# Regulation of p21$^{cip1}$ Expression by Growth Factors and the Extracellular Matrix Reveals a Role for Transient ERK Activity in G1 Phase

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*Abstract.* We have examined the regulation of p21$^{cip1}$ by soluble mitogens and cell anchorage as well as the relationship between the expression of p21$^{cip1}$ and activation of the ERK subfamily of MAP kinases. We find that p21$^{cip1}$ expression in G1 phase can be divided into two discrete phases: an initial induction that requires growth factors and the activation of ERK, and then a subsequent decline that is enhanced by cell anchorage in an ERK-independent manner. In contrast to the induction of cyclin D1, the induction of p21$^{cip1}$ is mediated by transient ERK activity. Comparative studies with wild-type and p21$^{cip1}$-null fibroblasts indicate that adhesion-dependent regulation of p21$^{cip1}$ is important for proper control of cyclin E–cdk2 activity. These data lead to a model in which mitogens and anchorage act in a parallel fashion to regulate G1 phase expression of p21$^{cip1}$. They also show that (a) growth factors and growth factor/extracellular matrix cooperation can have different roles in regulating G1 phase ERK activity and (b) both transient and sustained ERK signals have functionally significant roles in controlling cell cycle progression through G1 phase.

Key words: cell cycle • adhesion • ECM • MAP kinase • cdk inhibitors



THE majority of cells in the adult are thought to be in a resting quiescent state. When suitable extracellular cues are present, e.g., during a response to injury, cells leave this quiescent (G0) state and enter the G1 phase of the cell cycle. For most cell types, the extracellular cues that mediate progression through G1 phase can be divided into two general groups: soluble mitogenic growth factors and the extracellular matrix (ECM)[1] (reviewed in Assoian, 1997). The signaling potential of soluble mitogens and the ECM results from their ability to bind to and cluster specific cell surface receptors, typically receptor tyrosine kinases (RTKs) and integrins, respectively.

When RTKs and integrins are signaling, cells undergo a series of molecular events involving cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors (CKIs) (reviewed in Hunter and Pines, 1994; Sherr, 1994; Sherr and Roberts, 1995). Two cyclin-cdk activities, cyclin D–cdk4/6 and cyclin E–cdk2, are required for progression of cells through G1 phase. In large part, these enzymes are required because they phosphorylate the retinoblastoma protein (pRb); this event allows for the release of E2F and the induction of E2F-regulated genes such as cyclin A (Weinberg, 1995). The induction of cyclin A, with consequent formation of active cyclin A–cdk2 complexes, is thought to reflect entry into S phase of the cell cycle.

In fibroblasts and epithelial cells, mitogens and the ECM are jointly required to induce the expression of cyclin D1 mRNA (Böhmer et al., 1996; Zhu et al., 1996; Day et al., 1997; Radeva et al., 1997; Resnitzky, 1997). This effect has been linked to sustained ERK activity and the role of integrin signaling in sustaining ERK activity throughout G1 phase (Weber et al., 1997; Roovers et al., 1999). The translation of cyclin D1 mRNA is also dependent upon cell adhesion (Zhu et al., 1996; Huang et al., 1998). The combined mitogen/anchorage requirement for expression of cyclin D1 explains, in part, why hyperphosphorylation of pRb and expression of cyclin A are also jointly dependent upon exposure of cells to mitogens and an ECM.

In addition to their cooperative effects in the induction of cyclin D1, mitogens and the ECM are also jointly required for activation of cyclin E–cdk2 (reviewed in Assoian, 1997). The cyclin E–cdk2 complexes harvested from suspended cells are catalytically inactive when assayed in

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1. *Abbreviations used in this paper:* cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ECM, extracellular matrix; MEF, mouse embryo fibroblast; pRb, retinoblastoma protein; RTK, receptor tyrosine kinase.

vitro despite the fact that the cells had been exposed to growth factors. Suspended cells express elevated levels of two CKIs, $p21^{cip1}$ and $p27^{kip1}$, and the catalytically inactive cyclin E–cdk2 complexes isolated from suspended cells show increased association of both $p21^{cip1}$ and $p27^{kip1}$. Neither CAK activity nor the levels of cyclin E and cdk2 are significantly different in adherent vs. nonadherent cells, so it seems likely that mitogens and anchorage jointly regulate cyclin E–cdk2 activity by controlling the expression of $p21^{cip1}$ and $p27^{kip1}$. Changes in $p21^{cip1}$ expression are often associated with altered transcription of the gene, whereas changes in $p27^{kip1}$ levels have typically been associated with changes in protein translation or degradation (Hengst and Reed, 1996; Sheaff et al., 1997; Montagnoli et al., 1999). Thus, very different mechanisms likely underlie the effects of growth factors and the ECM on $p21^{cip1}$ and $p27^{kip1}$ levels.

In this study, we have examined the regulation of $p21^{cip1}$. $p21^{cip1}$ is poorly expressed in quiescent cells, it is rapidly induced when cells are stimulated with mitogens, and its expression then declines as cells reach mid-late G1 phase (Li et al., 1994; Macleod et al., 1995; Liu et al., 1996; Bosch et al., 1998). The early G1 phase induction of $p21^{cip1}$ plays a role in the assembly of cyclin D–cdk4/6 complexes (LaBaer et al., 1997; Cheng et al., 1999), and the mid-late G1 phase decline of $p21^{cip1}$ correlates with activation of cyclin E–cdk2. p53 is important for induction of the $p21^{cip1}$ promoter (El-Deiry et al., 1993), but p53-independent mechanisms also induce the $p21^{cip1}$ gene (Macleod et al., 1995; Zeng et al., 1996). In fact, activation of ERKs has been strongly implicated in the induction of $p21^{cip1}$ (Liu et al., 1996; Pumiglia and Decker, 1997; Auer et al., 1998).

We report here that $p21^{cip1}$ expression can be divided into two discrete phases: an initial induction that requires mitogens and ERK activity and a subsequent downregulation that is enhanced by cell anchorage and independent of ERK activity. Thus, growth factors and the ECM act in parallel to regulate the expression of $p21^{cip1}$ during G1 phase. We also report that, in contrast to the regulation of cyclin D1, a transient activation of ERK is sufficient to induce $p21^{cip1}$.

