Ras- and Mitogen-activated Protein Kinase Kinase Kinase-dependent and -independent Pathways in p21\textsuperscript{Cip1/Waf1} Induction by Fibroblast Growth Factor-2, Platelet-derived Growth Factor, and Transforming Growth Factor-\(\beta\)\textsuperscript{1}

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Abstract
p21\textsuperscript{Cip1/Waf1} (hereafter referred to as p21) is up-regulated in differentiating and DNA-damaged cells, but it is also up-regulated by serum and growth factors. We show here that fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and transforming growth factor-\(\beta\) (TGF-\(\beta\)) all induce p21 expression in mouse fibroblasts, but with markedly different kinetics. We link their effect on p21 to Ras and mitogen-activated protein kinase kinase-1/2 (MEK1/2)-regulated pathways using either a specific MEK1/2 inhibitor (PD 098059) or cells expressing conditionally activated Ras or dominant negative Ras. We demonstrate that p21 induction by PDGF and TGF-\(\beta\) requires MEK1/2 and, additionally, that the TGF-\(\beta\) effect on p21 depends on Ras, whereas the PDGF effect does not. In contrast, FGF-2 regulation of p21 is largely independent of MEK and Ras. However, PD 098059 efficiently inhibited S-phase entry of quiescent cells induced by either FGF-2 or PDGF, suggesting separate signaling pathways for FGF-2 in induction of p21 and in S-phase entry. The results suggest different but partly overlapping signaling pathways in growth factor regulation of p21.

Introduction
Growth factor receptor binding activates multiple intracellular pathways; the appropriate integration of these pathways in proliferation, differentiation, and growth arrest are crucial for the function of a cell. The final outcome of growth factor signaling is the result of complicated interplay between activated pathways and feedback loops, and it is also dependent on the amplitude and duration of the signals as well as type of cell involved. Ras protein is an important mediator of growth factor signals (reviewed in Ref. 1). Its activation to GTP-bound form is induced by various extracellular stimuli including EGF, PDGF, FGF, and NGF via activated tyrosine kinase receptors as well as by TGF-\(\beta\) and interleukins (reviewed in Ref. 2). Ras generates diverse cellular outcomes. Its essential role in eukaryotic cell growth has emerged from studies in which neutralizing Ras-antibodies or microinjection of dominant negative Ras into mouse fibroblasts prevent the S-phase entry of the cells by serum stimulation (3, 4). Furthermore, proliferation and transformation of mouse fibroblasts by tyrosine kinase oncogenes such as src, fms, and fes are blocked by dominant negative Ras (4). In mouse pheochromocytoma PC-12 cells, activated Ras induces differentiation in the absence of extracellular differentiation signals, whereas dominant negative Ras is sufficient to prevent differentiation of these cells by NGF or FGF (reviewed in Ref. 5). In addition to proliferation and differentiation signals, high amounts of Ras lead to senescence in primary cells via induction of growth-inhibitory proteins like p53, p16\textsuperscript{Ink4a}, and p21\textsuperscript{Cip1/Waf1} (hereafter referred to as p21; Ref. 6).

Ras activates a multitude of downstream pathways. The best characterized of these is Raf and MAPK (also called ERK) pathway. Like Ras, MAPK activation is essential for fibroblast proliferation (7), and most growth factors activating Ras also phosphorylate and activate MAPKs (reviewed in Ref. 8). In many cell types, activation of Ras leads to constitutive activation of MAPKs (9), whereas dominant negative Ras blocks MAPK phosphorylation by various extracellular signals such as NGF, FGF, EGF, and 12-O-tetradecanoylphorbol-13-acetate (10–12); PDGF (13, 14); and TGF-\(\beta\) (15). However, cell type differences in Ras regulation of MAPK phosphorylation, at least by 12-O-tetradecanoylphorbol-13-acetate and EGF, have been observed (13, 14). Activation of MEK leads to growth factor-independent proliferation and transformation of NIH 3T3 cells, whereas dominant negative MEK mutant restores normal growth of Ras-transformed cells (9). Furthermore, similar to dominant negative Ras, dominant negative MEK prevents neuronal differentiation of PC-12 cells, whereas constitutively active MEK induces differentiation of these cells in the absence of growth factor stimulation (9). Although these results imply a close link

