Regulation of p21<sup>cip1</sup> 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<sup>cip1</sup> by soluble mitogens and cell anchorage as well as the relationship between the expression of p21<sup>cip1</sup> and activation of the ERK subfamily of MAP kinases. We find that p21<sup>cip1</sup> 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<sup>cip1</sup> is mediated by transient ERK activity. Comparative studies with wild-type and p21<sup>cip1</sup>-null fibroblasts indicate that adhesion-dependent regulation of p21<sup>cip1</sup> 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<sup>cip1</sup>. 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) (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...
vitro despite the fact that the cells had been exposed to growth factors. Suspended cells express elevated levels of two CKIs, p21Cip1 and p27kip1, and the catalytically inactive cyclin-E-cdk2 complexes isolated from suspended cells show increased association of both p21Cip1 and p27kip1. 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 p21Cip1 and p27kip1. Changes in p21Cip1 expression are often associated with altered transcription of the gene, whereas changes in p27kip1 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 p21Cip1 and p27kip1 levels.

In this study, we have examined the regulation of p21Cip1, p21Cip1 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 p21Cip1 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 p21Cip1 correlates with activation of cyclin E–cdk2. p53 is important for induction of the p21Cip1 promoter (El-Deiry et al., 1993), but p53-independent mechanisms also induce the p21Cip1 gene (Macleod et al., 1995; Zeng et al., 1996). In fact, activation of ERK plays a strong role in the induction of p21Cip1 (Li et al., 1996; Pumiglia and Dicker, 1997; Auer et al., 1998).

We report here that p21Cip1 expression can be divided into two discrete phases: an initial induction that requires mitogens and E R K activity and a subsequent downregulation that is enhanced by cell anchorage and independent of E R K activity. Thus, growth factors and the ECM act in parallel to regulate the expression of p21Cip1 during G1 phase. We also report that, in contrast to the regulation of cyclin D1, a transient activation of E R K is sufficient to induce p21Cip1.

Materials and Methods

Cell Culture

Established mouse embryo fibroblasts (MEFs) from wild-type, p21 null, and p53-null C57BL/6 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 × 10^5 cells per 100-mm dish similarly to the procedure described in Böhm et al. (1996). To study the relative effects of growth factors and fibronectin, trypsinized quiescent cells (2 × 10^5 cells) were suspended in 10 ml defined medium (31 DME: Hams F-12, 15 mM Hepes, pH 7.4, 3 mM histidine, 4 mM glutamine, 8 mM sodium bicarbonate, 10 μM ethanolamine, 10 μM transferrin, 0.1 μM sodium selenite, 0.1 μM MgCl2, 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 mM EG F) that allowed for optimal induction of p21Cip1. 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 is performed as described (Zhao et al., 1999). In experiments with U O 126 (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 E R K activation in suspended cells, we used NIH 3T3 cells stably transfected with a constitutively active M E K-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 p21Cip1 Promoter Activity

MEFs (1.5 × 10^5 in 2 ml D ME, 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 p21Cip1-promoter-luciferase plasmid (0-luc; gift of Wafik El-D-Eiry, 0.1 μg of the renilla luciferase expression plasmid, pRL-SV 40 (Promega), and 5 μl lipofectamine (Life Technologies) in a total volume of 2 ml serum-free DME without antibiotics. A titer 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. A titer trypsinization, an aliquot of the cells was removed for determination of p21Cip1-promoter activity at quiescence; the remainder was resuspended in 10% FCS-DME and then replated in 100-mm dishes (0.5 × 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). p21Cip1 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 (Zhao et al., 1996). Immunoblotting for pRb, cyclins, M E K, and E R Ks was performed as described (Zhao and Asoian, 1995; Zhao 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 Coomasie binding (BioRad Protein assay), and equal amounts (100 μg for the analysis of cyclins, cdks, and CKIs, and 25 μg for the analysis of M E K and E R K) were fractionated on SD S gels. Cells destined for immunoblot analysis with anti-E R K or anti-phospho-E R K antibodies were extracted as described (Zhao et al., 1999).