## Materials and Methods

### Cell Culture

Established mouse embryo fibroblasts (MEFs) from wild-type, $p21^{cip1}$-null, and p53-null C57B6J mice were generous gifts from Jim Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA) and Tyler Jacks (MIT, Cambridge, MA). The cells had been maintained on a standard 3T3-like protocol which resulted in establishment of MEF cell lines. For G0-synchronization, MEFs were brought to confluence, washed, and then cultured in serum-free DME or defined medium (see below) for 2 d. To stimulate entry into the cell cycle, G0-synchronized cells were trypsinized, suspended in 10% FCS-DME, and reseeded on tissue culture dishes (monolayer) or agarose-coated petri dishes (suspension) using $2 \times 10^6$ cells per 100-mm dish similarly to the procedure described in Böhmer et al. (1996). To study the relative effects of growth factors and fibronectin, trypsinized quiescent cells ($2 \times 10^6$ cells) were suspended in 10 ml defined medium (1:1 DME: Hams F-12, 15 mM Hepes, pH 7.4, 3 mM histidine, 4 mM glutamine, 8 mM sodium bicarbonate, 10 μM ethanolamine, 10 μg/ml transferrin, 0.1 μM sodium selenite, 0.1 μM $MgCl_2$, and 1 mg/ml BSA). Some of the cells were treated with a cocktail of purified growth factors (10 ng/ml PDGF, 1 μM insulin, and 2 nM EGF) that allowed for optimal induction of $p21^{cip1}$. Cells were then plated on 100-mm dishes that had been coated (16 h at 4°C) with fibronectin (100 μg) or 2 mg/ml fatty acid–free, heat-inactivated BSA in PBS (which blocks cell adhesion in serum-free culture). Coating with fibronectin or BSA was performed as described (Zhu et al., 1999). In experiments with UO126 (Promega), G0-synchronized cells in 100-mm dishes were directly stimulated with purified growth factors in defined medium. For all experiments, cells were washed 2–3 times with PBS, collected by scraping (monolayer cultures) or low-speed centrifugation (suspension cultures), and extracted for Northern blotting, luciferase assays, and immunoblotting, or immunoprecipitation and in vitro kinase assays. In studies using serum as the mitogenic stimulus, the extracts of quiescent cells were prepared before incubation of the cells in monolayer or suspension. For studies in defined medium, extracts of quiescent cells (in growth factor-free defined medium) were prepared both before and after incubation of the cells in monolayer and suspension; similar results were obtained from both controls. Note that MEFs attached to fibronectin and cultured with growth factors in defined medium remained well spread for up to 12 h.

To study the consequence of ERK activation in suspended cells, we used NIH-3T3 cells stably transfected with a constitutively active MEK-1 (S218D/S222D) in a tetracycline-repressible expression system (Roovers et al., 1999). The transfectants were maintained at <50% confluence in DME, 10% calf serum, 0.5 mg/ml G418, and 0.4 mg/ml hygromycin. Tetracycline (2 μg/ml) was added daily.

### Analysis of $p21^{cip1}$ Promoter Activity

MEFs ($1.5 \times 10^5$ in 2 ml DME, 10% FCS) were plated in 35-mm dishes and incubated overnight. The resulting monolayers (~80% confluent) were washed with DME before transient cotransfection with 1 μg of $p21^{cip1}$ promoter-luciferase plasmid (0-luc; gift of Wafik El-Deiry), 0.1 μg of the renilla luciferase expression plasmid, pRL-SV40 (Promega), and 5 μl lipofectamine (Life Technologies) in a total volume of 1 ml serum-free DME without antibiotics. After a 5-h incubation, 1 ml of 20% FCS-DME was added to each well and the cultures were incubated overnight. 18 h after transfection, the cells were G0-synchronized by 2-d incubation in serum-free DME. After trypsinization, an aliquot of the cells was removed for determination of $p21^{cip1}$-promoter activity at quiescence; the remainder was resuspended in 10% FCS-DME and then replated in 100-mm dishes ($0.5–1 \times 10^5$ cells/10 ml) in monolayer and suspension. At selected times, cells were washed, collected, and extracted in 50 μl of Passive Lysis Buffer (Promega) before analysis of firefly luciferase and renilla luciferase activity using the Dual-Luciferase reporter assay system (Promega). $p21^{cip1}$ promoter activity was normalized to a constant amount of renilla-based luminescence to correct for differences in transfection efficiency.

### Other Procedures and Reagents

Northern blotting, immunoprecipitations, and in vitro kinase assays were performed as described (Zhu et al., 1996). Immunoblotting for pRb, cyclins, MEK, and ERKs was performed as described (Zhu and Assoian, 1995; Zhu et al., 1996) after fractionation of cell lysates on reducing SDS gels containing 7.5% acrylamide. Immunoblotting for CKIs was performed similarly except that the gels contained 15% acrylamide. The protein concentration of cell lysates destined for immunoblot analysis was determined by Coomassie binding (BioRad Protein Assay), and equal amounts (100 μg for the analysis of cyclins, cdks, and CKIs, and 25 μg for the analysis of MEK* and ERK) were fractionated on SDS gels. Cells destined for immunoblot analysis with anti-ERK or anti-phospho-ERK antibodies were extracted as described (Zhu et al., 1999).

Rabbit polyclonal antiserum against cyclin A was prepared in this laboratory, and the antiserum to $p21^{cip1}$ was the generous gift of Claudio Schneider (AREA Science Park, Trieste, Italy). All other antisera were purchased: anti-cyclin E (sc-481) and anti-cdk4 (sc-260) from Santa Cruz Biotechnology; anti-cyclin D1 (06-137), and anti-cdk2 (06-505) from Upstate Biotechnology; anti-MEK-1 (M17020), anti-$p27^{kip1}$ (K25020), and anti-ERK (M12320) from Transduction Laboratories, anti-pRb (100601) from Ciba-Corning; and anti-phospho-ERK (9101S) from New England BioLabs.

## Results

### G1 Phase Induction of $p21^{cip1}$ Requires Growth Factors, Does Not Require Cell Adhesion, and Is Dependent upon the Activation of ERKs

Our initial studies compared the temporal expression pat-

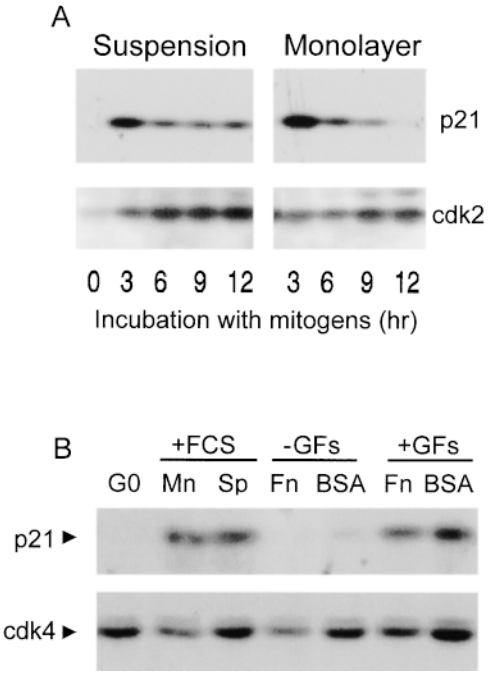


*Figure 1.* Biphasic regulation of p21$^{cip1}$ expression by growth factors and the extracellular matrix. In A, G0-synchronized MEFs were seeded in suspension and monolayer in 10% FCS-DME and incubated for 0–12 h. Cell extracts were prepared and equal amounts of protein were fractionated on reducing SDS gels and analyzed by immunoblotting and enhanced chemiluminescence (ECL) using antibodies against p21$^{cip1}$ and cdk2 (a control for loading). [Note: In this experiment the downregulation of p21 was essentially complete in 12 h, but other experiments indicate that strong downregulation usually occurs over a 12–18-h period.] In B, quiescent MEFs (G0) were cultured for 3 h in defined medium on dishes coated with BSA or fibronectin (Fn). The incubations were also performed in the absence (−GF) or presence (+GF) of purified growth factors. Duplicate cultures were incubated in monolayer (Mn) and suspension (Sp) in 10% FCS-DME for comparison. Collected cells were analyzed by immunoblotting with anti-p21$^{cip1}$ and anti-cdk4 (a control for loading).

