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Raf-1-Induced Cell Cycle Arrest in LNCaP Human Prostate Cancer Cells

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Abstract Prostate cancer is the most commonly diagnosed neoplasm in men. LNCaP cells continue to possess many of the molecular characteristics of in situ prostate cancer. These cells lack ras mutations, and mitogen-activated protein kinase (MAPK) is not extensively phosphorylated in these cells. To determine the effects of ras/raf/MAPK pathway activation in these cells, we transfected LNCaP cells with an activatable form of c-raf-1(ΔRaf-1:ER). Activation of ΔRaf-1:ER, with resultant MAPK activation, reduced plating efficiency and soft agarose cloning efficiency 30-fold in LNCaP cells. Cell cycle distribution showed an accumulation of cells in G1 and was associated with the induction of CDK inhibitor $p21^{WAF1/CIP1}$ at the protein and mRNA levels. $p21^{WAF1/CIP1}$ mRNA stability was increased after ΔRaf-1:ER activation. In addition, activated ΔRaf-1:ER induced the senescence associated-β-galactosidase in LNCaP cells. These data demonstrate that raf activation can activate growth inhibitory pathways leading to growth suppression in prostate carcinoma cells and also suggest that raf/MEK/MAPK pathway activation, rather than inhibition, may be a therapeutic target for some human prostate cancer cells. J. Cell. Biochem. 72:458–469, 1999.

Key words: activated Raf; MAP kinase; cell cycle; $p21^{WAF1/CIP1}$; SA-β-galactosidase

Activation of the ras/raf signal transduction pathway has been shown to contribute to tumorigenicity in numerous types of cancer [Morrison and Cutler, 1997; Marshall, 1995; Seger and Krebs, 1995; Avruch et al., 1994; Williams and Roberts, 1994; Cowley et al., 1994]. However in some types of cancer, ras mutations are rarely seen. In these cancers, ras mutations may not confer a growth advantage. For some cancer types, this has been confirmed in cell culture systems; at least for pheochromocytoma, medullary thyroid carcinoma and small cell lung cancer cells, introduction of an activated ras gene resulted in cell differentiation, accompanied by slower cell growth [Cowley et al., 1994; Mabry et al., 1989; Nakagawa et al., 1987].

In prostate cancer, the effect of ras mutations is less clear. In Western men, ras mutations are rarely found, but these mutations are more common in Japanese men [Pergolizzi et al., 1993; Carter et al., 1990]. The effect of ras mutations in prostate cancer has been studied in vitro in LNCaP cells. LNCaP is an androgen-dependent prostate carcinoma cell line which maintains many of the molecular characteristics of in situ prostate cancer [Davies and Eaton, 1991; Issacs, 1987]. LNCaP cells possess a point mutation in its androgen receptor and this point mutation does not alter the ligand binding specificity of androgen receptor [Elo et al., 1995]. LNCaP cells lack ras mutations, and MAP kinase is not phosphorylated in these cells. While introduction of an activated ras oncogene into LNCaP cells did not alter cell growth, it did confer androgen independence. Recently, several reports have shown that activation of raf can result in growth arrest even in cells not arrested by ras activation [Kuo et al., 1996; Carson et al., 1995; Wood et al., 1993]. To determine the effects of raf/MAPK activation in

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prostate carcinoma cells, we transfected LNCaP cells with an activatable form of human raf-1 (ΔRaf-1:ER). In this ΔRaf-1:ER construct, raf kinase (amino acids 305–648) fused to the estrogen receptor hormone binding domain, is activatable by addition of estradiol [Samuels et al., 1993]. Activated ΔRaf-1:ER is transforming in many cell systems and can differentiate some cells [Kuo et al., 1996; Carson et al., 1995; Samuels et al., 1993; Wood et al., 1993]. We show here that activation of Raf-1 can suppress growth in LNCaP cells, with an accumulation of cells in G1. This G1 arrest is associated with the induction of CDK inhibitor $p21^{WAF1/CIP1}$ and a concomitant decrease of G1 cyclin-dependent kinase (CDK4 and CDK2) expression and activity, reducing the phosphorylation of pRb in these prostate cancer cells.

MATERIALS AND METHODS

Cell Culture and Cell Lines

LNCaP human prostate cancer cells were cultured in RPMI-1640 medium without phenol red, 9% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin sulfate (GIBCO, Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were infected with equal volumes of retroviral supernatant from PA317 producer cells transfected with the retroviral vector pLNC hRaf-1:ER, containing the activatable ΔRaf-1:ER fusion construct [Samuels et al., 1993]. Infection of cells was augmented by 2 µg/ml polybrene (Sigma Chemical Co., St. Louis, MO) in the medium. After 48 h, the medium was replaced by selection medium containing 0.5 mg/ml of G418. Pooled cultures of G418 resistant cells were grown, total RNA was extracted and analyzed for the presence of ΔRaf-1:ER by Northern blot analysis. LNCaP cells expressing ΔRaf-1:ER were treated with 1 µM β-estradiol to activate the ΔRaf-1:ER fusion molecule.

Soft Agar Cloning Assay

Soft agar cloning assays were performed in 35-mm dishes over a bottom layer of 0.8% low-melting agarose in growth medium and 1.5×10^4 cells were plated in growth media containing 0.4% (w/v) agarose in the presence or absence of 1 µM estradiol. After 3 weeks of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, colonies with >30 cells were scored, and percent cloning efficiency was calculated.

Cell Cycle Analysis

Cells were washed with ice-cold 0.2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and suspended in sucrose/citrate buffer [Vindelov et al., 1983]. Nuclei were prepared, stained with propidium iodide and analyzed by an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with a gate that selects single nuclei within a normal size range. The cell cycle parameters from 10,000 gated nuclei were determined by multicycle software (Phoenix Flow Systems, San Diego, CA). To measure BrdU incorporation, cells were pulse-labeled with 1 µM bromodeoxyuridine (BrdU, Sigma) for 40 min at 37°C and were fixed in 70% ethanol/PBS. Extracted nuclei were stained with FITC-labeled anti-BrdU antibodies (Becton Dickinson, San Jose, CA) and propidium iodide. Flow cytometry analysis was performed as described above.

Western Blotting

Cells were lysed in PBS, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 2.5 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). After protein concentrations were determined, 50–100 µg of proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA). Membranes were probed with appropriate dilutions of primary antibodies anti-Raf-1 (C-12), anti-pRb (IF8), anti-cyclin E (HE12), anti-cyclin B (GNS1), anti-cyclin A (H-432), anti-CDK2 (M2), anti-CDK4 (C-22), anti-CDK6 (C-21), anti-PCNA (PC10), anti-$p34^{cdc2}$ (C-17), and Actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-$p21^{WAF1}$ (Oncogene Science, Cambridge, MA); anti-MAPK (phospho-specific MAPK from New England Biolabs, Beverly, MA). Immunoreactive protein complexes were detected by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL).

