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High-Intensity Raf Signal Causes Cell Cycle Arrest Mediated by $p21^{Cip1}$

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Activated Raf has been linked to such opposing cellular responses as the induction of DNA synthesis and the inhibition of proliferation. However, it remains unclear how such a switch in signal specificity is regulated. We have addressed this question with a regulatable Raf-androgen receptor fusion protein in murine fibroblasts. We show that Raf can cause a G_1-specific cell cycle arrest through induction of $p21^{Cip1}$. This in turn leads to inhibition of cyclin D- and cyclin E-dependent kinases and an accumulation of hypophosphorylated Rb. Importantly, this behavior can be observed only in response to a strong Raf signal. In contrast, moderate Raf activity induces DNA synthesis and is sufficient to induce cyclin D expression. Therefore, Raf signal specificity can be determined by modulation of signal strength presumably through the induction of distinct protein expression patterns. Similar to induction of Raf, a strong induction of activated Ras via a tetracycline-dependent promoter also causes inhibition of proliferation and $p21^{Cip1}$ induction at high expression levels. Thus, $p21^{Cip1}$ plays a key role in determining cellular responses to Ras and Raf signalling. As predicted by this finding we show that Ras and loss of p21 cooperate to confer a proliferative advantage to mouse embryo fibroblasts.

The serine-threonine protein kinase Raf is an essential component of the signal transduction pathway leading to mitogen-activated protein (MAP) kinase activation by growth factors which work through Ras (for reviews see references 32 and 53). Raf is a direct downstream effector of Ras (58, 90, 91) and phosphorylates MAP kinase–extracellular signal-regulated kinase (ERK) (MEK) (10, 38), which in turn activates the p42 and p44 ERK kinases (1, 25, 37, 60), resulting in their translocation into the nucleus (8, 42). This cascade of events leads to the activation of transcription factors and eventually to cell proliferation (for reviews see references 52 and 88). Blocking endogenous *raf* mRNA expression with antisense constructs or dominant negative Raf mutants interferes with mitogen-induced proliferation of NIH 3T3 cells (30, 36, 77), while microinjection of bacterially expressed c-Raf-1 protein is sufficient to induce DNA synthesis in quiescent cells (81).

Raf was first discovered as the transforming principle of avian and murine retroviruses (2, 34). Oncogenic activation of Raf can be achieved by truncation of its N-terminal regulatory domain (2, 33) or by constitutive translocation of Raf to the plasma membrane by adding a farnesylation signal, such as the CAAX motif (41). Activated Raf proteins readily transform established fibroblast cell lines and can confer growth factor independence to avian and murine macrophages (6, 26). Moreover, similar to Ras (39, 73), Raf cooperates with other cellular and viral oncogenes, such as the Myc and simian virus 40 large T antigen genes, and has been shown to be transforming in the absence of p53 function in various cell types (3, 26, 46, 54).

In addition to the notion that Raf signalling plays a key role in the stimulation of cell proliferation, Raf also has been linked to growth inhibition and differentiation. In primary rat Schwann cells, oncogenic Raf (46) and Ras (72) lead to G_1-specific cell cycle arrest. In PC12 cells, Raf induces growth arrest followed by neurite outgrowth (94), and in transgenic mice expressing activated Raf in the thymus, T-cell differentiation is accelerated (63).

In NIH 3T3 murine fibroblasts Raf can induce DNA synthesis (68, 81) as well as block platelet-derived growth factor and epidermal growth factor (EGF)-induced DNA synthesis (75). However, these apparently conflicting reports may be reconciled, as experiments with a series of activated Raf family proteins (68) indicate that Raf proteins able to induce proliferation activate ERK kinases only weakly whereas Raf molecules strongly activating ERKs inhibit proliferation. We wanted to explore this possibility further and investigate the molecular events involved. Using a conditional, steroid hormone regulatable Raf protein, we have investigated the mechanism through which Raf inhibits cell cycle progression and show how Raf signalling can specify opposing effects on cell proliferation in the same cell.

MATERIALS AND METHODS

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal calf serum or newborn calf serum. R1881 (methyltrienolone, NLP-005; Dupont) and testosterone (Fluka 86500) were made up as 1 mM stock solutions in ethanol and stored at −20°C. Doxycycline hydrochloride (Sigma) was dissolved in water (0.1 mg ml^{-1}) and stored at 4°C.

Recombinant retroviruses. A *Hin*dIII-*Eco*RI fragment encoding amino acids (aa) 305 to 647 of the human c-Raf-1 protein fused to the 20 carboxy-terminal amino acids of k-Ras was amplified with the c-Raf-CAAX-encoding sequence (41) in pCDNA3 (Stratagene) as a template (primer 1, 5′TGTAAGC-TTACG GAGTACTCACAGCCGAA3′; primer 2, SP6). This fragment was fused to the fragment encoding the C terminus of the hormone binding domain of the human androgen receptor (aa 646 to 917), which was amplified by PCR (primer 3, 5′TGTGGATCCGCCA-CCATGACTGAGGAGACAACCCAGAAG3′; primer 4, 5′TGTAAGCTTGGCCG-CTGCCTGGGTGTGGAAATAGATGG G3′) and cloned in frame with a fragment encoding an amino-terminal Myc tag (9E10 epitope [22]) into the retroviral vectors pLNSX3 and pLPSX3, which represent modified versions of the vector pLNSX (55). The v-Ha-Ras^{V12} cDNA was subcloned into the retroviral vector pBabePuroSTR1 under the control of a tetracycline repressible promoter (64). Replication-defective retroviruses were produced as previously described (59) with GP+E producer cells (51). Producer cell supernatants were used to infect NIH 3T3 cells or murine embryo fibroblasts (MEFs) for 90 to 360 min in the presence of 8 μg of polybrene (Sigma) ml^{-1}. Sixteen hours later the cultures were split and selected in 1 mg of Geneticin (Life

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Technologies) ml^{-1} or 4 μg (1.5 μg for MEFs) of puromycin ml^{-1}. Cells infected with derivatives of pBabePuroSTR1 were selected in the presence of 0.1 μg of doxycycline ml^{-1}. Several hundred colonies were pooled to derive polyclonal populations. Individual clones of cells infected with pBabePuroSTR1 v-Ha-Ras^{V12} were ring cloned and expanded. Several clones showing induction of Ras upon removal of doxycycline were identified. For further analysis clone C18 was used. As a control NIH 3T3 cells were infected with a pBabePuroSTR1 derivative lacking an insert.

Preparation of cell extracts and Western blotting. Cells were scraped in phosphate-buffered saline (PBS) and centrifuged, and the pellet was resuspended in lysis buffer IPB (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM NaF, 0.5 mM Na_3VO_4, 0.5% Nonidet P-40, 10 μg of aprotinin ml^{-1}, and 100 μg of phenylmethylsulfonyl fluoride ml^{-1}). Protein concentration was measured by the Bio-Rad protein assay. Protein (30 to 50 μg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred (Genie Blot II; Idea Sci.) onto Immobilon P membranes (Millipore). The following antibodies were used: polyclonal rabbit antibodies against cyclin E (rat, sc-481; human, sc-198), cyclin H (sc-609), cdk2 (sc-163), cdk4 (sc-260), ERK2 (sc-153), $p21^{Cip1}$ (sc-757), $p27^{Kip1}$ (sc-528) (all Santa Cruz), cyclin D1 (G. Peters, ICRF, London, United Kingdom), and p42 ERK kinase (122/2 [40]) and monoclonal mouse antibodies against cyclin D1 (DCS-11 [49]), pRB (14001A; Pharmingen), ras (pan-ras [AB-4; Oncogene Science]), and the Myc tag (9E10 [22]). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (antirabbit and antimouse) and visualized with the enhanced chemiluminescence detection kit (Amersham International PLC).

