Adhesion-dependent Cell Cycle Progression Linked to the Expression of Cyclin D1, Activation of Cyclin E-cdk2, and Phosphorylation of the Retinoblastoma Protein

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Abstract. Growth factors and cell anchorage jointly regulate transit through G1 in almost all cell types, but the cell cycle basis for this combined requirement remains largely uncharacterized. We show here that cell adhesion and growth factors jointly regulate the cyclin D1- and E-dependent kinases. Adhesion to substratum regulates both the induction and translation of cyclin D1 mRNA. Nonadherent cells fail to phosphorylate the retinoblastoma protein (Rb), and enforced expression of cyclin D1 rescues Rb phosphorylation and entry into S phase when G1 cells are cultured in the absence of substratum. Nonadherent cells also fail to activate the cyclin E–associated kinase, and this effect can be linked to an increased association of the cdk inhibitors, p21 and p27. These data describe a striking convergence in the cell cycle controls used by the two major signal transduction systems responsible for normal and abnormal cell growth. Taken together with our previous studies showing adhesion-dependent expression of cyclin A, they also establish the cell cycle basis for explaining the combined requirement for growth factors and the extracellular matrix in transit through the Rb checkpoint, entry into S phase, and anchorage-dependent growth.

With the exception of some cells in the hematopoietic lineage, adhesion to substratum is required for cell cycle progression through G1 and into S phase. Cell adhesion is largely mediated by the interaction of extracellular matrix proteins with integrins, a heterodimeric family of cell surface matrix protein receptors (Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990). Like growth factor receptors, the display of integrins varies in different cell types. Well studied anchorage-dependent cells, such as fibroblasts, express integrins that bind to collagen, fibronectin, and vitronectin. Although less well studied, syndecans, a distinct class of cell surface adhesion molecules, also play an important role in mediating cell adhesion and focal contact formation (Woods and Couchman, 1994).

Integrins act as signaling receptors and transmit growth regulatory signals from the extracellular matrix to the cell. They lack the intrinsic kinase activities characteristic of growth factor receptors, but signal-transducing molecules such as focal adhesion kinase (FAK) and IRS-1 can associate with integrin cytoplasmic tails (Vuori and Ruoslahti, 1994; Lewis and Schwartz, 1995; Miyamoto et al., 1995). These interactions likely explain the result that integrins, like growth factor receptors, can activate MAP kinase (Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995) and a number of other signal transduction events (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993; Miyamoto et al., 1995).

As opposed to these G0/G1 regulatory events that seem to be activated independently by integrins and growth factor receptors, the turnover of phosphoinositides (a hallmark of the G0/G1 transition) requires the coordination of signals from integrins and growth factor receptors. Integron signals lead to the production of PIP2, and signals from growth factor receptors lead to the activation of phospholipase C and the turnover of PIP2 (McNamee et al., 1993; Chong et al., 1994). Indeed, the cooperative regulation of phospholipid turnover by integrins and growth factor receptors provides a paradigm for explaining the fact that soluble mitogens and the extracellular matrix have nonredundant roles in cell cycle progression through G1.

Adherent cells irreversibly commit to cell cycle progression at a point in late G1 known as the restriction point (Pardee, 1989). Transit through the restriction point was originally defined as the switch from mitogen-dependent to -independent cell cycle progression. But the growth factor and adhesion requirements for proliferation are typically detected or lost in parallel; nontransformed cells are

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1. Abbreviations used in this paper: CAK, cyclin-activating kinase; cdk, cyclin-dependent kinase; CKI, cdk inhibitor; FAK, focal adhesion kinase; Rb, retinoblastoma.
mitogen and anchorage-dependent, and most transformed cells are mitogen- and anchorage-independent (Pardee, 1989). This overlap in growth requirements raises the possibility that adhesion-dependent signals may also be involved in cell cycle progression through the restriction point.

The molecular basis of restriction point regulation is not fully understood, but it correlates with the G1 hyperphosphorylation of the retinoblastoma protein (Rb). These phosphorylations are catalyzed by the cyclin-dependent kinase (cdk) family (Hinds et al., 1992; Ewen et al., 1993; Dowdy et al., 1993; Kato et al., 1993; Matsushima et al., 1992, 1994; Hatakeyama et al., 1994). Although several cyclin-cdk complexes can phosphorylate Rb in vitro, the timing of Rb phosphorylation in vivo indicates that cyclin D-cdk4/6 and cyclin E-cdk2 are likely to be the principal Rb kinases (Koff et al., 1992; Dulic et al., 1992; Meyerson and Harlow, 1994; Matsushima et al., 1994). Growth factors stimulate the expression of cyclins D and E and thereby activate the cdks that phosphorylate Rb (Matsushima et al., 1991; Lew et al., 1991; Koff et al., 1992; Ohsubo and Roberts, 1993). The phosphorylation of Rb in G1 phase inactivates its growth inhibitory effects, presumably by allowing for the release of E2F (for reviews see Nevins, 1992; Sherr, 1994; Johnson et al., 1993). Rb also binds to other proteins, including c-abl (Kim et al., 1992; Gu et al., 1993; Welch and Wang, 1993, 1995; Duniaef et al., 1994), and these interactions may also contribute to the control that Rb imposes on cell cycle progression.