tern of p21$^{cip1}$ when adherent and nonadherent MEFs were exposed to mitogens (Fig. 1 A). Consistent with studies by others (see above), we find that the level of p21$^{cip1}$ is low in quiescent MEFs, strongly induced within a few hours after exposure of adherent MEFs to mitogens (refer to 3 h, monolayer), and then declines to a barely detectable level (refer to 12 h, monolayer) as the cells progress into mid-late G1 phase. However, we also observed that if MEFs are stimulated with mitogens in the absence of substratum, the initial induction of p21$^{cip1}$ is preserved (3 h, suspension) while the subsequent downregulation is incomplete (compare monolayer and suspension cells at 12 h).

Addition of purified growth factors to quiescent MEFs in serum-free medium (Fig. 1 B) also resulted in a strong induction of p21$^{cip1}$ (similar to that seen with serum), and the degree of induction was similar whether cells were cultured on a substratum or in suspension (compare Mn to Sp and Fn+GFs to BSA+GFs). Moreover, p21$^{cip1}$ was not induced when MEFs were cultured on fibronectin-coated dishes in the absence of growth factors (Fn-GFs). These results strongly argue that growth factors, and not adhesion factors in serum, are responsible for the early G1 phase induction of p21$^{cip1}$. Together, the results of Fig. 1, A and B, indicate that G1 phase regulation of p21$^{cip1}$ can be divided into two discrete phases: an initial induction that is mediated by mitogens and a subsequent downregulation that is enhanced by cell adhesion to substratum.

Consistent with studies implicating ERK activation in the induction of p21$^{cip1}$ (see above), we found that the new generation MEK inhibitor, U0126 (Favata et al., 1998) completely blocked the induction of p21$^{cip1}$ when quiescent MEFs were treated with purified growth factors (Fig. 2 A). This effect was associated with the inhibition of ERK activation (determined by direct detection of phospho-ERK and ERK2 gel-shift). Treatment with rapamycin (a potent inhibitor of p70S6 kinase; Price et al., 1992) or LY294002 (a specific PI 3-kinase inhibitor; Vlahos et al., 1994) did not inhibit the activation of ERK or the induction of p21$^{cip1}$ (Fig. 2 A). Thus, activation of the MEK/ERK pathway is necessary for the induction of p21$^{cip1}$ by growth factors. NIH-3T3 transfectants expressing a constitutively active MEK-1 (MEK*) under control of a tetracycline-repressible promoter (tet-MEK*-3T3 cells) were then used to determine if activation of the MEK/ERK pathway would be sufficient to induce p21$^{cip1}$. Suspended MEFs were incubated in the presence and absence of tetracycline and a minimal mitogenic stimulus (0.5% serum, which permitted the synthesis of MEK* in the absence of tetracycline). In the presence of tetracycline, 0.5% serum had a small effect on ERK activation (assessed by direct detection of phospho-ERK) and little effect on the expression of p21$^{cip1}$ (Fig. 2 B; compare G0 to +tet). In the absence of tetracycline, MEK* was induced, resulting in a strong phosphorylation of ERKs and an induction of p21$^{cip1}$ (Fig. 2 B; compare G0 to −tet). Thus, MEK* is sufficient to induce p21$^{cip1}$ in the absence of both cell anchorage and a strong mitogenic stimulus. This result supports other studies showing that expression of constitutively active raf results in the induction of p21$^{cip1}$ (Lloyd et al., 1997; Pumiglia and Decker, 1997; Sewing et al., 1997; Woods et al., 1997; Auer et al., 1998; Kerkhoff and Rapp, 1998).

### *Transient ERK Activity Induces p21$^{cip1}$*

Next we examined the relationship between the duration of an ERK signal and the induction of p21$^{cip1}$. Addition of purified growth factors to MEFs in serum-free culture induced a complete activation of ERKs (determined by gel-shift and direct measurement of phospho-ERK) in both nonadherent and adherent cells (Fig. 3 A, compare BSA with FN at 1 h). However, ERK activity declined in suspended cells while it was sustained in adherent cells (Fig. 3 A, compare BSA with FN at 3 h). p21$^{cip1}$ was induced to the same degree in both the suspended and adherent cells (at 1–3 h in several independent experiments). In several experiments, the analysis of ERK activity by gel-shift, immunoblotting with an anti-phospho-ERK (specific for the dually phosphorylated form) and in vitro kinase assays (not shown) all demonstrated that transient ERK activity can be induced by growth factors in suspended cells while

