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Raf-Induced Proliferation or Cell Cycle Arrest Is Determined by the Level of Raf Activity with Arrest Mediated by $p21^{Cip1}$

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The Raf family of protein kinases display differences in their abilities to promote the entry of quiescent NIH 3T3 cells into the S phase of the cell cycle. Although conditional activation of ΔA-Raf:ER promoted cell cycle progression, activation of ΔRaf-1:ER and ΔB-Raf:ER elicited a G_1 arrest that was not overcome by exogenously added growth factors. Activation of all three ΔRaf:ER kinases led to elevated expression of cyclin D1 and cyclin E and reduced expression of $p27^{Kip1}$. However, activation of ΔB-Raf:ER and ΔRaf-1:ER induced the expression of $p21^{Cip1}$, whereas activation of ΔA-Raf:ER did not. A catalytically potentiated form of ΔA-Raf:ER, generated by point mutation, strongly induced $p21^{Cip1}$ expression and elicited cell cycle arrest similarly to ΔB-Raf:ER and ΔRaf-1:ER. These data suggested that the strength and duration of signaling by Raf kinases might influence the biological outcome of activation of this pathway. By titration of ΔB-Raf:ER activity we demonstrated that low levels of Raf activity led to activation of cyclin D1-cdk4 and cyclin E-cdk2 complexes and to cell cycle progression whereas higher Raf activity elicited cell cycle arrest correlating with $p21^{Cip1}$ induction and inhibition of cyclin-cdk activity. Using green fluorescent protein-tagged forms of ΔRaf-1:ER in primary mouse embryo fibroblasts (MEFs) we demonstrated that $p21^{Cip1}$ was induced by Raf in a p53-independent manner, leading to cell cycle arrest. By contrast, activation of Raf in $p21^{Cip1-/-}$ MEFs led to a robust mitogenic response that was similar to that observed in response to platelet-derived growth factor. These data indicate that, depending on the level of kinase activity, Raf can elicit either cell cycle progression or cell cycle arrest in mouse fibroblasts. The ability of Raf to elicit cell cycle arrest is strongly associated with its ability to induce the expression of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ in a manner that bears analogy to α-factor arrest in *Saccharomyces cerevisiae*. These data are consistent with a role for Raf kinases in both proliferation and differentiation of mammalian cells.

Biochemical and genetic strategies have implied that the Ras-activated extracellular ligand-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway is a key regulator of cell proliferation and differentiation in metazoan organisms (3, 4, 17–19, 21, 32, 46, 66–68). The binding of a variety of ligands to their cognate cell surface receptors elicits the activation of members of the Ras family of GTPases. Activation of Ras leads to the sequential activation of Raf, MEK, and p42 and p44 MAP-ERK kinases (16, 27, 35, 55, 103–105). Nuclear translocation of MAP kinases leads to the phosphorylation of transcription factors, such as Elk-1 and Ets-2, which regulate the expression of immediate-early genes, such as the c-Fos and HB-EGF genes, respectively (33, 34, 39, 53, 57, 58, 102). The loss of function of components of this pathway has severe developmental consequences for the organism (28, 50, 68, 77). Furthermore, activated forms of Ras are found frequently in human tumors, and activated forms of Ras, Raf, and MEK are oncogenic in a variety of cell types in vitro (28, 50, 68, 77).

Humans and mice possess three members of the Raf family of protein kinases (Raf-1, A-Raf, and B-Raf) that have the same structural organization. The amino-terminal region contains conserved regions CR1 and CR2, and these regions regulate the Raf kinase domain, which is contained in CR3 (66, 82, 100). Deletion of the amino terminus of Raf results in forms of the protein that are constitutively activated and which can elicit oncogenic transformation of NIH 3T3 cells and terminal differentiation of PC12 cells (97, 107).

We have previously described the utility of conditionally active forms of mammalian Raf kinases in which the kinase domain of either A-Raf, B-Raf, or Raf-1 has been fused to the hormone binding domain of the human estrogen receptor (ΔRaf:ER). Addition of estradiol or its analogs to NIH 3T3 cells expressing ΔRaf-1:ER leads to rapid activation of MEK and MAP kinase, phosphorylation of Ets-2, and transcriptional activation of a set of immediate-early target genes, leading ultimately to oncogenic transformation (57, 58, 78, 86, 87).

In general the rapidity and extent of the biological effects elicited by the activation of ΔRaf:ER proteins reflects their relative abilities to activate the MAP kinase pathway in vitro and in the intact cell. In this regard ΔB-Raf:ER is more active than ΔRaf-1:ER, which in turn is more active than ΔA-Raf:ER (7, 58, 78). An important exception to this general rule is the abilities of the different Rafs to promote the entry of quiescent NIH 3T3 cells into the S phase of the cell cycle. Activation of ΔA-Raf:ER efficiently promoted cell cycle progression in NIH 3T3 cells. By contrast, activation of either ΔRaf-1:ER or ΔB-Raf:ER failed to induce proliferation, inducing instead a G_1 arrest that was not overcome by the subsequent addition of growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor, and epidermal growth factor (78, 86).

In this paper we explore the differences among the Raf kinases that mediate their effects on cell proliferation. Consistent with a positive effect on cell proliferation, all of the active forms of ΔRaf:ER induced cyclin D1 and cyclin E expression and caused decreased expression of the cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$. However, all of the forms of

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ΔRaf:ER that elicited cell cycle arrest strongly induced the expression of the CKI p21^{Cip1}, with concomitant inhibition of the activity of cyclin E-cdk2 complexes. The mitogenic form of ΔA-Raf:ER induced cyclin D1 expression but failed to induce p21^{Cip1} significantly, with the consequence that cyclin-cdk complexes were active in these cells. Experiments performed with primary mouse embryo fibroblasts (MEFs) lacking p21^{Cip1} strongly support a role for this protein in Raf-induced cell cycle arrest. We further demonstrate that all three mammalian Raf kinases are capable of inducing both cell cycle progression and cell cycle arrest and that the appropriate biological outcome depends on the strength of signaling through the Raf kinase pathway. Low levels of Raf kinase activity elicited a proliferative response, whereas high levels of Raf kinase activity induced cell cycle arrest. These data indicate that in mouse cells, Raf-activated pathways are capable of eliciting distinctly different biological outcomes depending on the strength of the signal.

MATERIALS AND METHODS

Construction of retrovirus expression vectors. All of the work described here was conducted with NIH 3T3 cells transduced with the appropriate pBabepuro retrovirus stocks. Retrovirus vectors expressing ΔRaf:ER proteins have been described elsewhere (7, 65). ΔB-Raf:ER* consists of the protein kinase domain of mouse B-Raf described previously and a mutant form of the hormone binding domain of the mouse estrogen receptor that has been engineered to be nonresponsive to β-estradiol but retains responsiveness to 4-hydroxy-tamoxifen (4-HT) and the ICI series of estrogen receptor antagonists (13, 48, 78). Green fluorescent protein (GFP)-tagged forms of ΔRaf-1:ER were derived by ligating sequences encoding an enhanced form of GFP (EGFP; Clontech Labs, Palo Alto, Calif.) containing two point mutations (F64L and S65T) to either the [YY] or the [DD] form of ΔRaf-1:ER (7, 11, 76). The resulting constructs (GFPΔRaf-1:ER) encode chimeric proteins consisting of EGFP at the amino terminus, the [YY] or [DD] form of the catalytic domain of Raf-1 in the middle, and the hormone binding domain of the human estrogen receptor at the carboxy terminus. GFPΔRaf-1:ER coding sequences were subcloned into pBabepuro as described above. Precise details of these constructs are available on request.

Cell culture, retrovirus production, and infection. All cells were cultured in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a humidified atmosphere containing 6% (vol/vol) CO_2. Cells were photographed with a Nikon TMS photomicroscope as described previously (87). Primary mouse embryo fibroblasts were very kindly provided by J. Brugarolas and T. Jacks (MIT Cancer Center). 4-HT (Research Biochemicals) was prepared as a 1 mM stock in ethanol, stored at −20°C, and used at the appropriate concentrations. Retrovirus stocks were obtained by Lipofectamine (Gibco-BRL)-mediated transfection of the appropriate vectors into Bosc23 cells, as described previously (70). Target cells were infected and cultured in medium containing 2 to 10 μg of puromycin (Sigma) per ml to select for virus-infected cells. Standard virus stocks gave rise to $\geq 10^6$ puromycin-resistant colonies per ml of virus. Following selection in puromycin, cells were pooled, expanded, and tested for the expression of ΔRaf:ER and GFPΔRaf-1:ER proteins by Western blotting and, where appropriate, by FACScan at 490 nm. Under these conditions ≥95% of the pooled puromycin-resistant NIH 3T3 cells expressed the ΔRaf:ER and GFPΔRaf-1:ER fusion proteins. 3T3ΔB-Raf:ER* cells are a clonal population of ΔB-Raf:ER*-expressing NIH 3T3 cells that was isolated by ring cloning.

Confluent monolayers of NIH 3T3 cells were rendered quiescent by culture in deluxe serum-free Dulbecco's modified Eagle's medium containing penicillin, streptomycin, gentamicin, 4 μM $MnCl_2$, 10 μM ethanolamine, 500 mg of bovine serum albumin (BSA)-linoleic acid complex (Collaborative Research), 2 μM hydrocortisone (Sigma), 3 mM L-histidine, and 10 ml of insulin-transferrin-selenium medium supplement (ITS-X; Gibco-BRL) per liter for 24 to 48 h, as described previously (84). NIH 3T3 cells containing an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible H-Ras(V12) gene were a gift of H. Itoh and Y. Kaziro, Tokyo Institute of Technology (58).

Preparation of cell extracts and analysis by Western blotting. Triton X-100-soluble cell lysates were prepared as described previously (87). Cells were lysed in gold lysis buffer (GLB), and protein concentrations were measured with the bicinchononic acid (BCA) protein assay kit (Pierce). Aliquots of cell lysates were electrophoresed through polyacrylamide gels and Western blotted onto Immobilon P polyvinylidene difluoride membranes (Millipore). Western blots were probed with the appropriate dilutions of primary antibodies for at least 1 h at room temperature. Anti-estrogen receptor (α-hbER) and anti-cyclin E were from Santa Cruz Biotechnology, and anti-p21^{Cip1} and anti-p27^{Kip1} were from Pharmingen and Transduction Labs, respectively. Polyclonal antisera were raised against the carboxy-terminal 14 or 16 amino acids of human cyclin D1, cdk2, and cdk4 coupled to keyhole limpet hemocyanin (Pocono Rabbit Farms). Antisera were affinity purified and coupled to protein A-Sepharose 4B, as described previously (29). These antisera readily cross-react with the cognate mouse proteins (69a). The PSTAIRE antiserum was a kind gift of M. Yamashita (Hokkaido University). Antigen-antibody complexes were visualized with the enhanced chemiluminescence detection system (Amersham).