Cdk activity is negatively regulated by specific cdk inhibitors (CKIs). There are two families of CKIs: p21/p27/p57 (which bind to and inactivate all cyclin-cdk complexes; El-Deiry et al., 1993; Xiong et al., 1993; Harper et al., 1993; Polyak et al., 1994a,b; Toyoshima and Hunter, 1994; Lee et al., 1995; Matsuoka et al., 1995) and the INK4s (which only inhibit complexes containing cdk4/6; Serrano et al., 1993; Hannon and Beach, 1994; Guan et al., 1994; Hirai et al., 1995; Chan et al., 1995). Low levels of p21 and p27 can be found in association with active cyclin-cdk complexes, but increased association of p21 or p27 leads to the inhibition of cyclin-cdk activity (Zhang et al., 1994; Polyak et al., 1994a,b; Nourse et al., 1994; Harper et al., 1995). CKIs are regulated by mitogenic and anti-mitogenic signals (Hannon and Beach, 1994; Kato et al., 1994a; Polyak et al., 1994a,b; Nourse et al., 1994), thereby providing a clear link between mitogenic signal transduction pathways and the cell cycle. Activation of the cdks also requires phosphorylation by cyclin-activating kinase (CAK; Kato et al., 1994b; Fisher and Morgan, 1994; Mäkelä et al., 1994), but this enzyme seems to be constitutively expressed in an active form throughout the cell cycle (Matsuoka et al., 1994).

Although the molecular pathways that link mitogen action and cell cycle progression are beginning to be understood, there is relatively little insight into the pathways by which cell adhesion activates cell cycle progression. Nevertheless, our previous studies in NRK fibroblasts showed that the stimulatory effect of adhesion on cell proliferation can also be understood in terms of the cyclins and cyclin-dependent kinases. In this cell line, growth factors stimulate transit from G0 until late G1, and cell adhesion stimulates transit from late G1 into S phase (Guadagno and Assoian, 1991). NRK cells show an adhesion requirement for the expression of cyclin A mRNA, and infection with a cyclin A retrovirus allows for anchorage-independent expression of cyclin A-dependent kinase activity and anchorage-independent growth (Guadagno et al., 1993). We also found that the expression of cyclin D1 mRNA is dependent upon cell adhesion in normal human fibroblasts (Bohmer et al., 1996).

We now report that NRK cells express only a subset of the controls that normally link cell adhesion to cell cycle progression. In both NIH-3T3 cells and normal human fibroblasts, but not in NRK cells, we find that cell anchorage is required for the phosphorylation of the retinoblastoma protein. This effect can be linked to the adhesion-dependent expression of cyclin D1 and the adhesion-dependent activation of cyclin E-cdk2. Since cyclin D- and E-dependent kinase activities are also dependent upon mitogens, our results show that proper regulation of the G1 cdks requires the convergence of signals from growth factors and the extracellular matrix.

### Materials and Methods

#### Cell Culture

Confluent (NRK cells) or 50% confluent (NIH-3T3 cells) cultures were trypsinized and seeded in fresh 150-mm-tissue culture dishes at near confluence in medium containing 5% serum. After the cells had attached, the medium was removed, the cells were washed with DME and synchronized in G0 by incubation in 20 ml serum-free DME for 24–36 h (NIH-3T3) or 4 d (NRK). Early passage explant cultures of human foreskin fibroblasts were prepared by retroviral infection as described (Guadagno et al., 1993); pools of G418-resistant colonies (>100 colonies per infection) were selected and serum-starved using the conditions described for the parent.

The G0-synchronized cells were trypsinized, suspended in medium containing mitogens (5% FCS, 2–3 nM EGF for NRK and NIH-3T3 cells and 10% heat-inactivated FCS, 2 nM EGF for human fibroblasts) and cultured in monolayer or suspension using procedures similar to those described (Guadagno and Assoian, 1991; Han et al., 1993). At selected times after seeding, the cells were collected by centrifugation, either directly (suspended cells) or after trypsinization (monolayer cells). The collected cells (typically 1–5 × 10⁶ cells per time point) were washed twice with HBSS and extracted for total RNA or immunoblotting (see below). In most experiments, ~10% of each sample was suspended in 0.5 ml of a solution (25% ethanol in calcium-free PBS) containing 2 μg/ml HEOC151 33258. The cells were stored (4°C, >16 h) before flow cytometric analysis of DNA content. In several experiments, G0-synchronized NIH-3T3 cells were preincubated with mitogens in monolayer for 9 h (G1 phase cells) or 16 h (S phase cells) before trypsinization and incubation of the cells in monolayer and suspension. Fresh mitogen was added to these cultures during the incubation period.

#### Antibodies and Immunoblotting

NRK and NIH-3T3 cells (5 × 10⁶) and human fibroblasts (10⁶) were extracted in 0.1 ml lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM sodium fluoride, and 0.1 mM sodium orthovanadate). Unless noted in the figure legend, either 100 or 200 μg of each extract (determined by Coomassie binding; BioRad [Hercules, CA] protein assay) were fractionated on reducing SDS gels (7.5–12% acrylamide), and electrophoretically transferred to nitrocellulose filters. Immunoblotting was performed as described (Zhu and Assoian, 1995) using filters blocked in BSA (anti-Rb [Pharminigen, San Diego, CA or Ciba-Corning, Alameda, CA]) or nonfat milk (anti-cyclin D1 [Pharminigen or Upstate Biotechnology, Lake Placid, NY], anti-cdk2 [Upstate Biotechnology], anti-cdk4 [Pharminigen], anti-p21 [Pharminigen or Santa Cruz Biotechnology, Santa Cruz, CA], anti-
Immunoprecipitations and In Vitro Kinase Assays

Cell extracts (0.5 μg) were incubated in their lysis buffer with 5–10 μl of cyclin E antiserum (1 h, 4°C). The reaction volume was brought to 0.5 ml with fresh lysis buffer, and the immune complexes were collected by incubation (1 h at 4°C with rocking) with protein A agarose (50 μl; Life Technologies, Gaithersburg, MD). For subsequent immunoblotting, the collected immunoprecipitates were washed five times in ice-cold lysis buffer and fractionated on reducing SDS-gels as described above. For determination of cyclin E-associated kinase activity, the collected immunoprecipitates were washed three times with cold lysis buffer and twice with room temperature kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂). Kinase reactions were started by adding 2 μg histone H1, 25 μM ATP, and 10 μCi [γ-32P] ATP (3,000 Ci/mmol) in a final volume of 30 μl. The kinase reactions proceeded for 30 min at 30°C at which time they were stopped by addition of 2 × SDS-sample buffer (30 μl). The extent of histone H1 phosphorylation (the measure of kinase activity) was determined by SDS-gel electrophoresis (12% acrylamide) and autoradiography.