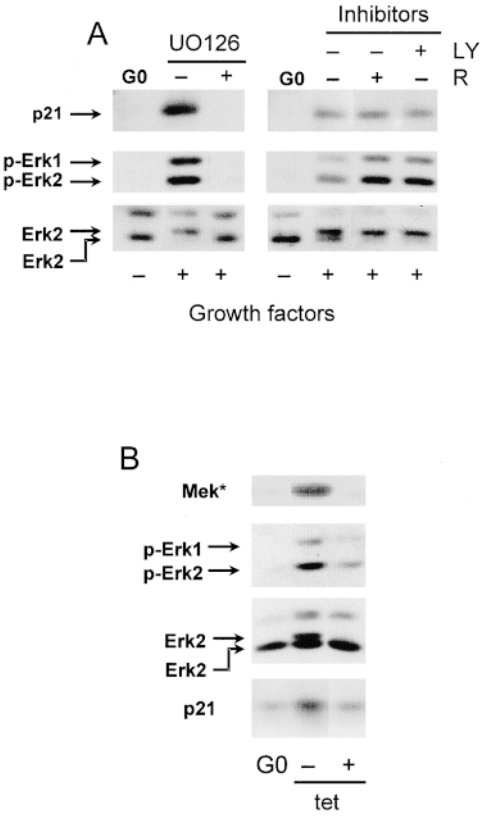


*Figure 2.* Activation of ERKs controls induction of $p21^{cip1}$ by mitogens. In A, G0-synchronized MEFs (G0) were washed and preincubated for 30 min in 10 ml defined medium with 100 μM U0126, 5 nM rapamycin (R), 25 μM LY294002 (LY), or solvent (dimethylsulfoxide) as control (–). Purified growth factors were directly added to the cultures. The cells were incubated for 1 h before collection and lysis. Equal amounts of cell extracts were analyzed by immunoblotting using antibodies against $p21^{cip1}$, phospho-ERK (p-ERK), and ERK. Controls (not shown) demonstrated that the concentrations of LY294002 and rapamycin we used completely inhibited phosphorylation of p70S6 kinase. In B, tet-MEK*-3T3 cells were grown to confluence in 10% FCS-DME with 2 μg/ml tetracycline. The medium was removed, replaced with serum-free DME, and the cultures were serum-starved for 1 d in the absence of tetracycline (G0). Serum-starved cells were trypsinized and reseeded ($2 \times 10^6$ in 10 ml 0.5% FCS) in 100-mm agarose-coated dishes in the absence and presence of tetracycline (tet). Cells were collected after 9 h and lysed. Equal amounts of protein were fractionated on an SDS gel and analyzed by immunoblotting with Mek-1, phospho-ERK (p-ERK), ERK and $p21^{cip1}$ antibodies. For both panels, upper and lower arrows in the ERK blots show the phosphorylated and unphosphorylated ERK2, respectively.

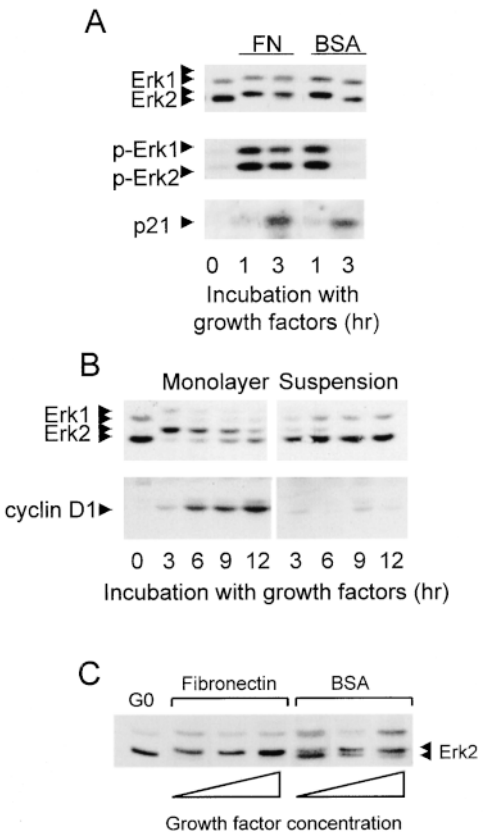


*Figure 3.* Anchorage-independent induction of $p21^{cip1}$ is associated with a short-term activation of ERK. In A, quiescent MEFs were trypsinized, suspended in defined medium, and reseeded on BSA- or fibronectin (FN)-coated dishes for 1 and 3 h in the presence of purified growth factors. Cells were collected, lysed, and analyzed by immunoblotting with anti-ERK (top), anti-phospho-ERK (middle panel), and anti-$p21^{cip1}$. In B, MEFs were rendered quiescent, trypsinized, suspended in 10% FCS-DME, and reseeded in monolayer and suspension for 0–12 h. Cells were collected, lysed, and analyzed by immunoblotting using antibodies to ERK and cyclin D1. In C, quiescent MEFs were trypsinized, suspended in defined medium, and reseeded on BSA- or fibronectin-coated dishes for 1 h in the presence of increasing concentrations of purified growth factors (the triangles represent a dose-response curve of a PDGF-insulin-EGF cocktail used at 25, 50, and 100% of the concentration described in Materials and Methods). For all panels, upper and lower arrowheads in the ERK blots show the phosphorylated and unphosphorylated ERK, respectively.

sustained ERK activity requires both growth factors and cell anchorage. Our interpretation of these results is that a transient ERK activation is sufficient to induce $p21^{cip1}$. These results also indicate that the induction of $p21^{cip1}$ is anchorage-independent because transient ERK activation does not require cell adhesion to substratum (see below). Note that the induction of cyclin D1, which requires a sustained ERK activation (Weber et al., 1997), does not occur when suspended MEFs (Fig. 3 B), NIH-3T3 cells (Zhu et al., 1996), or normal human fibroblasts (Zhu et al., 1996) are stimulated with mitogens.

Interestingly, a mitogen dose response curve showed that when MEFs are stimulated with our standard growth factor cocktail, fibronectin was not necessary for efficient activation of ERK (Fig. 3 C, compare ERK activation on BSA and fibronectin using the maximal concentration of growth factors). However, lower growth factor concentrations that partially activated ERK in suspended cells did activate ERK completely when the cells were attached to fibronectin (Fig. 3 C; compare ERK activation on BSA and fibronectin using the low and intermediate concentra-

tion of growth factors). Fibronectin alone minimally activated ERK under these conditions (data not shown). These results confirm other studies showing that fibronectin and growth factors can synergize to regulate ERK activity (see Discussion), but also show that growth factors alone can fully activate ERK if cells are provided with a sufficiently strong mitogenic stimulus. We emphasize, however, that this strong mitogenic stimulus allows for transient, but not sustained, ERK activation in suspended cells (refer to Fig. 3 A).

To assess directly the duration of the ERK signal that is required for the induction of $p21^{cip1}$, we activated ERK by stimulating quiescent MEFs with purified growth factors in defined medium and then treated the cultures with UO126 at 10, 20, and 40 min before collection at 1 h. Immunoblot analysis showed that (a) growth factors induced a complete activation of ERK by 10 min (Fig. 4 A), (b) this effect persisted for at least 60 min (Fig. 4 A), and (c) UO126 rapidly inhibited growth factor–dependent ERK activation (with ~50 and 90% inhibition in 10 and 20 min, respectively; Fig. 4 B). Importantly, the induction of $p21^{cip1}$ was completely blocked when UO126 was added either 10 or 20 min after stimulation with growth factors, but it was easily detected when UO126 was added after 40 min (Fig. 4 C). Considering that the inhibitory effect of UO126 on ERK activation is complete within 20 min, we conclude that an ERK signal of 40–60 min is sufficient to induce $p21^{cip1}$. The same experimental approach was applied to the analysis of cyclin D1 expression, and the results showed that cyclin D1 was not induced even when UO126 was added 60 min after growth factor stimulation. These data support and extend the results of Fig. 3, directly demonstrating that the ERK signal required for induction of $p21^{cip1}$ is transient relative to that required for the induction of cyclin D1.