Assays for cdk2-associated kinase activity. Quiescent NIH 3T3 cells expressing the different ΔRaf:ER proteins were stimulated as described above, and cell extracts were prepared by lysis either in GLB or in a buffer (TLB) containing 50 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM dithiothreitol, and 0.1% (vol/vol) Tween 20 containing protease and phosphatase inhibitors, as described previously for GLB (87). Cell extracts were subjected to two freeze-thaw cycles on dry ice and centrifuged at 12,000 × *g* to remove insoluble material. Protein concentration was measured by a Bradford assay (Bio-Rad). Aliquots (400 μg) of cell extract were precleared with normal rabbit serum and protein A-Sepharose 4B prior to being subjected to immunoprecipitation with an antiserum raised against the carboxy terminus of cdk2, which was covalently coupled to protein A-Sepharose 4B for 1 h at 4°C as described previously (29). Immune complexes were washed four times with lysis buffer and once with cdk reaction buffer containing 50 mM HEPES (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate. The kinase reaction mixtures were incubated in 30 μl of the same buffer containing 10 μCi of [γ-^{32}P]ATP, 50 μM ATP, and 2 μg of histone H1 (Boehringer Mannheim) as substrates for 10 to 15 min at 30°C. The reaction mixtures were denatured in sodium dodecyl sulfate sample buffer and analyzed by polyacrylamide gel electrophoresis and Western blotting onto a polyvinylidene difluoride membrane as described previously (87). Kinase activity was quantitated with a Molecular Dynamics Storm PhosphorImager. The presence of particular proteins in the immunoprecipitates was confirmed by probing a parallel immunoprecipitation-Western blot with the appropriate antiserum and secondary antibody, as described above.

Assays for cdk4-associated kinase activity. cdk4-associated kinase activity was measured by lysis of cells in TLB, as described above. After preclearing, 400-μg aliquots were subjected to immunoprecipitation with a protein A–Sepharose 4B-coupled antiserum raised against the carboxy terminus of human cdk4 in the absence or presence of an excess (10 mg/ml) of competing immunizing peptide. Immunoprecipitates of cdk4 were washed extensively and then incubated in 30 μl of cdk reaction buffer, as described above, containing 2 μg of bacterially expressed GST-Rb(CT) as a substrate (59). Kinase reactions were quantitated as described above. The presence of particular proteins in the immunoprecipitates was confirmed by probing a parallel immunoprecipitation-Western blot with the appropriate antiserum and secondary antibody, as described above.

DNA synthesis assays. DNA synthesis in quiescent NIH 3T3 cells plated in 96-well trays was measured at different times, following the addition of 4-HT or growth factors, by the addition of methyl-[^{3}H]thymidine to a final concentration of 1.5 μCi/ml for 24 to 48 h, as described previously (78, 86). Incorporation of methyl-[^{3}H]thymidine into DNA was measured with a Skatron Micro 96 cell harvester and a Betaplate 1205 liquid scintillation counter. Each measurement was performed at least in quadruplicate, and the averages of the values along with the standard deviations were determined. When appropriate, human PDGF (PDGF-BB; Upstate Biotechnology Inc.) was used at 10 ng/ml. The kinetics of induced DNA synthesis in response to the various stimuli was assessed as described above, except that the cells were plated on Cytostar-T 96-well scintillating microplates and labeled with [^{14}C]thymidine according to the manufacturer's instructions (Amersham Life Science). Incorporation of [^{14}C]thymidine was estimated with a Packard top-count scintillation machine.

DNA synthesis was also assessed by the incorporation of bromodeoxyuridine (BrdU) into cellular DNA. Briefly, cells were made quiescent by culture in deluxe serum-free Dulbecco's modified Eagle's medium for 36 to 48 h, at which time they were stimulated as described above. BrdU was added to the cells to a final concentration of 50 μM, and the incubation continued for a further 12 to 24 h. Paraformaldehyde-fixed cells were stained with an anti-BrdU antibody (Becton Dickinson) in the presence of 4 mg of dialyzed DNase I per ml. Following staining, the cells were washed and analyzed with a Becton Dickinson FACScan (101).

RESULTS

Effects of ΔRaf:ER activation on cell cycle progression in NIH 3T3 cells. We have previously demonstrated a reciprocal relationship between the ability of conditionally active forms of the mammalian Raf kinases (ΔRaf:ER) to activate MEK and MAP kinase and their ability to promote the entry of quiescent NIH 3T3 cells into the S phase of the cell cycle (78, 86). The protein kinase activities of Raf-1 and A-Raf are strongly influenced by two key tyrosine residues upstream of the ATP binding site (Y340 and Y341 in Raf-1 and Y299 and Y300 in A-Raf) (7, 23, 54). For ΔRaf-1, mutation of these residues to aspartic acid increased kinase activity approximately 10-fold to the level observed with ΔB-Raf, whereas mutation to phenyl-

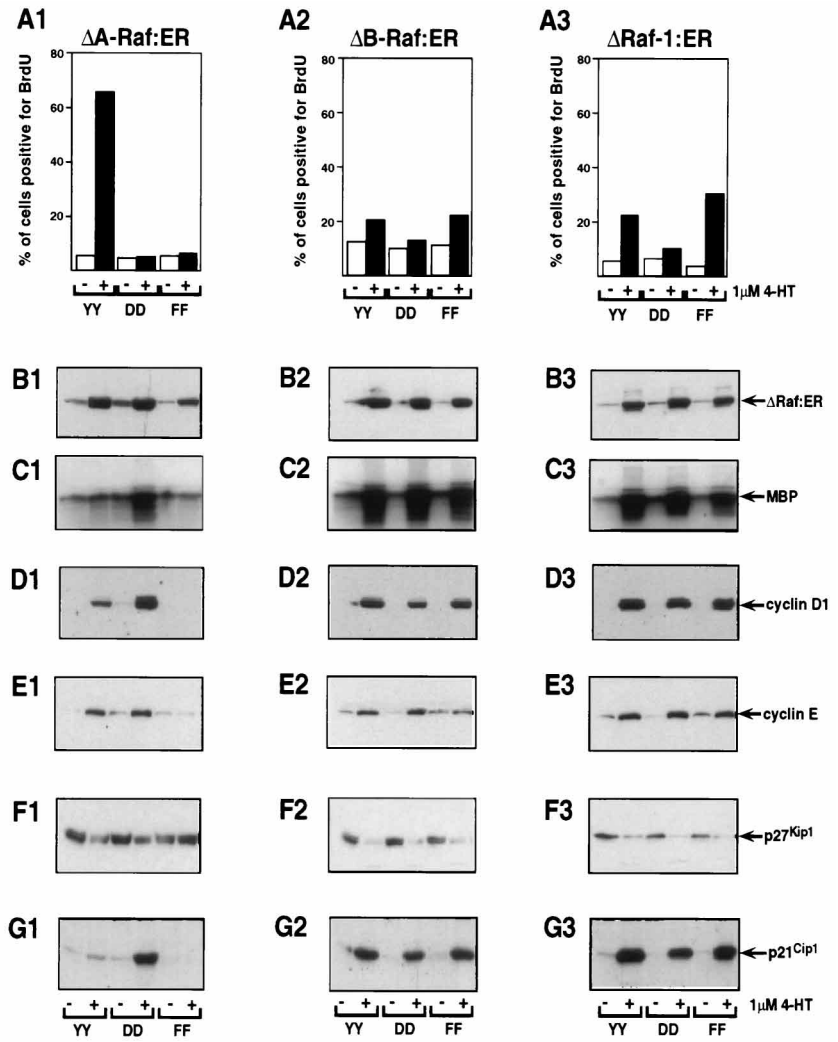


FIG. 1. Effects of Raf protein kinases on cell cycle progression and the expression of components of the cell cycle machinery. Quiescent populations of NIH 3T3 cells expressing the [YY], [DD], or [FF] forms of ΔA-Raf:ER (A1), ΔB-Raf:ER (A2), and ΔRaf-1:ER (A3) were either untreated or treated with 1 μM 4-HT for 48 h. DNA synthesis was assessed by the incorporation of BrdU and detected by immunofluorescence with a FACScan, as described in Materials and Methods. The results are expressed as the percentage of the total cell population that was labeled with BrdU either in the absence (−) or in the presence (+) of 1 μM 4-HT. Quiescent populations of NIH 3T3 cells expressing the [YY], [DD], or [FF] forms of ΔA-Raf:ER (B1 to G1), ΔB-Raf:ER (B2 to G2), and ΔRaf-1:ER (B3 to G3) were either untreated (−) or treated (+) with 1 μM 4-HT for 48 h, at which time cell extracts were prepared. The levels of expression of the ΔRaf:ER proteins (B1 to B3), cyclin D1 (D1 to D3), cyclin E (E1 to E3), $p27^{Kip1}$ (F1 to F3), and $p21^{Cip1}$ (G1 to G3) were determined by Western blotting with appropriate antisera. In addition the activity of p42 MAP kinase in the same extracts was determined by an immunoprecipitation kinase assay with myelin basic protein (MBP) as a substrate (C1 to C3).

alanine had no effect on kinase activity. Mutation of the analogous tyrosine residues to aspartic acid in ΔA-Raf increased kinase activity to the level observed with $\Delta Raf\text{-}1_{[YY]}$. Mutation to phenylalanine, however, gave rise to a form of ΔA-Raf than is both kinase inactive and nontransforming in NIH 3T3 and Rat1 cells. By contrast, the native sequence of ΔB-Raf already encodes aspartic acid residues in the analogous positions. Mutation of these residues to either tyrosine or phenylalanine had little or no effect on protein kinase activity (7).

We wished, therefore, to determine what the effects of these various mutations in ΔA-Raf:ER, ΔB-Raf:ER, and ΔRaf-1:ER would have on the ability of the different Raf protein kinases to promote the entry of quiescent NIH 3T3 cells into the S phase of the cell cycle. Pooled populations of NIH 3T3 cells expressing either the [YY], [DD], or [FF] forms of ΔRaf-1:ER, ΔA-Raf:ER, and ΔB-Raf:ER and consisting of $\geq 10^6$ individual infection events were derived as described in Materials and Methods (7, 78, 87). The extent of the conditionality of the ΔRaf:ER system makes analysis of cell proliferation in pooled populations possible (7, 87).