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\textsuperscript{3} The abbreviations used are: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; TGF-\(\beta\), transforming growth factor-\(\beta\); MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase; cdk, cyclin-dependent kinase; IPTG, isopropyl-\(\beta\)-thiogalactoactoside; BrdUrd, 5-bromo-2'-deoxyuridine; NBCS, newborn calf serum; GAPDH, glyceraldehyde phosphate dehydrogenase.
between Ras and MAPK pathway in cell proliferation and differentiation, additional pathways activated by Ras exist. MAPK-independent pathways, including guanine-nucleotide exchange factors Rho, its homologue Rac1, and Ral, have been characterized in Ras-mediated cellular transformation (16, 17). In addition, other candidates for Ras effector functions, including phosphatidyl inositol-3-OH kinase (18), GAP proteins, RalGDS, AF6, and Rin1 (reviewed in Ref. 19), have been described. These results suggest several Ras-induced pathways in different cellular responses. Furthermore, evidence of Ras-independent activation of MAPK pathway by protein kinase C exists (20, 21).

p21 cyclin kinase inhibitor is up-regulated in response to growth arresting and differentiation signals (22, 23), and it mediates cell cycle arrest by inhibiting cdk activity (24). Besides inhibiting the cell cycle machinery, p21 also functions as an assembly factor for cdk/cyclin complexes (25, 26). Thus, p21 action may be required for coordinated cell cycle progression. Indeed, p21 cyclin kinase inhibitor is induced by serum (27) and growth factors, including NGF (28, 29), EGF (30), TGF-β (31), PDGF, and FGF (32–34), as well as by the Ras (6, 35), Raf (36, 37), and MAPK (38) pathways. Rho, on the other hand, negatively controls Ras-mediated p21 induction (39). Evoking p21 action by both positive and negative growth-regulatory signals may, thus, serve to control the cell cycle in various cellular contexts. In addition, the duration and magnitude of p21 induction may determine the cellular response. Here, we have addressed the involvement of Ras and MAPK pathways in p21 induction by FGF-2, PDGF, and TGF-β1, with the use of conditional expression of activated or dominant negative Ras and MEK1/2 inhibitor PD 098059.

## Results

### Effects of TGF-β1, FGF-2, and PDGF on p21 Expression.

We and others have recently demonstrated that Ras, Raf, and MEK (6, 35–38) induce p21 by both transcriptional and posttranscriptional means. In addition, PDGF, FGF, EGF, and TGF-β induce p21 expression in mouse fibroblasts and HaCaT cells (31, 32). Because several of the growth factors are known to use Ras-mediated intracellular pathways for signaling, we wanted to evaluate the involvement of Ras and MEK/MAPK pathway in the growth factor action leading to p21 induction. NIH 3T3 cells were treated with increasing concentrations of FGF-2, PDGF, and TGF-β1 in low serum and incubated for 12 h. As shown by Western blot analysis, p21 protein was induced by all tested growth factors in a dose-dependent manner (3.0-fold induction by 7 ng/ml FGF-2, 5.5-fold by 10 ng/ml PDGF, and 4.2-fold by 250 pM TGF-β1; Fig. 1). Analysis of the same gels by anti-phospho-ERK1/2 antibodies showed also dose-dependent induction of active, phosphorylated ERK1 and ERK2 by FGF-2, PDGF, and TGF-β1 (Fig. 1). To test whether a p21-related inhibitor, p27Kip1 (40), is affected by the growth factors, we reprobed filters used for p21-probing with anti-p27Kip1 antibodies. However, the levels of p27Kip1 were unaffected by the growth factors (Fig. 1). Even sample loading was confirmed by reprobing the filters with an anti-ERK2 antibody, which showed invariant levels of ERK2 (see Figs. 1, 2, 4, and 5).