Biosynthetic Labeling

NIH-3T3 cells in late G1 (see above) were trypsinized, washed in DME lacking methionine and cysteine, and incubated (~2 × 10⁵ cells per 100-mm dish with 5 ml Met/Cys-free DME) in monolayer and suspension with mitogens. After 4 h, the cells were pulsed for 60 min with Translabel (1 μCi/dish; ICN Biomedicals, Costa Mesa, CA). The cells were collected and extracted in 0.3 ml using the procedures described above. Equal amounts of TCA-precipitable radioactivity (1000 cpm) were incubated with 5 μl of a rat monoclonal antibody to murine cyclin D1 (gift of C. Sherr) or CD44 (gift of L. Bourguignon) using the procedures similar to those described above except that the immune complexes were recovered by incubation with 50 μl protein G–plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA). The washed immunoprecipitates were fractionated on a reducing SDS-gel, and the amount of cyclin D1 was determined by fluorography.

RNA Blot Hybridization

Total RNA was isolated from cells (typically 2.5 × 10⁶ cells per time point), and equal amounts of RNA (~10 μg) were fractionated on denaturing agarose gels. The filters were incubated with a random-primed cDNA probe for murine cyclin D1 using standard conditions. The hybridized filters were washed with 0.2 × SSPE, 0.1% SDS at 65–68°C.

Results

To explore the effects of anchorage on cell cycle transit, monolayer cultures of anchorage-dependent fibroblasts in G0, late G1, and in S phase (Fig. 1) were trypsinized and transferred to suspension or reseeded in monolayer. We determined the effect of anchorage (or loss of anchorage) on the activity of cell cycle proteins, in particular the G1 and S phase cyclin-cdk complexes.

Adhesion-dependent Hyperphosphorylation of the Retinoblastoma Protein

Initial experiments tested the effects of anchorage on cell cycle progression from quiescence to S phase. Monolayer and suspension cultures of G0-synchronized NRK and NIH-3T3 cells were stimulated with soluble mitogenic growth factors (FCS/EGF). As previously reported, cells stimulated with soluble mitogens in suspension failed to complete G1 and enter S phase (Guadagno et al., 1993; Han et al., 1993). Expression of cyclin A is coincident with and necessary for the onset of S phase, and neither NRK nor NIH-3T3 fibroblasts synthesized cyclin A mRNA (Guadagno et al., 1993) or protein during an 18-h incubation in suspension (Fig. 2 A). Expression of cyclin A was also anchorage-dependent in human primary foreskin fibroblasts (see Fig. 8 A). We observed a modest induction of cyclin A (Fig. 2 A) and DNA synthesis (not shown) after prolonged incubation (>18 h) of NRK cells in suspension. However, this muted induction was not sufficient to stimulate cell proliferation in suspension as assessed by colony formation in soft agar (Guadagno et al., 1993).

The effect of cell anchorage on G1 progression in NIH-3T3 and normal human fibroblasts was more profound than in NRK cells. First, cyclin A was never induced during incubation in suspension (Figs. 2 A and 8). Second, growth factor–dependent hyperphosphorylation of the Rb protein was essentially anchorage-independent in NRK cells, whereas it was completely anchorage-dependent in NIH-3T3 (Fig. 2 B) and normal human fibroblasts (See Fig. 8 A). These results indicate that cell adhesion controls multiple cell cycle events during G1 and that the pathways causing Rb hyperphosphorylation had become largely anchorage-independent in NRK cells.

Adhesion-dependent Expression of Cyclin D1

The G1 cyclin-cdk complexes, cyclin D-cdk4 and cyclin E-cdk2, are thought to be the kinases that hyperphosphorylate Rb during G1 in fibroblasts (see Introduction). We compared the expression of cyclin D1 and cdk4 in monolayer and suspension cultures of G0-synchronized NRK and NIH-3T3 cells (Fig. 2 C) and found that cyclin D1 protein levels were growth factor–dependent in both cell lines. However, the growth factor–dependent induction of cyclin D1 protein was minimally affected by cell anchorage in NRK cells, consistent with the fact that Rb hyperphosphorylation was also anchorage-independent. In contrast, the induction of cyclin D1 was completely anchorage-dependent in NIH-3T3 cells, as was Rb hyperphosphorylation. Cyclin D1 is the predominant D-type cyclin expressed in both NRK and NIH-3T3 cells, as a monoclonal antiserum that recognizes all three D-type cyclins detected only cyclin D1 (not shown). Expression of cdk4 protein in both NRK and NIH-3T3 cells was neither anchorage- nor growth
Figure 2. Phosphorylation of the retinoblastoma protein is associated with adhesion-dependent expression of cyclin D1. NRK and NIH-3T3 cells were synchronized in G0, and trypsinized and seeded with soluble mitogens (FCS/EGF) in monolayer and suspension. Cells were collected and extracted at the times shown. The extracts were fractionated on SDS gels and analyzed by immunoblotting with antibodies to cyclin A (A), Rb (B), cyclin D1 (C), and cdk4 (C). The upper and lower arrowheads in B, respectively, show the hyper- and hypo-phosphorylated forms of Rb. In D, an anti-cdk4 antibody was used to harvest cyclin D-cdk4 complexes from extracts (0.5 mg) of monolayer (Mn) and suspension (Sp) NRK and NIH-3T3 cells. The immunoprecipitates were fractionated on reducing SDS gels, and the amount of associated cyclin D1 was determined by Western blotting. In E, equal amounts of total RNA (assessed by ethidium bromide staining of rRNA) were isolated from extracts of monolayer and suspension NIH-3T3 cells and analyzed by Northern blot hybridization with a murine cyclin D1 cDNA. Extracts from monolayer and suspension cells were always analyzed in parallel and exposed to film for the same times. Approximate exposure times: A and B (30 s); C (1 min); D (3 min); E (4 d).

factor-dependent, and cdk4 immunoprecipitations showed that cyclin D/cdk4 complex formation was unaffected by cell adhesion in NRK cells and strongly adhesion-dependent in NIH-3T3 cells (Fig. 2 D).