Quiescent cells expressing the different ΔRaf:ER proteins were treated with 1 μM 4-HT for 48 h, a concentration of hormone that is sufficient to fully activate each of the ΔRaf:ER fusion proteins. DNA synthesis was measured by the incorporation of either BrdU (Fig. 1A1 to A3) or [^{3}H]thymidine (data not shown). In the absence of any stimulus, approximately 5 to 12% of the cells in each of the pooled populations incorporated BrdU. As described previously (78), activation of $\Delta A\text{-}Raf_{[YY]}$:ER was strongly mitogenic in NIH 3T3 cells, leading to approximately 65% of the cells incorporating BrdU over the time course of stimulation (Fig. 1A1). In a parallel [^{3}H]thymidine-labeling experiment we observed a 12-fold stimulation of [^{3}H]thymidine incorporation (data not shown). In this experiment the mitogenic response to $\Delta A\text{-}Raf_{[YY]}$:ER activation was

equivalent to that observed in response to 10 ng of exogenously added PDGF per ml (data not shown) (78). The catalytically inactive, nontransforming ΔA-$Raf_{[FF]}$:ER had no effect on either BrdU or [^{3}H]thymidine incorporation (Fig. 1A1). These cells displayed levels of BrdU or [^{3}H]thymidine incorporation similar to those displayed by either unstimulated NIH 3T3 cells or cells expressing a catalytically inactive form of ΔRaf-1:ER (ΔRaf301:ER) (data not shown). Consistent with previous observations, the catalytically inactive ΔRaf:ER proteins had no effect on the cells' response to exogenously added PDGF and therefore do not display a dominant-negative effect in NIH 3T3 cells (40). Cells expressing catalytically inactive forms of ΔRaf:ER proteins therefore constitute ideal controls for any nonspecific effects of either ΔRaf:ER expression or the addition of 4-HT.

In contrast to ΔA-$Raf_{[YY]}$:ER, the catalytically activated ΔA-$Raf_{[DD]}$:ER failed to stimulate the entry of quiescent cells into the S phase and showed no more activity in this regard than the catalytically inactive ΔA-$Raf_{[FF]}$:ER or ΔRaf301:ER proteins (Fig. 1A1 and data not shown). Activation of ΔA-$Raf_{[DD]}$:ER elicited a G_1 arrest such that the cells were refractory to stimulation with PDGF in a manner similar to that described previously for ΔRaf-1:ER and ΔB-Raf:ER (data not shown) (78, 86). These data suggested that the ability of ΔRaf:ER proteins to promote or inhibit cell cycle progression is not necessarily an intrinsic property of each Raf isoform but may be a consequence of the level of catalytic activity.

Compared to NIH 3T3 cells expressing ΔA-$Raf_{[YY]}$:ER and to PDGF-stimulated cells, cells expressing the different forms of ΔB-Raf:ER and ΔRaf-1:ER displayed a markedly reduced mitogenic response to Raf activation, as measured by either BrdU (Fig. 1A2 and A3) or [^{3}H]thymidine incorporation (data not shown). Of the different forms of ΔRaf-1:ER the strongest mitogenic responses were observed with the forms of the kinase ([YY] and [FF]) that have the lowest intrinsic kinase activity (Fig. 1A3) (7). Extensive control experiments demonstrated that the addition of 4-HT, at 0.1 nM to 1 μM, to NIH 3T3 cells had no effect on NIH 3T3 cell proliferation or on the ability of cells to respond to exogenously added mitogens, as measured either by BrdU or [^{3}H]thymidine incorporation (78, 86).

These data support previous observations that indicated that the different forms of ΔRaf:ER displayed differential abilities to promote the entry of quiescent cells into DNA synthesis, with the least active form of Raf displaying the most potent mitogenicity. They further indicate that the mitogenicity of ΔA-$Raf_{[YY]}$:ER is lost when the kinase is either inactivated [FF] or when it is catalytically potentiated [DD] by appropriate point mutations.

Regulated expression of components of the cell cycle machinery. In order to understand the differential effects of Raf protein kinases on cell cycle progression we undertook a biochemical analysis of the effects of ΔRaf:ER activation on MAP kinase activity and the subsequent changes in expression of components of the cell cycle machinery. Quiescent populations of NIH 3T3 cells expressing the different ΔRaf:ER proteins were either untreated or treated with 1 μM 4-HT for 48 h, at which time the expression or activity of certain cellular proteins was determined.

The expression of all of the ΔRaf:ER proteins was readily detected and was elevated approximately 5-fold as a consequence of the selective protein stabilization that occurs following addition of 4-HT (Fig. 1B1 to B3) (87). The maximum levels of expression of the ΔRaf:ER proteins after 48 h of 4-HT treatment were approximately equal.

Activation of all of the isoforms of ΔRaf-1:ER and ΔB-Raf:ER gave rise to readily detectable activation of p42 MAP kinase activity (Fig. 1C2 and C3). This level of activity is similar to the sustained phase of p42 MAP kinase activity observed in PC12 cells treated with nerve growth factor (86). Addition of 4-HT to cells expressing the nontransforming ΔA-$Raf_{[FF]}$:ER had no effect on p42 MAP kinase activity, whereas activation of ΔA-$Raf_{[YY]}$:ER gave rise to a very modest increase in p42 MAP kinase activity that was no more than 2-fold above the basal level and similar to previous observations. By contrast, activation of ΔA-$Raf_{[DD]}$:ER elicited a level of p42 MAP kinase activation approximately the same as the levels elicited by all of the forms of ΔRaf-1:ER and ΔB-Raf:ER (Fig. 1C1). These data confirmed our previous findings that ΔRaf-1:ER and ΔB-Raf:ER are more potent activators of the MAP kinase pathway than ΔA-Raf:ER, which is at best a weak activator (78).

A crucial role for the activation of cyclin-cdk complexes in cell cycle progression has been inferred from numerous biochemical and genetic analyses (38, 45, 63, 64, 91–93). Since the regulated expression and activation of cyclin D1-cdk4 and cyclin E-cdk2 are a characteristic of proliferating cells, we examined the ability of the different ΔRaf:ER proteins to induce cyclin D1 and cyclin E expression.

Cyclin D1 was strongly induced by all of the ΔRaf-1:ER and ΔB-Raf:ER proteins (Fig. 1D2 and D3). Cyclin D1 was not induced in cells expressing the inactive ΔA-$Raf_{[FF]}$:ER protein, but both the [YY] and the [DD] forms of ΔA-Raf:ER induced cyclin D1. The more active [DD] isoform of ΔA-Raf:ER induced cyclin D1 more efficiently than the [YY] form (Fig. 1D1). In general the extent of cyclin D1 induction correlated with the relative abilities of the different ΔRaf:ER proteins to activate p42 MAP kinase.

Cyclin E was induced by all of the ΔRaf:ER proteins, with the exception of the inactive ΔA-$Raf_{[FF]}$:ER. Cyclin E induction did not show a correlation with p42 MAP kinase activation, as the [YY] form of ΔA-Raf:ER induced cyclin E to the same extent as the other forms of ΔRaf:ER (Fig. 1E1 to E3). These data indicate that, despite the strong induction of cyclin D1 and E expression by ΔRaf-1:ER and ΔB-Raf:ER, these cells fail to enter into the S phase of the cell cycle (91–93).

CKIs such as $p21^{Cip1}$ and $p27^{Kip1}$ regulate the activity of cyclin-cdk complexes by binding to and inhibiting cyclin-cdk activity (30, 31, 45, 64, 75, 85). We therefore assessed the effects of ΔRaf:ER activation on the expression of these proteins. Activation of ΔRaf-1:ER and ΔB-Raf:ER led to reduced expression of $p27^{Kip1}$ (Fig. 1F2 and F3). Activation of the [YY] and [DD] forms of ΔA-Raf:ER gave rise to no more than a 50% reduction in $p27^{Kip1}$ expression, whereas the inactive [FF] isoform had no effect on $p27^{Kip1}$ expression.

The most significant differences between the different ΔRaf:ER proteins were their relative abilities to induce the expression of $p21^{Cip1}$. All of the ΔRaf-1:ER and ΔB-Raf:ER proteins as well as the [DD] form of ΔA-Raf:ER strongly induced $p21^{Cip1}$ expression (Fig. 1G1 to G3). By contrast the [YY] form of ΔA-Raf:ER failed to induce $p21^{Cip1}$ significantly, and the inactive [FF] form had no effect on $p21^{Cip1}$ expression (Fig. 1G1). Treatment of parental NIH 3T3 cells with 4-HT had no effect on either Raf or MAP kinase activity nor on the expression of any of the cell cycle regulators that we have examined in this study (data not shown) (78, 86).

These data indicate that all of the active forms of ΔRaf:ER induced the expression of cyclin D1 and cyclin E and repressed the expression of $p27^{Kip1}$. The major difference between the mitogenic and nonmitogenic forms of ΔRaf:ER that we have observed is their relative abilities to induce the expression of $p21^{Cip1}$. Originally characterized as a gene induced both during

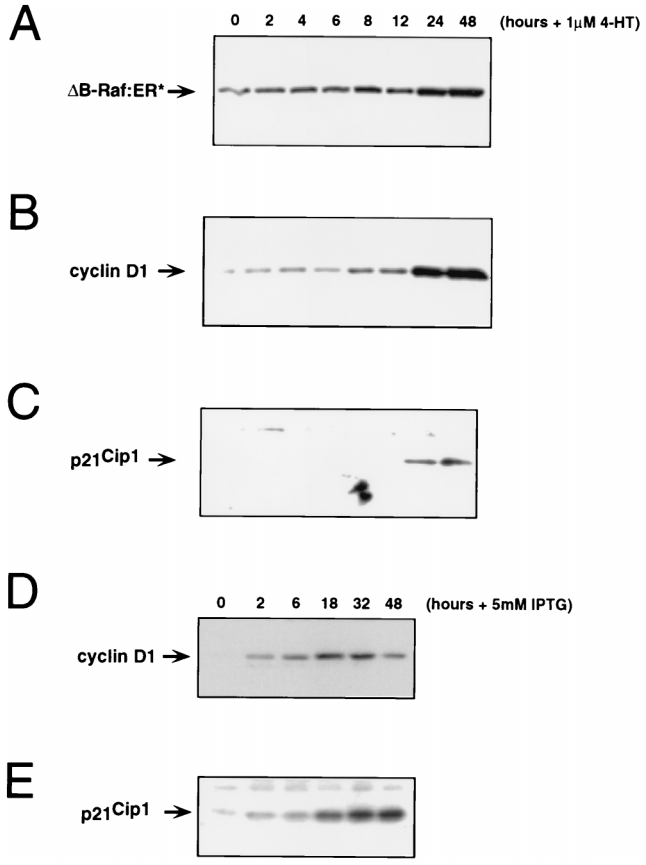


FIG. 2. Induction of cyclin D1 and $p21^{Cip1}$ mRNA and protein by ΔB-Raf:ER* and H-Ras(V12). Quiescent NIH 3T3 cells expressing ΔB-Raf:ER* were either untreated (0) or treated with 1 μM 4-HT for 2, 4, 6, 8, 12, 24, or 48 h as indicated, at which time cell extracts were prepared. The levels of expression of ΔB-Raf:ER* (A), cyclin D1 (B), and $p21^{Cip1}$ (C) were determined by Western blotting with the appropriate antisera. 3T3:iRas cells, cultured for 24 h in serum-free medium, were either untreated or treated with 5 mM IPTG for 2, 6, 18, 32, or 48 h as indicated (58). Cell extracts were prepared and Western blotted for the expression of cyclin D1 (D) and $p21^{Cip1}$ (E) as described above.

cell senescence and in response to activation of p53, $p21^{Cip1}$ has subsequently been shown to be subject to regulation by a variety of effectors (9, 22, 24, 25, 30, 69, 74, 98, 108). These data suggest that Raf-induced cell cycle arrest may result from the induction of $p21^{Cip1}$ expression with the subsequent inhibition of cyclin-cdk complexes.