Rabbit polyclonal antisera against cyclin A was prepared in this laboratory, and the antisera to p21Cip1 was the generous gift of Claudio Schneider (A R E A Science Park, Trieste, Italy). All other antisera were purchased: anti-cyclin E (sc-481) and anti-cdk4 (sc-280) from Santa Cruz Biotecnology; anti-cyclin D1 (0-163) and anti-cdk2 (0-505) from Upstate Biotechnology; anti-M E K-1 (M 17020) and anti-p21Cip1 (K 25020) and anti-E R K (M 12320) from Transduction Laboratories, anti-pRb (10609) from Ciba-Corning; and anti-phospho-E R K (91015) from New England Biolabs.

Results

G1 Phase Induction of p21Cip1 Requires Growth Factors, Does Not Require Cell Adhesion, and Is Dependent upon the Activation of ERKs

Our initial studies compared the temporal expression pat-
Bottazzi et al. Regulation of p21cip1 expression

Transient ERK Activity Induces p21cip1

Next we examined the relationship between the duration of an ERK signal and the induction of p21cip1. A duration 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), p21cip1 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
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 p21cip1. These results also indicate that the induction of p21cip1 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 p21cip1, 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 p21cip1 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 p21cip1. 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 p21cip1 is transient relative to that required for the induction of cyclin D1.

**Full Downregulation of p21cip1 in G1 Phase Is Dependent upon Cell Adhesion and Independent of ERKs**

Afer its initial induction, p21cip1 levels are strongly downregulated in mid-late G1 phase, and the completeness of this effect requires cell adhesion to substrate (refer to Fig. 1 A). To identify the basis by which adhesion affects p21cip1 downregulation, we compared the stability of p21cip1 protein by stimulating adherent and nonadherent MEFs with 10% FCS (to induce p21cip1) 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 p21cip1 was ~30 min under both culture conditions (Fig. 5). We were unable to find antibodies suitable for confirmation of this result by immunoprecipitation of p21cip1 from pulse-chase-labeled cells, but our results with cycloheximide indicate that the turnover of p21cip1 is not strongly affected by cell adhesion to substrate.

Consistent with the lack of a detectable effect on p21cip1 stability, both mRNA and promoter analyses showed that cell adhesion significantly enhances repression of p21cip1 gene expression. Northern blotting showed an initial induction of p21cip1 mRNA in both adherent and nonadherent MEFs (Fig. 6 A, 3 h), p21cip1 mRNA levels then declined, but the decline was much more pronounced in adherent cells (Fig. 6 A, 9 and 12 h). p21cip1 promoter-lucif-
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 p21cip1 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 p21cip1 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 p21cip1.

We examined the potential contribution of p53 in G1 phase regulation of p21cip1 gene expression by performing Northern blots with MEFs derived from p53-null mice. Consistent with many studies implicating p53 in the induction of the p21cip1 gene, we found that the levels of p21cip1 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 p21cip1 gene expression, it does show that p53 is not required.

Since the induction of p21cip1 requires ERK activity, we considered the possibility that the decay of ERK activity was responsible for downregulating p21cip1 in mid-late G1 phase of adherent cells. However, this potential mechanism is not compatible with the fact that p21cip1 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 p21cip1 gene expression. To address this potential mechanism, we forced sustained ERK activity in suspended cells and asked if p21cip1 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 p21cip1 failed to occur in the suspended cells, even when a sustained ERK signal had been enforced (compare the p21cip1 and phospho-ERK signals in Fig. 7 B; Sp ± tetr). We conclude that the downregulation of p21cip1 resulting from cell adhesion to ECM is independent of ERK.