Immunoprecipitation and kinase assays. Cell pellets were resuspended in IPB, vortexed, and left on ice for 5 min. For measurement of cyclin D1-associated kinase activity cells were resuspended in IPD (50 mM HEPES [pH 7.5], 10% glycerol, 150 mM NaCl, 10 mM NaF, 0.5 mM Na_3VO_4, 0.1% Tween 20) and sonicated for 10 s (25 W). After centrifugation the protein content was estimated and 50 to 300 μg of protein was used for immunoprecipitation. Cell lysates were incubated with the primary antibodies for 4 to 12 h at 4°C, and immunocomplexes were collected on protein A- or protein G-Sepharose beads for 1 h at 4°C. The complexes were washed four times with lysis buffer; for kinase assays two additional washes with kinase buffer (for cyclin E and cdk2, 50 mM Tris-Cl [pH 7.5]–10 mM $MgCl_2$; for cyclin D1 and cdk4, 50 mM HEPES [pH 7.5]–10 mM $MnCl_2$) followed. Kinase assays were performed in a final volume of 25 μl in the appropriate kinase buffer supplemented with 100 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 25 μg of histone H1 or 4 μg of GST-Rb (aa 300 to 928 [80]). The assays were incubated for 15 to 30 min at 30°C and analyzed on SDS–12.5% polyacrylamide gels.

Assay for p42 ERK kinase activity. p42 ERK kinase was immunoprecipitated from 100 μg of protein with a polyclonal antibody (122/2) specific for p42 ERK kinase for 2 h at 4°C. Immunocomplexes were collected with protein A-Sepharose 4B beads (Pharmacia), washed twice with IPB, and washed twice with kinase buffer (50 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol). The complexes were incubated for 20 min at 30°C in 25 μl of kinase buffer containing 100 μM ATP, 2 μCi of [γ-^{32}P]ATP, and 20 μg of myelin basic protein (MBP) as the substrate. The reactions were terminated by addition of 5× SDS sample buffer, and the reaction mixtures were analyzed by SDS–15% PAGE.

FACS and DNA synthesis analysis. Proliferation was measured with the SPA [^{3}H]thymidine uptake assay (Amersham), with [^{3}H]thymidine (0.2 μCi ml^{-1}) present in the medium for 2 h prior to harvesting. For fluorescence-activated cell sorter (FACS) analysis, cells were incubated with 50 μM bromodeoxyuridine (BrdU) for 2 h, trypsinized, and fixed in 70% ethanol (−20°C) for 30 min on ice. Detection of BrdU incorporation with an anti-BrdU-isothiocyanate conjugate (1202 693; Boehringer Mannheim) was carried out according to the manufacturer's protocol. Cells were stained for total DNA with 10 μg of propidium iodide ml^{-1} containing 20 μg of RNase H ml^{-1} and analyzed by bivariate flow cytometry.

RNA isolation and PCR analysis. RNA was isolated according to a modified method of Chirgwin et al. (9). cDNA synthesis and PCR reactions were carried out as previously described (79) with primers for L7 (P1, 5′ATTCGCATG GCTAGGATGGC3′; P2, 5′ACCAAGAGATCGAGCAACCA3′) (29) and p21 (P1, 5′GATGTCCGACCTGTTCCG-CA3′; P2, 5′CTCC-GTTTTCGGCCCTG AGA3′).

Transfection and luciferase assays. A full-length human p21 promoter-luciferase construct (21) was used to determine the response of the p21 promoter to activation of the MAP kinase pathway. NIH RafAR cells in 28-cm^2 (70 to 80% confluent) dishes were transfected with 3.5 μg of reporter plasmid and 2.5 μg of CH110 *lacZ* plasmid by a standard calcium phosphate transfection protocol. The precipitate was removed after 12 h, and after another 12 h the cells were treated with 0.5 μM R1881 for 24 h and the controls were treated with ethanol. PBS-washed cells were harvested in 250 mM Tris-Cl, pH 7.5, and lysed by three cycles of freeze thawing. After brief centrifugation, 40 μl of each supernatant was assayed in 350 μl of luciferase assay buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 5 mM ATP, 3 mM Luciferin [Sigma]) or β-galactosidase buffer (100 mM NaH_2PO_4 [pH 7.5], 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 4 μg of *o*-nitrophenyl-β-D-galactopyranoside [OPNG; Sigma] ml^{-1}) to determine luciferase and β-galactosidase activity. Luciferase activity was measured in a Luminometer (LKB) and the results were normalized for β-galactosidase activity.

In situ detection of β-galactosidase activity. Cells were washed with PBS and fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature. Staining solution (PBS, 3 mM $K_4Fe[CN]_6$, 3 mM $K_3Fe[CN]_6$, 2 mM $MgCl_2$, 2% X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Stratagene]) was added, and cells were incubated for 2 to 4 h at 37°C until a blue color developed.

RESULTS

Raf induces G_1-specific cell cycle arrest in fibroblasts. NIH 3T3 cells can serve as a simple model to explore how a specific cellular response to a Raf-dependent signal is determined, as Raf can both stimulate and inhibit the proliferation of these cells. The detailed biochemical analysis of such a process requires the use of a conditional Raf molecule. For this purpose we fused the hormone binding domain of the androgen receptor to the kinase domain of c-Raf-1. This chimera (RafAR) also contains an N-terminal Myc tag and a C-terminal CAAX motif supporting membrane localization (Fig. 1a). NIH 3T3 cells infected with a recombinant retrovirus encoding RafAR were selected and shown to express a protein of the expected size upon probing a Western blot with the Myc tag-specific monoclonal antibody 9E10 (22) (Fig. 1b). RafAR can be activated by androgens such as the synthetic testosterone analog R1881, which can be monitored by means of a specific and rapid increase in ERK kinase activity (Fig. 1c) and by means of morphological transformation (data not shown). Thus, the activity of RafAR can be regulated by steroid hormones in a fashion similar to the manner of regulation of the previously described Raf-estrogen receptor chimera ΔRaf-1:ER (76).

In proliferating NIH 3T3 cells activation of RafAR with 0.5 μM R1881 leads to an inhibition of DNA synthesis, while cells infected with an empty retrovirus are not affected by the hormone (Fig. 2a). FACS analysis shows that this inhibition of DNA synthesis corresponds to a G_1-specific cell cycle arrest (Fig. 2b). To determine the nature of the G_1 arrest we compared the expression levels and activities of G_1-specific cyclins and cyclin-dependent kinases as well as the levels of cdk inhibitors and the phosphorylation status of the Rb protein in RafAR-arrested and proliferating control cells in the presence of serum. In the arrested cells expression of cyclins D1 and E as well as the cell cycle inhibitor $p21^{Cip1}$ is induced, while the levels of cdk2, cdk4, and $p27^{Kip1}$ remain virtually unchanged (Fig. 2c). Similar to the Raf-induced G_1 arrest in Schwann cells (46), the increase in $p21^{Cip1}$ levels correlates with an inhibition of cyclin D1- and cyclin E-dependent kinase activities. In contrast, cyclin H-dependent kinase activity, which is not affected by $p21^{Cip1}$ (28), remains unchanged (Fig. 2d). At the same time Rb accumulates in its hypophosphorylated form (Fig. 2c).

