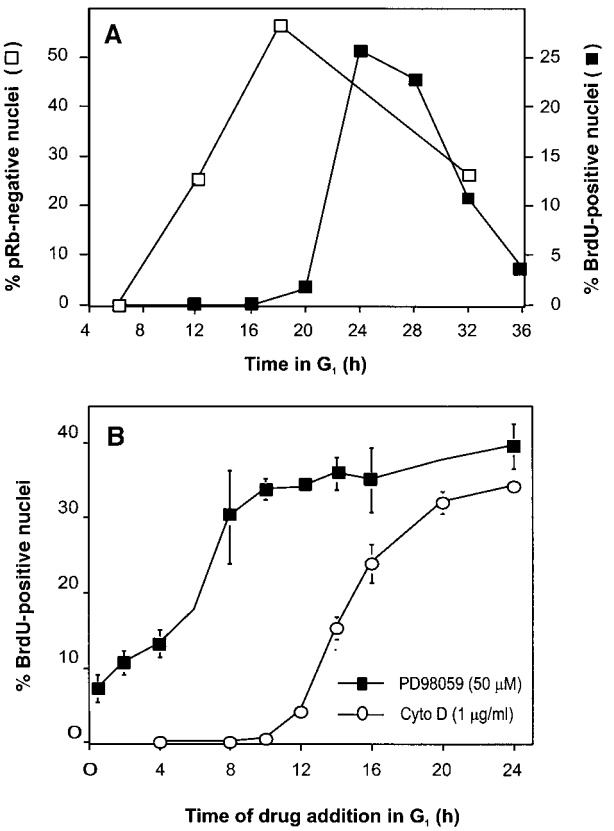

FIG. 1. Distinct temporal requirements for MAPK/Erk activation and the actin CSK during G_1 progression. (A) Baseline kinetics of pRb hyperphosphorylation (open squares) and entry into S phase (solid squares) in lovastatin-synchronized CE cells. Hyperphosphorylated pRB was monitored by counting nuclei with extractable pRb; DNA synthesis was measured by nuclear incorporation of BrdU after 4 h pulse-labeling and fixing at the indicated times. These results indicate that cells pass through the late G_1 restriction point at approximately 12 h and enter into S phase about 20 h after release into G_1 by addition of mevalonate and growth factors (see Materials and Methods). (B) Effects of inhibiting MAPK/Erk activation using PD98059 (50 μM; solid squares) and of disruption of the actin CSK using Cyto D (1 μg/ml; open circles) on S-phase entry as measured by cumulative BrdU incorporation at 30 h.

kinetics of S-phase entry (Fig. 2A). The action of Cyto D was reversible, as cells recovered from the arborized state, spread, and resumed G_1 progression after a delay. Specifically, when the Cyto D pulse was added in early–mid G_1 (6–9 h in G_1) or just after passage through the late G_1 restriction point (12–15 h in G_1), a similar fraction of cells entered S phase as in the untreated controls, but with a 6- to 8-h time lag (Fig. 2B). In contrast, when Cyto D was added between 9 and 12 h, just prior to the restriction point, the cells failed to enter S phase even when followed for extended times. Instead, they typically showed a slow rise of basal BrdU incorporation, reflecting a progressive loss of synchronization (Fig. 2B). The requirement for actin