Cyclin D1 mRNA was present in both monolayer and suspension cultures of NIH-3T3 cells (Fig. 2 E), and control studies (not shown) indicated that the mRNA was cytoplasmic in both conditions. Cyclin D1 mRNA levels were induced 3–5-fold by soluble mitogens in adherent NIH-3T3 cells, and this induction was greatly reduced in the suspended cells (Fig. 2 E). We concluded that the decreased expression of cyclin D1 protein in suspended NIH-3T3 cells was at least partly due to the 3–5-fold lower levels of its mRNA, but that this effect might not be sufficient to completely account for the apparent absence of the cyclin D1 protein.

Indeed, posttranscriptional control of cyclin D1 protein levels could be demonstrated by transferring NIH-3T3 cells to suspension late in G1, after growth factor-dependent induction of the cyclin D1 mRNA had occurred. Quiescent NIH-3T3 cells were preincubated with soluble mitogens in monolayer for 9 h, at which time cyclin D1 mRNA (Fig. 3 A) and protein (Fig. 3 B) were almost fully induced. As determined by densitometric scanning, the subsequent incubation of these cells in monolayer and suspension had less than a twofold effect on the expression of cyclin D1 mRNA (Fig. 3 A), but the amount of cyclin D1 protein decreased almost fivefold and became almost undetectable within 5 h after the transfer of cells to suspension (Fig. 3 B). In contrast, expression of cyclin D1 was constant during the incubation of cells in monolayer (Fig. 3 B).

Cycloheximide was added to the late G1 cells in order to compare the rates of cyclin D1 degradation in monolayer and suspension cells. The preaccumulated cyclin D1 protein (shown as 9 h) had a half-life of less than 1 hour in both culture conditions (Fig. 3 C). Thus, the turnover of cyclin D1 appeared to be anchorage-independent. Cyclin E levels were unaffected by cell adhesion, both in the ab-
Figure 3. Adhesion-dependent translation of cyclin D1 mRNA. G0-synchronized NIH-3T3 cells were preincubated with soluble mitogens (FCS/EGF) in monolayer for 9 h. These late G1 cells were trypsinized and reseeded in monolayer (Mn) and suspension (Sp) in the continued presence of mitogens. At the times indicated in the figure, the cells were collected and extracted for RNA blot and immunoblot analysis. A shows the expression of cyclin D1 mRNA (equal loading was confirmed by ethidium bromide staining of rRNA; shown as 28S). B and C show the expression of cyclin D1 and E (control) proteins. For the experiment shown in C, quiescent NIH-3T3 cells were preincubated with mitogen for 9 h to accumulate cyclin D1 protein. These preincubated cells were trypsinized, and then seeded in monolayer or suspension in the presence of cycloheximide (10 μg/ml); collected cells were extracted at 1, 3, and 5 h, and the decay of cyclin D1 (accumulated during the preincubation) was determined by immunoblotting. For the experiments shown in D, the late G1 cells were pulsed (for the last 60 min of a 5-h incubation) with Translabel. As expected from the results of others (Benecke et al., 1978), total protein synthesis was decreased slightly by incubation in suspension (20% over four separate experiments as determined by TCA precipitation). Therefore, equal amounts of TCA-insoluble radioactivity were incubated with rat monoclonal antibodies to murine cyclin D1. Duplicate extracts from adherent cells were also incubated in parallel with monoclonal antibodies to cyclin D1 and CD44 (CON) to identify nonspecifically immunoprecipitated proteins. The collected immunoprecipitates were fractionated on reducing SDS gels and analyzed by fluorography. E shows the results obtained when protein blots (prepared as described for B) were incubated with an antibody against Rb. The positions of hypo- and hyperphosphorylated Rb are shown by the lower and upper arrowheads, respectively. Approximate exposure times: A (18 h); B and C (2 min); D (3 d); E (30 s).
Figure 4. Adhesion-dependent expression of cyclin D1 is linked to G1, but not S phase, phosphorylation of Rb. G0-synchronized NIH-3T3 cells (A and C) or NIH-3T3 cells overexpressing human cyclin D1 (hD1/NIH-3T3; B) were preincubated with soluble mitogens in monolayer for 9 h (A and B) or 16 h (C). The cells were then trypsinized and incubated with soluble mitogens in monolayer (Mn) and suspension (Sp) for 1–5 h. Collected cells were extracted and the extracts were analyzed by immunoblotting with anti-Rb and anti-cyclin D antibodies. The hypo- and hyperphosphorylated forms of pRb are indicated by the lower and upper arrows, respectively. Exposure times: 30 s for each panel. Aliquots of each sample were also analyzed by flow cytometry to assess the effect of adhesion and the expression of cyclin D1 on cell cycle progression. D shows the percent of S phase cells in the initial G0-synchronized cultures ("0"), the cultures after preincubation with mitogens in monolayer ("9" and "16" h), and the cultures after final incubation in monolayer and suspension for 5 h (Mn and Sp, respectively).

