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Induction of p202, A Modulator of Apoptosis, During Oncogenic Transformation of NIH 3T3 Cells by Activated H-Ras (Q61L) Contributes to Cell Survival

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Abstract Previous studies have revealed that p202 (52 kDa), an interferon (IFN) and differentiation-inducible protein, negatively regulates cell proliferation and modulates cell survival. However, the role of p202 in transformed cells remains to be investigated. Here we report that constitutive expression of oncogenic H-Ras (Q61L) in NIH 3T3 cells, which resulted in cell transformation, was associated with increases in the steady-state levels of 202 RNA and protein. Interestingly, the increase in p202 levels in transformed cells correlated with increases in the activity of the transcription factor c-Jun/AP-1, which bound to the two potential AP-1 DNA binding sites (the AP-1CS1 and AP-1CS2) in the 5′-regulatory region of the *202* gene in gel mobility shift assays. Furthermore, the site-directed mutagenesis, coupled with promoter-reporter analyses, revealed that these two AP-1 DNA binding sites contribute to the regulation of the *202* gene in Ras transformed cells. Because treatment of transformed cells with a specific inhibitor of MEK (PD 98059) resulted in significant decreases in the levels of p202, these observations raise the possibility that in transformed cells Ras/Raf/MEK pathway regulates the transcriptional activation of the *202* gene. Significantly, decreases in the levels of p202 in Ras transformed NIH 3T3 cells under reduced serum conditions increased the susceptibility to apoptosis. Collectively, our observations support the idea that the transcriptional increases in the levels of p202 by oncogenic H-Ras in NIH 3T3 cells are needed for cell survival. J. Cell. Biochem. 88: 191–204, 2003.

Key words: interferon-inducible p202; c-Jun/AP-1; transformation; H-Ras; p21; cell survival

The protein, p202, is an interferon (IFN)-inducible murine phosphoprotein (52 kDa) whose basal levels are detectable in a variety of mouse tissues and cultured cells [Choubey et al., 1989; Choubey and Lengyel, 1993; Wang et al., 1999; Choubey, 2000; Choubey and Kotzin, 2002]. p202 levels increase in mouse embryonic fibroblast cell lines under reduced serum conditions (independent of IFNs) and the presence of serum growth factors inhibits the increase [Geng et al., 2000]. Similarly, the levels of p202 also increase during differentiation of murine skeletal muscle cells in vitro [Datta et al., 1998]. Interestingly, p202 levels decrease in fibroblasts after increases in the levels of wild-type p53 [D'Souza et al., 2001]. Together, these observations support the idea that p202 levels can be regulated independent of IFNs, thus, raising the possibility that p202 also plays a role in the regulation of cell growth independent of IFNs.

Slight overexpression of p202 (about twofold above the basal levels) in a variety of cultured cells results in retardation of cell proliferation [Choubey and Lengyel, 1995; Choubey et al., 1996; Min et al., 1996; Gutterman and Choubey, 1999; Choubey, 2000; Choubey and Kotzin, 2002]. Moreover, retardation of cell proliferation after the inducible expression of p202 in mouse embryonic fibroblasts accompanies increases in the levels of $p21^{WAF1/CIP1}$, a potent inhibitor of cyclin-dependent kinases, and

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hypophosphorylation of retinoblastoma protein (Rb) [Gutterman and Choubey, 1999]. p202 contains the Rb binding motif LxCxE and binds to pRb and other members of the "pocket" protein family [Choubey and Lengyel, 1995; Choubey and Gutterman, 1997]. Interestingly, the decreased levels of p202 under reduced serum conditions (in consequence of the expression of antisense to the 202 RNA) in mouse AKR-2B fibroblasts increase the susceptibility to apoptosis [Koul et al., 1998], suggesting a potential role of p202 in the modulation of programmed cell death. Consistent with this notion, ectopic expression of p202 under reduced serum conditions results in inhibition of c-Myc [Wang et al., 2000] and adenovirus E1A-induced apoptosis [Xin et al., 2001]. Additionally, ectopic expression of p202 significantly delays c-Myc/p53-induced apoptosis [D'Souza et al., 2001]. Furthermore, increased expression of p202 in splenocytes derived from a congenic strain of mice (congenic for the *Nba2* locus) correlates with accumulation of splenocytes, resulting in splenomegaly and defects in apoptosis after ligation with anti-IgM in vitro [Rozzo et al., 2001]. However, overexpression of p202 in human MCF-7 cells sensitizes them to TNF-alpha induced apoptosis [Wen et al., 2000]. Together, these observations support the notion that increased levels of p202 in a variety of cells both in vitro and in vivo negatively regulate cell proliferation and modulate apoptosis. In light of these observations, it is important to determine how p202 expression and its functions are regulated in transformed cells.