Immunoprecipitations

Whole cell lysates were prepared in 50 mM Tris-HCl, pH 7.5, 137 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50 mM Na β-glycerophosphate, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml

leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF. A total of 300–500 μg of whole cell lysates were incubated for 2 h to overnight at 4°C with 1 μg/ml primary antibody. The immune complexes were immunoprecipitated with protein A-Sepharose beads (Pharmacia, Piscataway, NJ) for 1 h; the immune complexes bound to the beads were washed three times with same lysis buffer and twice with buffer containing 10 mM Tris-HCl, pH 7.5. The beads were boiled with 25 μl of 2× Laemmli buffer, resolved on SDS-PAGE gels and transferred to Immobilon-P PVDF membrane (Millipore). The membranes were Western blotted with the appropriate antibodies, and immunoreactive protein complexes were detected by ECL.

In Vitro Kinase Assays

Whole cell lysates were prepared as described above. A total of 100 μg of whole cell lysates was incubated for 2 h with 1 μg/ml anti-CDK2, anti-CDK4, anti-cyclin E, or anti-MAPK. The immune complexes were immunoprecipitated with protein A-Sepharose beads (Pharmacia) for 1 h; the immune complexes bound to the beads were washed twice with the same lysis buffer and three times with kinase buffer. Kinase assays were performed using histone H1 for CDK2 and cyclin E; GST-pRb (Santa Cruz Biotechnology, Santa Cruz, CA) for CDK4, and myelin basic protein (MBP) (Sigma) for MAPK. The immunoprecipitates and substrates were incubated in a volume of 40 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.125 μCi/μl γ-^{32}P ATP at 30°C for 20 min. Substrates for these kinase assays were either 20 μg histone H1 for CDK2, 5 μg of GST-pRb for CDK4, 10 μg MBP for MAPK assays. The reactions were stopped by the addition of 40 μl of 2× Laemmli buffer, boiled for 3 min and resolved on 12.5% SDS-PAGE gels and transferred to Immobilon-P PVDF membrane. Phosphorylated proteins were visualized by autoradiography and quantitated on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Northern Blotting

Total RNA was extracted with an acid phenol-guanidinium isothiocyanate method [Chomczynski and Sacchi, 1987]. Total RNA (20 μg/lane) was separated on 1.2% agarose/2.2 M formaldehyde-denaturing gels and transferred to Zeta-Probe (Bio-Rad, Hercules, CA). Probes used in Northern analysis were a 2.1-kb p21^{WAF1} cDNA insert (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University), and human β-actin, *Bam*H1 fragment. These probes were labeled with [α-^{32}P]-dCTP (Dupont, New England Nuclear, Boston, MA) by random primer labeling (Boehringer Mannheim). Hybridizations were done using radiolabeled probe (1×10^6 CPM/ml) at 42°C for 16–18 h, then rinsed twice at room temperature and washed once for 30 min at 65°C with 1× SSC and 1% SDS. Membranes were then exposed to x-ray film (Kodak X-0-MAT) at −80°C with intensifying screens. Autoradiograms were quantitated on a phosphorimager (Molecular Dynamics).

Nuclear Run-off Assay

Nuclei from control LNCaP cells and ΔRaf-1:ER activated LNCaP cells were isolated by lysing cells in lysis buffer containing 0.2% Nonidet-P (NP)-40, 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM DTT, and 0.3 M sucrose. The nuclear pellet was washed once in NP-40 lysis buffer and resuspended in 150 μl of storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA) containing 1×10^7 nuclei. The transcription reactions were performed as described by Celano et al. [1989]; 2 μg of denatured, cDNA inserts of p21^{WAF1}, GAPDH, and β-actin (denatured by heat and alkalization, 0.4 N NaOH) were immobilized onto a Zeta-probe membrane. Equal number of counts/ml of nascent RNA were added to hybridization buffer (0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA, and 1% BSA) and hybridized to these membranes for 24 h at 65°C. The membranes were washed as described above for Northern blotting. The signal intensity was quantitated using phosphorimager (Molecular Dynamics) and normalized relative to β-actin loading control.

Senescence-Associated β-Galactosidase

Cells were stained for senescence-associated β-Galactosidase (SA-β-Gal) as described by Dimri et al. [1995]. Cells were washed in PBS, fixed for 5 min (room temperature) in 2% formaldehyde, 0.2% glutaraldehyde in PBS, washed, and incubated with freshly made SA-β-Gal stain solution (1 mg/ml X-gal in dimethylformamide, 40 mM citric aid/Na phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride) overnight at 37°C.

RESULTS

ΔRaf-1:ER Activation Altered Morphology and Growth of LNCaP Cells

Pooled G418-resistant clones of LNCaP cells transfected with ΔRaf-1:ER were used for the study. Activation of ΔRaf-1:ER in LNCaP cells resulted in phosphorylation of raf-1 and sustained phosphorylation and activation of MAP kinases in these cells for ≥7 days after estradiol treatment (Fig. 1A,B). Since estradiol was used to activate the Raf fusion molecule, we examined LNCaP cells, LNCaP cells treated with estradiol, and ΔRaf-1:ER transfected cells without estradiol as controls. In all these control cells, MAPK phosphorylation was not seen (Fig. 1A). There were no morphological effects of transducing LNCaP cells with ΔRaf-1:ER, and estradiol treatment of the parental LNCaP cells had no effect on LNCaP cell morphology (Fig. 2A,B,C).