Induction of cyclin D1 and $p21^{Cip1}$ by Ras and Raf oncogenes. In order to investigate the kinetics of cyclin D1 and $p21^{Cip1}$ induction by Raf, we prepared cell extracts from a clone of NIH 3T3 cells expressing ΔB-Raf:ER*, a fusion protein consisting of the kinase domain of B-Raf (ΔB-Raf) and a form of the hormone binding domain of the mouse estrogen receptor (ER^{TM}) which responds to 4-HT but not to β-estradiol (13, 48). Quiescent 3T3ΔB-Raf:ER* cells were treated with 1 μM 4-HT for different lengths of time, cell extracts were prepared, and the expression of cyclin D1 and $p21^{Cip1}$ was assessed by Western blotting. The expression of the ΔB-Raf:ER* protein increased with time following the addition of 4-HT to cells, as we have previously described (Fig. 2A) (78). Activation of ΔB-Raf:ER* led to induction of cyclin D1 expression, which was detected 6 to 8 h after ΔB-Raf:ER* activation (Fig. 2B). Cyclin E induction lagged behind that of cyclin D1, being detectable 12 to 24 h after ΔB-Raf:ER* activation (data not shown). The kinetics of $p21^{Cip1}$ induction were similar to those of cyclin E in that little or no $p21^{Cip1}$ was detectable until 12 to 24 h after ΔB-Raf:ER* activation (Fig. 2C).

In order to determine if the induction of cyclin D1 and $p21^{Cip1}$ was unique to activation of Raf, we prepared extracts of NIH 3T3 cells in which the expression of oncogenic H-Ras(V12) is induced by the addition of IPTG to the culture medium. Induced expression of H-Ras(V12) leads to activation of the Raf/MEK/MAP kinase cascade and induction of HB-EGF mRNA within 1 to 2 h (58). Cell extracts were prepared from cells that were either untreated or treated with IPTG for 2, 6, 18, 32, or 48 h. Western blot analysis revealed the anticipated induction of both cyclin D1 and $p21^{Cip1}$ expression (Fig. 2D and 2E, respectively). As was observed with ΔB-Raf:ER*, the maximal induction of cyclin D1 appeared to precede that of $p21^{Cip1}$ by several hours. The kinetics and extent of induction of $p21^{Cip1}$ and cyclin D1 by H-Ras(V12) were identical in the absence and presence of fetal calf serum and similar to those induced by ΔB-Raf:ER* (data not shown).

Raf-induced $p21^{Cip1}$ binds to cyclin E-cdk2 complexes. $p21^{Cip1}$ has previously been shown to bind and inhibit the activity of complexes containing cyclin E-cdk2 (30, 31). To determine if Raf-induced $p21^{Cip1}$ inhibited cyclin E-cdk2 activity in cells, we measured cdk2-associated kinase activity in the extracts of NIH 3T3 cells expressing the different ΔRaf:ER proteins shown in Fig. 1, as described in Materials and Methods (Fig. 3A and B). The presence of $p21^{Cip1}$ and cdk2 in parallel immunoprecipitates was confirmed by Western blotting (Fig. 3C and D).

Immunoprecipitates of cdk2 from cells expressing activated ΔA-$Raf_{[YY]}$:ER were four- to fivefold more active than cdk2 immunoprecipitates from cells expressing either ΔRaf:ER or ΔB-Raf:ER. The level of cdk2 activity in these latter cells was identical to that observed in quiescent NIH 3T3 cells (data not shown) and cells expressing the kinase-inactive [FF] form of ΔA-Raf:ER (Fig. 3A and B). Moreover immunoprecipitates of cdk2 from cells expressing the more active [DD] form of ΔA-Raf:ER also displayed low cdk2 kinase activity. Consistent with this observation we detected little or no $p21^{Cip1}$ in cdk2 immunoprecipitates from ΔA-$Raf_{[YY]}$:ER-expressing NIH 3T3 cells, whereas $p21^{Cip1}$ was readily detected in cdk2 immunoprecipitates from ΔRa-1:ER and ΔB-Raf:ER-expressing cells. We could not detect $p21^{Cip1}$ in immunoprecipitates of cdk2 from cells expressing the inactive ΔA-$Raf_{[FF]}$:ER protein. The low level of cdk2 activity in these extracts was most likely due to the fact that there is little cyclin E expressed in these cells (Fig. 1E1). These data indicate that Raf-induced $p21^{Cip1}$ binds to cyclin E-cdk2 complexes and is further consistent with a role for $p21^{Cip1}$ in the inhibition of cdk2 kinase activity and Raf-induced cell cycle arrest.

Different biological outcomes as a consequence of differential activation of the Raf/MAP kinase pathway. A hypothesis to explain the differential ability of Raf kinases to promote cell cycle progression in NIH 3T3 cells is that low levels of Raf activity elicit a mitogenic response whereas high levels of Raf activity elicit cell cycle arrest. To test this hypothesis we took advantage of the fact that activation of ΔRaf:ER proteins is dependent on the dose of 4-HT added to the culture medium (87). Quiescent 3T3ΔB-Raf:ER* cells were treated with a range of 4-HT concentrations from 0 to 67 nM, and DNA synthesis was measured by incorporation of [^{3}H]thymidine. Consistent with our hypothesis, the cells displayed a bell-shaped mitogenic response to the addition of different concentrations of 4-HT, with the peak of DNA synthesis occurring at 1.7 nM. The peak response of these cells was equivalent to

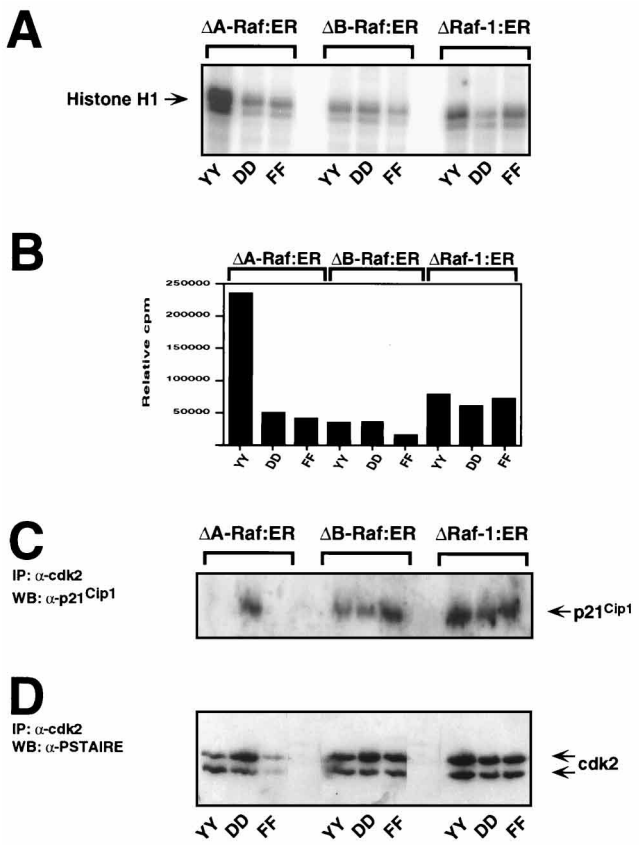


FIG. 3. Inhibition of cdk2-associated kinase activity by Raf-induced p21^{Cip1}. Immunoprecipitates of cdk2 were prepared from the cell extracts of quiescent cells in which the various ΔRaf:ER proteins were activated for 48 h, as described in the legend to Fig. 1. cdk2-associated kinase activity was measured with histone H1 as a substrate (A) and quantitated with a Molecular Dynamics Storm PhosphorImager (B). After kinase activity was measured, the amounts of p21^{Cip1} (C) and cdk2 (D) in these immunoprecipitates were assessed by Western blotting with the appropriate antisera. IP, antiserum for immunoprecipitation; WB, antiserum for Western blotting.

their response to the addition of 20% (vol/vol) fetal calf serum and about 65% of their response to 10 ng of exogenously added PDGF per ml (Fig. 4, upper left panel).

Photomicrographs demonstrated that in the absence of 4-HT, 3T3ΔB-Raf:ER* cells displayed a flat, nonrefractile morphology that is characteristic of normal NIH 3T3 cells (Fig. 4). The addition of 4-HT to 67 nM caused the striking changes in cell shape and refractility that are characteristic of NIH 3T3 cells transformed by oncogenic Ras and Raf, but no increase in cell number was observed. Cells treated with 1.7 nM 4-HT displayed more subtle changes in cell shape and a loss of contact inhibition; however, we observed an approximately two- to threefold increase in cell number, consistent with the observed induction of [^{3}H]thymidine incorporation. These data support previous observations that dissociate the effects of oncogenes, such as the Raf and Fos genes, on cell morphology from their effects on cell proliferation (60, 86).

To confirm these results we measured the induction of DNA synthesis in pooled populations of NIH 3T3 cells expressing either the [YY] or the [DD] forms of ΔA-Raf:ER. Consistent with our previous observations, DNA synthesis was induced in cells expressing ΔA-Raf$_{[YY]}$:ER at concentrations of 4-HT greater than 3 nM, with no diminution of the response with concentrations of 4-HT up to 1 μM. The kinetics of DNA synthesis induced by ΔA-Raf$_{[YY]}$:ER lagged behind the response to acute stimulation with either PDGF or serum by about 6 to 12 h (data not shown) (78). In contrast, cells expressing ΔA-Raf$_{[DD]}$:ER displayed a bell-shaped mitogenic response, similar to that described for ΔB-Raf:ER*. We have subsequently observed bell-shaped mitogenic responses in numerous clones and pooled populations of NIH 3T3 cells expressing ΔRaf-1:ER and ΔB-Raf:ER, including C2 cells, the cell line in which Raf-induced cell cycle arrest was first characterized (86). These data strongly suggest that Raf is able to elicit distinctly different biological outcomes in NIH 3T3 cells, namely, cell proliferation or cell cycle arrest, depending on the level of Raf kinase activity within the cell.

Differential activation of cdk2 and cdk4 kinase activity by ΔRaf:ER proteins. To investigate the effects of different levels of Raf activation on cyclin-cdk complex activity, we prepared cell extracts from quiescent 3T3:ΔB-Raf:ER*-expressing cells that were either untreated or treated with 0.5, 2.0, 100, or 1,000 nM 4-HT for 48 h. The activities of ΔB-Raf:ER*, p42 MAP kinase (data not shown), and cdk2- and cdk4-associated kinase were measured with the appropriate substrates, as described in Materials and Methods (Fig. 5 [cdk2] and 6 [cdk4]).