Cells constitutively expressing cyclin D1 differed from control cells in two important ways. First, the enforced expression of cyclin D1 allowed Rb to remain hyperphosphorylated when G1 cells were transferred to suspension (Fig. 4 B), indicating that cyclin D1 was necessary to maintain the hyperphosphorylated state of Rb during G1. (Note that cyclin E–dependent kinase activity was induced during the 9-h preincubation with mitogens in these experiments, and the activity persisted throughout the subsequent incubation in monolayer and suspension [data not shown]. Thus, in this protocol the G1 phosphorylation of Rb is specifically controlled by the expression of cyclin D.) Second, the forced expression of cyclin D1 rescued entry into S phase. Aliquots of the cells used for the immunobots above were processed for a flow cytometric analysis of cell cycle progression (Fig. 4 D). Consistent with the dephosphorylation of Rb, entry into S phase was inhibited when G1 control cells were incubated in suspension (Fig. 4 D, left) and expression of cyclin D1 overcame this G1 block (Fig. 4 D, middle). Since cyclin A is necessary for entry into S phase, these results also indicate that the forced expression of cyclin D1 affects the expression of cyclin A in nonadherent NIH-3T3 cells (see Discussion).

In contrast to the results obtained with G1 cells, cyclin D1 was not necessary to maintain Rb hyperphosphorylation or cell cycle progression once cells had entered S...
phase. Quiescent NIH-3T3 cells were preincubated with soluble mitogens in monolayer for 16 h to generate a population of S phase cells (refer to Fig. 1). The cells were then trypsinized and placed in suspension or returned to monolayer. Cyclin D1 protein still decreased when these S phase cells were incubated in suspension, but Rb remained hyperphosphorylated (Fig. 4 C). This result is in contrast to the behavior of Rb in late G1 cells, and it suggests that once cells have completed G1, other kinases maintain Rb in its hyperphosphorylated state. Cyclin A-cdk2, which remained active in the suspended S phase cells (not shown), is one candidate for the S-phase Rb kinase. The suspended S-phase cells were also able to progress into G2/M (Fig. 4 D, right) despite the fact that the expression of cyclin D1 remained adhesion dependent (Fig. 4 C). Overall, Fig. 4 shows that the expression of cyclin D1 protein is anchorage dependent throughout the cell cycle, but the biological consequence of this restriction (for both Rb phosphorylation and cell cycle progression) is evident only in G1 cells.

**Cell Adhesion Regulates Cyclin E-cdk2 Activity**

Growth factor–dependent induction of cyclin E kinase activity was modestly delayed when NRK cells were cultured in suspension (Fig. 5 A), but it was completely blocked in suspended NIH-3T3 cells (Fig. 5 A) and primary human fibroblasts (see Fig. 8 B). The effects of adhesion on cyclin E–associated kinase activity could not be explained by changes in the levels of cyclin E or its catalytic partner, cdk2: the expression of these proteins was anchorage-independent in all three of these fibroblasts (Fig. 5 B and refer to Fig. 8 B). Thus, the activities of both G1 cyclin-cdk complexes (cyclin D-cdk4 and cyclin E-cdk2) are tightly regulated by cell adhesion in NIH-3T3 and human fibroblasts. In large part, both of these controls are absent in NRK cells.

Cyclin E was immunoprecipitated from extracts of quiescent and growth factor–stimulated NIH-3T3 cells in monolayer and suspension, and all of the immunoprecipitates contained similar amounts of cdk2 (Fig. 5 C). Thus, formation of the cyclin E-cdk2 complex is unaffected by cell adhesion. Cyclin E-cdk2 complexes are activated by a CAK-mediated phosphorylation at threonine 160, and this phosphorylation can be detected as an increase in the electrophoretic mobility of cdk2 on SDS gels (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). This CAK-phosphorylated form of cdk2 was readily detected in the cyclin E complexes harvested from NIH-3T3 cells (compare total and cyclin E–associated cdk2 in Fig. 5 C), and the degree of CAK phosphorylation was unaffected by incubation of the cells in suspension (Fig. 5 C). Moreover, extracts of monolayer and suspended NIH-3T3 cells contained readily detectable CAK activity, and the amount of activity was largely unaffected by cell adhesion (Fig. 5 D). Thus, the strict adhesion requirement for cyclin E-kinase activity in NIH-3T3 cells could not be explained by differences in complex formation or by the regulation of CAK. CAK phosphorylation of cdk2 was also anchorage-independent in NRK cells (data not shown).

Extracts from monolayer and suspension cultures of NRK and NIH-3T3 cells (Fig. 6) were analyzed by immunoblotting to determine whether cell adhesion controlled