**Effect of p21cip1 on Anchorage-dependent Cyclin E–cdk2 Activity**

Cyclin E–cdk2 activity is not induced when fibroblasts are
treated with mitogens in the absence of a substratum, and the lack of kinase activity correlates with an increased expression of p21<sup>cip1</sup> and p27<sup>kip1</sup> (reviewed in Assoian, 1997). To determine if this increased expression of p21<sup>cip1</sup> 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<sup>cip1</sup>. G0-synchronized MEFs derived from wild-type and p21<sup>cip1</sup>-null mice were mitogen-stimulated in monolayer and suspension before collection and analysis of cyclin E–cdk2 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<sup>cip1</sup>-null cells, at ~50% of the value seen in lysates from the adherent p21<sup>cip1</sup>-null cells. (Because the p21<sup>cip1</sup>-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<sup>kip1</sup>, phosphorylation of pRb, and expression of cyclin A) were adhesion dependent in both wild-type and p21<sup>cip1</sup>-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<sup>cip1</sup>-null cells. Thus, the cyclin E–cdk2 kinase activity detected in suspended p21<sup>cip1</sup>-null MEFs can be attributed specifically to the loss of p21<sup>cip1</sup>. In turn, this result indicates that downregulation of p21<sup>cip1</sup> participates in the activation of cyclin E–cdk2. The incomplete rescue of cyclin E–cdk2 activity in suspended p21<sup>cip1</sup>-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<sup>kip1</sup> 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 A ssioan, 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 (Rovers et al., 1999). In contrast, our data with p21<sup>cip1</sup> show that a strong mitogenic stimulus is sufficient to induce p21<sup>cip1</sup> in early G1 phase and that cell anchorage subsequently allows for full repression of p21<sup>cip1</sup> 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<sup>cip1</sup> 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<sup>cip1</sup> by growth factors. This effect probably contributes to the assembly of cyclin D–cdk4/6 complexes (Cheng et al., 1999). Indeed, a p21<sup>cip1</sup>-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<sup>cip1</sup> whether or not cells are attached to a substratum. This anchorage independency of p21<sup>cip1</sup> induction reflects the fact that a transient activation of ERK is sufficient to induce p21<sup>cip1</sup>. In our studies and some others (this report; Zhu and A ssioan, 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;
Independence from MAP Kinase

Our data also suggest that the effect of cell anchorage on mid-late G1 repression of p21cip1 gene expression is important for cell cycle progression. We and others have previously reported that p21cip1 and p27kip1 levels increase when fibroblasts are cultured with mitogens in suspension, and this increase in CKI levels is associated with inactivation of the cyclin E–cdk2 complex. Cyclin E–cdk2 is partially anchorage independent in cells lacking p21cip1, implying that downregulation of p21cip1 in normal cells contributes to the activation of cyclin E–cdk2. The increased expression of p21cip1 in suspended cells has typically been interpreted as an induction, but the results shown here indicate that impaired downregulation of p21cip1 is the proper explanation.

Brugarolas et al. (1998) have also examined the effect of p21cip1 on the anchorage dependency of cyclin E–cdk2 activity. Consistent with our results, they found that cyclin E–cdk2 activity in suspended pRb/p21cip1-null MEFs was higher than that in wild-type MEFs. However, we found that cyclin E–cdk2 activity in suspended p21cip1-null MEFs was half of that seen in the adherent p21cip1-null MEFs while they reported that the cyclin E–cdk2 activity of suspended pRb/p21cip1-null cells was only 20% of that observed with adherent pRb/p21cip1-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 p21cip1 protein by rho (Olson et al., 1998). Negative regulatory motifs have also been mapped to the 3′ untranslated region in the p21cip1 mRNA (Rishi et al., 1997). Downregulation of the p21cip1 promoter may also be specific to positional cues in the cell cycle because the p21cip1 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 p21cip1 observed in response to cell adhesion is independent of E RK activity and can even occur in the presence of a sustained E RK signal. This allows the cell to fully downregulate p21cip1 expression in the presence of the sustained E RK signal needed for the induction of cyclin D1.

Several laboratories have shown that high-intensity raf signals induce p21cip1 and also result in a p21cip1-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 p21cip1 by raf is probably the result of E RK activation and is consistent with our studies. The persistently elevated expression of p21cip1 characteristic of cell cycle arrest in high intensity raf
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 p21cip1 by the extracellular matrix.

**Integrated Growth Factor and ECM-dependent Signaling and Its Consequence for Cyclin D1, p21cip1, 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 p21cip1 while sustained ERK activity, mediated by growth factor/ECM cooperation, and results in the induction of cyclin D1 (Fig. 9). The induction of p21cip1 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, p21cip1 inhibits cyclin E–cdk2; our results indicate that full downregulation of p21cip1 gene expression requires cell adhesion to ECM and that this effect contributes to the control of cyclin E–cdk2 activity.

The importance of p21cip1 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 p21cip1 are anchorage-independent for growth. Nevertheless, several studies do indicate that ECM-dependent regulation of p21cip1 also plays a role in regulating cyclin E–cdk2 activity. p21cip1 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 p21cip1 is likely to be very different from that of p21cip1 and an interesting matter for investigation.

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**References**