Ras is required throughout the G_1 phase of the cell cycle and is essential for S phase progression of fibroblasts [Pardee, 1989; Schlessinger and Bar-Sagi, 1994; Campbell et al., 1998; Malumbres and Pellicer, 1998]. The expression of activated Ras leads to an increase in cyclin D1 expression, accumulation of Cdk4/cyclin D1 complexes, and shortening of the G_1 phase [Malumbres and Pellicer, 1998]. Cyclin D1 together with its respective Cdks target phosphorylation of retinoblastoma protein and thus inactivate the G_1-restriction point control [Pardee, 1989; Malumbres and Pellicer, 1998]. The presence of pRb is needed for the arrest of cells by Ras-antibodies or dominant negative Ras [Malumbres and Pellicer, 1998].

In contrast to positively driving the cell proliferation, Ras also arrests the proliferation of some cell types, and induces cellular senescence of human diploid fibroblasts and primary mouse fibroblasts [Lloyd, 1998]. These opposing effects of Ras are at least partly dependent on cell type and functions of tumor suppressor genes. Thus, Ras activity stimulates transcription of the negative regulator of cell proliferation, such as $p21^{WAF1/CIP1}$ and p16 [Serrano et al., 1995; Lloyd et al., 1997]. Moreover, the absence or functional inactivation of negative regulators of cell growth like p53, p16, or pRb renders the cells subject to cellular transformation by Ras rather than to growth arrest [Clark et al., 2000]. Similarly, in the absence of Cdk inhibitor $p21^{WAF1/CIP1}$ or presence of cooperative oncogenes, such as Myc, Ras causes transformation of cells [Clark et al., 2000]. While it is becoming clear that Ras activation of the Raf/MEK/ERK mitogen-activated protein kinase (MAPK) cascade is important for Ras transformation, it is not clear which downstream genes participate in Ras transformation.

The Ras oncogene activates multiple effector pathways that give rise to different outputs depending on the cell context [Campbell et al., 1998; Downward, 1998]. Of particular relevance for cell survival are shown to be phosphatidalinositol 3-kinase and the PKCs. In addition, ERK that is activated by Raf-1 and PKC also appears to trigger anti-apoptotic signals [Downward, 1998]. Another pathway whereby oncogenic Ras promotes cell survival involves NF-kB, which is necessary for Ras-induced transformation by suppressing the p53-independent induction of apoptosis [Mayo et al., 1997]. Collectively, these observations would be in keeping with the notion that cell transformation by Ras is associated with the activation of pathways that promote cell survival and inhibit apoptosis. However, the molecular mechanisms by which oncogenic Ras promotes cell survival remain to be established.

MATERIALS AND METHODS

Cell Culture

NIH 3T3 cells (from American type culture collection) were maintained at very low density in Dulbecco's modified Eagle's medium (with high glucose) (DMEM) supplemented with 10% calf serum and antibiotics in an incubator with 5% CO_2. If so indicated, cells were treated with either recombinant IFN (1,000 μ/ml), DMSO or PD-98059 (50 μm in DMSO) for the indicated time.