### *Full Downregulation of $p21^{cip1}$ in G1 Phase Is Dependent upon Cell Adhesion and Independent of ERKs*

After its initial induction, $p21^{cip1}$ levels are strongly downregulated in mid-late G1 phase, and the completeness of this effect requires cell adhesion to substratum (refer to Fig. 1 A). To identify the basis by which adhesion affects $p21^{cip1}$ downregulation, we compared the stability of $p21^{cip1}$ protein by stimulating adherent and nonadherent MEFs with 10% FCS (to induce $p21^{cip1}$) before the addition of cycloheximide. Immunoblot analysis was performed on lysates of cells collected 0–120 min after addition of cycloheximide. The results showed that the half-life of $p21^{cip1}$ was ~30 min under both culture conditions (Fig. 5). We were unable to find antibodies suitable for confirmation of this result by immunoprecipitation of $p21^{cip1}$ from pulse–chase-labeled cells, but our results with cycloheximide indicate that the turnover of $p21^{cip1}$ is not strongly affected by cell adhesion to substratum.

Consistent with the lack of a detectable effect on $p21^{cip1}$ stability, both mRNA and promoter analyses showed that cell adhesion significantly enhances repression of $p21^{cip1}$ gene expression. Northern blotting showed an initial induction of $p21^{cip1}$ mRNA in both adherent and nonadherent MEFs (Fig. 6 A, 3 h). $p21^{cip1}$ mRNA levels then declined, but the decline was much more pronounced in adherent cells (Fig. 6 A, 9 and 12 h). $p21^{cip1}$ promoter-lucif-

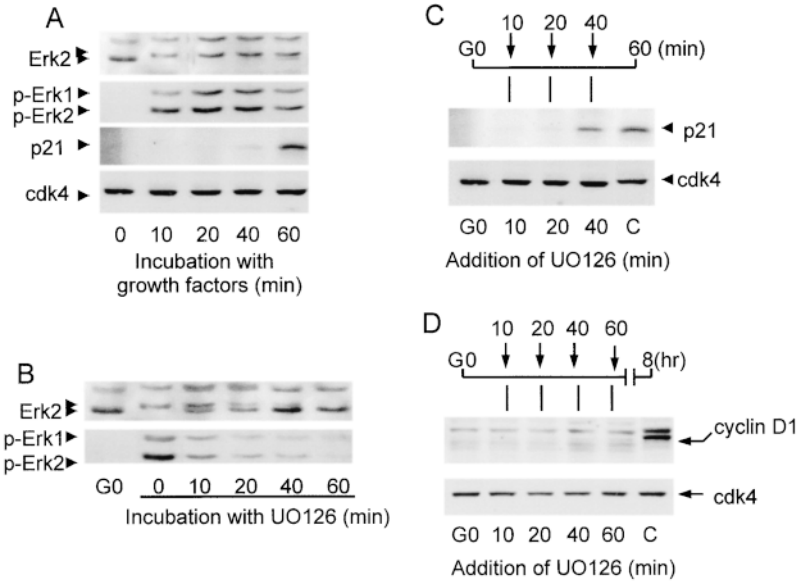


*Figure 4.* A transient ERK signal induces $p21^{cip1}$. MEFs were G0-synchronized in defined medium and stimulated with purified growth factors for the times shown. In A, cells were collected, lysed, and analyzed by immunoblotting with anti-ERK (ERK), anti-phospho-ERK (p-ERK), anti-$p21^{cip1}$, and anti-cdk4 (loading control). In B, the cells were pretreated with purified growth factors for 10 min (time 0) before the addition of U0126 (100 μM final concentration). After incubation with UO126 for 10–60 min, cells were collected, lysed, and the degree of ERK activation was determined by gel-shift and direct assessment of dually phosphorylated ERK by immunoblotting with anti-ERK (ERK) and anti-phospho-ERK (p-ERK), respectively. For A and B, the upper and lower arrowheads in the ERK blots show the phosphorylated and unphosphorylated ERK2, respectively. In C, the G0-synchronized cells were stimulated with purified growth factors, and U0126 was added to the cultures 10, 20, and 40 min after the stimulation with growth factors. All cells were collected at 60 min (a time sufficient for $p21^{cip1}$ induction in cells lacking UO126), lysed, and analyzed for the expression of $p21^{cip1}$ by immunoblotting. MEFs stimulated in the absence of U0126 for 60 min were used as a positive control (C) for the induction of $p21^{cip1}$. In D, G0-synchronized MEFs were stimulated with purified growth factors and U0126 was added to the cells 10, 20, 40, and 60 min after stimulation with growth factors. All cultures were collected at 8 h (a time sufficient for cyclin D1 induction in cells lacking UO126), lysed and analyzed for the expression of cyclin D1 by immunoblotting. MEFs stimulated in the absence of U0126 for 8 h were used as a positive control (C) for the expression of cyclin D1 (detected as the doublet migrating slightly above cdk4). In C and D, immunoblotting with anti-cdk4 was used to control for protein loading.

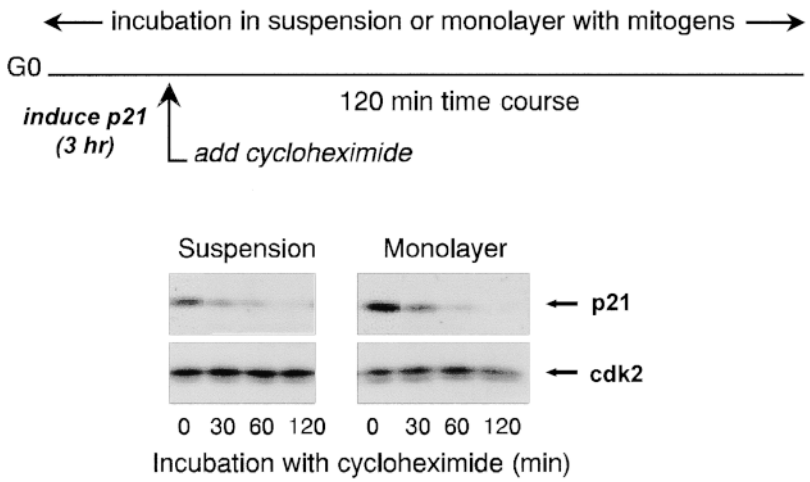


*Figure 5.* Turnover of $p21^{cip1}$ in adherent and nonadherent cells. G0-synchronized MEFs were trypsinized and replated in suspension or monolayer for 3 h with 10% FCS-DME before the addition of cycloheximide (10 μg/ml final concentration). Cells were collected at the indicated times spanning 120 min and lysed. Equal amounts of protein were fractionated on SDS gels and immunoblotted using anti-$p21^{cip1}$ and anti-cdk2 (loading control).

erase assays gave similar results (Fig. 6 B) except that there was essentially no decline in promoter activity when cells were cultured in suspension. This difference in $p21^{cip1}$ promoter activity vs. $p21^{cip1}$ mRNA or protein (refer to Fig. 1 A) levels in suspended MEFs suggests that a constitutive, anchorage-independent turnover of $p21^{cip1}$ mRNA may also play a role in setting the steady state level of $p21^{cip1}$ protein. Nevertheless, the combined results of Figs. 5 and 6 strongly indicate that changes in gene expression play a major role in the adhesion-dependent repression (as well as the growth factor–dependent induction) of $p21^{cip1}$.