In order to follow the kinetics of cyclin E, cyclin D1, and $p21^{Cip1}$ induction we monitored the respective protein levels at various times after hormone application (0.5 μM) to quiescent cell cultures in 0.1% serum. In parallel, the cells were stimulated with serum as a control. Under these conditions Raf activation is sufficient to induce cyclin D1 expression, which increases with kinetics similar to those induced by serum stimulation. In contrast, induction of cyclin E expression does not occur at low serum concentrations and can only be observed 8 h after serum stimulation. The induction of $p21^{Cip1}$ is specific to Raf activation and is evident already after 4 h of hormone stimulation (Fig. 3a). In these experiments only the cells stimulated with serum enter the S phase (data not shown).

The induction of $p21^{Cip1}$ is at least in part due to stimulation of $p21^{Cip1}$ transcription. Following hormone application the levels of $p21^{Cip1}$ mRNA increase with kinetics similar to those of the protein (Fig. 3b), although this induction is not an immediate early response, as it requires protein synthesis (data not shown). In agreement with similar experiments with HeLa

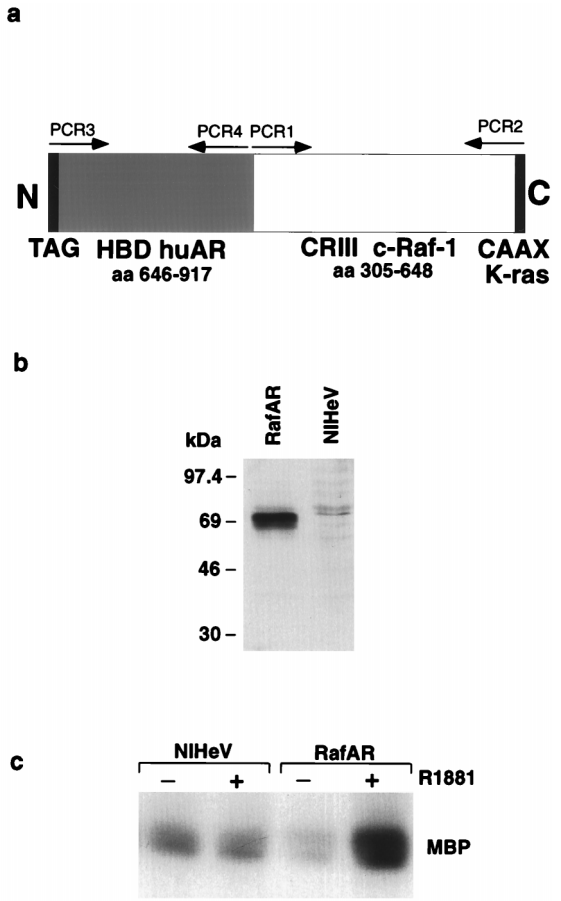

FIG. 1. Construction and characterization of TARΔ305RC (RafAR). (a) PCR-amplified fragments encoding the kinase domain of c-Raf-1 (CRIII c-Raf-1, aa 305 to 648) fused to the CAAX motif from k-Ras (K-ras, aa 169 to 188) and the hormone binding domain of the human androgen receptor (HBD huAR, aa 646 to 917) were cloned, in frame with an amino-terminal 9E10 Myc epitope (TAG), into the replication-defective retroviral vector pLNSX3 (see Materials and Methods). PCR1, -2, -3, and -4 indicate the locations of the primers used for PCR amplification. (b) Expression of RafAR in NIH 3T3 cells infected with RafAR- and empty vector (NIHeV)-expressing retroviruses. Protein (50 μg) was separated on SDS–10% polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and probed with a monoclonal antibody directed against the Myc tag. (c) Activation of p42 MAP kinase in NIH 3T3 cells infected with RafAR or empty vector (NIHeV). Confluent cells were kept in 0.1% serum for 48 h and stimulated with 0.01% ethanol (−) or 0.5 μM R1881 (+) for 30 min. p42 ERK kinase was immunoprecipitated from 100 μg of protein. Immunocomplexes were collected on protein A beads and assayed for kinase activity with MBP as the substrate. Reactions were stopped by adding SDS sample buffer, and the proteins were separated on SDS–15% polyacrylamide gels.

and PC12 cells (44), the activity of a transiently transfected $p21^{Cip1}$ promoter-luciferase reporter construct (21) is stimulated by Raf (Fig. 3c). Furthermore, we have not been able to detect any changes in $p21^{Cip1}$ half-life in experiments using inhibitors of protein synthesis following Raf activation (data not shown), although an increased half-life of $p21^{Cip1}$ has been observed in human fibrosarcoma cells, in which C/EBPα can induce a $p21^{Cip1}$-dependent cell cycle arrest (85).

$p21^{Cip1}$ induction is essential for Raf-dependent arrest. The fact that cells show increased levels of cyclin D1 and E but low associated kinase activity after activation of Raf raises the possibility that the induction of $p21^{Cip1}$ expression may be causally involved in the observed G_1 arrest. In order to test this possibility we analyzed the association of $p21^{Cip1}$ with cdk4 and cdk2 complexes in proliferating and Raf-arrested cell cultures 18 h after exposure of the cells to the hormone. Consistent with the elevation in $p21^{Cip1}$ levels, we found an increase in the levels of $p21^{Cip1}$ coimmunoprecipitated with both cdk4- and cdk2-containing complexes (Fig. 4a). However, as both cyclin D1 and cyclin E levels are also increased under these conditions (Fig. 2c), we wanted to address whether the Raf-induced $p21^{Cip1}$ levels are sufficient to inhibit cdk activity. For this purpose we mixed extracts from proliferating Rat1 cells expressing human cyclin E (66) with native extracts from RafAR-expressing cells before and after hormone application and used an antibody specific for human cyclin E to measure the associated kinase activity. The extracts from arrested cells were able to suppress the kinase activity, while those from proliferating cells were unable to do so. Importantly, the inhibition seen with the extract from RafAR-arrested cells was due to $p21^{Cip1}$, as depletion of the extract with $p21^{Cip1}$-specific antibodies prior to mixing with the target extract virtually eliminated the inhibitory activity (Fig. 4b and c). These results are consistent with the idea that $p21^{Cip1}$ induction is causally involved in Raf-dependent G_1 arrest.

We next wanted to confirm the role of $p21^{Cip1}$ with a genetic approach. At the same time we wanted to explore the role of p53 in this context, as the induction of $p21^{Cip1}$ can be dependent on p53 (20, 46, 50), and to assess the relevance of cyclin D1 in Raf-induced cell cycle arrest. Cyclin D1 is also dramatically induced by Raf and has been associated with growth inhibition (14) and cellular senescence (19, 48). We therefore compared the effects of RafAR on the proliferation of MEFs from wild-type mice to its effects on the proliferation of MEFs from mice in which the $p21^{Cip1}$ (4), cyclin D1 (23), or p53 (45) gene is ablated by homologous recombination. For this purpose low-passage cell populations were infected with a recombinant retrovirus and selected in the absence of Raf activity. The effect of RafAR activation was then monitored in polyclonal cell populations. While activation of RafAR led to an inhibition of DNA synthesis in cells from wild-type, $p53^{-/-}$, and cyclin $D1^{-/-}$ mice, the proliferation of $p21^{-/-}$ cells was not affected by RafAR activation (Fig. 5a). Compared to our results for NIH 3T3 cells the inhibition of DNA synthesis in MEFs is not dramatic. It is likely that this is due to lower efficiency of virus expression in these cells. Nevertheless, similar results have been obtained consistently in several independent experiments. As a control we monitored the induction of cyclin D1 or $p21^{Cip1}$ expression in response to Raf activation in the three cell populations. As in the NIH 3T3 cells, Raf activation induced cyclin D1 and $p21^{Cip1}$ levels in MEFs. These results show that RafAR-dependent induction of $p21^{Cip1}$ does not require the presence of p53 in murine fibroblasts. As expected, cyclin D1 and $p21^{Cip1}$ are not expressed in the respective null cells, while cyclin D1 and $p21^{Cip1}$ inductions are intact in $p21^{-/-}$ and cyclin $D1^{-/-}$ cells, respectively (Fig. 5b). In agreement with the results mentioned above, Raf activation in $p21^{-/-}$ cells does not lead to inhibition of cyclin E-dependent kinase activity, while in $p53^{-/-}$ cells this effect is clearly detectable (Fig. 5c). Thus, $p21^{Cip1}$ induction is required for Raf-dependent inhibition of proliferation. In addition, a major role of cyclin D1 in this process appears unlikely, since cyclin D1 induction in $p21^{-/-}$ cells remains unaltered and Raf is able to inhibit the proliferation of cyclin $D1^{-/-}$ cells.