Consistent with previous observations (78), the activities of ΔB-Raf:ER* and p42 MAP kinase increased proportionally in response to the concentration of added 4-HT, such that cells treated with 1,000 nM 4-HT displayed the highest level of Raf and MAP kinase activity. Similar to our findings with cells transformed by ΔA-Raf$_{[YY]}$:ER, we could detect only a modest activation of Raf, MEK, and MAP kinase activity in cells treated with the concentration of 4-HT that elicited the most potent mitogenic response (data not shown).

The activity of cdk2 was low in untreated cells and was elevated six- to sevenfold in cells treated with 0.5 nM 4-HT, but in contrast to Raf and MAP kinase activity, it decreased to basal levels in cells treated with 100 and 1,000 nM 4-HT (Fig. 5A and 5B). Consistent with the ability of ΔB-Raf:ER* to induce cyclin E expression, we detected low levels of cyclin E associated with cdk2 in extracts of untreated cells. Following activation of ΔB-Raf:ER* by all concentrations of 4-HT, cyclin E was readily detected in cdk2 immunoprecipitates (Fig. 5C). Little or no p21^{Cip1} was detected in cdk2 immunoprecipitates from cells treated with 0.5 nM 4-HT, consistent with the elevated activity of the cyclin E-cdk2 complexes. As p21^{Cip1} became detectable in cdk2 immunoprecipitates, the level of cdk2 kinase activity decreased (Fig. 5D). These data indicate that the ability of ΔB-Raf:ER* to activate cdk2 activity was indirectly proportional to the extent of Raf and MAP kinase activation. Activation of ΔB-Raf:ER* to a low level elicited the strongest activation of cdk2 kinase activity, whereas cells with the highest level of Raf and MAP kinase activity had low cdk2 kinase activity. The low level of cdk2 kinase activity in these latter samples correlated with the presence of induced p21^{Cip1} in the cyclin E-cdk2 immunoprecipitates.

We next assessed the effects of ΔB-Raf:ER* activation on cdk4 activity (Fig. 6A and B). Activation of ΔB-Raf:ER* with 0.5 to 100 nM 4-HT induced cdk4 kinase activity approximately two- to threefold. When ΔB-Raf:ER* was fully activated by 1 μM 4-HT, the level of cdk4 activity was reduced almost to the level found in untreated cells. At all concentrations of 4-HT, cyclin D1 was readily detectable in cdk4 immunoprecipitates. p21^{Cip1} was detected in cdk4 immunoprecipitates from cells treated with 4-HT concentrations of 2 nM and above (Fig. 6C and D, respectively). The specificity of the cdk4 kinase assays was ascertained by peptide competition experiments, which demonstrated that in the presence of competing immunogen 90% of the cdk4 activity was lost (Fig. 6B).

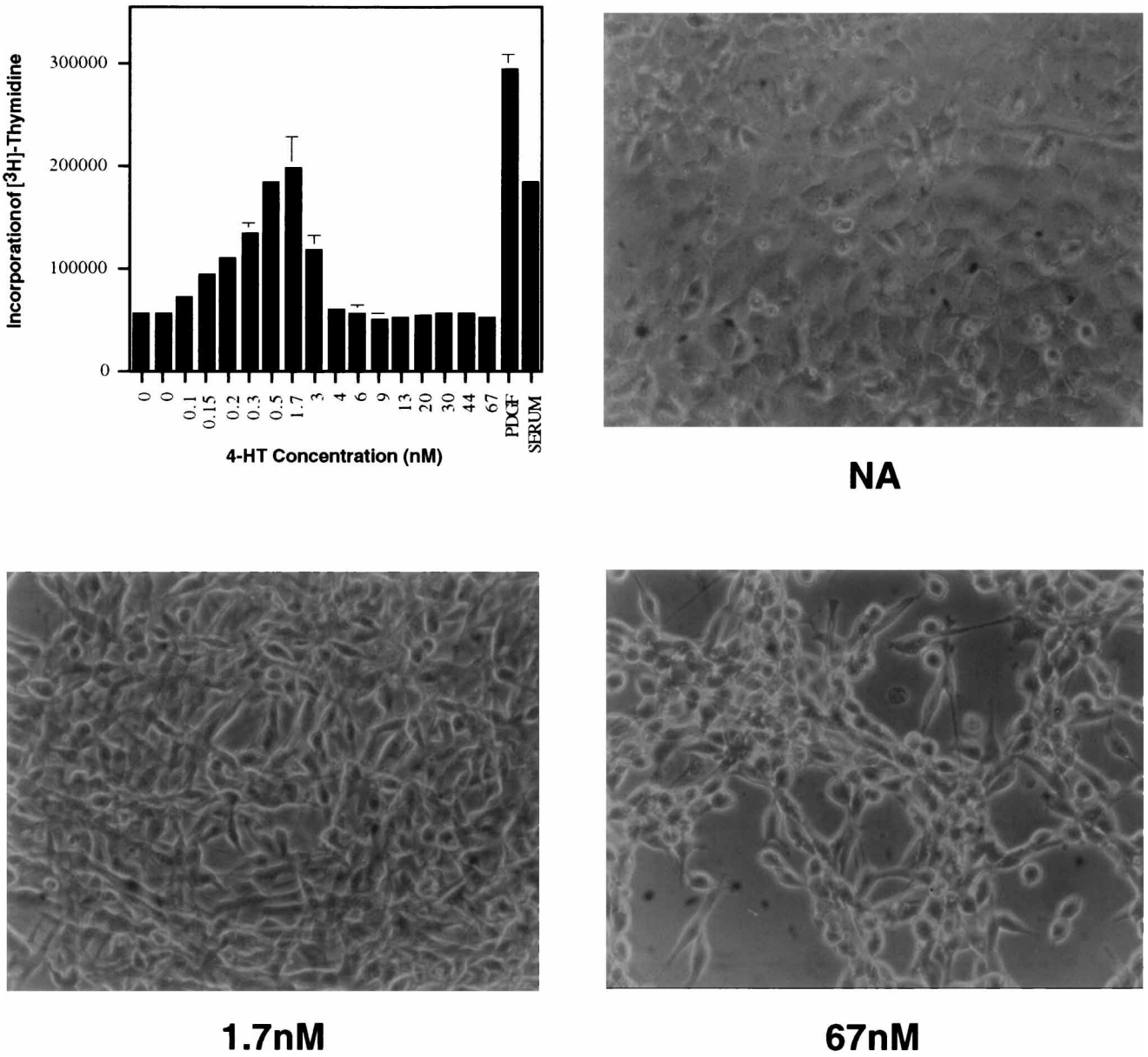


FIG. 4. Cell cycle progression and arrest induced by different levels of ΔB-Raf:ER* activity. Quiescent 3T3ΔB-Raf:ER* cells in a 96-well dish were treated with different 4-HT concentrations from 0 to 67 nM or with 10 ng of PDGF per ml or 20% (vol/vol) fetal calf serum, as indicated on the abscissa of the graph. DNA synthesis was measured by the incorporation of methyl-[^{3}H]thymidine, as described in Materials and Methods. Each point is the average of quadruplicate measurements, with the standard deviations indicated by the error bars. The absence of an error bar indicates that the error was too small to register on the graph at the scale used. Representative photomicrographs of 3T3ΔB-Raf:ER* cells either untreated (NA) or treated with 1.7 or 67 nM 4-HT were taken as described in Materials and Methods.

These data strongly suggest that low-level activation of ΔB-Raf:ER* (0.5 nM 4-HT) leads to activation of both cyclin D1-cdk4 and cyclin E-cdk2 kinase activity, whereas at higher-level ΔB-Raf:ER* activation the activity of cyclin-cdk complexes is inhibited. It is interesting to note the apparent differential sensitivity of cdk4 and cdk2 complexes to the effects of $p21^{Cip1}$ induction. Cyclin E-cdk2 complexes appear to be more sensitive to $p21^{Cip1}$-mediated inhibition than cyclin D-cdk4 complexes. Although the significance of these latter observations is unclear, they warrant further investigation. These experiments also do not preclude a possible role for CKIs other than $p21^{Cip1}$ in the regulation of cyclin-cdk complexes by Raf. Although the cyclin-dependent kinase inhibitor $p16^{INK4a}$ is encoded by a Ras-responsive gene, NIH 3T3 cells have sustained a deletion of the gene that extinguishes its expression; hence, it plays no role in the inhibition of cyclin D-cdk4 activity observed here (47, 81). We cannot rule out a possible role for other INK4 family CKIs in Ras- or Raf-mediated cell cycle arrest (45, 93).

Construction and characterization of GFP-tagged forms of ΔRaf-1:ER. In order to facilitate the analysis of the effects of Raf activation in primary cells from mice lacking specific cell signaling and cell cycle components, we fused sequences encoding either the [YY] or the [DD] forms of ΔRaf-1:ER to sequences encoding EGFP (11, 76). Pooled populations of NIH 3T3 cells expressing the resulting chimeric gene product (GFPΔRaf-1:ER) were derived by retrovirus infection, as de-

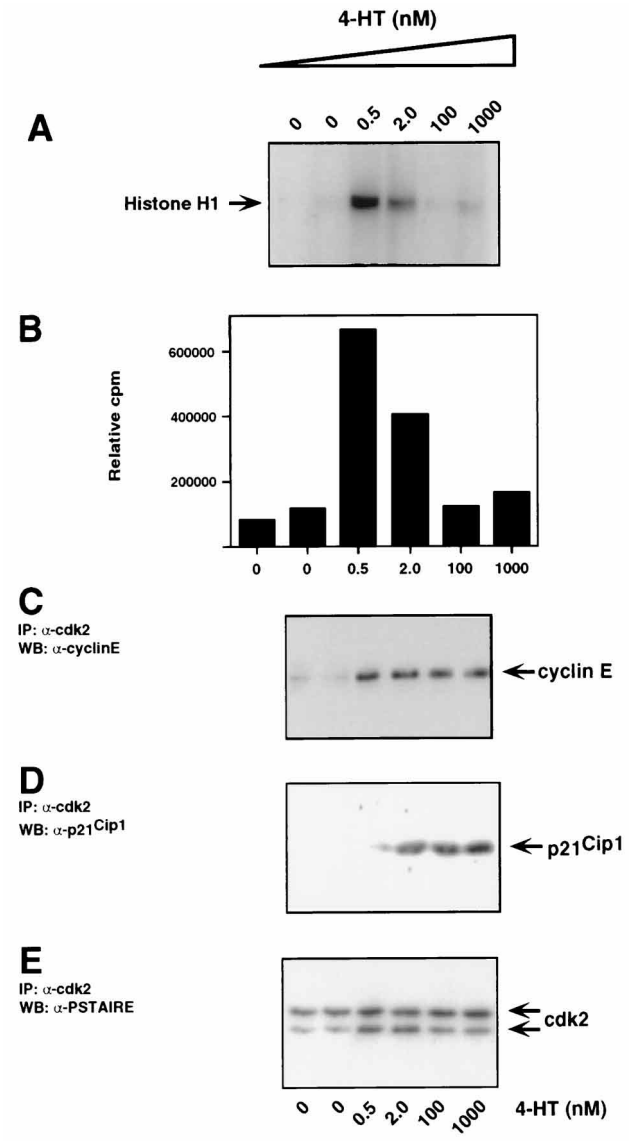


FIG. 5. Activation of cdk2 in NIH 3T3 cells by ΔB-Raf:ER*. Quiescent NIH 3T3 cells expressing ΔB-Raf:ER* were either untreated (0) or treated with 0.5, 2.0, 100, or 1,000 nM 4-HT for 48 h, at which time cell extracts were prepared and cdk2-associated kinase activity was measured with histone H1 as a substrate (A), as described in Materials and Methods. cdk2-associated kinase activity was quantitated with a Molecular Dynamics Storm PhosphorImager (B). The presence of cyclin E (C), p21[Cip1] (D), and cdk2 (E) was quantitated in parallel immunoprecipitates by Western blotting with the appropriate antisera. IP, antiserum for immunoprecipitation; WB, antiserum for Western blotting.