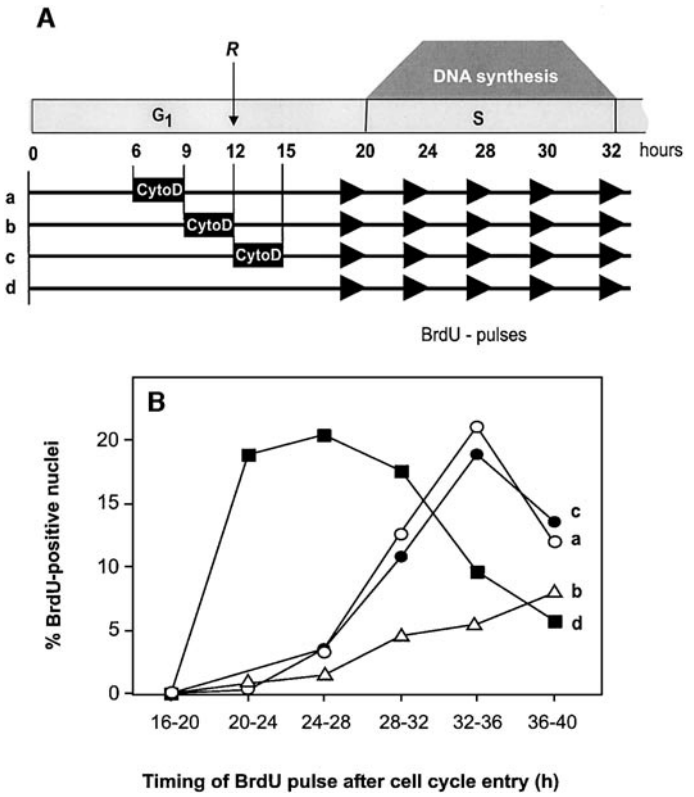

FIG. 2. A discrete CSK-dependent checkpoint in late G_1 is essential for the G_1/S transition in CE cells. (A) Diagrammatic representation of the experimental design involving four different treatment schedules (a–d) used to localize the CSK-sensitive checkpoint during cell cycle progression using pulses of Cyto D. *R*, late G_1 restriction point associated with hyperphosphorylation of pRb; solid triangles indicate time period when cells were exposed to BrDU. (B) Effects of CSK disruption at different times during G_1 on subsequent S-phase entry. Each time point represents the percentage of nuclear labeling obtained after a 4-h pulse with BrDU during the time period indicated. Open circles, treatment schedule a (exposure to Cyto D at 6 to 9 h); open triangles, schedule b (Cyto D at 9 to 12 h); solid circles, schedule c (Cyto D at 12 to 15 h); solid squares, schedule d, control without Cyto D. Note the failure of schedule b cells to exhibit synchronized entry into S phase.

CSK in the early phase (first few hours of G_1) was not examined here because Cyto D prevents cell spreading and alters immediate–early signaling. Taken together, these findings reveal that a critical time window exists just prior to the hyperphosphorylation of Rb in which the integrity of the actin CSK is absolutely essential for cell cycle progression. This time window does not overlap with the time period in the first part of G_1 in which MAPK/Erk activation was found to be most critical (Fig. 1B).

Effects on Cyclin D1, p27, and pRb

We have previously shown that the G_1 block produced by inhibiting cell spreading or disrupting the actin CSK in CE cells correlates with changes in the levels of critical G_1 cell cycle proteins, including cyclin D1 and the cdk inhibitor p27 [3]. Similarly, when Cyto D was added in early or mid G_1 (2 or 8 h) it inhibited cyclin D1 synthesis and prevented down-regulation of p27, as revealed by measuring the levels of these proteins in late G_1 (18 h) (Fig. 3A). It has been suggested that the MAPK/Erk pathway is necessary for G_1 progression because of its role in cyclin D1 induction, but is not sufficient for S-phase entry because it fails to down-regulate p27 [21, 22]. In CE cells, MAPK/Erk is activated by growth factors and integrins independently of cell spreading and it remains detectable in an activated form throughout all of G_1; yet, this high level of MAPK/Erk activity is not sufficient to promote S-phase entry in round cells [3]. Interestingly, when we added the specific MAPK/Erk inhibitor, PD98059, as early as 2 h into G_1, cyclin D1 induction was only minimally reduced and p27 down-regulation was unaffected (Fig. 3A), whereas cell cycle progression was inhibited by 75% (Fig. 2B). Blocking MAPK/Erk in later G_1 (8 h) had no effect on cyclin D1 and p27 levels (Fig. 3A).

The finding that addition of PD98059 at 2 h had little effect on cyclin D1 suggested that the MAPK/Erk response may proceed extremely rapidly. In fact, when cells were pretreated with PD98059 1 h prior to release from the lovastatin block and restimulation with growth factors, MAPK/Erk activation did not recover even at the time of cell harvest in late G_1 (18 h), nor was cyclin D1 protein detectable (Fig. 3B). In contrast, when the drug was added only 1 min after restimulation, a low level of MAPK/Erk phosphorylation could be detected which correlated with a significant induction of cyclin D1 synthesis (Fig. 3B). When PD98059 was added at 8 h (when MAPK/Erk is normally high), MAPK/Erk phosphorylation recovered in late G_1 (18 h) and enhanced cyclin D1 induction was observed.