Upon addition of estradiol to LNCaP:ΔRaf-1:ER cells, the cells became rounded, and formed multinucleated giant cells (Fig. 2D). Growth of LNCaP cells after ΔRaf-1:ER activation was markedly diminished (28-fold) compared with their parental LNCaP cells (data not shown). To confirm this reduced growth, we measured the plating efficiency of LNCaP:ΔRaf-1:ER cells in either the presence or absence of 1 μM estradiol. The plating efficiency of LNCaP cells with activated ΔRaf-1:ER was decreased 30-fold compared with LNCaP:ΔRaf-1:ER cells without estradiol (Fig. 3A). We also measured the soft agarose cloning efficiency of the LNCaP parental cells and LNCaP:ΔRaf-1:ER cells in the presence and absence of estradiol. Parental LNCaP cells grew well in soft agarose in the presence or absence of 1 μM estradiol, and LNCaP:ΔRaf-1:ER also cloned well in soft agarose in the absence of estradiol. However, the soft agar cloning efficiency of LNCaP:ΔRaf-1:ER cells was reduced 40-fold upon activation of the ΔRaf-1:ER fusion protein by addition of estradiol (Fig. 3B). No effect of ΔRaf-1:ER activation was observed in other androgen-independent prostate cancer cells like DU145 and PC3, which already have active raf/MAP kinase pathway (R. Ravi, B. Nelkin, and M. Mabry, unpublished results). The proportion of apoptotic cells was increased 5–10% after ΔRaf-1:ER activation when compared with their parental control cells. Thus, Raf kinase activation strongly decreased both the plating and soft agarose cloning efficiency of human LNCaP prostate cancer cells.

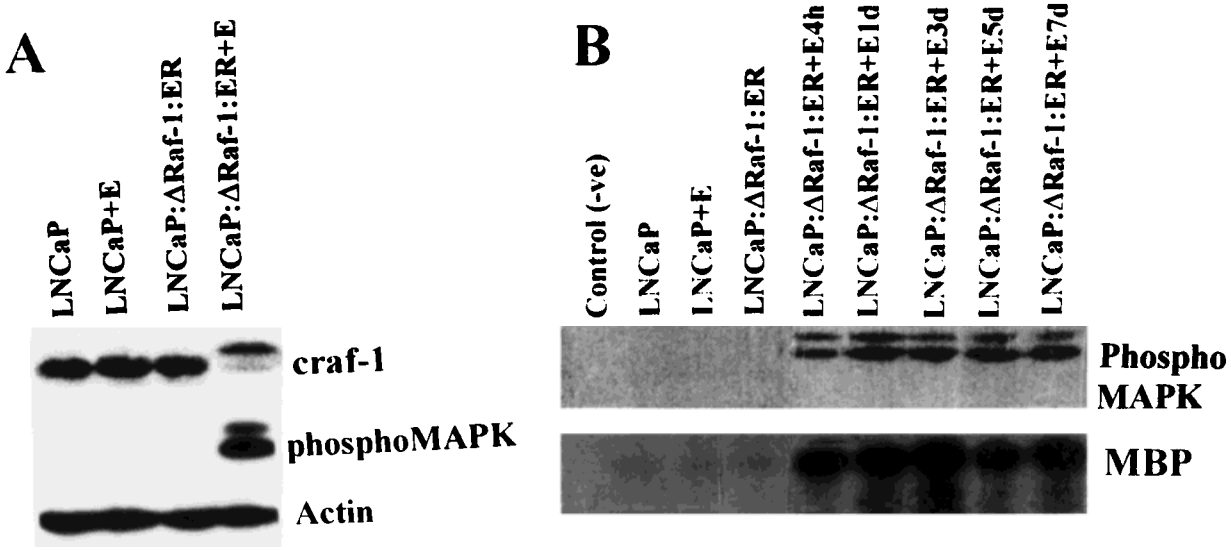

Fig. 1. ΔRaf-1:ER activation resulted in the phosphorylation of raf-1 and mitogen-activated protein kinases (MAPK) in LNCaP cells. **A:** Lysates from parental LNCaP cells, LNCaP cells treated with estradiol (+E) for 48 h; ΔRaf-1:ER-transduced LNCaP cells either untreated or treated with estradiol for 48 h were immunoblotted with antibodies to phosphorylated MAPK and actin. Expression of actin serve as loading control. **B:** Phosphorylation and enzymatic activity of MAPK after ΔRaf-1:ER activation in LNCaP cells. Lysates from parental control cells and LNCaP:ΔRaf-1:ER cells with ΔRaf-1:ER activated for 4 h for ≤7 days were immunoblotted with phosphospecific MAPK antibody. The same cell lysates were immunoprecipitated with anti-MAPK, and kinase activity was measured using MBP as a substrate, as described under Materials and Methods. Phosphorylation and enzymatic activity of MAPK remained the same from 24 h to 7 days after ΔRaf-1:ER activation.