Signal strength determines cellular response to Raf and Ras. The results described above apparently contradict the finding that activated Raf can transform NIH 3T3 cells and is involved in mitogenic signalling. How can such opposing cellular behavior in response to Raf be explained? Recently Pritchard and colleagues (68) observed that the ability of various

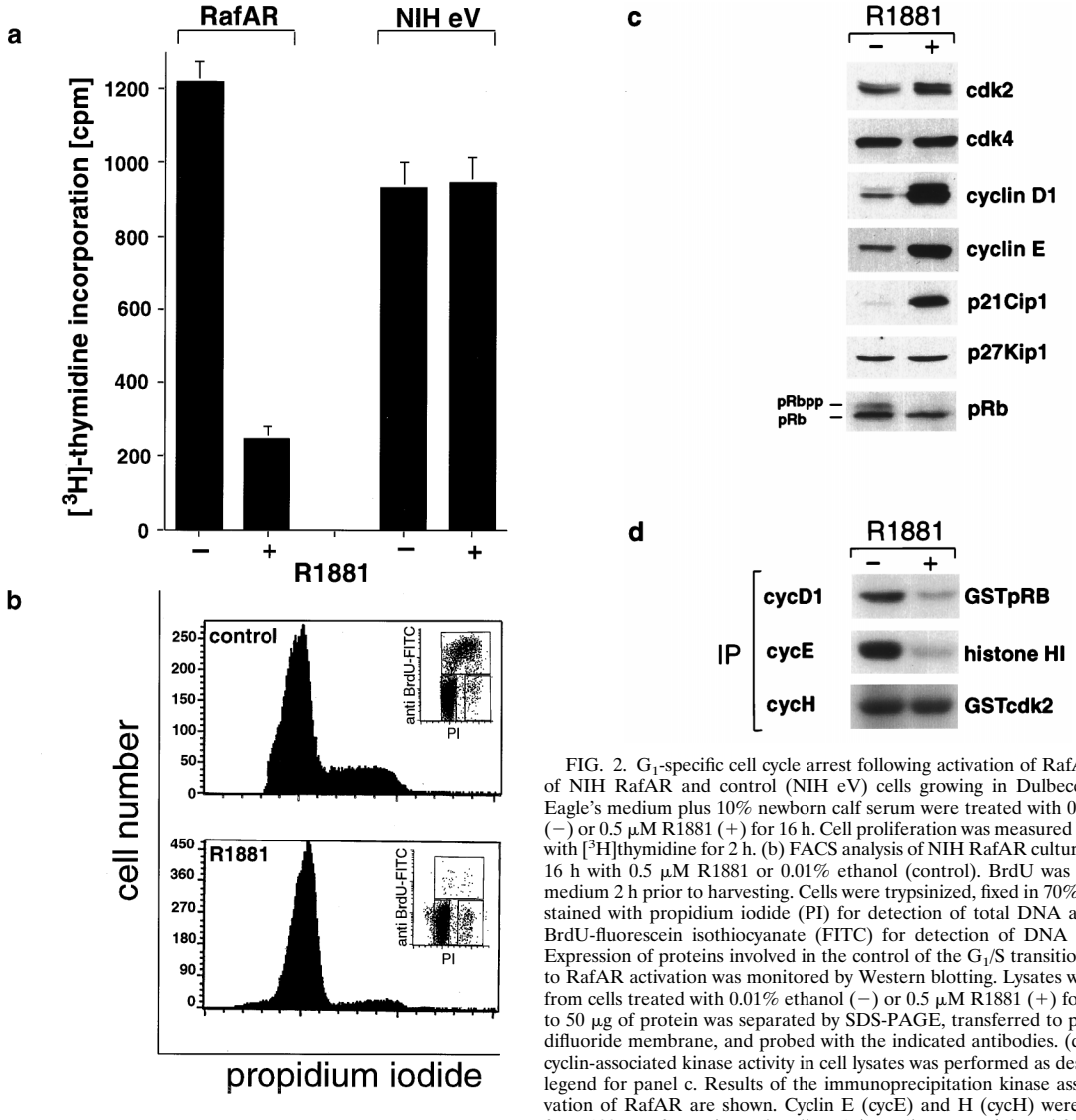

FIG. 2. G_1-specific cell cycle arrest following activation of RafAR. (a) Pools of NIH RafAR and control (NIH eV) cells growing in Dulbecco's modified Eagle's medium plus 10% newborn calf serum were treated with 0.01% ethanol (−) or 0.5 μM R1881 (+) for 16 h. Cell proliferation was measured by incubation with [^{3}H]thymidine for 2 h. (b) FACS analysis of NIH RafAR cultures treated for 16 h with 0.5 μM R1881 or 0.01% ethanol (control). BrdU was added to the medium 2 h prior to harvesting. Cells were trypsinized, fixed in 70% ethanol, and stained with propidium iodide (PI) for detection of total DNA and with anti-BrdU-fluorescein isothiocyanate (FITC) for detection of DNA synthesis. (c) Expression of proteins involved in the control of the G_1/S transition in response to RafAR activation was monitored by Western blotting. Lysates were prepared from cells treated with 0.01% ethanol (−) or 0.5 μM R1881 (+) for 16 h and 30 to 50 μg of protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the indicated antibodies. (d) Analysis of cyclin-associated kinase activity in cell lysates was performed as described in the legend for panel c. Results of the immunoprecipitation kinase assay after activation of RafAR are shown. Cyclin E (cycE) and H (cycH) were precipitated from 100 μg of protein, and cyclin D1 (cycD1) was precipitated from 300 μg of protein. Immunocomplexes were collected on protein A-Sepharose beads (cyclin D1-protein G), and the associated kinase activity was analyzed with either histone H1 (cyclin E), GST-cdk2 (cyclin H), or GST-pRB (cyclin D1) as the substrate. IP, immunoprecipitates.

Raf family members and mutant RafER proteins to inhibit DNA synthesis correlates well with their ability to activate ERK kinases: Raf proteins that are able to induce proliferation activate ERK kinase only weakly. We therefore tested whether differences in Raf signal intensity may be responsible for the opposing effects on cell cycle progression. For this purpose we stimulated cells expressing RafAR with different concentrations of testosterone, which has a lower affinity to the androgen receptor than R1881 (35). Raf activity was monitored at the level of ERK kinase activation and was found to be dependent on the testosterone concentration (Fig. 6a). Remarkably, the ability of Raf to induce DNA synthesis is restricted to a sharp optimum at a concentration of 10 nM testosterone (Fig. 6b), which leads to a moderate activation of p42 ERK kinase and induction of cyclin D1 (Fig. 6a). In contrast, induction of $p21^{Cip1}$ and inhibition of proliferation require high testosterone concentrations (Fig. 6). Thus, the amplitude of the Raf signal can direct distinct patterns of protein expression and determine whether cells will initiate DNA synthesis or arrest in the G_1 phase of the cell cycle.