Together, these findings indicate that the MAPK/Erk pathway acts very quickly to activate cyclin D1 synthesis following stimulation by external factors that trigger CE cell reentry into the cell cycle. Thus, in these CE cells, the regulation of cyclin D1 by MAPK/Erk exhibits hysteresis: the MAPK/Erk signal which triggers cyclin D1 induction in early G_1 acts immediately after growth factor stimulation but once initiated, cyclin D1 accumulation in late phases of G_1 does not require sustained MAPK/Erk activity. Conversely, an intact actin CSK does not appear to be required for MAPK/Erk activation in these cells when they are already attached [3]; yet CSK integrity must be maintained to sustain high levels of cyclin D1 and suppress p27 throughout late G_1.

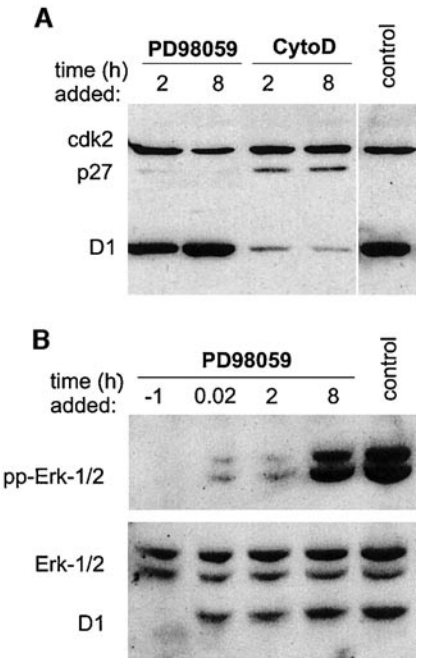

FIG. 3. Effects of MAPK/Erk pathway inhibition and actin CSK disruption on the key cell cycle regulator proteins cyclin D1 and p27. (A) Western blot showing effects of adding PD98059 (50 μM) and Cyto D (1 μg/ml) at 2 or 8 h after release from lovastatin block on expression of cdk2, cyclin D1 (D1), and p27, as measured in late G_1 at 18 h (time of cell harvest). (B) Western blot showing effects of adding PD98059 1 h before or 0.02, 2, or 8 h after release of cells from lovastatin block on cyclin D1 protein levels and MAPK/Erk-1/2 activation, as measured by Erk-1/2 phosphorylation (pp-Erk-1/2), at 18 h. Control indicates untreated cells harvested at the same time point. Note that due to the extremely rapid activation of MAPK/Erk after cell cycle reentry, pretreatment with drug was required for complete inhibition of its activity.

CSK-Dependent Effects on Cyclin D1 and p27 Are Independent of Cell Cycle Phase

As previously reported [3, 9–11, 35], Cyto D blocked pRb phosphorylation if added before the late G_1 restriction point (at 12 h for our CE cells), whereas, if added after this point, cell cycle progression proceeded normally and cells entered S phase at 18–20 h. The inhibitory effect of Cyto D correlated with its ability to up-regulate p27 protein, since the effects of Cyto D on both p27 and pRb hyperphosphorylation were reduced when it was added at a later time (16 h) in G_1 (Fig. 4). One explanation for the effect of Cyto D on p27 and cyclin D1 is that it merely reflects a global cell cycle block in early G_1 and the maintenance of low levels of cyclin D1 and high p27 that are characteristic of this early time. To explore this in greater detail, synchronized CE cells were treated with Cyto D for 6 h either in late G_1 (14–20 h) or after the G_1/S transition (20 to 26 h). These studies revealed that Cyto D increased p27 levels and reduced cyclin D1 independently of the stage of the cell cycle in which cells were exposed to the drug (Fig. 5A). This effect of Cyto D was also observed when the treatment period was at S/G_2 (>30 h) (data not shown). Thus, the effect of Cyto D on p27 and cyclin D1 appears to be a direct consequence of the disruption of the actin CSK, and not just a genetic manifestation of G_1 arrest.