### Kinetics of p21 Induction and ERK1/2 Phosphorylation by Growth Factors.

To analyze the kinetics of p21 induction, we treated starved NIH 3T3 cells with FGF-2, PDGF, and TGF-β1 for various times in low serum. The induction of p21 was most rapid by FGF-2, being detectable as early as 30 min after growth factor stimulation and peaking at 2 h (1.6- and 4.8-fold inductions; Fig. 2A). The PDGF effect on p21 was also rapid, with the induction peaking at 2 h (2.5-fold induction; Fig. 2B). Comparison of the p21 levels showed that, after 2 h, the levels of FGF-2- and PDGF-induced p21 gradually started to decline (Fig. 2, A and B). In contrast to the effect by FGF-2 and PDGF, the TGF-β1-induction of p21 was significantly slower reaching maximal levels only 12 h after growth factor addition (2.1-fold; Fig. 2C). Longer starvation of the cells was observed to decrease.
the basal p21 levels (data not shown), thereby affecting the magnitude of p21 induction by the growth factors. Therefore, because the control cells were starved for 16 h in Fig. 1 as compared to 4 h in Fig. 2, the decrease in basal p21 level yielded an apparently greater p21 induction by, e.g., PDGF in Fig. 18 versus Fig. 2B (5.5-fold by 12 h as compared with 2.5-fold by 2 h). Induction of ERK1/2 phosphorylation by FGF-2 and PDGF was maximal 30 min after growth factor treatment, followed by a decrease 2 h after FGF-2 and 6 h after PDGF addition (Fig. 2, A and B). Similar to the induction of p21, the induction of ERK1/2 phosphorylation by TGF-β1 was slower than that by FGF-2 or PDGF peaking at 12 h (Fig. 2C). Again, there was no variation in p27Kip1 or total ERK2 levels.

Requirement of MEK1/(2)-dependent Pathways in Growth Factor Regulation of p21. The involvement of MAPK pathway in p21 induction by FGF-2, PDGF, and TGF-β1 was studied by pretreating the cells with MEK1/(2) inhibitor PD 098059 (80 μM) for 4 h in low serum followed by addition of growth factors and analysis of p21 levels and ERK1/2 phosphorylation by Western blotting at different timepoints. PD 098059 was found to inhibit phosphorylation of ERK1/2 by FGF-2 by 58% 0.5 h after the growth factor addition, with prominent inhibition at later timepoints (72 and 59% inhibition at 2 and 6 h, respectively; Fig. 2A). However, the induction of p21 by FGF-2 was unaffected, although the level of phospho-ERK1/2 was decreased (Fig. 2A). Similar results were obtained in cells incubated with PD 098059 and increasing concentrations of FGF-2 for 12 h (data not shown). This further confirmed that FGF-2 at concentrations 3–7 ng/ml increased p21 independently of MEK1/(2). PD 098059, which efficiently inhibited PDGF-stimulated ERK1/2 phosphorylation (64 and 85% inhibition by 30 min and 2 h, respectively; Fig. 2B), also inhibited fully the PDGF induction of p21 (Fig. 2B). TGF-β1 induction of phospho-ERK1/2 was clearly reduced by the MEK1/(2) inhibitor at all timepoints, and at the same time, TGF-β1 induction of p21 after 12 h treatment was abolished (Fig. 2C). The results suggest that active MEK1/(2) is required for induction of p21 by PDGF, and TGF-β1, whereas the induction by FGF-2 seems to largely use MEK-independent pathways. Unexpectedly, simultaneous treatment of the cells with PD 098059 and TGF-β1 for 2 h repeatedly induced p21 by 2-fold (Fig. 2C), whereas at the same timepoint, PD 098059 alone had no effect (data not shown). This amplified p21 induction by PD 098059 in cells treated for 2 h with TGF-β1 may imply MEK1/(2)-dependent negative regulation of p21 in response to TGF-β1. Reprobing of the filters used for detection of p21 and phospho-ERK1/2 showed no significant change in the levels of either p27Kip1 or total ERK2 (Fig. 2).

To investigate whether the growth factor effects are exerted via regulation of p21 mRNA, we cultured NIH 3T3 cells in low serum in the presence of TGF-β1, FGF-2, and PDGF or without PD 098059 followed by Northern blotting. Incubation of the cells for 1 h with FGF-2 or PDGF induced p21 mRNA by 2.8- and 2.5-fold, respectively, whereas TGF-β1 had no effect (Fig. 3A). PD 098059 reduced the p21 mRNA induction by FGF-2 and PDGF by 50 and 30%, respectively. In contrast, p21 mRNA induction was absent in cells incubated with the growth factors for 16 h (Fig. 3B), suggesting a transient effect by the growth factors. The results indicate that the increase in p21 protein by FGF-2 or PDGF involves an increase in the amount of p21 mRNA, whereas TGF-β1 may act by enhancing translation and/or protein stabilization. However, as shown in Fig. 2A, down-regulation of FGF-2-induced p21 mRNA by PD 098059 does not lead to a corresponding decrease in p21 protein level. This would suggest that FGF-2 may increase p21 also by means other than affecting its mRNA expression. Furthermore, although no evidence exists of major MEK1/(2)-dependent p21 regulation by FGF-2 at the protein level, p21 mRNA induction by FGF-2 seems to be partially under MEK1/(2) control.