In order to test whether $p21^{Cip1}$-dependent growth arrest upon activation of RafAR may be specific for this and other related Raf-steroid hormone fusion proteins (68, 75), or whether similar effects may be induced by other components of the Ras/Raf MAP kinase pathway, we tested the cellular response to an activated Ras protein with an inducible expression system. The Harvey virus *ras* gene was inserted into the retrovirus pBabe Puro STR1, which harbors both the tetracycline repressor (fused to VP16) and a tetracycline-sensitive promoter (64). NIH 3T3 cells were infected with this virus, and clones in which the level of expression of the v-Ha-Ras^{V12} protein is inversely proportional to the concentration of doxycycline in the culture medium were isolated (for an example see Fig. 7a). Upon removal of doxycycline such clones become

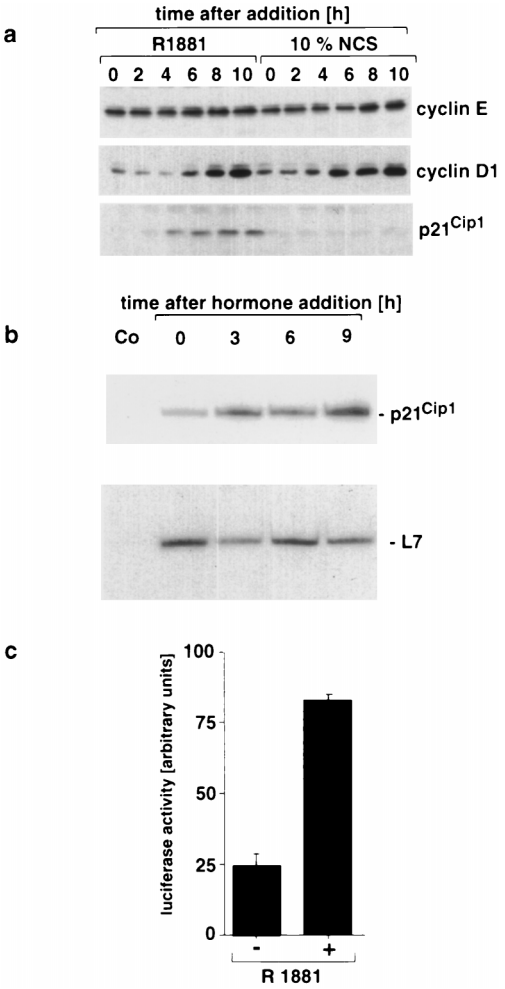

FIG. 3. Induction of $p21^{Cip1}$ and cyclin D1 after activation of RafAR in quiescent cells. (a) Confluent NIH RafAR cells were kept in 0.1% serum for 48 h and stimulated with 0.5 μM R1881 or 10% newborn calf serum (FCS). At the indicated times cell lysates were prepared, and 30 μg of total protein was separated on SDS–12.5% polyacrylamide gels, transferred onto polyvinylidene difluoride membrane, and probed with antibodies to cyclin E, cyclin D1, and $p21^{Cip1}$. (b) PCR analysis of $p21^{Cip1}$ mRNA expression. Serum-starved NIH RafAR cells were treated with 0.5 μM R1881. At the indicated times cells were harvested, total RNA was isolated, and expression of $p21^{Cip1}$ and the ribosomal protein L7, as a control (Co), was analyzed by reverse transcription-PCR. Five micrograms of RNA was used for cDNA synthesis in a total volume of 25 μl. One microliter of cDNA was used for the PCRs amplifying L7 (18 cycles) and $p21^{Cip1}$ (21 cycles). (c) $p21^{Cip1}$ promoter activity. NIH RafAR cells were transfected with a $p21^{Cip1}$ promoter-luciferase construct. Twelve hours after the removal of the DNA-$CaPO_4$ precipitate, cells were stimulated with 0.01% ethanol (−) or 1 μM R1881 (+), and cell lysates were prepared by three cycles of freeze thawing and assayed for β-galactosidase and luciferase activity.

morphologically transformed (data not shown). Similar to RafAR, high levels of Ras are able to induce $p21^{Cip1}$ expression (Fig. 7a). Thus, induction of $p21^{Cip1}$ appears to be a consequence of strong activation of the Ras/Raf pathway in general. Moreover, when the effect of Ras induction is monitored in proliferating cells, we observe an inhibition of DNA synthesis which tightly correlates with the increase in $p21^{Cip1}$ expression (Fig. 7b). In contrast, Ras induction in confluent cultures in the absence of serum leads to S phase entry (data not shown). Thus, similar to RafAR, Ras signals can cause induction and inhibition of DNA synthesis in NIH 3T3 cells.

Ras confers proliferative advantage to $p21^{Cip1-/-}$ cells. One obvious question arising from these observations is the relevance of the link between the Ras/Raf pathway and $p21^{Cip1}$ for cell transformation and tumorigenesis. Given our data one should predict that the transformation induced by Ras and Raf oncogenes should be affected by the levels of $p21^{Cip1}$ expression. Although, as shown above, RafAR activation does not inhibit the growth of early-passage $p21^{Cip1-/-}$ cells, RafAR is