p27 is regulated largely by ubiquitin-mediated degradation in proteosomes [40], although translational regulation also plays a role [41, 42]. To reveal which mode of regulation is affected by actin disruption, Cyto D treatment was carried out under conditions in which protein synthesis or degradation was blocked. We found that the induction of p27 accumulation by Cyto D could be fully abolished by inhibiting ongoing protein synthesis with cycloheximide (CHX) during the period of Cyto D treatment (Fig. 5B). This indicates that Cyto D does not fully block p27 degradation and that ongoing protein synthesis is necessary for maintaining high levels of p27 when the actin CSK is disrupted. Conversely, in the case of cyclin D1, the effect of Cyto D was fully abolished when calpain inhibitor 1 LLnL, which blocks proteosome-dependent protein degradation [38], was administered concomitantly with Cyto D. LLnL alone even led to the accumulation of cyclin D1 above the untreated controls, and Cyto D prevented this accumulation (Fig. 5B). Thus, cyclin D1 protein synthesis depends on an intact actin CSK, however, the loss of cyclin D when the actin network is disrupted requires ongoing protein degradation. LLnL also prevented the normal degradation of p27, and concomitant treatment with Cyto D increased the accumulation of p27. This points to the contribution of protein synthesis in the accumulation of p27 following Cyto D treatment. Together these findings suggest that both p27 and cyclin D1 are regulated by a dynamic balance

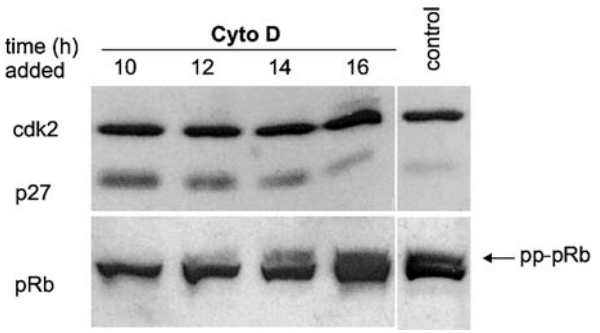

FIG. 4. Western blot showing effects of Cyto D (1 μg/ml), when added at the indicated times after release of synchronized cells into the cell cycle, on protein levels of cdk2, p27, and phosphorylation status of pRb, as determined at 18 h (time of harvest). Arrow indicates the slower migrating, hyperphosphorylated band of pRb (pp-pRb). Control represents untreated cells harvested at the same 18-h time point.

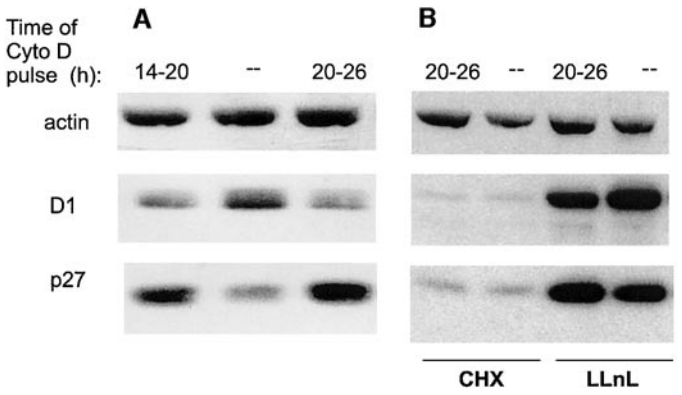

FIG. 5. Effects of CSK disruption on cyclin D1 and p27 protein levels are independent of cell cycle phase. (A) Western blots showing effects of exposing cells to a 6-h pulse with Cyto D (1 μg/ml) either during late G_1 (14–20 h after release from lovastatin block) or in S phase (20–26 h) on cyclin D1, p27, and actin protein levels, as measured at the end of the treatment period; the untreated control was harvested at 20 h. (B) Western blot showing the effects of treatment of cells with cycloheximide (5 μg/ml, CHX) or LLnL (50 μM) in the presence or absence (—) of Cyto D (1 μg/ml) during the 20- to 26-h period on cyclin D1, p27, and actin.

between protein synthesis and degradation, and that the actin cytoskeleton modulates both processes in opposite directions for p27 and cyclin D.

DISCUSSION

We report here that a mitogenic signal emanating from the intact actin CSK of spread cells is necessary for progression through the restriction point *R* of the cell cycle and that this signal can be temporally separated from the early G_1 MAPK/Erk signal. This finding supports the concept that multiple, parallel but time-shifted signals during G_1 are necessary for entry into S phase. It has long been recognized that while most of the biochemical signaling events triggered by growth factor receptors are only transient, growth factor stimulation must be maintained for many hours until the restriction point in late G_1 for cells to proceed into S phase [18]. Thus, there has been a recent shift of emphasis from the identification of immediate–early pathways to the characterization of mitogenic signals that are important in late G_1. This work has led to the identification of distinct pathways, typically effector cascades downstream of Ras, whose activity is required beyond the activation of the MAPK/Erk pathway in early G_1 [20, 26–30].