Effects of Oncogenic Ras on p21 Induction by FGF-2, PDGF, and TGF-β1. To study the possible modulation of p21 induction by Ras, we used NIH 3T3 cells stably transfected with lactose analogue (IPTG) inducible Ha-Ras(Val-12) expression vector (ras8 cells). Ras induction is detectable within 6 h after IPTG treatment, being maximal by 16 h (35).
Due to basal expression of Ha-Ras(Val-12) in uninduced cells, the levels of p21 and phospho-ERK1/2 were constitutively higher in ras8 cells as compared with parental NIH 3T3 cells (data not shown). However, induction of Ras expression (7-fold induction by IPTG; Fig. 4A), accompanies induction of ERK1/2 phosphorylation (mean induction of ERK1/2 phosphorylation, 1.9-fold; Fig. 4) and p21 expression (mean induction of p21, 3.2-fold; in Fig. 4). The induction of Ras expression was analyzed in each experiment and was found to be similar in all growth factor-treated cells (data not shown): Ras expression in FGF-2-treated cells is shown (Fig. 4A). All growth factors stimulated phosphorylation of ERK1/2 in the Ras-expressing cells (Fig. 4), and a synergistic effect on p21 expression was observed by FGF-2 and PDGF (Fig. 4A, A and B). The effect was detected 2 h after FGF-2 and PDGF addition, being maximal at 6 h, indicating prolonged induction of p21 expression and ERK1/2 phosphorylation in Ras-expressing cells as compared with NIH 3T3 cells (Fig. 4, A and B). In FGF-2- treated Ras-expressing cells, phosphorylation of ERK1/2 was sustained for 12 h (7.9-fold increase; Fig. 4A), whereas in PDGF-treated cells, phospho-ERK1/2 levels decreased to the basal levels by 12 h (Fig. 4B). In FGF-2- and PDGF-treated Ras-expressing cells, however, the synergistic effect on p21 disappeared by 12 h (Fig. 4, A and B). In contrast to the kinetics seen in NIH 3T3 cells, TGF-β1 treatment of Ras-expressing cells increased phosphorylation of ERK1/2 2 h after growth factor addition (Fig. 4C). Similarly, p21 was induced by TGF-β1 with somewhat faster kinetics starting at 6 h in Ras-expressing cells as compared with NIH 3T3 cells (Fig. 4C). However, p21 was not further increased by TGF-β1 in Ras-expressing cells at any timepoint (Fig. 4C). The specificity of p21- and phospho-ERK1/2 effects in Ras-expressing cells were confirmed by analyzing the levels of p27Kip1 (data not shown) and ERK2 (Fig. 4), which were unaffected by Ha-Ras or the growth factors at all timepoints. We, therefore, suggest that, because FGF-2 and PDGF synergistically affect p21 levels in Ras-expressing cells, they may activate also other, Ras- independent negative Ras(Asn-17) mutant. Induction of Ras(Asn-17) expression after a 30-h treatment with IPTG was prominent (mean induction, 5.5-fold; Fig. 5), and was not affected by the growth factors. Expression of Ras(Asn-17) was able to abolish the induction of p21 by TGF-β1, with concomitant inhibition of ERK1/2 phosphorylation by 47% (Fig. 5). Induction of p21 by FGF-2 was slightly attenuated (33%) whereas phospho-ERK1/2 was reduced by 66%. However, there was no effect of Ras(Asn-17) expression on p21 induction by PDGF, although phosphorylation of ERK1/2 was greatly reduced (75%; Fig. 5). The levels of p27Kip1 (data not shown)