Plasmids and Generation of Stable Cell Lines

The 202-reporter plasmid (202-luc), containing the 5′-flanking sequence (0.8 kb) from the *202* gene has been described [D'Souza et al., 2001]. Mutations in the 202AP-1 DNA binding sites (the 202AP-1CS1 and 202AP-1CS2) were introduced using QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) as suggested by supplier. The 202AP-1CS1 site (TGACTAA) was changed to AACCTAA and the 202AP-1CS2 site (TGATTCA) was changed to AACTTCA. The reporter plasmid AP-1-luc was purchased from Clontech Inc., (Paolo Alto, CA). pRL-TK reporter vector allowing expression of *Renilla* luciferase was purchased from Promega (Madison, WI). Plasmid pUSE, pUSE-H-Ras (WT), and pUSE-H-Ras (Q61L) were purchased from Upstate Biotechnology, NY.

To establish stable cell lines of NIH 3T3 cells, cells were transfected with either empty plasmid pUSE or plasmid pUSE-H-Ras (Q61L) using Calcium phosphate precipitation method as suggested by supplier (Gibco-BRL, Grand Island, NY). The transfected cells were selected in G418 (500 μg/ml). After two weeks, the drug-resistant colonies (>100) were pooled and maintained at 250 μg/ml of G418.

Colony Formation Assays

For colony formation assays, NIH 3T3 cells were transfected with a pUSE-H-Ras (Q61L) plasmid or, as a control, with empty vector (pUSE). Transfected cells were selected in G418 (500 μg/ml). Pooled cell clones were subsequently transfected with pCDNA3.1-202 plasmid or, as a control, pCDNA3.1 plasmid. Transfected cells were selected in G418 and Zeocin (100 μg/ml) for two weeks. Colonies (1–1.5 mm size) were stained with crystal violet and counted. The number of colonies in pCDNA3.1 transfected control and transformed cells were indicated as 100%.

Flow Cytometry Analyses

Flow cytometry was performed on single cell suspensions on adherent (after trypsin and EDTA treatment) as well as the floating cells after pooling them. Briefly, for cell cycle analysis cells were stained with propidium iodide (50 μg/ml, Sigma) and subjected to flow cytometry using Coulter Epics XL-MCL flow cytometer as described previously [D'Souza et al., 2001]. Apoptosis was measured by the accumulation of cells with a sub-G_1 DNA content.

Immunoblotting

Cells were collected from plates in PBS and resuspended in a modified RIPA lysis buffer (50 mM Tris-HCl, pH 8.0; 250 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (leupeptin, 50 μg/ml; Pepstatin A, 50 μg/ml; PMSF, 1 mM), and incubated at 4°C for 30 min. The cell lysates were sonicated briefly before centrifugation at 14,000 rpm in a microfuge for 10 min. The supernatants were collected and equal amounts of proteins were processed for immunoblotting as described previously [Choubey and Lengyel, 1993]. The p202 polyclonal antiserum has been described previously [Choubey and Lengyel, 1993]. The protein p68 is detected by antiserum to p202 (Choubey and Lengyel, 1993). Because its levels do not change under conditions tested so far, we have used the levels of p68 to determine whether equal amounts of proteins were loaded. Antibodies to Ras, cyclin D1, p21, p27, Mdm2, and Rb were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). For gel shift assays, concentrated (100 ×) anti-JunD, JunB, and c-Jun were purchased from Santa Cruz.

Northern Blotting

Total cytoplasmic RNA was isolated from cells and subjected to northern blotting followed by hybridization with the 202-specifc-cDNA probe (Hinc II-fragment) [D'Souza et al., 2001].

Reverse Transcriptase Polymerase Chain Reaction

To study the expression of the *202* gene after transformation of NIH 3T3 cells, total RNA was isolated from cells transfected with vector or expressing oncogenic H-Ras and subjected to the 202-specific RT-PCR using the 202 gene specific forward (5′-GGTCATCTACC AACTCA GAAT-3′) and backward primer (5′-CTCTAGGATGC-CAC TGCTGTTG-3′) and Superscript one-step RT-PCR system from Gibco-BRL (Grand Island, NY). As a control, to determine the amounts of input RNA in the above RT-PCR reactions, beta-actin-specific primers from Gibco-BRL (Grand Island, NY) were used for RT-PCR. The 202-specific (358 bp) and beta-actin specific (353 bp) RT-PCR products were analyzed by agarose gel electrophoresis.