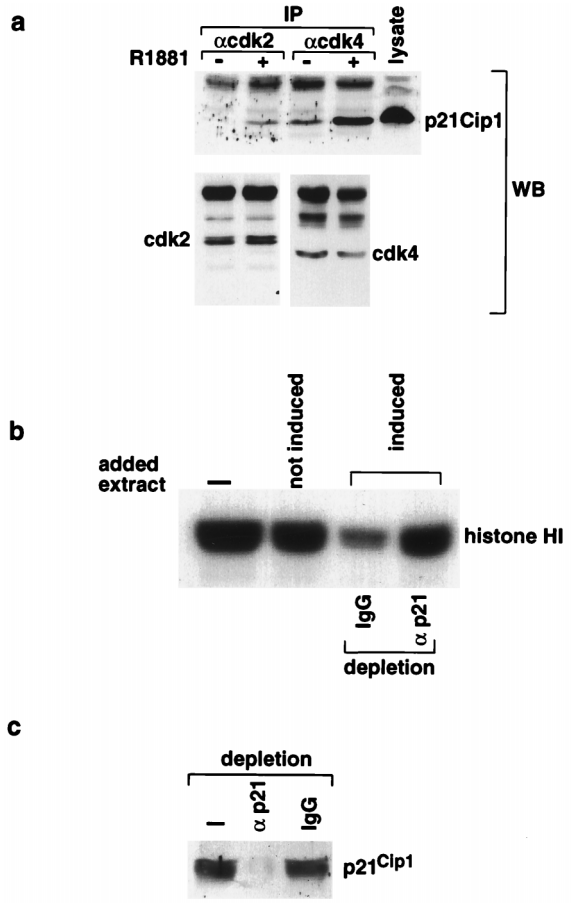

FIG. 4. Binding and inhibition of cyclin-cdk complexes by $p21^{Cip1}$ in response to RafAR activation. (a) cdk2 and cdk4 were immunoprecipitated from NIH RafAR cells growing in Dulbecco's modified Eagle's medium plus 10% newborn calf serum and treated with 0.01% ethanol (−) or 0.5 μM R1881 (+) for 16 h. A total of 250 μg of protein was used to immunoprecipitate cdk2 and cdk4. Immunocomplexes were collected on protein A-Sepharose beads, boiled in SDS sample buffer, separated on SDS–12.5% polyacrylamide gels, transferred onto polyvinylidene difluoride membrane, and analyzed for cdk2- and cdk4-associated $p21^{Cip1}$ as well as for the amounts of precipitated cdk2 and cdk4. IP, immunoprecipitates; WB, Western blots. (b) $p21^{Cip1}$ causes inhibition of cyclin E-associated kinase activity. The inhibitory activity of lysates before and after RafAR activation was tested with human cyclin E-cdk2 complexes expressed in Rat1 cells (66) as a target. Lysates were prepared from NIH RafAR cells treated with ethanol (not induced) or 0.5 μM R1881 (induced) for 24 h. Samples of the lysate from cells treated with R1881 were immunodepleted either with an antibody to $p21^{Cip1}$ (αp21) or with unrelated immunoglobulin G (IgG) (2 μg). Target extract (10 μg) containing human cyclin E-cdk2 complexes was mixed with 25 μg of extract either from control cells (not induced) or from cells treated with R1881 depleted with IgG or αp21. As a control, target extract alone was used (−). The mixed extracts were incubated at 20°C for 30 min, cyclin E-cdk2 complexes were immunoprecipitated with an antibody specific for human cyclin E, and kinase activity was analyzed as described previously. (c) Analysis of $p21^{Cip1}$ levels in the lysates used for the experiment described in the legend to panel b. Lysates of cells after activation of RafAR immunodepleted with antibodies to $p21^{Cip1}$ (αp21) and unrelated IgG and before depletion (−) were analyzed by Western blotting with an antibody against $p21^{Cip1}$.

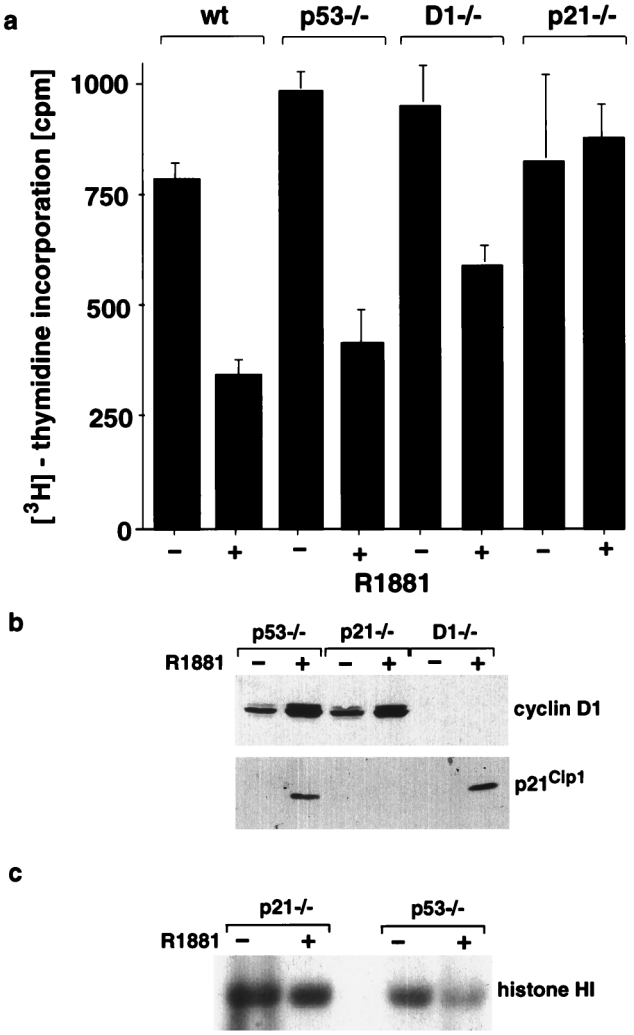

FIG. 5. Analysis of RafAR function in knockout MEFs. wt MEFs lacking either p53 ($p53^{-/-}$), $p21^{Cip1}$ ($p21^{-/-}$), or cyclin D1 ($D1^{-/-}$) were infected with an RafAR-expressing virus. Pools of puromycin-resistant cells growing in 10% fetal calf serum were treated with ethanol (−) or 0.5 μM R1881 for 16 h (+). (a) Growth inhibition after activation of RafAR assayed by incorporation of [^{3}H]thymidine. (b) Induction of $p21^{Cip1}$ and cyclin D1 after hormone treatment. Lysates of knockout MEFs were analyzed by Western blotting for expression of $p21^{Cip1}$ and cyclin D1. (c) Cyclin E-associated kinase activity in $p53^{-/-}$ and $p21^{Cip1-/-}$ cells. Immunoprecipitation kinase assays were performed as described previously.

unable to induce morphological transformation and focus formation in these cells (data not shown). Conversely, infection of wild-type and $p21^{Cip1-/-}$ cells with the same stock of a recombinant retrovirus expressing activated Ras (Zip-ras/neo) (18) showed a more than 10-fold induction of focus formation (43 and 572 macroscopically detectable foci/10^5 cells exposed to virus, respectively). However, because of the variable and generally small clonal expansion of MEFs, the frequency of viral infection could not be appropriately monitored in this experiment. Therefore, we carried out a similar infection with a virus expressing the v-Ha-*ras* gene together with β-galactosidase (Zip-*ras*/β*gal*) (84). The cell cultures were split 16 h later and the respective infection frequencies were determined by β-galactosidase staining of parallel cultures after a further 24 h. We found that the susceptibilities to retroviral infection of wild-type and $p21^{Cip1-/-}$ MEFs were similar, as 22.4 and 26.9% of the cells were β-galactosidase positive, respectively. However, when we monitored the proportion of the infected cells again after a further 4 days we noted that the frequency of Zip-*ras*/β*gal*-infected cells within the $p21^{Cip1-/-}$ cell population had expanded (51.3%) while their frequency within the wild-type population had decreased (7.3%) (see also Fig. 8). Thus, the loss of $p21^{Cip1}$ provides cells expressing an activated Ras oncoprotein with a selective proliferative advantage. This indicates that the link between Ras and Raf signalling and $p21^{Cip1}$ expression can also play an important role in determining the cellular response to Ras oncogenes. As such, the loss of $p21^{Cip1}$ cooperates with activated Ras in cell transformation.

DISCUSSION

Mechanism of Raf-dependent cell cycle arrest. We have demonstrated genetically and biochemically that highly active Raf kinase induces cell cycle arrest through the induction of the cell cycle inhibitor $p21^{Cip1}$ (see also reference 95). The induction of $p21^{Cip1}$ by Raf in NIH 3T3 cells occurs at least in part at the level of transcription, and binding of $p21^{Cip1}$ to cdk4 and cdk2 complexes inhibits cyclin D- and E-dependent kinase