Fig. 3. FGF-2 and PDGF induce p21 mRNA levels. A and B, Northern blotting analyses of p21 mRNA levels. A, NIH 3T3 cells were pretreated in low serum with or without PD 098059 (80 μM) for 4 h followed by incubation with FGF-2 (5 ng/ml), PDGF (10 ng/ml), or TGF-β1 (250 pm) for 1 h. B, NIH 3T3 cells were cultured in low serum in the absence (Lanes ) or presence (Lanes ) of 50 μM PD 098059 and TGF-β1 (250 pm). FGF-2 (7 ng/ml), or PDGF (10 ng/ml) for 16 h. Poly(A)+ mRNAs were prepared, and Northern blotting analyses were performed using 32P-labeled mouse p21 and GAPDH CDNAs as probes. The signals were quantitated by PhosphoImager analyzer and normalized against GAPDH. Fold induction of p21 mRNA as compared with nontreated controls are shown below.

Fig. 4. Effect of Ha-Ras expression on p21 regulation by growth factors. ras8 cells were cultured with or without IPTG (10 mM) for 8–12 h to induce Ras expression. The cells were transferred to low serum for 4 h, followed by culturing the cells for indicated times with FGF-2 (5 ng/ml); A, PDGF (10 ng/ml); B, or TGF-β1 (250 pm); C in the presence or absence of IPTG. Immunoblotting was performed with anti-p21, anti-phospho-ERK1/2, anti-ERK2, and anti-Ras antibodies as indicated. Fold inductions of p21 as compared with non-IPTG-treated cells grown in low serum for 6 h are shown below.
DNA (Fig. 6), and a 12-h incubation with TGF-
and ERK2 (Fig. 5) analyzed from the same filters remained unaltered by Ras(Asn-17) expression or the growth factor treatments. These results implicate that Ras function is required for p21 induction by TGF-β1, whereas PDGF, and, in large part, also FGF-2 activate pathways involving Ras.

**MEK1(2) Inhibitor Prevents S-Phase Entry Induced by FGF-2 and PDGF.** To correlate the p21 regulation to the cell cycle effects of TGF-β1, FGF-2, and PDGF in NIH 3T3 cells, we analyzed DNA replication of the cells and cell cycle distribution by BrdUrd incorporation and flow cytometry of cells cultured in low serum with or without growth factors and PD 098059. In low serum, only 1% of the cells were replicating DNA (Fig. 6), and a 12-h incubation with TGF-β1 (250 pm) was unable to induce S-phase entry of the cells (1.6% replication; Fig. 6). In contrast, treatment with FGF-2 or PDGF increased the amount of S-phase cells to 23 and 18%, respectively (Fig. 6). Similar results were obtained by flow cytometry (Table 1). Simultaneous treatment of the cells with PD 098059 prevented the S-phase entry of the cells by FGF-2 and PDGF as shown by BrdUrd incorporation (89 and 72% inhibition of DNA replication, respectively), with similar results obtained by fluorescence-activated cell sorting analysis (64 and 62% inhibition, respectively; Table 1). The results, therefore, indicate that inhibition of MEK1(2)-activated pathway(s) significantly decreases the mitogenic effects of both FGF-2 and PDGF, regardless of differences in the level of p21 or pathways required for its induction.