Gel Mobility Shift Assays

Nuclear extracts from NIH 3T3 cells stably transfected with empty vector or a plasmid encoding H-Ras (Q61L) were prepared as described previously [Geng et al., 2000]. Equal amounts of nuclear proteins were used for binding to labeled oligonucleotides (labeled using T4 polynucleotide kinase and annealed into double stranded oligonucleotides) containing either the AP-1 DNA binding consensus sequence (purchased from Santa Cruz Biotech.) or the *202* gene AP-1 DNA binding sequence AP-1CS1 (5′-GTGAGCCTGACTA AGCTGTGA-3′ and the complementary sequence) or AP-1CS2 (5′-GCCTGGCT GATTCAGCACTCC-3′ and the complementary sequence). For supershift assays, nuclear extract proteins were incubated with anti-JunD, JunB, or c-Jun antibodies (1 μg) for 20 min at room temperature. Binding reactions were subjected to gel mobility shift assays as described previously [Geng et al., 2000].

Reporter Assays

For reporter assays, sub-confluent cells in six-well plates were transfected with the reporter plasmids 202-luc (5 μg) and pRL-TK (0.5 μg) using the Calcium phosphate transfection system (Gibco-BRL Life Tech., Rockville, MD), as suggested by the supplier. Cells were harvested 42–48 h after transfections and the firefly luciferase and *Renilla* luciferase activities were determined as described previously. The luciferase activity in control vector transfected cells is shown as 1.

RESULTS

Transformation by Oncogenic Ras is Accompanied by Increases in the Levels of p202

To investigate the regulation of p202 expression during transformation of cells by oncogenic Ras, we generated a stable cell line of NIH 3T3 cells constitutively expressing the activated H-Ras (Q61L). As expected, the expression of oncogenic Ras in cells resulted in remarkable alterations in cell morphology and cells looked transformed (Fig. 1A). However, transfection of cells with empty vector did not result in such morphological changes. Consistent with the previous report [Jacobsen et al., 2002], at lower cell densities, cultures derived from vector-transfected cells and Ras transformed cells grew at the comparable cell proliferation rate for the first three days (Fig. 1B). However, after three days upon reaching higher cell densities, the Ras transformed cells continued to proliferate whereas the vector transfected cells (control cells) slowed down significantly. Furthermore, the cell cycle analysis of asynchronous cultures of control and Ras transformed cells at higher cell densities under normal culture conditions and under reduced serum conditions (0.1% serum) indicated that relatively more cells were in S phase of cell cycle in cultures of Ras transformed cells than in cultures of vector transfected cells (Fig. 1C). Consistent with the previous reports [Liu et al., 1995; Lloyd et al., 1997; Pumiglia and Decker, 1997], the expression of oncogenic Ras in NIH 3T3 cells resulted in marked increases in the levels of cyclin D1 and p21 (Fig. 1D).

Next, we tested whether the levels of p202 are affected during the transformation of NIH 3T3 cells by oncogenic Ras. As shown in Figure 2A and B, the steady-state levels of 202 RNA were significantly higher in Ras transformed cells than vector transfected cells (Fig. 2A and B, compare lane 2 with lane 1). Notably, in experiments shown in Figure 2A, the basal levels of the 202 RNA were detectable only after very long exposure of the film (not shown). Furthermore, the increase in the levels of the 202 RNA also correlated with about threefold increases in the steady-state levels of p202 (Fig. 2C, compare lane 4 with lane 3). Notably, the increase in the levels of p202 was reproducibly less than what we detected after IFN treatment of NIH 3T3 cells (compare lane 4 with lane 2). Consistent with the above observations, we noted that the activity of the 202-luc-reporter was reproducibly higher (2–3 fold) in Ras transformed cells than the vector-transfected cells (Fig. 2D). Together, these observations support the idea that transformation of NIH 3T3 cells by activated H-Ras results in increase in the steady-state levels of p202 and the increase is, in part, due to the transcriptional activation of the *202* gene.