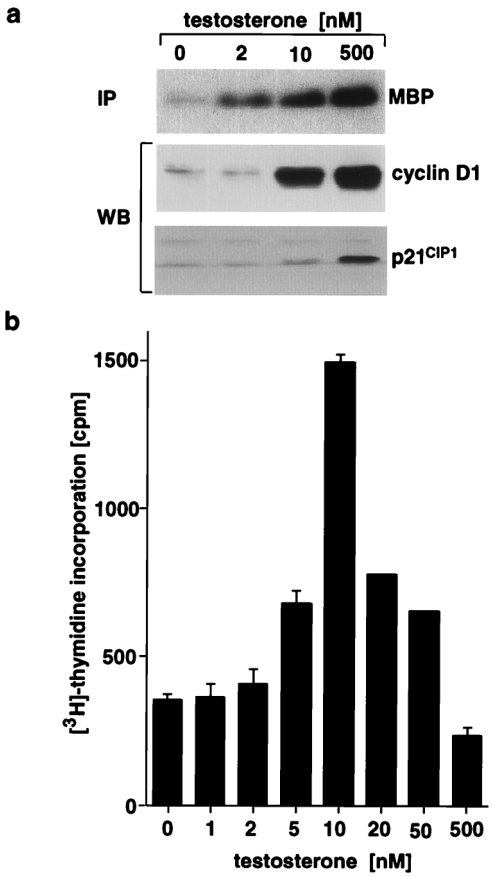

FIG. 6. Titration of RafAR activity. (a) Confluent cultures of NIH RafAR cells were kept in 0.1% serum for 48 h. Thereafter, cells were stimulated with the indicated concentrations of testosterone, and lysates were prepared 16 h after hormone addition. p42 ERK kinase activity (with MBP as the substrate) as well as $p21^{Cip1}$ and cyclin D1 protein expression was measured as described before. IP, immunoprecipitates; WB, Western blots. (b) Induction of DNA synthesis by RafAR. Confluent, serum-starved cultures of NIH RafAR cells were treated with different amounts of testosterone, and DNA synthesis was measured by [^{3}H]thymidine incorporation 16 to 18 h after hormone addition.

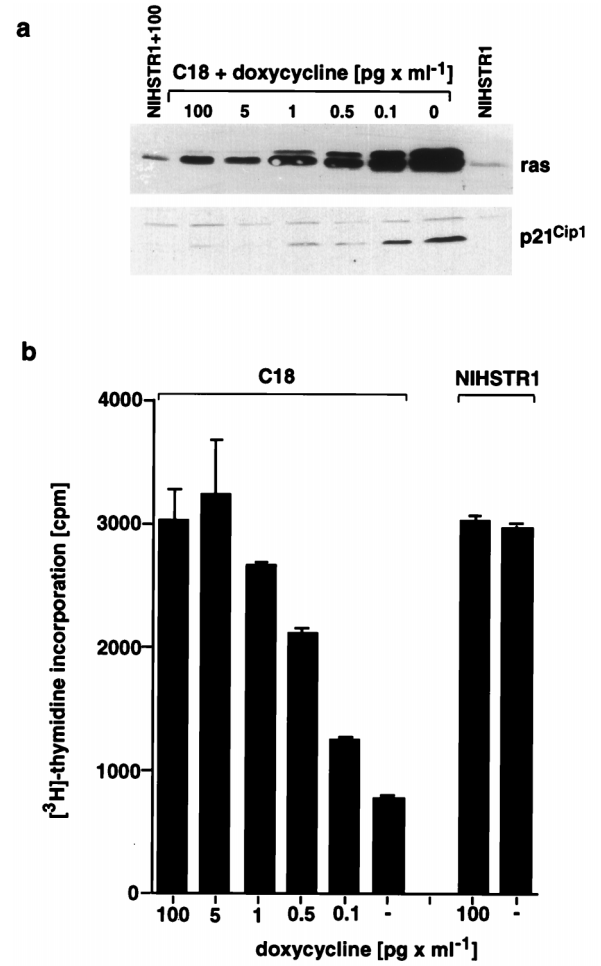

FIG. 7. Growth inhibition by v-Ha-Ras^{V12}. NIH-C18 (C18) or control cells (NIHSTR1) were grown in Dulbecco's modified Eagle's medium plus 10% newborn calf serum in the presence of the indicated amounts of doxycycline for 60 h. (a) Expression of v-Ha-Ras^{V12} and p21^{Cip1} was analyzed by Western blotting. (b) DNA synthesis was measured by [^{3}H]thymidine incorporation.

activities followed by G_1 arrest. p21^{Cip1} induction via activation of neuronal growth factor (NGF) receptors, Ras, or Raf followed by growth arrest has recently been observed in several cell types (13, 46, 78, 89) and depends on MEK activity in at least NIH 3T3 (69) and Schwann cells (61a). Moreover, pheromone-mediated G_1 arrest in *Saccharomyces cerevisiae* is executed via a parallel mechanism involving the ERK homolog Fus3, which regulates the Cln-Cdc28 kinase inhibitor Far1 via phosphorylation as well as transcriptional activation (7, 62, 67), suggesting general involvement of the ERK kinase pathway in negative cell cycle control.

In mammalian cells the Ras/Raf signalling pathway is utilized for multiple purposes. It plays an essential role in the induction of proliferation, cell cycle arrest, and differentiation (for reviews see references 47 and 53). For example, in PC12 cells activated Ras, Raf, and MEK or the application of NGF induces inhibition of proliferation and neurite outgrowth (10, 61, 94). In contrast, EGF and insulin support cell division (11, 31). Yet, in response to elevated EGF or insulin signalling mediated by an increased number of EGF or insulin receptors, PC12 cells begin to form neurites (17, 87). Since EGF stimulation induces a transient activation of ERK, while NGF causes prolonged ERK activity (86), the specificity of ERK signalling appears to be modulated by the duration and/or strength of MEK activity (10), although an involvement of distinct signalling pathways affected by EGF or NGF cannot be excluded. As in PC12 cells, myoblasts, or preadipocytes, differentiation is often coupled with proliferation arrest accompanied by p21^{Cip1} induction (27, 85, 89), although the latter appears to be involved in regulation of the arrest process and not required for the differentiation process per se (82, 89).

Raf signal specificity is determined by signal intensity. Using a regulatable Raf kinase, we provide direct evidence that modulation of Raf signal strength is sufficient to determine the specificity of the cellular response in NIH 3T3 cells. Moderate activation of RafAR, at low testosterone concentrations, leads to stimulation of DNA synthesis. In contrast, strong activation of Raf causes cell cycle arrest. The switch in signal specificity appears to be due to the fact that distinct signal quantities establish different patterns of gene expression. The p21^{Cip1} induction responsible for cell cycle arrest requires high Raf activity. In contrast, cyclin D1 expression, which is also induced by the NGF receptor, Ras, or Raf (43, 46, 89, 93), is sensitive to moderate as well as high Raf activity.

The induction of cyclin D1 by Raf does not play a significant role in establishing the Raf-dependent cell cycle arrest, as following Raf activation the proliferation of embryo fibroblasts derived from cyclin D1$^{-/-}$ mice is inhibited. Although high levels of cyclin D1 have been found in senescent fibroblasts (19, 48) and cyclin D1 has been associated with growth inhibition (14), induction of cyclin D1 is also known to accelerate the entry of cells into the S phase (70, 71). Moreover, the ability of neutralizing Ras antibodies or the dominant negative mutant RasASN17 to inhibit DNA synthesis is dependent on functional Rb, indicating that Rb phosphorylation is an important functional target of Ras signalling (57, 65). In NIH 3T3 cells this regulation involves Ras-dependent modulation of cyclin D1 levels, since in cells expressing RasASN17 cyclin D1 levels are low and the G_1 arrest can be blocked by expression of exogenous cyclin D1-cdk4 complexes (65). It thus appears that cyclin D1 induction in response to Ras (43, 93) or Raf (46) is part of a Ras- or Raf-dependent signal stimulating cell cycle progression.