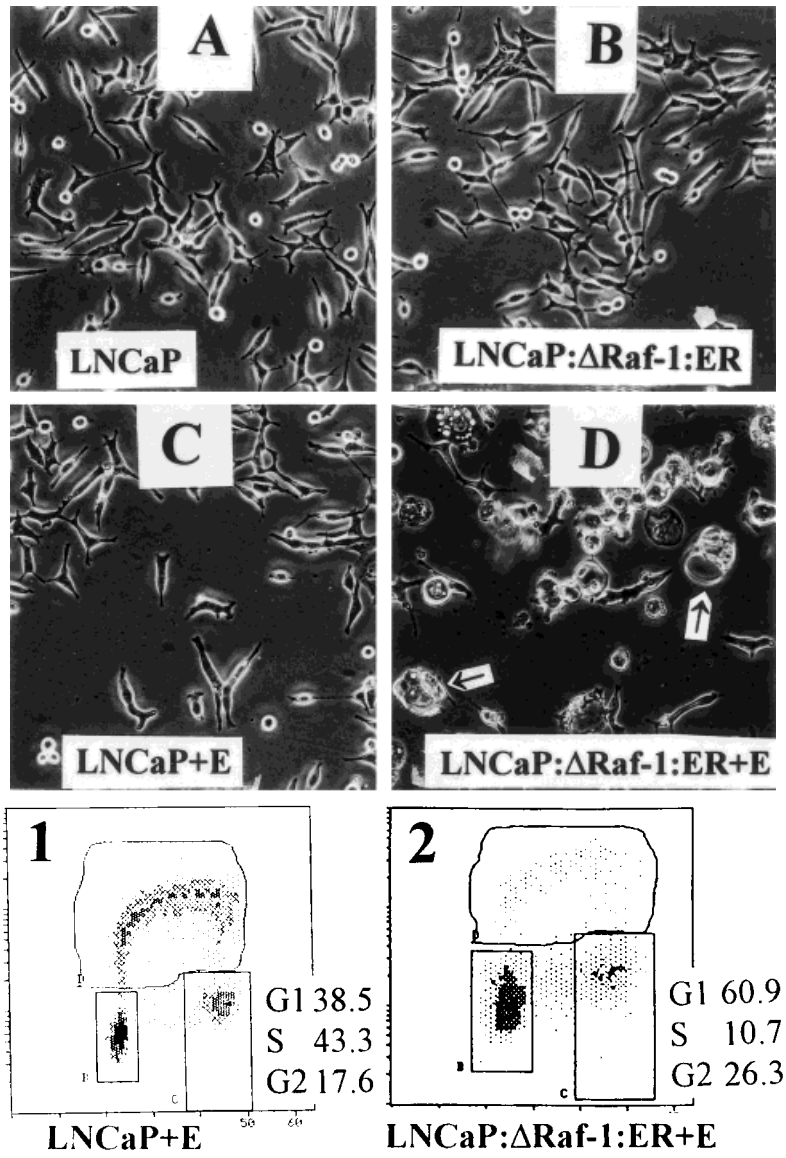

Fig. 2. A–D: Effect of activated ΔRaf-1:ER on LNCaP cell morphology. Cells were grown in the presence of 1 μM estradiol (+E) for 6 days. Photographs of parental cells and transduced cells were taken by phase-contrast light microscopy at 100× magnification. No morphological changes were observed in parental cells grown in the absence or presence of estradiol. Activation of ΔRaf-1:ER caused many of the LNCaP:ΔRaf-1:ER cells (D) to become round and form giant cells (arrows). 1,2: Cell cycle distribution of ΔRaf-1:ER activated LNCaP cells. LNCaP cells, their transduced ΔRaf-1:ER cells were exposed to 1 μM estradiol (+E) for 4 days and were pulsed with bromodeoxyuridine (BrdU). Cells were stained with FITC-anti-BrdU to detect BrdU incorporation (vertical axis) and propidium iodide to detect total DNA (horizontal axis). Bitmaps were set using 10,000 FITC-unlabeled cells. The percentage of cells in S phase (upper box), G0/G1 cells (lower left box), and G2/M cells (lower right box) were measured.

Effect of ΔRaf-1:ER Activation on Cell Cycle

The reduced growth of LNCaP:ΔRaf-1:ER cells in estradiol suggested that ΔRaf-1:ER activation might have induced a cell cycle arrest. To investigate this possibility, we examined cell cycle progression by measuring the incorporation of BrdU and DNA content of the cells by flow cytometry. Cell cycle progression from G1 to S, measured by BrdU incorporation, showed that ΔRaf-1:ER activation reduced the proportion of cells in S phase to 24.7% of parental LNCaP cells, with an associated accumulation of cells in G1 (Fig. 2.1, 2.2). Parental LNCaP cells, parental cells exposed to 1 μM estradiol, and ΔRaf-1:ER transduced LNCaP cells without estradiol were similar in their cell cycle distribution.

Effect of ΔRaf-1:ER Activation on Cell Cycle Proteins

To determine whether ΔRaf-1:ER-induced growth arrest was associated with changes in expression of cell cycle proteins, we analyzed the expression of these proteins in LNCaP cells with activated ΔRaf-1:ER by immunoblotting. Consistent with the growth arrest we have seen, expression of many of these cell cycle proteins is diminished. These include cyclins A and B, the retinoblastoma family protein pRb, CDC2, CDK2, CDK4, E2F-1, DP-1, and PCNA

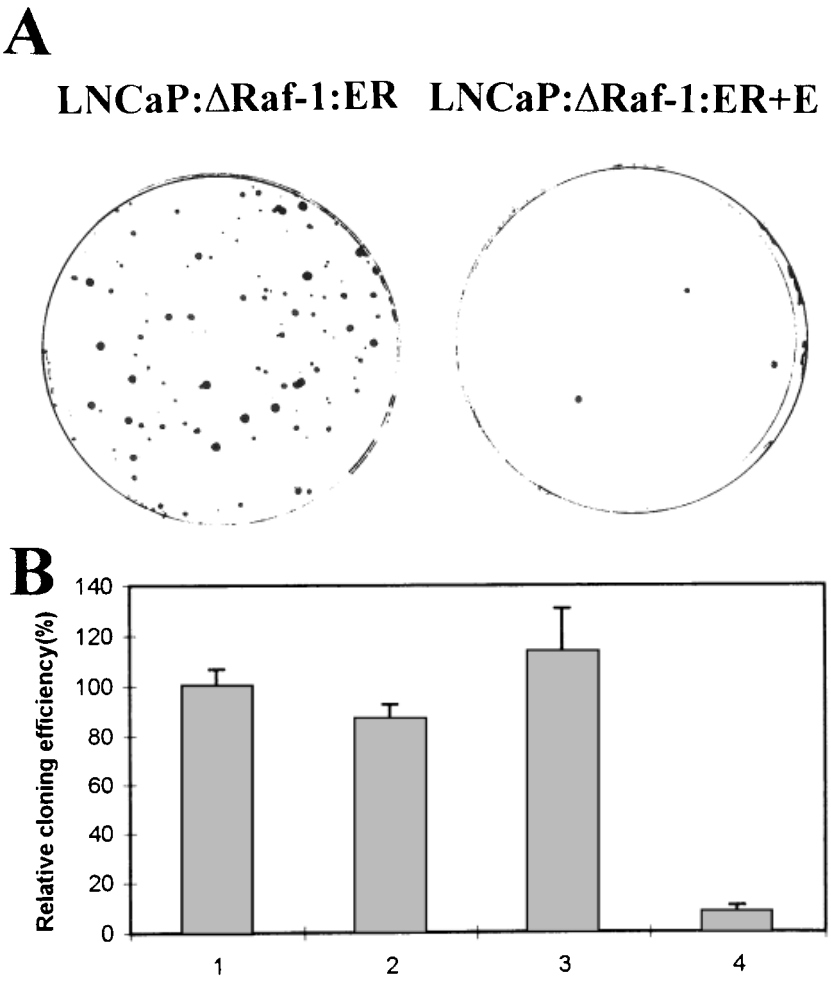

Fig. 3. ΔRaf-1:ER activation inhibited plating and soft agar cloning efficiency of LNCaP cells. **A:** Plating efficiency of ΔRaf-1:ER-transfected LNCaP cells in the absence or presence of estradiol (+E). ΔRaf-1:ER-transfected LNCaP cells were grown in the presence or absence of estradiol for 2 weeks and stained with crystal violet. Photographs were taken using phase-contrast light microscope. **B:** Parental LNCaP cells (1), LNCaP cells in the presence of estradiol (2), ΔRaf-1:ER transfected LNCaP cells (3) without added estradiol cloned well in soft agarose. ΔRaf-1:ER activation (4) markedly reduced the soft agarose cloning efficiency of LNCaP cells. Data are representative of three independent experiments and were represented as mean cloning efficiency with standard deviation from a set of three plates.