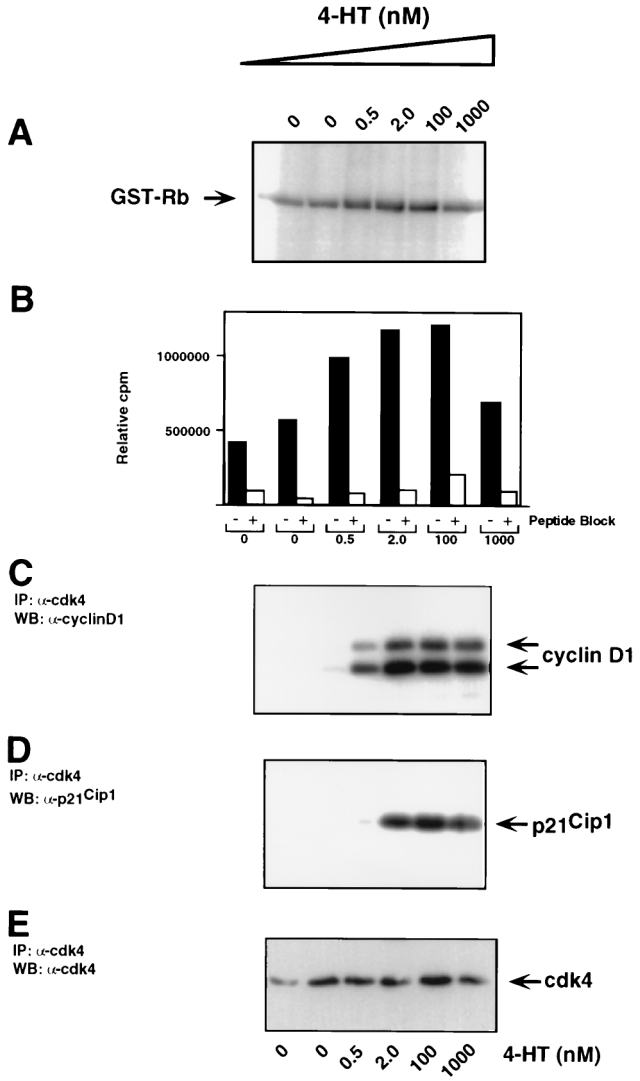
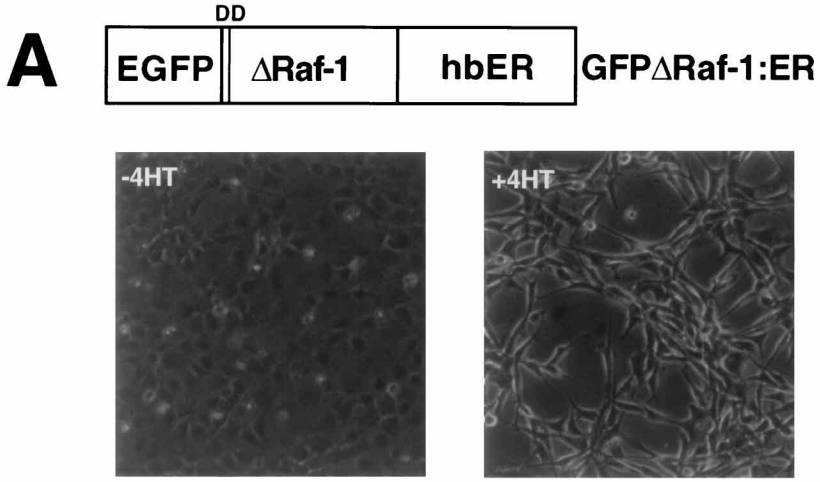


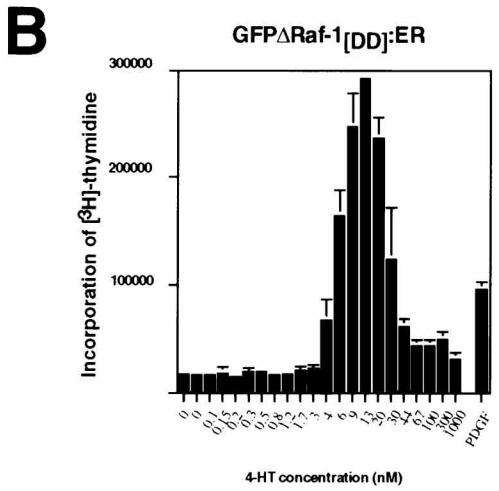
FIG. 6. Activation of cdk4 in NIH 3T3 cells by ΔB-Raf:ER*. Quiescent NIH 3T3 cells expressing ΔB-Raf:ER* were either untreated (0) or treated with 0.5, 2.0, 100, or 1,000 nM 4-HT for 48 h, at which time cell extracts were prepared and cdk4-associated kinase activity was measured in the absence (−) or presence (+) of an excess of competing immunizing peptide (Peptide Block) with GST-Rb(CT) (59) as a substrate (A), as described in Materials and Methods. cdk4-associated kinase activity was quantitated with a Molecular Dynamics Storm PhosphorImager (B). The presence of cyclin D1 (C), p21[Cip1] (D), and cdk4 (E) was confirmed in parallel immunoprecipitates by Western blotting with the appropriate antisera. IP, antiserum for immunoprecipitation; WB, antiserum for Western blotting.

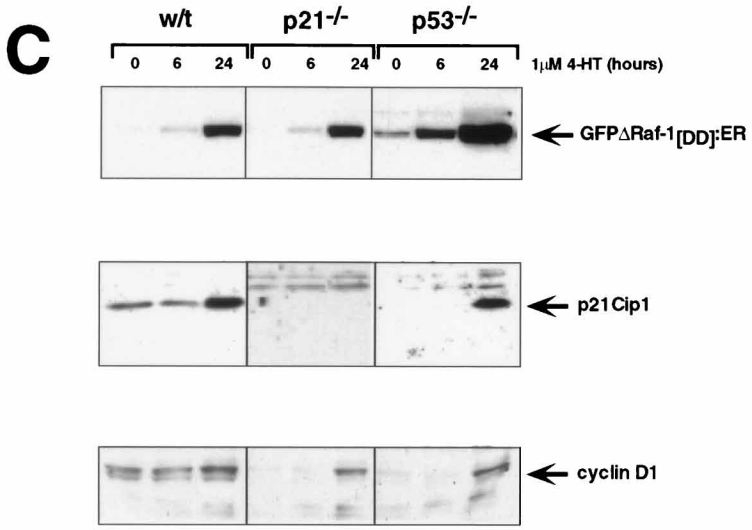
scribed in Materials and Methods (Fig. 7A). Consistent with previous immunofluorescence analysis of NIH 3T3 cells expressing ΔRaf-1:ER, the GFPΔRaf-1:ER proteins were expressed in the cytoplasm of the cells, with little or no nuclear fluorescence detected in the absence or presence of 4-HT (107a). FACScan analysis of these cells revealed that they displayed 10- and 100-fold greater fluorescence than parental NIH 3T3 cells in the absence and presence of 4-HT, respectively (data not shown).

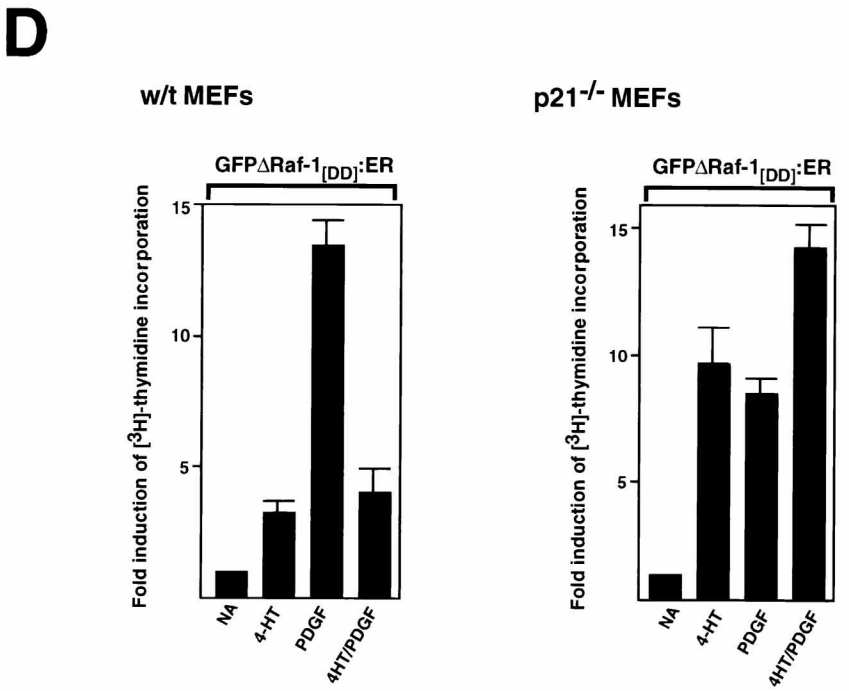
Addition of 1 μM 4-HT to cells expressing GFPΔRaf-1$_{[DD]}$:ER led to striking alterations in cell morphology in the absence or presence of fetal calf serum, consistent with our previous observations with ΔRaf-1:ER (Fig. 7A) (86). Furthermore, addition of 1 μM 4-HT to these cells led to rapid activation of MEK and p42 MAP kinase and to the alterations in expression of HB-EGF, cyclin D1, cyclin E, p21[Cip1], and p27[Kip1] that we have described previously for the various forms of ΔRaf:ER and that are shown in Fig. 1 (58, 87). Moreover, addition of a range of 4-HT concentrations to quiescent NIH 3T3 cells expressing GFPΔRaf-1$_{[DD]}$:ER gave rise to a pronounced bell-shaped mitogenic response similar to that described above (Fig. 7B). The 20-fold induction of DNA synthesis was, in this experiment, stronger than the response to PDGF. Similar results were obtained with cells expressing the [YY] form of GFPΔRaf-1:ER (data not shown).