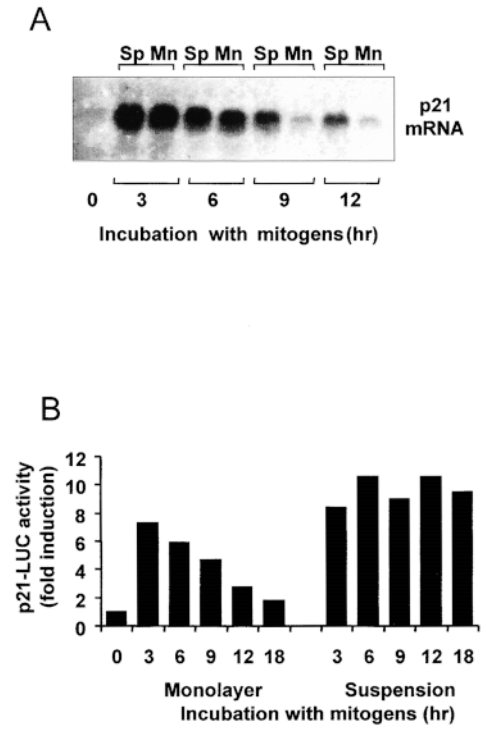


*Figure 6.* Full downregulation of $p21^{cip1}$ gene expression requires cell adhesion. In A, MEFs were rendered quiescent, trypsinized, and then replated in monolayer (Mn) and suspension (Sp) in the presence of 10% FCS-DME. At the times shown, cells were collected and lysed for collection of RNA. Northern blotting was performed using a $^{32}P$-labeled, random-primed probe for murine $p21^{cip1}$ and equal loading of samples was confirmed by ethidium-bromide staining of rRNA (not shown). In B, MEFs were transiently transfected with a $p21^{cip1}$ promoter-luciferase construct as described in Materials and Methods. Transfected cells were G0-synchronized and stimulated with 10% FCS-DME in monolayer and suspension for the times shown. B shows fold-induction of luciferase activity, relative to G0-synchronized cells and normalized to constant activity of the Renilla luciferase control reporter vector.

We examined the potential contribution of p53 in G1 phase regulation of $p21^{cip1}$ gene expression by performing Northern blots with MEFs derived from p53-null mice. Consistent with many studies implicating p53 in the induction of the $p21^{cip1}$ gene, we found that the levels of $p21^{cip1}$ mRNA were generally reduced about three- to fivefold when compared with those observed in wild-type MEFs (not shown). However, the pattern of mitogen-dependent induction and adhesion-enhanced repression was retained in the p53-null MEFs (Fig. 7 A). Although this result does not exclude a potential role for p53 in the ECM-dependent downregulation of $p21^{cip1}$ gene expression, it does show that p53 is not required.

Since the induction of $p21^{cip1}$ requires ERK activity, we considered the possibility that the decay of ERK activity was responsible for downregulating $p21^{cip1}$ in mid-late G1 phase of adherent cells. However, this potential mechanism is not compatible with the fact that $p21^{cip1}$ is poorly downregulated in suspended cells where the ERK signal decays quickly. We then considered the possibility that sustained ERK activity might be phosphorylating a repressor of $p21^{cip1}$ gene expression. To address this potential mechanism, we forced sustained ERK activity in suspended cells and asked if $p21^{cip1}$ expression was repressed, as it would be in monolayer cells. tet-MEK*-3T3 cells were cultured in monolayer and suspension in the presence and absence of tetracycline. We found that downregulation of $p21^{cip1}$ failed to occur in the suspended cells, even when a sustained ERK signal had been enforced (compare the $p21^{cip1}$ and phosho-ERK signals in Fig. 7 B; Sp ± tet). We conclude that the downregulation of $p21^{cip1}$ resulting from cell adhesion to ECM is independent of ERK.

### *Effect of $p21^{cip1}$ on Anchorage-dependent Cyclin E–cdk2 Activity*

Cyclin E–cdk2 activity is not induced when fibroblasts are

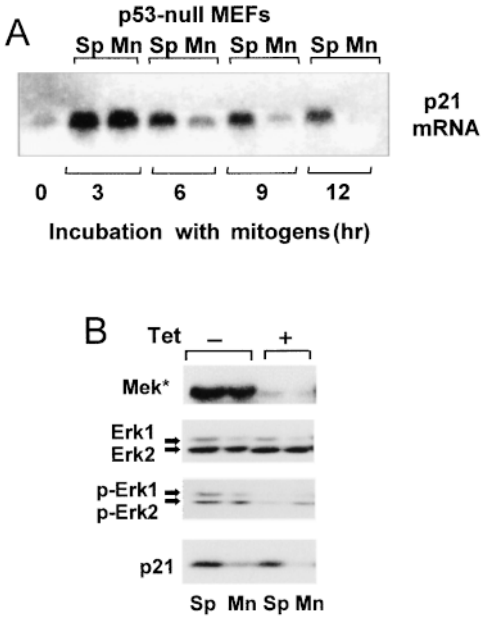


*Figure 7.* Downregulation of $p21^{cip1}$ by ECM does not require p53 and is independent of ERK. In A, serum-starved p53-null MEFs were cultured in monolayer (Mn) and suspension (Sp) and analyzed by Northern blotting as described in the legend to Fig. 6 A. Equal RNA loading was confirmed by ethidium-bromide staining of rRNA (not shown). In B, asynchronous tet-mek*-3T3 cells ($2 \times 10^6$ cells per 100-mm dish) were incubated with 10 ml of 10% FCS-DME in suspension (Sp) and monolayer (Mn) for 24 h in the absence (−) and presence (+) of tetracycline (tet). Cells were collected and lysed for immunoblotting analysis using antibodies to mek-1, ERK, phospho-ERK (p-ERK), and $p21^{cip1}$. In this experiment, lysates were fractionated on a 5–15% gradient SDS gel that does not permit complete resolution of unphosphorylated and phosphorylated ERKs by gel-shift.

treated with mitogens in the absence of a substratum, and the lack of kinase activity correlates with an increased expression of $p21^{cip1}$ and $p27^{kip1}$ (reviewed in Assoian, 1997). To determine if this increased expression of $p21^{cip1}$ is causally related to the inhibition of cyclin E–cdk2 activity, we asked if cyclin E–cdk2 activity would be anchorage independent in suspended cells lacking $p21^{cip1}$.