(Fig. 4A). The cyclin D family members were unchanged (data not shown) and cyclin E was modestly increased (Fig. 4A), perhaps reflecting their expression in the G1 phase of the cell cycle. There was no change in p53 protein in LNCaP cells after ΔRaf-1:ER activation (data not shown).

The decreased levels of CDK2 and CDK4 were accompanied by lower kinase activities. In vitro kinase assays, using pRb and histone H1 as substrates for CDK4 and CDK2 activity, respectively, showed that CDK4 activity was reduced to 69% and CDK2 activity was reduced to 11% of control parental levels, after ΔRaf-1:ER activation in LNCaP cells (Fig. 4B). Since cyclin E forms complex with CDK2 in late G1 phase, we measured the cyclin E-associated kinase activity. Cyclin E-associated kinase (phosphorylation of histone H1) was reduced to 39% of LNCaP parent levels after ΔRaf-1:ER activation (Fig. 4B). These reduced CDK4 and CDK2 activities are consistent with their reduced protein levels after ΔRaf-1:ER activation. Since previous studies have been shown that cyclin E/CDK2 activity is necessary for cells to exit from late G1 to S [Ohtsubo et al., 1995; Tsai et al., 1993], our data suggest that ΔRaf-1:ER activation in LNCaP cells may block G1-S progression by reducing G1-CDK (CDK4 and CDK2) protein expression and activity. ΔRaf-1:ER activation in LNCaP cells also reduced cyclin A protein expression (Fig. 4A) and activity (data not shown), consistent with the reduced fraction of cells in S phase of the cell cycle.

Activation of ΔRaf-1:ER-Induced cdk Inhibitor $p21^{WAF1/CIP1}$

The activities of cyclin-CDKs are controlled by CDK inhibitors that are induced by external stimuli. Overexpression of CDK inhibitors has been shown to cause decreased CDK activity and cell cycle arrest in several cell types [Sherr and Roberts, 1995]. We investigated whether

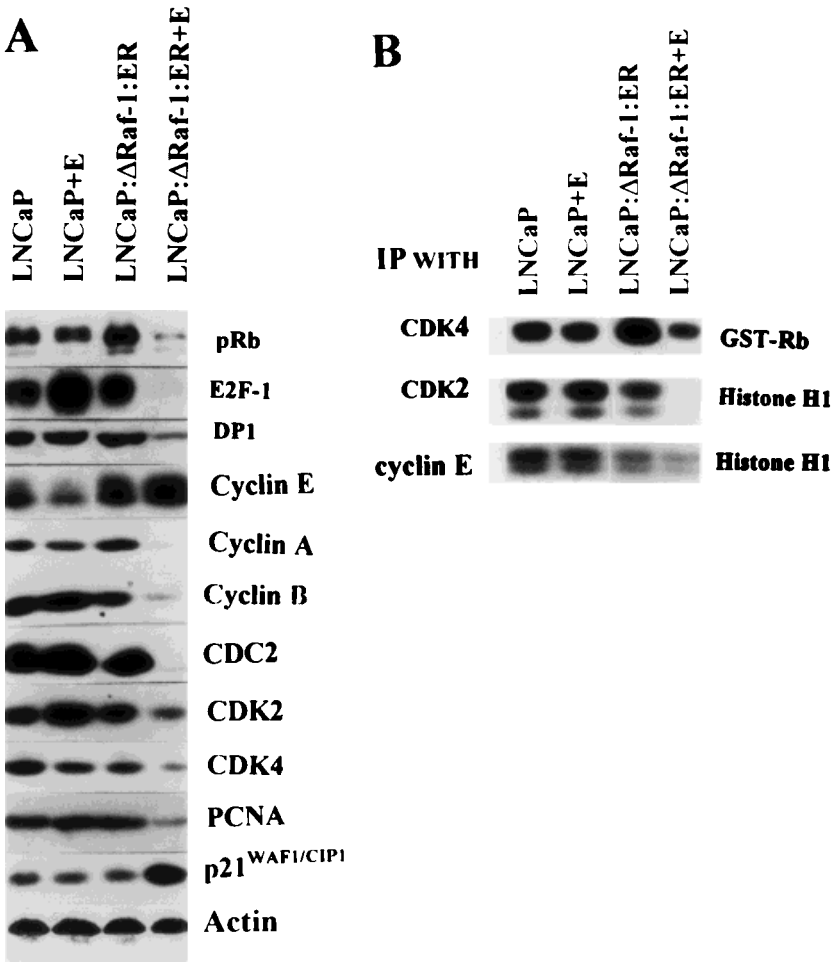

Fig. 4. Effect of ΔRaf-1:ER activation on cell cycle proteins in LNCaP cells. **A:** Parental LNCaP and LNCaP:ΔRaf-1:ER cells were grown in the presence (+E) or absence of 1 μM estradiol for 4 days, harvested for proteins and immunoblotted with appropriate antibodies. ΔRaf-1:ER activation reduced pRb, E2F-1, DP1, cyclin A, cyclin B, CDC2, CDK2, CDK4, and PCNA proteins. CDK inhibitor $p21^{WAF1/CIP1}$ was markedly induced in ΔRaf-1:ER-activated LNCaP:ΔRaf-1:ER cells. Expression of actin serve as loading control. **B:** Immunoprecipitates of CDK2, CDK4, and cyclin E were made from the same cell lysates used for protein expression. Kinase assays were performed using their substrates, histone H1 for CDK2 and cyclin E-associated kinase, and pRb for CDK4.

ΔRaf-1:ER activation altered the expression of the CDK inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$. Activation of ΔRaf-1:ER had no effect on $p27^{Kip1}$ (data not shown). However, $p21^{WAF1/CIP1}$ protein was induced 4-fold in growth arrested LNCaP:ΔRaf-1:ER cells after ΔRaf-1:ER activation (Fig. 4A).