**Figure 5.** Adhesion-dependent activity of cyclin E-cdk2. G0-synchronized NRK and NIH-3T3 cells were incubated in monolayer (Mn) and suspension (Sp) in the presence of soluble mitogens (FCS/EGF). At the indicated time, cells were collected and extracted for the analysis of cyclin E–associated kinase activity using histone H1 as substrate (A). Identically prepared extracts were fractionated on reducing SDS gels and immunoblotted with antisera against cyclin E and cdk2 (B). To assess assembly of the cyclin E-cdk2 complex and its activation by CAK, extracts were prepared from quiescent NIH-3T3 cells (0) and cells that had been treated with soluble mitogens for 12 h in monolayer and suspension. Cyclin E complexes immunoprecipitated from the extracts were fractionated on SDS gels and immunoblotted with an antibody to cdk2 (C, left side). The migration of total cdk2 from the same extracts is shown by immunoblotting (C, right side). Note that the samples analyzed in C were run on one gel and exposed to film for the same time. For direct measurement of CAK activity (D), extracts (0.2 mg protein) from adherent and nonadherent cells were incubated with 1, 3, or 5 µl of an antibody to MO15 (the catalytic subunit of CAK, Upstate Biotechnology Inc.) or normal serum (NRS, negative control), and the amount of CAK activity was determined by the ability of the immunoprecipitates to activate H1 histone kinase activity (H1) of a recombining cyclin A-cdk2 complex (see Nourse et al., 1994 for procedures). The dose-dependent increase in CAK activity (with increasing amounts of anti-MO15) confirms that this analysis was performed in the linear range. Approximate exposure times: A and D (2 min); B (1 min); C (30 s).
Figure 6. Cell adhesion affects the association of p21 and p27 with cyclin E-cdk2 complexes in NIH-3T3 cells. The figure shows immunoblots of total and cyclin E-associated p27 and p21 (as well as cyclin E and cyclin E-associated kinase activity) from quiescent (0) and mitogen-treated (12 h) NRK and NIH-3T3 cells in monolayer (Mn) and suspension (Sp). The levels of total and cyclin E-associated proteins were determined with 100 and 500 μg extract, respectively. Approximate ECL exposure times for both NRK and NIH-3T3 cells: (30 s for total cyclin E, 30 s for total and cyclin E-associated p27, 5 min for total and cyclin E-associated p21).

Figure 7. Stoichiometry of CKI binding to cyclin E-cdk2 complexes. G0-synchronized NIH-3T3 cells were cultured in monolayer and suspension for 12 h in the presence of mitogens. Collected cells were lysed and equal aliquots (0.5 mg) of each sample were incubated with anti-cyclin E. The immunoprecipitates were thoroughly washed before fractionation on reducing SDS gels. Selected amounts of recombinant GST-murine p21, his-tagged murine p27, and GST-cdk2 were electrophoresed in parallel. The immunoprecipitates and standards were analyzed simultaneously by immunoblotting with antibodies that detect murine p21, p27, and cdk2. We visually matched band intensities to the standard curves and confirmed the results by densitometry (to correct for potential nonlinearity in the ECL signals). The recombinant standards were quantified by Coomassie blue staining (Bio-Rad assay) and purity was assessed on SDS gels. Quantification of GST-cdk2 and GST-p21 was confirmed with anti-GST immunoblots using a preparation of homogeneous GST as standard. From this analysis, we calculated the moles of cdk2, p21, and p27 in the cyclin E-cdk2 complexes from adherent and nonadherent cells. The results are presented as the moles of p21 and p27 per mol cdk2 and show the mean of two separate experiments. Error bars indicate the ranges observed.

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suspension. Flow cytometry (not shown) indicated that the adherent cells first began to enter S phase after 20-22 h and that the suspended cells failed to enter S phase even after 72 h in suspension. The hyperphosphorylation of Rb and the expression of cyclins D1 and A were adhesion-dependent in human fibroblasts (Fig. 8 A). Cyclin E-associated kinase activity was also adhesion-dependent despite the fact that cyclin E and cdk2 were present at similar levels in monolayer and suspended cells (Fig. 8 B). p21 mRNA (not shown) and protein (Fig. 8 B) were both induced approximately twofold when human fibroblasts were cultured in suspension. p27 (Fig. 8 B) is present in quiescent human fibroblasts, its levels decrease when monolayer cells are exposed to growth factors, and several independent experiments indicated that this decrease is somewhat less efficient (approximately twofold in several independent experiments) when cells are cultured in suspension (Fig. 8 B). The inactive cyclin E-cdk2 complexes harvested from mitogen-treated cells in suspension consistently contained increased amounts of both p21 and p27 relative to complexes harvested from mitogen-treated cells in monolayer (Fig. 8 B).

Overall, the phenotype of normal human fibroblasts essentially recapitulates that observed with NIH-3T3 cells and clearly differs from the phenotype of NRK cells. In turn, these results suggest that the subset of the adhesion-dependent events regulating the expression of cyclin D1, activity of cyclin E-cdk2, and phosphorylation of Rb have been lost in the NRK fibroblast cell line. Note that the small upregulation of p21 mRNA that we observed upon loss of cell adhesion in NIH-3T3 cells and human fibroblasts is likely to be independent of p53: suspended fibroblasts.

Figure 8. The adhesion-dependent phenotype of human fibroblasts. G0-synchronized human skin fibroblasts (h-fib; A and B) or human skin fibroblasts overexpressing cyclin E (E/h-fib; C and D) were incubated with soluble mitogens (FCS/EGF) for 0 or 20-22 h in monolayer (Mn) and suspension (Sp). Collected cells were extracted, and the extracts were analyzed by immunoblotting with antibodies to Rb, cyclin D, cyclin A, cyclin E, cdk2, p21, and p27. Cell extracts were also incubated with a cyclin E antibody to determine the level of cyclin E-associated H1 kinase activity (B and D). Duplicate samples of the cyclin E immunoprecipitates were fractionated on reducing SDS gels and the amounts of associated cdk2, p21, and p27 were determined by immunoblotting (B and D). The levels of total and cyclin E-associated proteins were determined with 100 and 500 μg extract, respectively. Approximate ECL exposure times: A (30 s); B (5 m for cyclin E, 3 m for cdk2, 1 m for cyclin E-associated cdk2, 20 s for total and cyclin E-associated p21); C (30 s); D (30 s).
blasts derived from wild-type and p53-deficient mouse embryos showed similar increases in the expression of p21 mRNA (data not shown).