Our results are consistent with a model (Fig. 9) in which Raf can activate a growth promoting and a growth inhibitory signal. The growth promoting signal, i.e., cyclin D1 activation, is activated at any significant signal intensity. In contrast, the induction of p21^{Cip1} requires high Raf signal activity. In the presence of high levels of p21^{Cip1}, cyclin D-cdk4 and cyclin E-cdk2 complexes are inhibited and p21^{Cip1} appears dominant over other Raf signalling involved in promoting cell cycle progression. As a consequence Rb remains in its hypophosphorylated state and cells arrest in the G_1 phase of the cell cycle.

Although p53 is known to directly target the p21^{Cip1} gene (20, 21), the induction of p21^{Cip1} by Raf in fibroblasts is p53 independent. In early-passage MEFs from p53$^{-/-}$ mice, induction of p21^{Cip1} by Raf is still intact. Moreover, expression of a dominant negative mutant of human p53 (175H) in NIH 3T3-derived RafAR cells did not affect the ability of Raf to induce p21^{Cip1} levels (data not shown). There are a variety of examples for p53-independent regulation of the Cip1 gene (12, 50, 83). Nevertheless, p53 ablation in rat Schwann cells leads to the abrogation of Raf-induced p21^{Cip1} expression (46). Thus, the mechanism of p21^{Cip1} regulation by Raf appears to differ in various tissues.

Role of p21^{Cip1} in growth control. The regulation of p21^{Cip1} expression is pivotal for determining how cells respond to high Ras or Raf activity and thereby cooperates with Ras in cell transformation. Activated Ras confers a growth advantage to

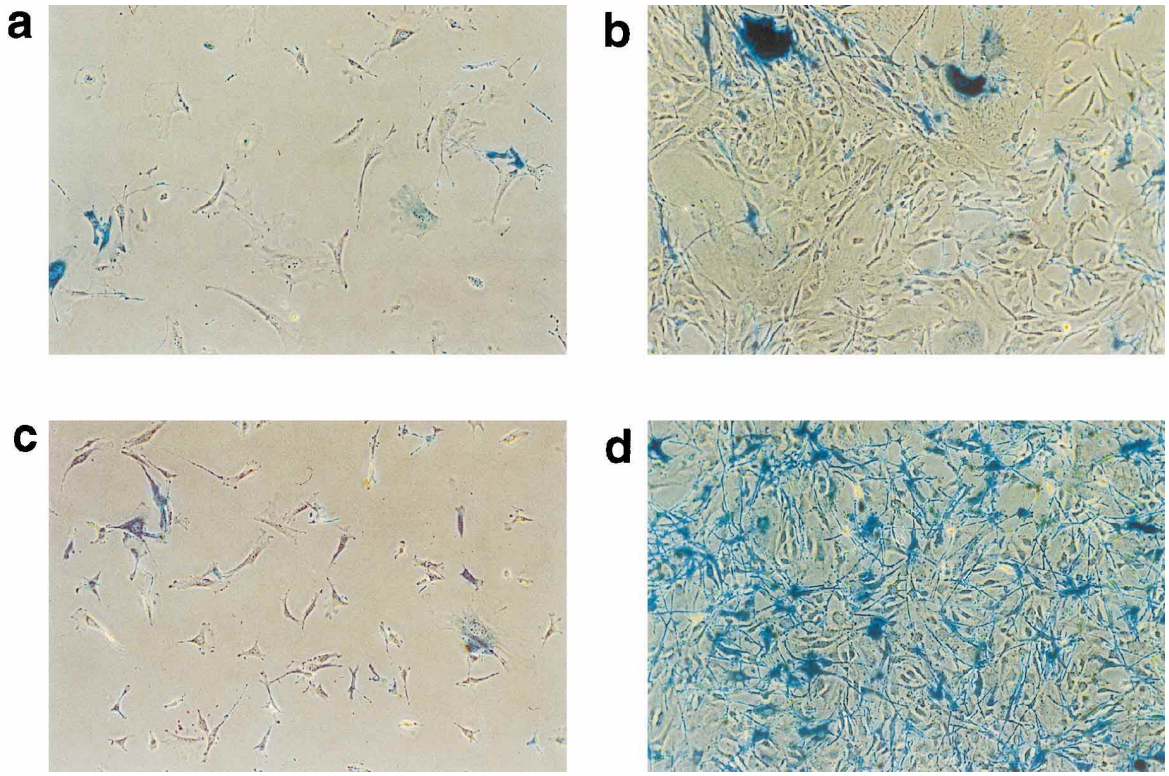

FIG. 8. Infection of wt (a and b) and $p21^{Cip1-/-}$ (c and d) MEFs with the *ras* and β-galactosidase-expressing retrovirus Zip-*ras*/β*gal*. Cells were fixed and stained for β-galactosidase activity 40 h (a and c) and 5.5 days (b and d) after infection.

$p21^{Cip1-/-}$ MEFs, while it has the opposite effect on wild-type cells (see also reference 78). Similarly, murine $p21^{Cip1-/-}$ keratinocytes become tumorigenic when expressing activated Ras, a property that is not shared by wild-type and $p27^{Kip1-/-}$ keratinocytes (56), although $p21^{Cip1-/-}$ mice appear not to exhibit an increased rate of spontaneous tumor incidence (15). The distinct behavior of $p21^{Cip1-/-}$ and $p27^{Kip1-/-}$ keratinocytes in response to activated Ras may in fact be due to the ability of Ras or Raf signalling to induce $p21^{Cip1}$ rather than $p27^{Kip1}$ expression, as seen in fibroblasts (Fig. 2) and Schwann cells (46). Overexpression of $p21^{Cip1}$ may also be involved in certain diseases. For example, in thanatophoric dysplasia type II dwarfism the constitutive tyrosine kinase activity of a mutant fibroblast growth factor receptor 3 leads to increased $p21^{Cip1}$ expression and growth arrest in the cartilage of affected individuals (96).

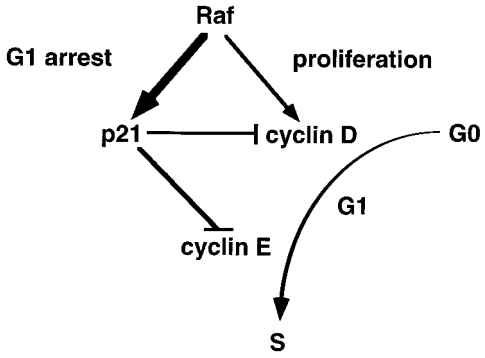

FIG. 9. Raf activation induces signals activating and inhibiting G_1/S progression. The specificity of the cellular response is determined by modulation of signal intensity, leading to distinct patterns of gene expression. Moderate activation of Raf (thin arrow) is sufficient to induce cyclin D1, which ultimately triggers Rb phosphorylation and S-phase entry. In contrast, strong activation of Raf (thick arrow) causes an arrest in G_1 via induction of $p21^{Cip1}$, which inhibits the activity of G_1-specific cyclin-cdk complexes.

There is precedence that differences in signal quantity cause distinct cellular responses during development. Gradients of transcription factor expression determine patterning in the early *Drosophila melanogaster* embryo through induction of distinct programs of gene expression along the body axis (for a review see reference 74). Moreover, Ras and Raf signalling is required for proliferation and for differentiation of many cell types in *Drosophila* (5, 16, 24, 92). It will be important to identify the essential downstream signalling components determining signal specificity and to explore which mechanisms control signal specificity switching during normal development. The latter may involve modulations of signal strength and cellular sensitivity towards signals and/or convergent signalling of different pathways (46). In addition, the fact that different signal intensities control distinct effectors predicts the existence of oncogene or tumor-specific signalling targets which may prove useful for therapeutic intervention.

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