How does the signal from the actin CSK fit into this paradigm? In the present study, we identified a distinct CSK-sensitive restriction point which corresponds to a 3-h period (9–12 h) in mid G_1, just prior to the restriction point *R* as measured by pRb hyperphosphorylation. In contrast, temporary disruption of the CSK network in mid-G_1 (6–9 h) or between the restriction point and S-phase entry (12–15 h) only produced a reversible pause in G_1 progression. As the cells recovered and respread, they entered S phase with a delay of 6–8 h, which may reflect the time period of Cyto D treatment (3 h) plus 3–5 h for structural recovery. Alternatively, this time lag may be due to inhibition of protein synthesis which has been shown to produce a similar time lag during cell cycle progression in other cell types [18]. In contrast, the 9- to 12-h time period appeared to be qualitatively distinct, representing a unique window of opportunity in which specific CSK signaling events occur that cannot be compensated for at later times. Thus, cells treated with Cyto D during this critical period failed to enter S phase, even after the cells rebuilt their actin filament network (Fig. 2B). These observations suggest that the intact actin CSK is an integral part of the mitogenic signaling cascade in late G_1. In addition, our time window scanning experiment suggests the presence of irreversibility and a "memory effect" in the control system: lack of CSK signals during this critical time window amounts to a "missed opportunity," indicating the existence of other parallel processes which must integrate with CSK signals in order to proceed and which lose this ability after a discrete time period (3 h before *R*). This points to previously unappreciated layers of complexity in cell cycle progression that involve parallel rather than sequential events and nonlinear control mechanisms.

We show here that the actin CSK affects cyclin D1 and p27 levels directly rather than as a consequence of a general block of cell cycle progression in early G_1 (Fig. 4). While synthesis of many proteins requires an intact actin CSK scaffold [43], it is not likely that the cell cycle inhibitory effect of Cyto D is solely due to a general suppression of protein synthesis. First, p27 synthesis was increased in the presence of Cyto D, although its accumulation appeared to be caused by both inhibition of degradation and enhanced synthesis. Second, synthesis of cyclin D1 has been shown to be affected more than the synthesis of other proteins when the actin CSK is disrupted in human fibroblasts [44]. Moreover, the requirement for protein synthesis for G_1/S transition extends beyond the restriction point to the onset of S phase ([18], and our unpublished data) in contrast to the requirement for an intact actin CSK which lasts only to the restriction point. On the other hand, the finding that treatment with a short pulse of Cyto D produces a time lag in cell cycle progression similar to that produced when fibroblasts are exposed to a pulse of CHX suggests that this effect of Cyto D may be in part due to inhibition of synthesis of a subset of critical cellular proteins.

Cells must extend on an ECM substratum that supports adhesion and subsequent distortion of the CSK

before they can enter S phase [3]. Studies on the mitogenic role of ECM in early G_1 have shown that adherence to the ECM and associated integrin activation are necessary to generate sustained activation of MAPK/Erk for >6 h during early to mid-G_1 which, in turn, is necessary for induction of cyclin D1 synthesis in fibroblasts [45]. In our CE cells, a brief pulse of MAPK/Erk activation of 1 min was sufficient to induce normal levels of cyclin D1 synthesis. This differs from previous reports in fibroblasts showing that short MAPK/Erk activity pulses are unable to induce cyclin D1 [24, 29, 30]. This disparity might stem from the difference in cell type (endothelial cells vs fibroblast lines). Moreover, we used lovastatin to synchronize cells at the G_0/G_1 border rather than serum starvation which blocks cells within G_0. Although in our CE cells the time for G_1 passage was similar after these two methods of cell cycle synchronization (data not shown), the nature of the cell cycle arrest by lovastatin is different from that after starvation [36, 46, 47]. Thus, this could account for some of the discrepancies regarding the requirement for cyclin D1 induction. However, our finding that a short pulse of MAPK/Erk activity was insufficient to stimulate successful passage through G_1/S, despite induction of cyclin D, is functionally consistent with these previous reports.