**Discussion**

We show here that FGF-2, PDGF, and TGF-β1 induce p21 expression in NIH 3T3 cells, which is consistent with results obtained by using other cell types (31, 32, 34, 41). Our results indicate, however, that pathways leading to p21 induction by these growth factors vary, as suggested by their requirement of Ras and MEK1(2) and by their different kinetics. In NIH 3T3 cells, by using a MEK1(2) inhibitor and conditional expression of Ha-Ras or dominant negative Ras(Asn-17), p21 induction by FGF-2 was shown to be mainly MEK1(2) independent, whereas PDGF-regulation of p21 was MEK1(2) dependent and, like FGF-2-regulation, independent of Ras (Fig. 7). In contrast, TGF-β1 regulation of p21 expression was found to require both MEK1(2) and Ras (Fig. 7). However, based on these results, we cannot exclude that also FGF-2 and PDGF could use intact Ras and MEK signaling pathways for p21 induction. In addition, in differing cellular contexts, the signaling pathways used by the growth factors may vary. Reprobing of all filters used for detection of p21 with anti-p27kip1 antibodies demonstrated that p27kip1 expression was unaffected by the growth factors, MEK1(2) inhibitor, Ha-Ras, or dominant negative Ras as well as their combination and confirmed the specificity of the observed p21 effect. FGF-2, at the highest doses used, was found to function largely in a manner independent of Ras and MEK1(2) and to rapidly induce p21 in NIH 3T3 cells (Fig. 7). However, we cannot exclude that residual MEK1(2) activity stimulated by FGF-2 exists, which leads to partial p21 induction. Still, p21 induction by FGF-2 was not down-regulated by PD 098059, although the MEK1(2) inhibitor inhibited ERK1/2 phosphorylation and reversed S-phase entry of the cells. Similarly, dominant negative Ras decreased p21 induction by FGF-2 somewhat, but not significantly. Thus, we cannot fully exclude the utilization of MEK/Ras pathway by FGF-2 in p21 regulation, but based on the studies here, neither MEK nor Ras seems to be exclusively required. Previous studies show that FGF activates both Ras- and MAPK-dependent as well as -independent pathways in execution of its various cellular actions, suggesting that diverse activated pathways specify different outcomes in the cell. Indicating the complexity of the FGF signaling, activation of prolactin promoter by FGF is a Ras-independent but MAPK-dependent event (42), whereas transcriptional activation of cyclic AMP-responsive element-binding protein by FGF requires Ras but not MAPK (43). In primary endothelial cells FGF has been shown to induce p21 transiently in a MEK1(2)-dependent manner fol-
loops along the MAPK pathway are known to exist, e.g., PD 098059 enhances Raf activity by various extracellular stimuli (46), which could lead to additional p21 induction. TGF-β1 was found to elicit its effects on p21 through both Ras and MEK1/2 (Fig. 7), whereas requirement of ERK1/2 by TGF-β1 is not conclusive from these experiments. The TGF-β effects on MAPK pathway appear to be cell type specific. In rat hepatic stellate cells TGF-β induces a rapid activation of Ras, Raf, MEK1, and ERK1/2 (47), whereas in Swiss 3T3 cells, there is no ERK activation by TGF-β (48). In epithelial cells sensitive to growth inhibition by TGF-β, TGF-β activates Ras (49) and ERK1 (50) as well as induces p21 and p27 cyclin kinase inhibitors (41). The TGF-β effects on ERK1, p21 and p27 are abolished by dominant negative Ras expressed in these cells (15, 41). The results on epithelial cells support our data on Ras dependency of p21 induction by TGF-β, whereas the MEK1/2 dependency by TGF-β1 appears to be a novel finding. Unlike its behavior in epithelial cells, TGF-β did not regulate p27 levels in NIH 3T3 cells (Figs. 1C and 2C).

p21 mRNA levels were increased shortly after FGF-2 and PDGF treatments in NIH 3T3 cells, similarly to other studies (32, 34). TGF-β1 was unable to induce p21 mRNA in NIH 3T3 cells, although in HaCaT keratinocytes, it induces p21 at least partially through transcriptional activation (31). Cell type differences may explain these conflicting results. However, several studies support posttranscriptional regulation of p21 by growth factors and serum (27, 34, 35, 51).

Whereas p21 regulation by FGF-2 and PDGF is accompanied by S-phase entry of quiescent NIH 3T3 cells, the late p21 induction by TGF-β1 appears not to be coupled with cell cycle progression, because TGF-β1 is unable to induce S-phase entry of quiescent NIH 3T3 cells. Several studies have demonstrated transient p21 induction by either FGF-2 or PDGF at both protein and mRNA levels, which is accompanied by cell cycle entry of quiescent cells (27, 32, 34), whereas a sustained p21 induction takes place in growth-arrested cells (32–34). For example, in breast cancer cells growth arrested by FGF, a sustained p21 induction is detected, which is lacking in a breast cancer cell line resistant to growth inhibition (33). Thus, S-phase entry is probably feasible due to the transient nature of p21 expression by FGF-2 and PDGF found also here. Inhibition of MEK action prevented the S-phase entry by both PDGF and FGF-2, although the MEK-inhibition was unable to down-regulate FGF-2-induced p21. This suggests separate pathways for FGF-2 in regulation of cell cycle progression and p21 induction. It is possible that the transient induction of p21 by

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**Table 1  Cell cycle distribution of growth factor-treated NIH 3T3 cells in the presence and absence of PD 098059**

<table>
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*Starved NIH 3T3 cells were treated with TGF-β1 (250 pM), FGF-2 (7 ng/ml), or PDGF (10 ng/ml) in the presence or absence of 50 μM PD 098059 for 12 h followed by flow cytometry.
growth-stimulating factors serves as a regulatory step in cell cycle progression, e.g., by modulating the assembly of cdk4/ 
cyclin D complexes (25, 26), and that S-phase entry occurs 
when the amount of active cdk/cyclin complexes exceeds 
the amount of their inhibitors, such as p21.