Regulation by the Transcription Factor c-Jun/AP-1

Consistent with the previous studies [Deng and Karin, 1994; Karin, 1995], we noted that the transcriptional activity of AP-1 was about

threefold higher in Ras transformed NIH 3T3 cells as compared to the vector transfected cells (Fig. 3A). Because the 5′-regulatory region (between nucleotide − 817 and +1) of the *202* gene contains potential DNA binding sites for the transcription factor AP-1 (Fig. 3B), one of which (the CS1 site) bound to the JunD/AP-1 complex in gel mobility shift assays [Geng et al., 2000], we tested whether oligonucleotides containing the potential AP-1 DNA binding sites (see Fig. 3B for sequences) in the *202* gene could bind to the transcription factor AP-1 in gel mobility shift assays. For this purpose, we first compared the specific DNA binding activity of AP-1 factor in nuclear extracts prepared from vector transfected cells and Ras transformed cells using equal amounts of proteins. As shown in Figure 3C, an oligonucleotide containing the AP-1 DNA binding consensus site (AP-1CS) bound to an AP-1 protein complex selectively. As expected, more DNA binding activity was detected in nuclear extracts prepared from Ras transformed cells than vector-transfected cells (compare lane 5 with lane 4). Interestingly, an oligonucleotide containing either the 202AP-1CS1 site or 202AP-1CS2 site also bound to the transcription factor AP-1 (Fig. 3D, see lanes 6 and 9). However, no appreciable binding was detected using an oligonucleotide containing the 202AP-1CS3 site (data not shown). Furthermore, competitions between an oligonucleotide containing the consensus sequence for AP-1 DNA binding site and the binding site 202AP-1CS1 or 202AP-1CS2 revealed that both AP-1 sites in the *202* gene exhibited relatively less affinity (50–100-fold less) to AP-1 as compared to the AP-1 consensus sequence in gel mobility shift assays (Fig. 3E, compare lanes 2 to 4 with lanes 5 to 7 or with lanes 8 to 10). Additionally, an incubation of the nuclear extracts derived from Ras transformed cells with the specific antibodies to c-Jun, JunD, or JunB before binding to the 202AP-1CS1 or 202AP-1CS2 oligonucleotide revealed that the AP-1 complexes, which bound to the 202-sequences containing the AP-1 DNA binding site in gel mobility shift