G0-synchronized MEFs derived from wild-type and $p21^{cip1}$-null mice were mitogen-stimulated in monolayer and suspension before collection and analysis of cyclin E–cdk2 kinase activity in vitro. As expected, the activation of cyclin E–cdk2 was completely blocked when wild-type MEFs were cultured in suspension (Fig. 8). In contrast, cyclin E–cdk2 activity was readily detectable in lysates from suspended $p21^{cip1}$-null cells, at ~50% of the value seen in lysates from the adherent $p21^{cip1}$-null cells. (Because the $p21^{cip1}$-null and wild-type MEFs are independent isolates, comparisons of cyclin E–cdk2 activity in lysates of quiescent, adherent, and suspended cells should only be made within each established line.) Note that other established markers of G1 phase cell cycle progression (induction of cyclin D1, complete downregulation of $p27^{kip1}$, phosphorylation of pRb, and expression of cyclin A) were adhesion dependent in both wild-type and $p21^{cip1}$-null MEFs, consistent with our previous studies (Zhu et al., 1996). The expression of cyclin E and cdk2 was also similar in the wild-type and $p21^{cip1}$-null cells. Thus, the cyclin E–cdk2 kinase activity detected in suspended $p21^{cip1}$-null MEFs can be attributed specifically to the loss of $p21^{cip1}$. In turn, this result indicates that downregulation of $p21^{cip1}$ participates in the activation of cyclin E–cdk2. The incomplete rescue of cyclin E–cdk2 activity in suspended $p21^{cip1}$-null MEFs supports previous studies by us and others (Fang et al., 1996; Schulze et al., 1996; Zhu et al., 1996) which indicate that adhesion-dependent downregulation of $p27^{kip1}$ also contributes to the adhesion dependency of cyclin E–cdk2 activity.

## *Discussion*

Several studies have shown that growth factors and the ECM cooperate to regulate cell cycle progression. Phosphorylation/activation of the ERKs and induction of cyclin D1 are two well-established examples of this cooperation (reviewed in Assoian, 1997; Giancotti, 1997; Howe et al., 1998). These events are causally related because cyclin D1 expression is induced by sustained ERK activity (Weber et al., 1997). Although the exact mechanisms by which growth factors and ECM signals cooperate to regulate ERK activity are still under investigation, it is generally thought that the regulation of G1 phase ERK activity by growth factors and the ECM reflects a convergence of RTK and integrin signals upstream of ERK (Lin et al., 1997; Renshaw et al., 1997). This convergence is important for expression of cyclin D1 (Roovers et al., 1999). In contrast, our data with $p21^{cip1}$ show that a strong mitogenic stimulus is sufficient to induce $p21^{cip1}$ in early G1 phase and that cell anchorage subsequently allows for full repression of $p21^{cip1}$ expression in mid-late G1 phase. These results show that growth factor/ECM cooperation involves parallel as well as convergent signaling.

### *Transient Activation of ERKs Induces $p21^{cip1}$ in G1 Phase*

Our results with pharmacologic inhibitors and conditional expression of constitutively active MEK show that the activation of ERKs plays a major role in the induction of $p21^{cip1}$ by growth factors. This effect probably contributes to the assembly of cyclin D–cdk4/6 complexes (Cheng et al., 1999). Indeed, a $p21^{cip1}$-mediated assembly of cyclin-cdk complexes could explain the results of Cheng et al. (1998), which indicate that activation of the MEK/ERK pathway is sufficient to override the mitogen requirement for assembly of cyclin D–cdk4/6 complexes. In contrast to the induction of cyclin D1, growth factors can induce $p21^{cip1}$ whether or not cells are attached to a substratum. This anchorage independency of $p21^{cip1}$ induction reflects the fact that a transient activation of ERK is sufficient to induce $p21^{cip1}$. In our studies and some others (this report; Zhu and Assoian, 1995; Renshaw et al., 1997; Clark et al., 1998), transient ERK activation occurred in response to growth factors and in the absence of cell adhesion. However, others have also reported that growth factors poorly activate ERK when cells are cultured in the absence of a substratum (e.g., Miyamoto et al., 1996; Lin et al., 1997;

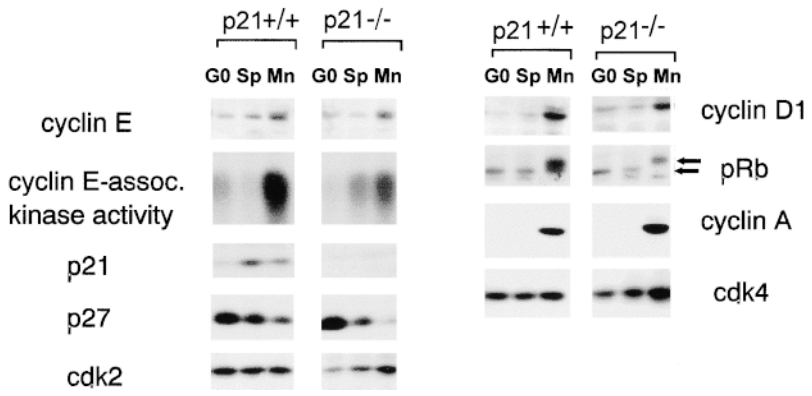


*Figure 8.* $p21^{cip1}$ is important for the anchorage dependency of cyclin E–cdk2 activity. G0-synchronized (G0) MEFs from wild-type (p21+/+) and $p21^{cip1}$-null (p21−/−) mice were trypsinized, reseeded in suspension (Sp) and monolayer (Mn), and incubated in 10% FCS-DME for 18 h. The cells were collected and lysed. Equal amounts of cellular protein (200 μg) were loaded on a reducing SDS gradient gel (5–15% acrylamide) and analyzed by immunoblotting with antibodies to cyclin E, $p21^{cip1}$, $p27^{kip1}$, cdk2, cyclin D1, pRb, cyclin A, and cdk4. The immunoreactive proteins were visualized by ECL. The upper and lower arrows on the pRb blot show the phosphorylated and hypophosphorylated pRb, respectively. Duplicate aliquots of 200 μg from the same experiment were incubated with anti-cyclin E in order to collect cyclin E–cdk2 complexes for determination of cyclin E–cdk2 kinase activity in vitro using histone H1 as substrate as described (Zhu et al., 1996). p21−/− MEFs progressed through G1 phase several hours faster than p21+/+ MEFs (data not shown), and the difference in cyclin E–cdk2 kinase activity detected in monolayer cultures of p21+/+ cells vs. p21−/− cells most likely reflects the fact that the two lines were not in identical positions within G1 phase when assayed 18 h after exposure to mitogens.



Renshaw et al., 1997; Short et al., 1998; Aplin and Juliano, 1999). The different results may reflect how long cells are kept in suspension before growth factor stimulation (Renshaw et al., 1997) or whether the cells have received a sufficiently strong mitogenic stimulus as described in Fig. 3 C.