Materials and Methods

Cells and Cell Culture. Mouse NIH 3T3 fibroblasts (ATCC CRL 1658, 
Rockville, MD) were maintained in DMEM containing 10% NBCS (Life 
Technologies, Inc., Grand Island, NY). Conditionally Ha-Ras(Val-12) ex-
pressing NIH 3T3 cells have been described previously (52). For condi-
tional expression of dominant negative Ras, Ras(Asn-17) kindly provided 
by Dr. L. A. Feig (Tufts University, Boston, MA) was cloned into pSVlacO-
vector [pSVlacO-Ras(Asn-17)], and NIH 3T3 cells were transfected by 
calcium phosphate precipitation with equal amount of pHPJINL.sneo and 
pSVlacO-Ras(Asn-17) plasmids followed by selection with 0.6 mg/ml 
G418 and ringcloning of single-cell colonies. All NIH 3T3 transfectants 
were maintained in DMEM containing 10% NBCS supplemented with 0.4 
mg/ml G418. Ha-Ras(Val-12) and Ras(Asn-17) expression was induced by 
addition of 10 mM lactose analogue IPTG (Promega, Madison, WI) to 
the culture medium.

Immunoblotting Analyses. Preparation of cell lysates for immuno-
blotting analyses has been described previously (53). Bio-Rad DC 
protein assay was used to determine protein concentration of the lysates, and 150 
µg of protein were electrophoretically resolved in 12.5% SDS-PAGE 
and transferred to Immobilon P membranes (Millipore, Bedford, MA). Mouse 
p21 was detected with anti-p21 antibody (13436E; PharMingen), p27 with 
anti-p27(18) antibody (K25020; Transduction Laboratories, Lexington, 
KY), ERK2 with anti-ERK2 antibody (E16220; Transduction Laboratories), 
and Ras with anti-Ras antibody (Abx1; Oncogene Science, Cambridge, 
MA). For detection of phosphorylated ERK1 and ERK2, phosphospecific 
and Ras with anti-Ras antibody (Abx1; Oncogene Science, Cambridge, 
MA). For detection of phosphorylated ERK1 and ERK2, phosphospecific 
anti-MAPK antibody (V667A; Promega) were used. Bound immunoglobu-
lins were detected with horseradish peroxidase-conjugated antirabbit 
(for p27, ERK2, and Ras) and antirabbit IgG (for p21 in Figs. 2, 6, and 7; 
Dako, Glostrup, Denmark), or with biotinylated antirabbit IgG (for phos-
pho-ERK1/2 and for p21 in Fig. 1; Dako) followed by streptavidin- 
biotinylated antirabbit IgG (for p21, ERK2, and Ras) and antirabbit antibody (Abx1; Oncogene Science, Cambridge, 
MA). For detection of phosphorylated ERK1 and ERK2, phosphospecific 
anti-MAPK antibody (V667A; Promega) were used. Bound immunoglobu-
lins were detected with horseradish peroxidase-conjugated antirabbit 
(for p27, ERK2, and Ras) and antirabbit IgG (for p21 in Figs. 2, 6, and 7; 
Dako, Glostrup, Denmark), or with biotinylated antirabbit IgG (for phos-
pho-ERK1/2 and for p21 in Fig. 1; Dako) followed by streptavidin- 
conjugated horseradish peroxidase (Amersham, Buckinghamshire, United 
Kingdom). Final detection step was performed by chemiluminescence 
(ECL; Amersham). The quantitation of the signals was carried out using 
Bio-Rad MultiAnalyser Version 1.0.

mRNA Isolation and Hybridization. Poly(A)+ mRNAs were prepared, 
and Northern blotting was performed as described previously (52). 
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50. Gorospe, M., Wang, X., and Holbrook, N. J. p53-dependent elevation of p21Waf1 expression by UV light is mediated through mRNA stabili-