We reasoned that the inhibitory threshold imposed by p21 and p27 on cyclin E-cdk2 might be overcome by increasing the expression of cyclin E, so we infected human fibroblasts with a cyclin E retrovirus in an attempt to rescue cyclin E kinase activity in nonadherent cells. The expression of cyclins D1 and A and the hyperphosphorylation of Rb remained anchorage-dependent (Fig. 8 C), indicating that constitutive expression of cyclin E had not deregulated adhesion-dependent cell cycle control in general. Although cyclin E was overexpressed about fivefold, cyclin E immunoprecipitates isolated from suspended cells still lacked kinase activity (Fig. 8 D). As assessed by the increased electrophoretic mobility of cdk2, the cyclin E-cdk2 complexes in the transfecteds were phosphorylated by CAK during serum-starvation, and the activated form persisted during incubation in suspension. A modest increase in CAK-phosphorylated cdk2 was detected in the growth factor-treated monolayers (Fig. 8 D), but in general, the strict adhesion requirement for cyclin E kinase activity could not be explained by the regulation of CAK.

Interestingly, the overexpression of cyclin E in human fibroblasts led to a fivefold increase in total p21, and, as a consequence, we could readily detect enhanced association of p21 in the cyclin E-cdk2 complexes harvested from suspended cells (Fig. 8 D). p27 levels, and its association with cyclin E-cdk2, were minimally affected by the overexpression of cyclin E (data not shown). Thus, these normal human fibroblasts responded to a cyclin E challenge by increasing the steady-state levels of p21, and our inability to rescue kinase activity by overexpression of cyclin E is the likely consequence of this response. Note that nonadherent cyclin E-human fibroblasts show an increased association of p21 with cyclin E-cdk2 (relative to the adherent cells) even though total p21 levels have become anchorage-independent (Fig. 8 D).

Discussion

Cell adhesion to substratum is required for cell cycle progression through G1 phase, but the basis for this requirement has been poorly understood. We show here that cell adhesion is necessary for activation of all three cyclin-cdk complexes required for cell cycle progression from G0 to S phase. First, cell adhesion is required for the induction and translation of cyclin D1 mRNA. Second, cell adhesion regulates the activity of cyclin E-cdk2 by determining the extent of its association with the cdk inhibitors p21 and p27. Third, the expression of cyclin A mRNA and protein requires cell adhesion. We also show that these effects are not only causally related to the level of cyclin E-associated kinase activity, but also causally related to the ability of cells to phosphorylate Rb and progress into S phase. The cell cycle effects we observed by incubating cells in suspension were not changed by enriching the mitogenic cocktail (with 1 μM insulin; refer to Massagué et al., 1985) (data not shown). Moreover, mitogens do function in nonadherent cells as assessed by the phosphorylation of their receptors and receptor substrates (McNamee et al., 1993) and the induction of c-myc mRNA (Böhmer et al., 1996).

We therefore conclude that the G1 cell cycle events that have been attributed to growth factors actually require the convergence of signals from growth factors and the extracellular matrix.

Adhesion-dependent Induction and Translation of Cyclin D1 mRNA

Cell adhesion controls the abundance of cyclin D1 protein at two levels. First, in the absence of cell adhesion, cyclin D1 mRNA levels are 3–5-fold lower than in adherent cells. c-myc has been implicated as a positive regulator of cyclin D1 gene expression (Daksis et al., 1994). However, at least in normal human fibroblasts, adhesion-dependent control of cyclin D1 mRNA does not appear to involve a direct effect of c-myc because c-myc mRNA is induced by mitogenic growth factors independently of cell adhesion (Böhmer et al., 1996). Second, direct measurements showed that synthesis, but not degradation, of cyclin D1 was also dependent upon cell adhesion. Adhesion-dependent synthesis of cyclin D1 may be related to the fact that the binding of cells to substratum is required for the persistent activation of MAP kinase (Zhu and Assoian, 1995; our unpublished observations), and that activation of MAP kinase in mid-G1 has the potential to selectively stimulate the translation of cyclin D1 mRNA by regulating the activity of PHAS-1 (Rosenwald et al., 1993; Lin et al., 1994). Note also that the synthesis of cyclin D1 is less affected by adhesion if the transcript lacks UTRs (Zhu and Assoian, our unpublished observations).

Adhesion-dependent Activity of Cyclin E-cdk2

Cell adhesion is also required for cyclin E kinase activity in NIH-3T3 cells and human fibroblasts. This effect is not related to assembly of cyclin E-cdk2 complexes nor can it be explained by an adhesion-dependent regulation of CAK. Rather, our data indicate that an increased association of p21 and p27 inhibits cyclin E kinase activity in suspended cells. This interpretation is supported by our stoichiometric analysis which indicates that the CKI/cdk2 ratio changes from about one to two when NIH-3T3 cells are cultured in suspension. Others have shown that a change of this magnitude is sufficient to inhibit cyclin E kinase activity (Harper et al., 1995), and we have obtained similar results.

While this manuscript was under review, Fang et al. (1996) reported that cell adhesion was required for cyclin E-cdk2 kinase activity in human fibroblastic cell lines most likely because the levels of total and cyclin E-associated p21 and p27 were increased (approximately fourfold) when the cells were cultured in suspension. Although smaller in magnitude, we have observed similar effects with NIH-3T3 cells and normal human fibroblasts. However, our data also suggest that the absence of cyclin D1 protein in nonadherent NIH-3T3 cells and human fibroblasts (with the consequent lack of cyclin D-cdk4 complexes) should allow for an altered distribution between the total p21/27 pool and cyclin E-cdk2, and that this effect can contribute to the increased association of these CKIs with cyclin E-cdk2. (Cyclin E-cdk2 should be targeted in suspended fibroblasts because cyclin A and the mitotic cyclins are not present in G1 cells.) This idea is supported by
Adhesion-dependent Expression of Cyclin A mRNA

Expression of the third cyclin necessary for S phase entry, cyclin A (Girard et al., 1991; Pagano et al., 1992), is also dependent upon cell adhesion. During a normal cell cycle, cyclin A expression begins after cells have accumulated active cyclin D- and cyclin E-cdk complexes (for a review see Heichman and Roberts, 1994). The cyclin A promoter contains E2F sites (Henglein et al., 1994) and overexpression of E2F induces anchorage-independent growth of NIH-3T3 cells (Xu et al., 1993). It has therefore been suggested that cyclin A expression may be activated indirectly by cyclin D-cdk4 and cyclin E-cdk2, as a consequence of their ability to phosphorylate Rb, release E2F, and induce E2F-dependent gene transcription (Xu et al., 1995). We find that forced expression of cyclin D1 rescues Rb phosphorylation and entry into S phase when G1 NIH-3T3 cells are cultured in suspension, and this result is consistent with a role for an Rb/E2F pathway in the adhesion-dependent expression of cyclin A.