ΔRaf-1:ER-Increased $p21^{WAF1/CIP1}$ mRNA Stability

We have examined the control of $p21^{WAF1/CIP1}$ in LNCaP:ΔRaf-1:ER cells. Northern blot analysis showed that $p21^{WAF1/CIP1}$ mRNA was increased in ΔRaf-1:ER activated LNCaP cells (Fig. 5A). To investigate whether the ΔRaf-1:ER induction of $p21^{WAF1/CIP1}$ mRNA was due to increased gene transcription, we performed nuclear run-off assays. ΔRaf-1:ER activation increased $p21^{WAF1/CIP1}$ transcription 1.4-fold compared with LNCaP control cells (Fig. 5B). This minimal increase in the $p21^{WAF1/CIP1}$ transcription does not account for the large increase in $p21^{WAF1/CIP1}$ protein and mRNA in LNCaP cells. This finding suggests that the ΔRaf-1:ER-induced increase in $p21^{WAF1/CIP1}$ expression may largely be regulated posttranscriptionally. We therefore determined whether initiation of raf signaling stabilizes the previously transcribed $p21^{WAF1/CIP1}$ mRNA. To measure the $p21^{WAF1/CIP1}$ mRNA stability, LNCaP control cells and LNCaP:ΔRaf-1:ER cells were exposed to estradiol for 2 days and then exposed to 5 μg/ml actinomycin D, to inhibit nascent RNA synthesis. This short treatment with actinomycin D did not have any discernible cytotoxic effects on these cells. $p21^{WAF1/CIP1}$ mRNA levels were determined after different time periods (Fig. 6A) and decay rates estimated using Lotus Freelance (Fig. 6B). mRNA half-life studies ($t_{1/2}$) demonstrated a 2.7-fold increase in $p21^{WAF1/CIP1}$ mRNA half-life (from 4.3 h to 11.5 h) after ΔRaf-1:ER activation. Thus, mRNA stabilization may account for much of the increase in $p21^{WAF1/CIP1}$ expression mediated by ΔRaf-1:ER activation.

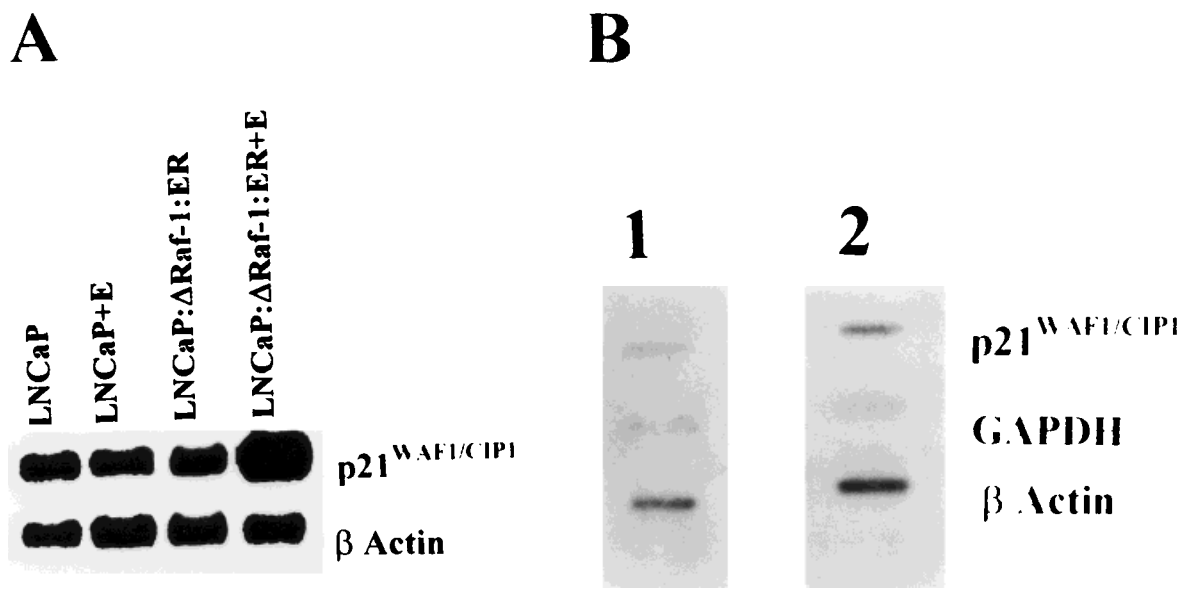

Fig. 5. Induction of p21$^{WAF1/CIP1}$ mRNA and transcriptional analysis of p21$^{WAF1/CIP1}$ after ΔRaf-1:ER activation in LNCaP:ΔRaf-1:ER cells. **A:** Total RNA was extracted from untreated LNCaP cells, and LNCaP:ΔRaf-1:ER cells exposed to estradiol for 4 days. A total of 20 μg of RNA was electrophoresed through a 1.2% formaldehyde gel, transferred to a nylon membrane, and hybridized with a p21$^{WAF1/CIP1}$ cDNA probe. **B:** Nuclear run-off assay using nuclei from LNCaP cells and LNCaP:ΔRaf-1:ER cells treated with estradiol for 4 days. Radiolabeled nascent RNA was hybridized to membranes containing immobilized p21$^{WAF1/CIP1}$ cDNA, GAPDH, and β-actin probes. GAPDH and β-actin were used as positive controls, and β-actin was used for normalization of signal.

Raf-Induced Cell Cycle Block Resulted from Activation of the MEK/MAPK Pathway

To determine whether the ΔRaf-1:ER induced cell cycle block resulted from the activation of MEK/MAPK signal transduction pathway, we pretreated LNCaP and LNCaP:ΔRaf-1:ER cells with the MEK inhibitor, PD098059. PD098059 selectively blocks the activation of MEK, and thereby inhibits phosphorylation and activation of MAPK in vitro [Alessi et al., 1995]. Exposure of LNCaP and LNCaP:ΔRaf-1:ER cells to 10 μM PD098059 for 45 min before ΔRaf-1:ER activation partially inhibited phosphorylation (Fig. 7) and enzymatic activity of MAPK (data not shown). PD098059 allowed LNCaP:ΔRaf-1:ER cells with activated ΔRaf-1:ER to proliferate, abrogating the cell cycle block (data not shown) and inhibited the induction of CDK inhibitor p21$^{WAF1/CIP1}$ (Fig. 7), suggesting that these effects require the activation of MEK and MAPK.

The Cell Phenotype Induced by ΔRaf-1:ER Activation Includes a Specific Marker of Cellular Senescence

After a finite number of cell divisions, primary human cells in culture cease proliferation at subconfluent densities, despite the presence of serum. This state has been called replicative senescence. Recent studies have identified numerous changes in gene expression that occur in such senescent cells [Dimri et al., 1995; Smith and Pereira-Smith, 1996; Stein and Dulic, 1995]. These include overexpression of the growth inhibitory genes p21$^{WAF1/CIP1}$ and p16^{INK4a}, and decreased expression of the growth stimulatory genes c-myc, cyclin A, cdc2, histone H1, PCNA, DHFR (dihydrofolate reductase), TK (thymidine kinase) and E2F. As shown above, ΔRaf-1:ER activated LNCaP cells exhibited most of these gene changes. However, quiescent and terminally differentiated cells can also exhibit similar changes in gene expression. Recently, it has been shown that senescence-associated-β-galactosidase (SA-β-gal) is a specific marker for senescent, but not by presenescent, quiescent or terminally differentiated cells [Dimri et al., 1995]. SA-β-gal activity was detected in 70–80% of the growth arrested ΔRaf-1:ER activated LNCaP cells (Fig. 8). By contrast, only 5–10% of LNCaP parent control cells were positive for SA-β-gal. Hence, these data suggest that ΔRaf-1:ER-induced growth arrest may be associated with a phenotype similar to cellular senescence.