Others have shown that the induction of $p21^{cip1}$ requires a stronger ERK signal than does the induction of cyclin D1 (Sewing et al., 1997; Woods et al., 1997). In agreement with these studies, our results do show that the persistent ERK signal seen throughout G1 phase and associated with cyclin D1 expression is not as strong as the transient, early G1 ERK signal associated with the induction of $p21^{cip1}$. Some studies also suggest that sustained ERK activity mediates the induction of $p21^{cip1}$ (Pumiglia and Decker, 1997; Auer et al., 1998), while our results show that a transient activation is sufficient. A likely explanation for these different results is that most of the studies by others have relied on overexpression of activated rafs, and the nature of this experimental approach precludes an analysis of effects mediated by transient ERK activity.

### *Adhesion-dependent Downregulation of $p21^{cip1}$ and Its Independence from MAP Kinase*

Our data also suggest that the effect of cell anchorage on mid-late G1 repression of $p21^{cip1}$ gene expression is important for cell cycle progression. We and others have previously reported that $p21^{cip1}$ and $p27^{kip1}$ levels increase when fibroblasts are cultured with mitogens in suspension, and this increase in CKIs is associated with inactivity of the cyclin E–cdk2 complex. Cyclin E–cdk2 is partially anchorage independent in cells lacking $p21^{cip1}$, implying that downregulation of $p21^{cip1}$ in normal cells contributes to the activation of cyclin E–cdk2. The increased expression of $p21^{cip1}$ in suspended cells has typically been interpreted as an induction, but the results shown here indicate that impaired downregulation of $p21^{cip1}$ is the proper explanation.

Brugarolas et al. (1998) have also examined the effect of $p21^{cip1}$ on the anchorage dependency of cyclin E–cdk2 activity. Consistent with our results, they found that cyclin E–cdk2 activity in suspended pRb/$p21^{cip1}$-null MEFs was higher than that in wild-type MEFs. However, we found that cyclin E–cdk2 activity in suspended $p21^{cip1}$-null MEFs was half of that seen in the adherent $p21^{cip1}$-null MEFs while they reported that the cyclin E–cdk2 activity of suspended pRb/$p21^{cip1}$-null cells was only 20% of that observed with adherent pRb/$p21^{cip1}$-null cells. The difference between their results and ours may reflect the fact that our analysis was performed with cells in the first G1 phase while their studies used cells incubated in suspension for three days before analysis. The cell cycle blocks in their system and ours may be of a different nature and not directly comparable. Moreover, we can not exclude the possibility that the pRb-null phenotype of the MEFs used by Brugarolas et al. (1998) may also contribute to the different results.

There is precedent for negative regulation of $p21^{cip1}$ protein by rho (Olson et al., 1998). Negative regulatory motifs have also been mapped to the 3′ untranslated region in the $p21^{cip1}$ mRNA (Rishi et al., 1997). Downregulation of the $p21^{cip1}$ promoter may also be specific to positional cues in the cell cycle because the $p21^{cip1}$ promoter is downregulated in mid-late G1; cells treated with mitogens in suspension fail to reach mid-late G1 (Schwartz et al., 1991; Böhmer et al., 1996). The effect of cell anchorage on these different modes of regulation remains to be elucidated. Importantly, the downregulation of $p21^{cip1}$ observed in response to cell adhesion is independent of ERK activity and can even occur in the presence of a sustained ERK signal. This allows the cell to fully downregulate $p21^{cip1}$ expression in the presence of the sustained ERK signal needed for the induction of cyclin D1.

Several laboratories have shown that high-intensity raf signals induce $p21^{cip1}$ and also result in a $p21^{cip1}$-dependent cell cycle arrest (Lloyd et al., 1997; Pumiglia and Decker, 1997; Sewing et al., 1997; Woods et al., 1997; Auer et al., 1998). As discussed above, the induction of $p21^{cip1}$ by raf is probably the result of ERK activation and is consistent with our studies. The persistently elevated expression of $p21^{cip1}$ characteristic of cell cycle arrest in high intensity raf

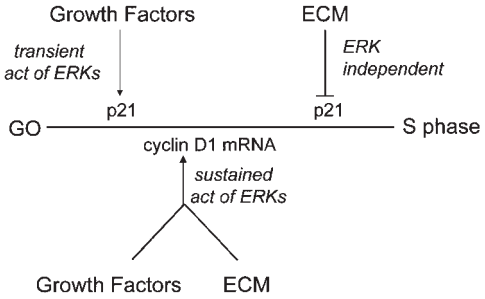


*Figure 9.* Cooperative effects of growth factors and the ECM in G1 phase. The model shows that the induction of $p21^{cip1}$ and cyclin D1 in G1 phase are differentially regulated by growth factors and the ECM largely because $p21^{cip1}$ induction occurs in response to transient ERK activation while cyclin D1 induction requires sustained ERK activation. The figure also shows that the ECM-enhanced downregulation of $p21^{cip1}$ in mid-late G1 phase is independent of ERK activity.

transformants suggests that overexpression of constitutively activated raf can override the normal ERK-independent signaling mechanism(s) that control the mid-late G1 phase downregulation of $p21^{cip1}$ by the extracellular matrix.

### *Integrated Growth Factor and ECM-dependent Signaling and Its Consequence for Cyclin D1, $p21^{cip1}$, and Cell Cycle Progression through G1 Phase*

While others have shown that sustained ERK activation is required for cell cycle progression, our results indicate that transient ERK activation is not without effect. Rather, we propose that transient ERK activity results in the induction of $p21^{cip1}$ while sustained ERK activity, mediated by growth factor/ECM cooperation, and results in the induction of cyclin D1 (Fig. 9). The induction of $p21^{cip1}$ and cyclin D1 are both important for the assembly of cyclin D–cdk4/6 complexes (LaBaer et al., 1997; Cheng et al., 1999). In addition, $p21^{cip1}$ inhibits cyclin E–cdk2; our results indicate that full downregulation of $p21^{cip1}$ gene expression requires cell adhesion to ECM and that this effect contributes to the control of cyclin E–cdk2 activity.

The importance of $p21^{cip1}$ in regulating adhesion-dependent G1 phase progression is highlighted by the study of Brugarolas et al. (1998) which showed that MEFs null for pRb (the only essential substrate for cyclin D1-cdk4/6; Lukas et al., 1995) and $p21^{cip1}$ are anchorage-independent for growth. Nevertheless, several studies do indicate that ECM-dependent regulation of $p27^{kip1}$ also plays a role in regulating cyclin E–cdk2 activity. $p27^{kip1}$ levels are typically regulated posttranscriptionally, and ubiquitin-mediated degradation is thought to play a critical role in this process (see introduction). Thus, the mechanism by which the ECM controls the steady state expression of $p27^{kip1}$ is likely to be very different from that of $p21^{cip1}$ and an interesting matter for investigation.

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