The finding that induction of cyclin D1 is not sufficient for S-phase entry emphasizes the importance of p27 down-regulation as a critical event for G_1 progression [16]. p27 can suppress the cyclin D/cdk complexes formed in response to MAPK/Erk activation [22]. Thus, up-regulation of cyclin D1 and down-regulation of p27 are both required downstream of MAPK/Erk activation for S-phase entry. In fact, overexpression of Raf, which activates the upstream MAPK Mek1, has been reported to suppress p27 and trigger S-phase entry [48, 49]. Inhibition of MAPK/Erk activation with PD98059 also inhibits Ras-induced p27 down-regulation in fibroblasts [50]. However, the role of MAPK/Erk in control of p27 levels remains controversial. For example, other studies suggest that sustained MAPK/Erk activation is not sufficient for down-regulation of p27 [21, 22]. In any case, our data clearly demonstrate that MAPK/Erk activation is not necessary for p27 down-regulation in CE cells, a result that is consistent with previous work in fibroblasts [21].

Importantly, our findings suggest that the MAPK/Erk pathway must therefore have other targets in mid-to late G_1, in addition to cyclin D1 (and p27), in order to promote S-phase entry. Based on our results, the actin CSK and associated signaling complexes could represent such a target. Our data also indicate that MAPK/Erk activity becomes superfluous after mid-G_1 in CE cells even though its activity can be detected throughout G_1. In contrast, at this stage in late G_1, the CSK signal must be sustained to maintain high levels of cyclin D1, down-regulate the p27 degradation, and, hence, promote passage through G_1/S. Thus, late G_1 signals that are critical for cell cycle progression involve CSK-mediated events that act independently of the sustained MAPK/Erk signals.

The biochemical nature of the signals elicited or mediated by the intact actin CSK of spread cells remains to be determined. The requirement for Ras activity in late G_1 phase [20, 26, 27, 51] might point to a synergism between the structural (CSK-dependent) and soluble signal pathways required for passage through late G_1—in analogy to the situation with early G_1 signals. Late G_1 Ras activity has been shown to be required for p27 down-regulation by suppression of p27 synthesis and induction of its degradation [27]. While MAPK/Erk activity is apparently not sufficient for p27 down-regulation [21, 22], another target of Ras, the small GTPase RhoA, appears to trigger p27 down-regulation in fibroblasts and epithelial cells [21, 52]. Rho also plays an important role in the actin CSK rearrangements that mediate cell spreading [53]. Interestingly, RhoA activation appears to be necessary for sustained activation of MAPK/Erk in fibroblasts [54]. This activity would give Rho a central role in integrating early and late signals as well as soluble and structural signals, although the full picture is likely to be more complicated because of the multiphasic behavior of RhoA activity upon adherence [55, 56].

Other downstream targets of Ras that have been shown to be important in late G_1 include Rac and PI3K. For example, both proteins have been implicated in control of cyclin D1 transcription and/or translation in late G_1 [29, 57–60]. PI3K activity has also been shown to be essential for p27 down-regulation in response to growth factors in various cell types [61–63].

G_1 phase can be temporally subdivided into two distinct, nonoverlapping phases with respect to growth factor-dependent signaling [30]. While the first, early-G_1, phase is driven by MAPK/Erk and Myc, the second phase, which begins at about 8 h in G_1, critically depends on PI3K. This time period would correspond to the window that we found to be CSK-dependent. Given the importance of PI3K activity precisely in this late G_1 time window it is reasonable to assume that PI3K may be a target of the intact CSK within extended cells or vice versa. PI3K has been shown to be linked with the actin CSK via its localization in focal adhesion complexes [64, 65] whose formation requires actin-dependent tension generation [66]. In insulin-dependent activation of PI3K in adipocytes, the actin CSK is essential for PI3K translocalization to the membrane [67]. Moreover, our laboratory has recently found that PI3K activity was inhibited by treatment with Cyto D in CE cells [68]. Further studies will be needed to

corroborate the relationship between actin CSK and PI3K or other signaling molecules, including the Rho family of small GTPases in the regulation of G_1 completion.

In conclusion, our findings underscore the temporal heterogenity of the G_1 phase, and identify the actin CSK as an important source of late G_1 signals that are essential for entry into S phase. The observation of a separate signal that depends on CSK integrity provides a handle for future studies on the important role of structural parameters in cell cycle regulation [4, 69].

This work was supported by National Health Institutes Grant CA58833.

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Received December 3, 2001
Revised version received February 6, 2002