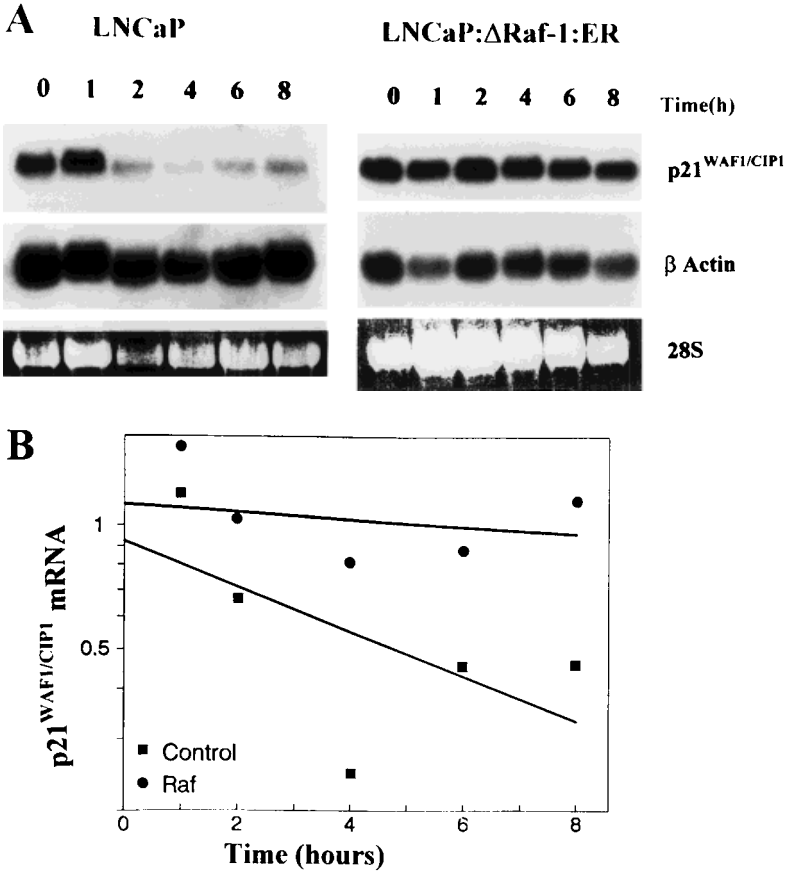

Fig. 6. **A:** p21$^{WAF1/CIP1}$ mRNA half-life was increased after ΔRaf-1:ER activation in LNCaP cells. LNCaP cells and LNCaP:ΔRaf-1:ER cells were exposed to estradiol for 4 days before the addition of actinomycin D (5 μg/ml). Cells were harvested at 0, 1, 2, 4, 6, and 8 h after the addition of actinomycin D for total RNA extraction. Ethidium bromide staining of 28S RNA before transfer and β-actin hybridization serve as loading controls. **B:** p21$^{WAF1/CIP1}$ mRNA stability after ΔRaf-1:ER activation in LNCaP cells. Semilogarithmic plot of p21$^{WAF1/CIP1}$ RNA decay in LNCaP parental cells (control) and ΔRaf-1:ER-activated LNCaP: ΔRaf-1:ER cells (Raf). p21$^{WAF1/CIP1}$ mRNA decay was plotted using values of p21$^{WAF1/CIP1}$ RNA expression normalized relative to β-actin expression. Data are representative of two independent experiments.

DISCUSSION

The mammalian cell cycle is regulated by cyclins and their associated cyclin-dependent kinases (CDK) [Sherr, 1996; Morgan, 1995]. The retinoblastoma (Rb) family proteins, pRb, p107 and p130 are phosphorylated by cyclin/CDK complexes, and their phosphorylation is necessary for G1/S transition [Paggi et al., 1996; Weinberg, 1995]. In cells with functional Rb family molecules, members of the cyclin D family complex with either CDK4 or CDK6 and phosphorylate pRb in early G1. Cyclin D/CDK4 complex phosphorylates p107 in mid-G1. In late G1, the cyclin E/CDK2 complex associates with p130 and controls progression beyond the restriction point. Cyclin A, which is required for transit through S-phase of the cell cycle, complexes with CDK2 in S phase and with p34^{cdc2} (CDC2) near the G2/M portion of the cell cycle. For cells to pass from G2 to M in the cell cycle, p34^{cdc2}/cyclin B activity is needed [Sherr, 1996; King et al., 1994]. LNCaP cells possess a functional Rb molecule and express wild-type p53 [Carroll et al., 1993; Bookstein et al., 1990]. Our results show that ΔRaf-1:ER activation inhibits the growth of human prostate cancer

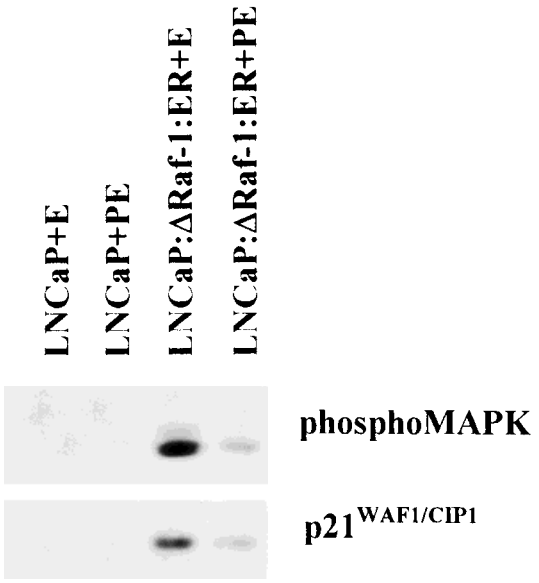

Fig. 7. PD098059 inhibited the phosphorylation of MAPK and induction of p21$^{WAF1/CIP1}$. LNCaP control cells and their ΔRaf-1:ER-transfected cells were treated with 10 μM PD098059 for 45 min before the addition of 1 μM estradiol and grown for 48 h in the presence of PD098059 and estradiol (+EP: E, estrogen; P, PD098059). Western blot analyses showed that PD098059 inhibited the phosphorylation of mitogen-activated protein kinase (MAPK) and induction of cdk inhibitor p21$^{WAF1/CIP1}$ after ΔRaf-1:ER activation in LNCaP:ΔRaf-1:ER cells.