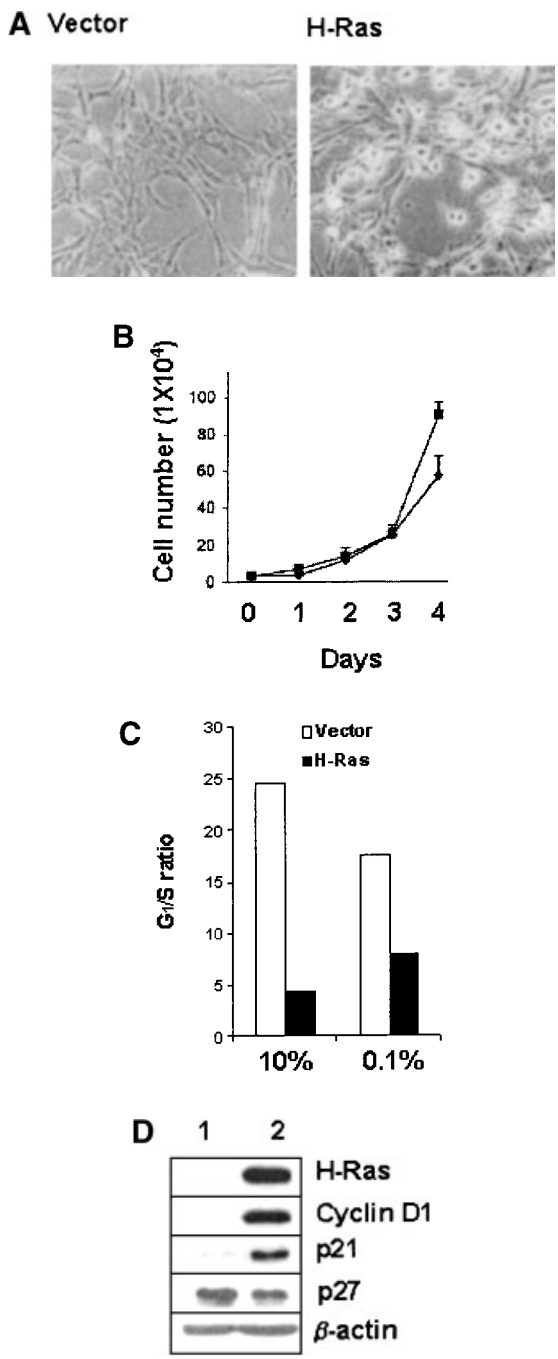


Fig. 1. Expression of oncogenic H-Ras (Q61L) in NIH 3T3 cells results in cell transformation. **A**: NIH 3T3 cells stably transfected with vector (control cells; left panel) or H-Ras (Q61L)-encoding plasmid (transformed cells; right panel) were cultured in medium supplemented with 10% serum. The phase contrast photographs are shown. **B**: Control and transformed cells were cultured at a low density (4×10^4 cells per plate) in medium supplemented with 10% serum. Cells were harvested on indicated days and counted (in triplicates). The calculated standard deviation is shown. Diamond box, control cells; Square box, transformed cells. **C**: Control and transformed cells (at higher cell densities) were incubated in medium containing either 10% serum or 0.1% serum for two days and cells were subjected to flow-cytometry to determine cell cycle distribution. The G_1/S ratio is shown. White bar, control cells; black bar, transformed cells. **D**: Cell extracts derived from control (**lane 1**) and H-Ras (Q61L) transformed (**lane 2**) cells were subjected to immunoblotting using the indicated specific antibodies.