These data indicate that the addition of the GFP moiety to


A
DD
EGFP
ΔRaf-1
hbER
GFPΔRaf-1:ER
-4HT
+4HT


B
GFPΔRaf-1[DD]:ER
Incorporation of [3H]-thymidine
300000
200000
100000
4-HT concentration (nM)
PDGF


C
w/t
p21-/-
p53-/-
0
6
24
1μM 4-HT (hours)
GFPΔRaf-1[DD]:ER
p21Cip1
cyclin D1


D
w/t MEFs
p21-/- MEFs
GFPΔRaf-1[DD]:ER
Fold induction of [3H]-thymidine incorporation
15
10
5
NA
4-HT
PDGF
4HT/PDGF

ΔRaf-1:ER did not alter the well-established biological and biochemical properties of the ΔRaf-1:ER fusion protein. Such GFP-tagged forms of Raf will be useful for studying the effects of Raf activation in a variety of primary and established cell lines. Cells expressing the chimeric proteins can be readily visualized and isolated by FACS (8a).

Induced $p21^{Cip1}$ inhibits the mitogenic response to Raf activation in primary MEFs. To investigate the role of $p21^{Cip1}$ in Raf-induced cell cycle arrest, we introduced the [YY] and [DD] forms of GFPΔRaf-1:ER into either wild-type (wt) primary MEFs or MEFs that lack the genes encoding the tumor suppressor protein p53 ($p53^{-/-}$) or the CKI $p21^{Cip1}$ ($p21^{Cip1-/-}$) by retrovirus infection. The efficiency of retrovirus infection of primary MEFs (as measured by conversion to puromycin resistance) was almost 100%, and by FACScan analysis we estimated that ≥40 to 50% of the infected cells expressed the GFPΔRaf-1:ER proteins. Each cell population was derived from approximately 10^5 independent infection events.

Quiescent primary MEFs (wt, $p53^{-/-}$, and $p21^{-/-}$) expressing the [DD] form of GFPΔRaf-1:ER were either untreated or treated with 1 μM 4-HT for 6 or 24 h, at which time GFPΔRaf-1:ER, $p21^{Cip1}$, and cyclin D1 expression was assessed by Western blotting (Fig. 7C and data not shown).

Addition of 4-HT to all of the populations of MEFs described above led to increased expression of GFPΔRaf-$1_{[DD]}$:ER. The level of GFPΔRaf-$1_{[DD]}$:ER expression was similar in the wt and $p21^{Cip1-/-}$ MEFs and was highest in $p53^{-/-}$ MEFs (Fig. 7C). Prolonged exposure of the Western blot shown in Fig. 7C revealed that GFPΔRaf-$1_{[DD]}$:ER was expressed prior to the addition of 4-HT in the wt and $p21^{Cip1-/-}$ MEFs, which is consistent with the FACScan analysis of these cells (data not shown). Similar observations were made with cells expressing the [YY] form of GFPΔRaf-1:ER (data not shown).

Activation of GFPΔRaf-$1_{[DD]}$:ER in wt MEFs led to induction of $p21^{Cip1}$ expression that was readily detected 24 h after the addition of 4-HT (Fig. 7C). As expected, no $p21^{Cip1}$ was detected in cells derived from the $p21^{Cip1-/-}$ mice, even after prolonged exposure of the Western blot (Fig. 7C). Interestingly, although there is evidence that the induction of $p21^{Cip1}$ by Raf in primary Schwann cells is dependent on p53 (49), we observed Raf-induced $p21^{Cip1}$ expression in $p53^{-/-}$ cells (Fig. 7C). Consistent with our observations obtained with NIH 3T3 cells, GFPΔRaf-$1_{[DD]}$:ER activation in primary MEFs led to induction of cyclin D1 that was detected 24 h after the addition of 4-HT. The absence of $p21^{Cip1}$ or p53 had no significant effect on the ability of Raf to induce cyclin D1 expression (Fig. 7C). The experiments described above were also conducted in parallel with cells expressing the [YY] form of GFPΔRaf-1:ER, with similar results (data not shown). These data indicate that the ability of Raf to induce $p21^{Cip1}$ and cyclin D1 expression is not restricted to established cell lines but is observed in nonimmortalized primary mouse fibroblasts.

To assess the mitogenic effects of GFPΔRaf-$1_{[DD]}$:ER activation, quiescent populations of wt or $p21^{Cip1-/-}$ MEFs expressing GFPΔRaf-$1_{[DD]}$:ER were either untreated or treated with 1 μM 4-HT, 10 ng of PDGF per ml, or a combination of both 4-HT and PDGF, as indicated on the abscissas of the graphs in Fig. 7D. DNA synthesis was measured by [^{3}H]thymidine incorporation, as described in Materials and Methods.

In wt MEFs activation of GFPΔRaf-$1_{[DD]}$:ER induced DNA synthesis approximately threefold. We assume that in pooled populations of primary MEFs there is a subpopulation of cells in which Raf activation elicits a mitogenic response, either because of variations in GFPΔRaf-$1_{[DD]}$:ER expression or activation or because of the heterogeneity of primary MEF cultures. The response of these cells to GFPΔRaf-1:ER activation was approximately 25% of the response to stimulation with PDGF. However, activation of GFPΔRaf-$1_{[DD]}$:ER consistently inhibited the response of wt MEFs to PDGF stimulation (Fig. 7D). We interpret this to mean that the majority of cells that respond to PDGF have a level of Raf activity that inhibits cell cycle progression. Such a hypothesis explains the somewhat counterintuitive observation that the combination of two mitogenic stimuli gives rise to a response that is less than the sum of the individual responses.

In contrast to the results obtained with wt MEFs, activation of GFPΔRaf-$1_{[DD]}$:ER in $p21^{Cip1-/-}$ MEFs gave rise to a 12-fold induction of DNA synthesis that was equal to the response to PDGF (Fig. 7D). In these cells we saw no evidence that GFPΔRaf-$1_{[DD]}$:ER activation had any inhibitory effect on the response of the cells to PDGF; rather, we observed an additive response when the cells were treated with both agents (Fig. 7D). These data were not a consequence of a difference in the frequency or level of expression of GFPΔRaf-$1_{[DD]}$:ER (Fig. 7C and data not shown). In addition, qualitatively similar data were obtained in parallel experiments using pooled populations of cells expressing the [YY] form of GFPΔRaf-1:ER (data not shown). We were unable to assess the effects of Raf activation in $p53^{-/-}$ cells, as even after prolonged culture under serum-free conditions (16 days), $p53^{-/-}$ MEFs displayed a very high basal level of [^{3}H]thymidine incorporation. Interestingly, this high basal level of [^{3}H]thymidine incorporation was not inhibited by the activation of GFPΔRaf-1:ER.

These data indicate that the loss of $p21^{Cip1}$ has a significant effect on the mitogenic response of cells to Raf activation and lend support to the hypothesis that the ability of Raf to elicit a G_1 arrest is linked to its ability to induce $p21^{Cip1}$ expression.

DISCUSSION

Distinct biological outcomes are determined by different levels of Raf activity. In this paper we demonstrate that activation of Raf in NIH 3T3 cells can promote either cell proliferation or cell cycle arrest, depending on the level of Raf

FIG. 7. Construction and expression of GFP-tagged forms of ΔRaf-1:ER. (A) The coding sequences for EGFP were fused in frame to sequences encoding either the [YY] or [DD] forms of ΔRaf-1:ER to generate GFPΔRaf-1:ER in the retrovirus vector pBabepuro, as described in Materials and Methods. Pooled populations of NIH 3T3 cells expressing the [DD] form of GFPΔRaf-1:ER were tested for hormone-dependent morphological transformation in serum-free medium in the absence (−4HT) or presence (+4HT) of 1 μM 4-HT. hbER, hormone-binding domain of the estrogen receptor. (B) Quiescent populations of NIH 3T3 cells expressing GFPΔRaf-$1_{[DD]}$:ER were treated with different 4-HT concentrations from 0.1 to 1,000 nM or 10 ng of PDGF per ml, as indicated on the *x* axis of the graph. DNA synthesis was measured by the incorporation of methyl-[^{3}H]thymidine, as described in Materials and Methods. Each point is the average of quadruplicate measurements, with the standard deviation indicated by the error bar. The absence of an error bar indicates that the error was too small to register on the graph at the scale used. (C) Primary MEFs, from either wild-type mice (w/t) or mice lacking the genes encoding $p21^{Cip1}$ ($p21^{Cip1-/-}$) or p53 ($p53^{-/-}$), expressing GFPΔRaf-$1_{[DD]}$:ER were derived by retrovirus infection. Cells were made quiescent by culture in serum-free medium for 3 days, at which time they were either untreated (0 h) or treated with 1 μM 4-HT for 6 or 24 h as indicated. Cell extracts were prepared and analyzed for the expression of GFPΔRaf-$1_{[DD]}$:ER, $p21^{Cip1}$, and cyclin D1 as indicated. (D) Quiescent populations of w/t or $p21^{Cip1-/-}$ cells expressing GFPΔRaf-$1_{[DD]}$:ER were either untreated (NA) or treated with 1 μM 4-HT, 10 ng of PDGF per ml, or a combination of both 4-HT and PDGF, as indicated on the abscissas of the graphs. DNA synthesis was measured by the incorporation of methyl-[^{3}H]thymidine, as described in Materials and Methods. Each point for w/t cells is the average of 16 replicate measurements, and each point for the $p21^{Cip1-/-}$ cells is the average of 9 replicate measurements, with the standard deviations indicated by the error bars. The absence of an error bar indicates that the error was too small to register on the graph at the scale used.

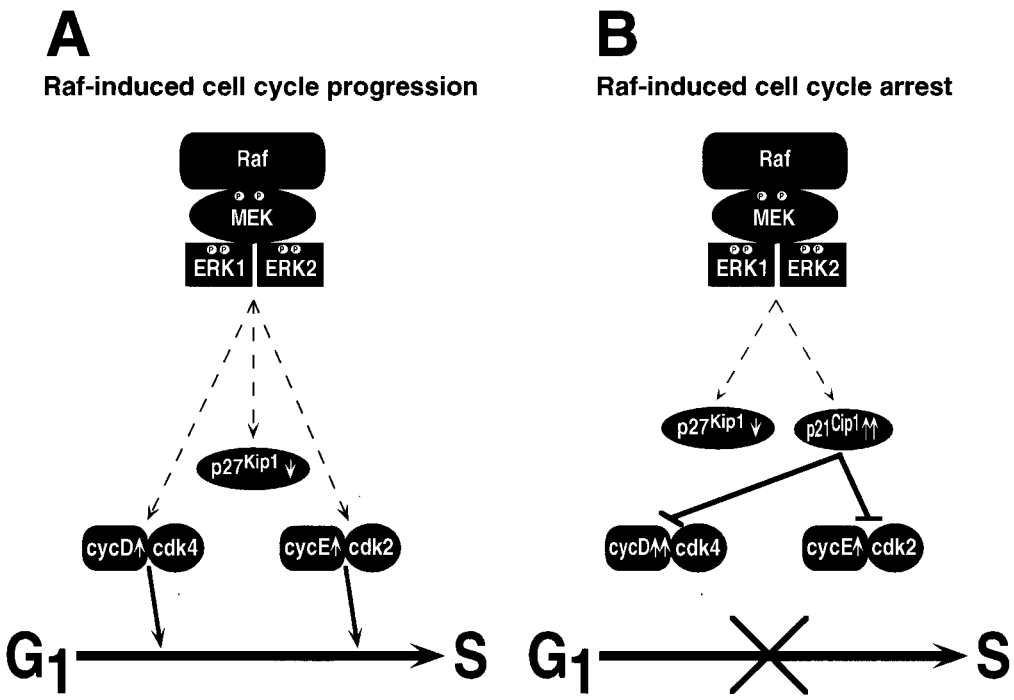


FIG. 8. Model of cell cycle progression and arrest induced by Raf. (A) Low-level activation of Raf leads to the induction of cyclin D1 and cyclin E and to reduced $p27^{Kip1}$ expression, thereby promoting the entry of quiescent cells into the cell cycle. (B) Higher levels of Raf activity lead to induction of cyclin D1 and cyclin E and to $p27^{Kip1}$ expression but also lead to induced expression of $p21^{Cip1}$, which inhibits both cyclin D-cdk4 and cyclin E-cdk2 complexes, thereby eliciting cell cycle arrest. P, phosphorylation of MEK and MAP kinase that occurs when the enzymes are activated.

activity. By eliciting cell cycle arrest, changes in cell morphology, and the expression of cell surface markers, such as the transmembrane form of HB-EGF and certain integrins (107b), it appears that Raf is capable of inducing a "quasi-differentiated" state in NIH 3T3 cells. By an analogous strategy others have come to similar conclusions (90a).