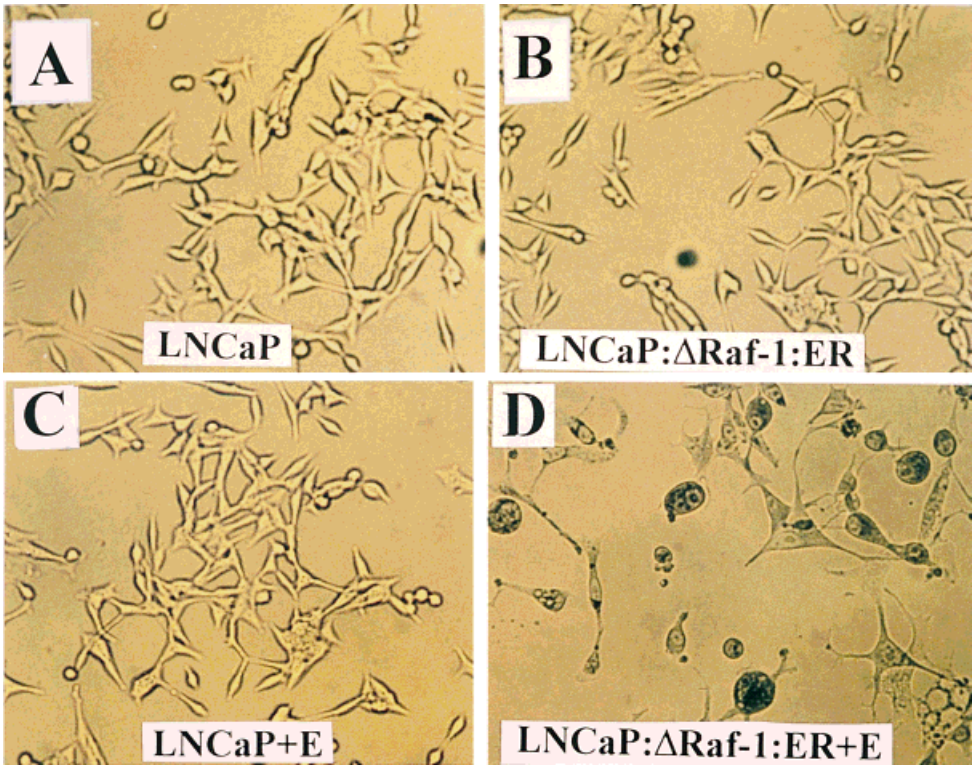

Fig. 8. Expression of senescence-associated-β-galactosidase in LNCaP:ΔRaf-1:ER cells after ΔRaf-1:ER activation. LNCaP control cells and their ΔRaf-1:ER-transfected cells were exposed to estradiol for 6 days and stained with X-gal. Photographs were taken by phase-contrast light microscopy.

cells and is associated with the accumulation of cells in G1 of the cell cycle. This cell cycle block is associated with the induction of CDK inhibitor $p21^{WAF1/CIP1}$, and reduced G1-cyclin/CDK activity. This suggests that, in LNCaP:ΔRaf-1:ER cells, raf-1 mediated induction of $p21^{WAF1/CIP1}$ may be responsible for the G1 block, by interacting with cyclin-CDK complexes, thereby reducing the phosphorylation-dependent inactivation of Rb family proteins [Harper et al., 1993; Waga et al., 1994]. Similarly, Woods et al. [1997] and Sewing et al. [1997] showed that, depending on the level of raf kinase activity, mouse fibroblasts can progress or arrest in the cell cycle, and this raf-induced cell cycle arrest was associated with the induction of CDK inhibitor $p21^{WAF1/CIP1}$. Activation of raf also resulted in growth arrest via the induction of $p21^{WAF1/CIP1}$ in primary rat Schwann cells and in NIH 3T3 cells expressing TrkA [Lloyd et al., 1997; Pumiglia and Decker, 1997]. A role for the ras/raf/MAPK pathway in growth arrest or cellular differentiation has also been suggested by earlier studies. Ectopic expression of v-ras causes growth inhibition in Schwann cells and REF52 cells [Ridley et al., 1988]. Human medullary thyroid carcinoma cells [Carson et al., 1995; Nakagawa et al., 1987], small cell lung cancer cells [Mabry et al., 1989], pheochromocytoma cells [Wood et al., 1993], and hippocampal neuronal cells [Kuo et al., 1996] are differentiated by ras and raf. In pheochromocytoma cells, ras-regulated hypophosphorylation of pRb mediates growth inhibition and neuronal differentiation [Li et al., 1996]. Our results are consistent with those of Liu et al. [1996], who showed that in HL 60 cells treated with TPA, $p21^{WAF1/CIP1}$ induction is dependent on the raf/MAPK signal transduction pathway. Blagosklonny et al. [1997] have also shown that LNCaP cells are also growth arrested by TPA treatment, with induction of $p21^{WAF1/CIP1}$, and suggested that the raf/MAPK signal transduction pathway may be involved. Recently, Olson et al. [1998] showed that ras-related GTPase Rho overcomes the ras/raf/MAPK-induced cell cycle arrest by suppressing $p21^{WAF1/CIP1}$. Our results directly demonstrate the ability of the raf/MAPK signal transduction pathway to induce $p21^{WAF1/CIP1}$ in LNCaP cells. Posttranscriptional regulation of $p21^{WAF1/CIP1}$ through p53-independent pathways has been reported in several cell types,

including breast carcinoma cells [Li et al., 1996], HL-60 cells [Zeng et al., 1996; Schwaller et al., 1995], and p53 null KG-1 cells [Akashi et al., 1995]. Our results indicate that $p21^{WAF1/CIP1}$ induction in LNCaP cells is posttranscriptional, while the level of control of $p21^{WAF1/CIP1}$ expression in HL 60 cells was transcriptional [Zeng et al., 1996]; thus, the mechanism of raf-induced growth arrest may be cell type specific.

Upon activation of the raf/MAPK pathway in LNCaP cells, the senescence marker SA-β-gal was expressed. This finding suggests that raf activation induced a cell senescence program in these cells. Serrano et al. [1997] observed that expression of an activated ras oncogene result in premature cell senescence in nonimmortalized fibroblasts. Our data extend this finding to suggest that at least some cancer cells may retain the ability to initiate a program of cell senescence that can be commenced by raf/MAPK signaling.

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