However, our results with NRK fibroblasts indicate that other mechanisms also contribute to adhesion-dependent cyclin A expression. These cells phosphorylate Rb in suspension but remain unable to induce cyclin A expression to a level that is sufficient for colony formation in soft agar (Guadagno et al., 1993). The cyclin A promoter contains a variant E2F site that seems to bind to p107/E2F4 complexes specifically (Schulze et al., 1995); this site may be involved in adhesion-dependent cyclin A expression. Alternatively, Fang et al. (1996) have suggested that adhesion-dependent expression of cyclin A may merely reflect the anchorage requirement for induction of cyclin E-cdk2 activity. This proposal is not supported by our results which show that cyclin E-dependent kinase activity is readily detected in suspended NRK cells, even though they poorly express cyclin A. Moreover, the enforced expression of cyclin A (but not cyclin E) is sufficient to stimulate soft agar growth of NRK fibroblasts (Guadagno et al., 1993).

Phosphorylation of the Retinoblastoma Protein Reflects the Converging Actions of Mitogenic Growth Factors and the Extracellular Matrix

Rb phosphorylation is dependent upon mitogenic growth factors, and phosphorylation of Rb during G1 correlates with the transition from the growth factor-dependent to the growth factor-independent portion of the cell cycle. Thus, Rb phosphorylation is viewed as a key molecular target by which growth factors control cell cycle progression. Our results fully support the essential role of growth factors in regulating G1 cyclin activity and Rb phosphorylation. But the data we obtained in NIH-3T3 cells and human fibroblasts also show that phosphorylation of the Rb protein during the G1 phase of the cell cycle requires information provided by both mitogenic growth factors and the extracellular matrix. Neither signal alone is sufficient for Rb phosphorylation.

In nonadherent cells both cyclin D1 and cyclin E kinase are inactive, and Rb is not phosphorylated. When late G1 cells are transferred to suspension, cyclin D1 mRNA is not translated and Rb is dephosphorylated. Enforced expression of cyclin D1 protein in late G1 rescues Rb phosphorylation and entry into S phase in nonadherent cells. These results establish a causal relationship between the control of cyclin D1 translation and adhesion-dependent cell cycle progression. On the other hand, enforced expression of cyclin D1 was not sufficient to allow fully quiescent cells to phosphorylate Rb or activate cyclin E-cdk2 in the absence of adhesion, suggesting that there are additional controls on cyclin D1/cdk4 activity that specifically act in early G1. This result may also explain why the overexpression of cyclin D1 in NIH-3T3 cells fails to induce colony formation of NIH-3T3 cells in soft agar despite its causal role in Rb phosphorylation (Quelle et al., 1993 and our unpublished observations).

Cyclin E-cdk2 is an Rb kinase both in vitro and in vivo, and it is activated in vivo at a time when Rb phosphorylation is occurring (refer to Introduction). Our results showing adhesion-dependent cyclin E-cdk2 activity indicate that the effect of cyclin E-cdk2 on Rb phosphorylation will also be dependent upon cell adhesion. Involvement of cyclin E-cdk2 in Rb phosphorylation may explain the observation that suspended NRK cells have similar lags in the onset of cyclin E-kinase activity and Rb hyperphosphorylation (compare Figs. 2 and 5). Thus, through their complementary effects on cyclin D and E kinase activities, the signals generated by mitogenic growth factors and the extracellular matrix converge, and this convergence is manifested in the phosphorylation state of the retinoblastoma protein and cell cycle progression through G1.

Multistep Progression towards Anchorage-independent Growth

Our data emphasize that there is redundancy in adhesion-dependent cell cycle control. For example, nonadherent NIH-3T3 weakly upregulate the protein levels of p21 in comparison to human fibroblasts (this report and Fang et al., 1996), but their adhesion-dependent expression of cyclin D1 is sufficient to render them anchorage-dependent. NRK cells have largely lost their adhesion controls on cyclin D1 and E kinase activities, but adhesion control of cyclin A is relatively intact so these cells still appear anchorage-dependent. Thus, the phenotype of anchorage-independence reflects a multistep deregulation of adhesion-controlled cell cycle events. The variable effects of tumor viruses, oncogenes, and chemical mutagens in inducing anchorage independence in different cell lines may well re-
reflect the extent to which adhesion-dependent cell cycle controls have been lost in the host. Multistep progression to anchorage independence may also explain the fact that this phenotype is the best cell culture correlate to tumorigenicity in animals (Shin et al., 1975).

The transforming effects of DNA tumor viruses, of oncogenes, and of deletions and mutations in tumor suppressor genes have all been linked to the deregulated function of the cdk complexes (D-cdk4, E-cdk2, and A-cdk2) that guide cells through G1 and S phase (for review see Hunter and Pines, 1994). Since growth factors regulate the G1 cyclins, these transforming events have typically been viewed in terms of escape from the requirement for growth factors. Our results show that they must be equally viewed in terms of escaping the requirement for cell adhesion.

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