It seems likely that Raf-induced cell cycle progression is associated with the ability of Raf to induce the expression and activation of cyclin D1-cdk4 and cyclin E-cdk2 activity and to decrease the expression of $p27^{Kip1}$ (Fig. 8A). Although the mechanisms of cyclin E and $p27^{Kip1}$ regulation by Raf are not known, we have observed that cyclin D1 mRNA is induced 10-fold following ΔB-Raf:ER* activation (4a). Furthermore, a role for MAP kinases and Ets transcription factors has been suggested in the ability of Ras to induce the expression of cyclin D1 (1, 44, 106).

The ability of Raf to elicit cell cycle arrest in NIH 3T3 cells correlates well with the induction of $p21^{Cip1}$. Support for a role for $p21^{Cip1}$ in Raf-induced cell cycle arrest came from the observation that activation of Raf is strongly mitogenic in $p21^{Cip1-/-}$ MEFs (Fig. 8B). Furthermore, it has recently been demonstrated that the ability of ΔRaf-1:ER to induce $p21^{Cip1}$ expression is inhibited by the MEK1 inhibitor PD98059 (79). These data do not, however, rule out the possibility of branch points on the Raf pathway that may influence other components of the cell cycle machinery.

Consistent with the data described here, we have observed that a conditionally active form of oncogenic MEK1 (ΔMEK1:ER) displayed biochemical and biological properties in NIH 3T3 cells similar to those displayed by $\Delta A\text{-}Raf_{[YY]}$:ER (52). Activation of ΔMEK1:ER in NIH 3T3 cells elicited a robust proliferative response that was accompanied by weak activation of p42 and p44 MAP kinase, modest induction of cyclin D1, and a failure to induce $p21^{Cip1}$ expression (107c). This is consistent with the observation that constitutively activated MEK1, when microinjected into NIH 3T3 cells, induced DNA synthesis (12).

It is important to note that since the effects of Ras or Raf on cell cycle progression or cell cycle arrest appear to be rather delicately tuned to the level of Raf activity in cells, it is likely that differences in the choice of expression vector, gene transfer system, form of Ras or Raf used, and cell background could explain the plethora of contradictory reports that have been published on the relative ability of Raf to induce cell proliferation or arrest (8, 10, 78, 86, 95). These results underscore the utility of conditional forms of Raf in such analyses.

The data described in this report are in accord with recent observations that demonstrated that expression of oncogenic Ras or activation of ΔRaf-1:ER in primary Schwann cells elicits a $p21^{Cip1}$-mediated cell cycle arrest. Under these circumstances the ability of Raf to induce cell cycle arrest is abrogated by the expression of either large T antigen, dominant negative p53, or antisense $p21^{Cip1}$ (49, 83). In addition it has recently been shown that keratinocytes lacking $p21^{Cip1}$ are more highly susceptible to Ras-mediated tumorigenesis than their normal counterparts (61). Finally, recent evidence has suggested that the Ras pathway is also capable of inducing premature cell senescence as a consequence of the induced expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$, a specific inhibitor of cyclin D-cdk complexes (90). These data are therefore consistent with a model in which the Ras/Raf pathway can induce cell cycle arrest and/or senescence via the induction of cyclin-dependent kinase inhibitors and moreover indicate that these observations are features common to a number of different cell types.

Considerable evidence has accumulated indicating that the

Rb tumor suppressor protein is a key substrate for cyclin-cdk complexes. It is not presently clear if Ras or Raf is capable of arresting cells from the $Rb^{-/-}$ mouse; however, it has been demonstrated that ΔRaf-1:ER can elicit a G_1 arrest in human small-cell lung cancer cell lines which lack a functional Rb gene (82a). It will therefore be of interest to investigate the effects of Raf activation on cell cycle progression in cells from mice lacking either Rb or the related proteins p107 and p130 and various combinations thereof.

MAP kinase pathways in proliferation and differentiation. A wealth of biochemical and genetic evidence indicates that MAP kinase signaling modules are important in the control both of normal eukaryotic cell proliferation and differentiation and of the aberrant cell proliferation associated with neoplastic transformation in mammals (6, 17, 32, 66).

In *Saccharomyces cerevisiae*, α-factor-mediated activation of the Ste11/Ste7/Fus3/Kss1 MAP kinase pathway elicits a G_1 cell cycle arrest as a consequence of the induction and activation of a cyclin-dependent kinase inhibitor, Far1 (32, 73). In *Drosophila melanogaster* the Ras-activated MAP kinase pathway is involved in cell proliferation, survival, and differentiation in response to signaling through the *Drosophila* epidermal growth factor receptor homolog (72). Furthermore, flies lacking a functional Raf homolog (lethal-1-polehole) display defects in both cell proliferation and differentiation (67, 68). Amongst the best-characterized roles for the Raf/MAP kinase pathway in *Drosophila* are the terminal differentiation of the R7 photoreceptor cell and the specification of terminal cell fates, which are mediated by the Sevenless and the torso protein tyrosine kinase receptors, respectively (3, 18, 20, 96). Finally, in *Caenorhabditis elegans* the Raf homolog (Lin45) is involved in the transmission of an inductive signal(s) that elicits the migration and terminal differentiation of vulval precursor cells through the activation of homologs of the EGF receptor and Ras (Let-23 and Let-60, respectively) (28, 99).

In the case of mammalian cells a variety of experiments indicate that activated Ras mediates both mitogenic and differentiation signals (56). In thymocytes the MAP kinase pathway appears to be most closely associated with thymocyte differentiation and not cell proliferation per se (2). However, in PC12 cells the Ras pathway is required for both mitogenic and differentiation responses (56). In addition Ras has been shown to be required for the propagation of transformation signals from upstream oncogenes such as v-*src* (94). The observation that Ras mutations are common in human cancer and that Ras-transformed human tumor cells have a reduced requirement for serum growth factors further supports the hypothesis that Ras is a major mediator of proliferative signaling in mammalian cells (6). However, it is clear from the multistage nature of human cancer and from cell culture experiments involving cooperating oncogenes that expression of activated Ras is insufficient for oncogenic transformation (36, 41, 42). It seems likely, therefore, that some of the additional events required for Ras-induced oncogenic transformation may function by influencing the way in which a sustained Ras signal is interpreted within the cell, perhaps by converting what might normally be interpreted as a differentiation signal into a proliferation signal (36, 37).

Evidence for such a situation has been garnered from transgenic mouse model systems where activated Ras has been shown to elicit both cell proliferation and terminal differentiation, depending on the cell type. Expression of activated H-Ras under the control of the keratin 10 promoter results in increased differentiation leading to hyperkeratosis of the skin and forestomach. Benign papillomas appear only after subsequent secondary events (5). By contrast, transgenic expression of oncogenic Ras in the pancreas, liver, or epithelial cells of the Haderian gland results in increased cell proliferation (51, 80, 88).

It seems likely, therefore, that normal Ras and Raf signaling is involved in both proliferative and differentiative signaling. It appears that the appropriate biological response may be influenced by the ligand-receptor pair, the cell type, and the status of other signaling pathways within the cell. Such a model is consistent with previous observations in PC12 and in A431 cells and with the data presented here (24, 56).

Signaling thresholds for mitogenesis and differentiation. Considerable interest has focused on connections between signal transduction pathways and the cell cycle. In this report we have demonstrated that differential activation of Raf-1 in NIH 3T3 cells leads to distinct biological outcomes. The ability of low levels of Raf to induce the expression of cyclin D1 and cyclin E and to reduce the level of $p27^{Kip1}$ expression is entirely consistent with the recent description of a requirement for the RAS pathway to abrogate the effects of pRb in cells (62, 71). The observation that high-level activation of Raf causes cell cycle arrest suggests that these cells have a "mitogenic window" which, in NIH 3T3 cells, requires low-level activation of the Raf/MAP kinase pathway. The size of the mitogenic window may be influenced considerably by a variety of other factors that include, but are not limited to, the level of expression of other cyclins and CKIs, the level of expression of tumor suppressor proteins such as p53 and pRb, the presence of antiproliferative agents such as transforming growth factor β or synergizing growth factors, and the expression of proteins such as c-Myc that may influence the ability of a cell to pass through a particular cell cycle checkpoint. It appears that in NIH 3T3 cells a threshold for cell cycle arrest is defined by the amount of Raf activity required for the induction of $p21^{Cip1}$. It is interesting to note, however, that activation of c-Myc:ER^{TM} (48) overcomes Raf-induced cell cycle arrest (107d).

What is the significance of the ability of Ras and Raf to either promote or inhibit cell cycle progression, depending on the level of activation? It has been suggested that this may represent a cellular monitoring system for the presence of Ras mutations (90). Alternatively it may be a reflection of the fact that the Ras/Raf pathway serves double duty in both the cell proliferation and the cell differentiation pathways. The generation of mice and cell lines lacking the expression of components of the cell cycle machinery will facilitate the analysis of the role of these proteins in both normal and aberrant cell proliferation and differentiation (14, 15, 26, 43, 89).

Ultimately it will be of considerable interest to determine if parallel MAP kinase modules, such as those leading to JNK, SAPK, and $p38^{HOG1}$ activation, are capable of influencing cell cycle progression and what effect these pathways have on the cell cycle machinery (44). In addition a precise molecular understanding of how both normal and aberrant signal transduction pathways impinge on the cell cycle will be crucial to further attempts to understand the role of the Ras pathway in human tumorigenesis.

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