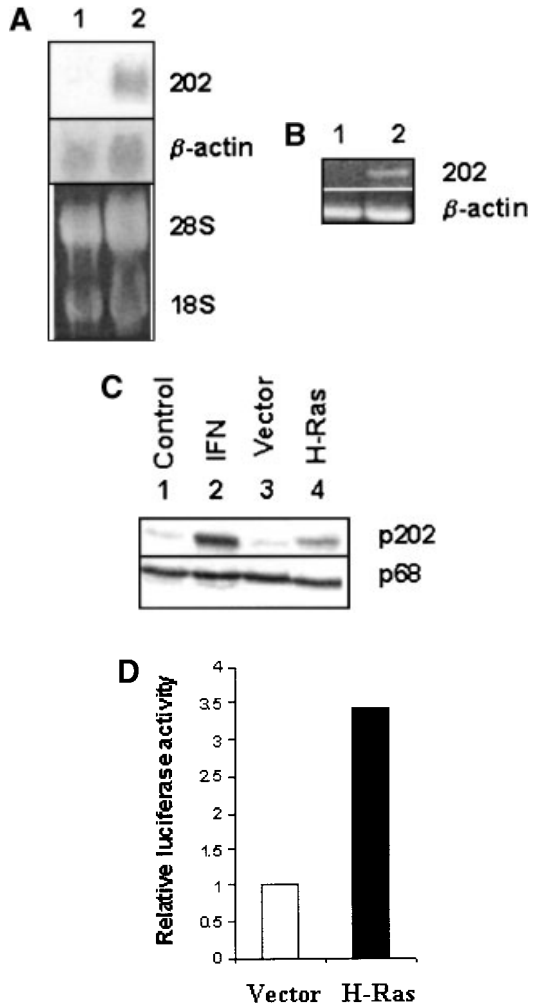
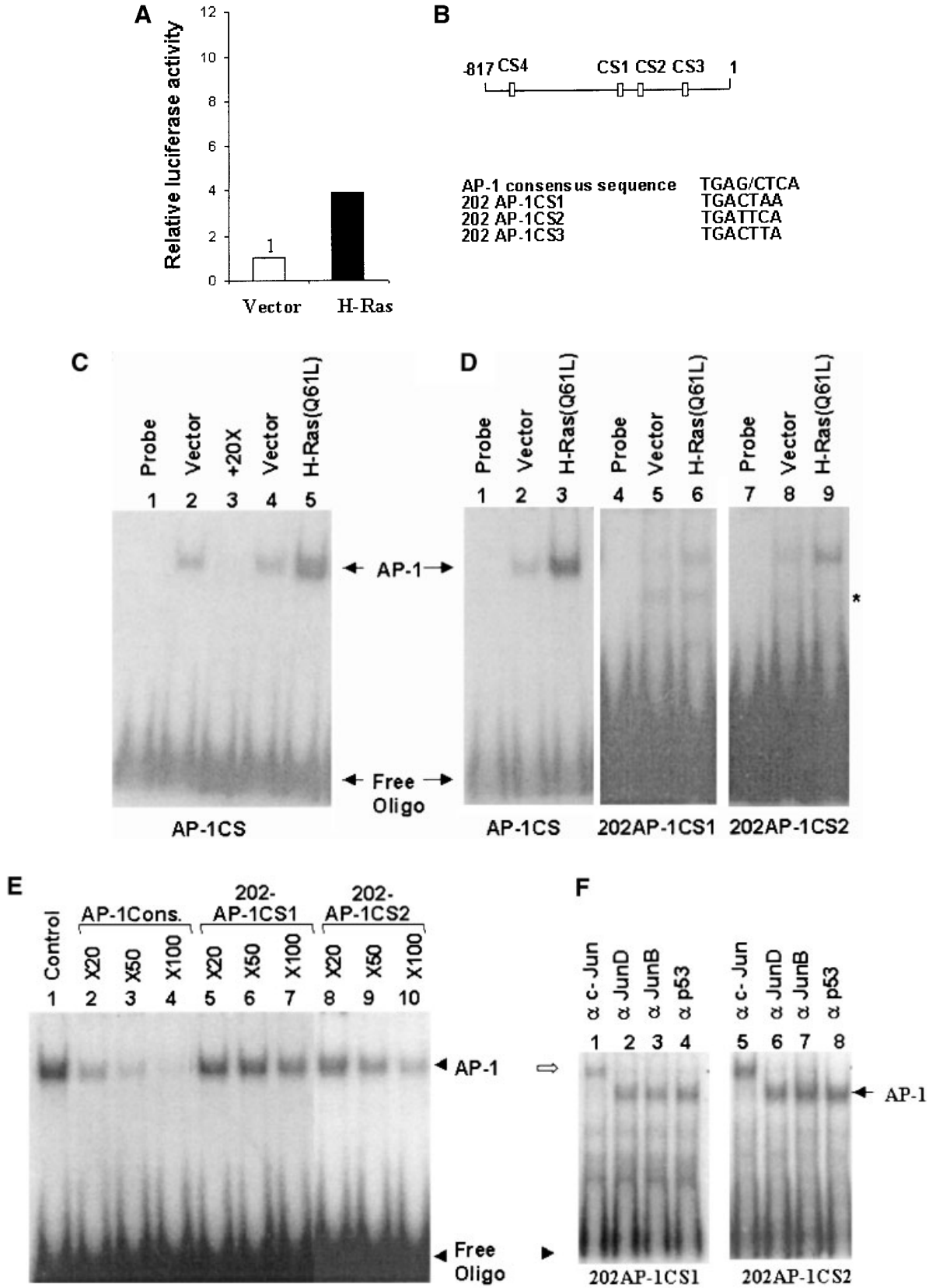


Fig. 2. The levels of the 202 RNA and protein increase in H-Ras transformed NIH 3T3 cells. **A**: Cytoplasmic RNA isolated from control (**lane 1**) and H-Ras transformed (**lane 2**) cells (cultured in medium supplemented with 10% serum) was subjected to Northern hybridization (upper panel) using a cDNA probe specific to the 202 RNA. The same membrane was stripped and reprobed with the beta-actin specific probe as mentioned in the procedure. The lower panel shows a photograph of RNA gel stained with dye ethidium bromide for RNA amounts used. **B**: RNA samples prepared from control (lane 1) and transformed (lane 2) cells (also used in Fig. 2A) were subjected to semi-quantitative RT-PCR using a pair of primers specific to the *202* gene (upper panel). The same reverse transcription reaction products were subjected to PCR using a pair of primers specific to beta-actin (same number of cycles). The PCR products after 25 cycles were analyzed by agarose gel electrophoresis. **C**: Total cell extracts were prepared from control (**lane 3**) and transformed (**lane 4**) cells. As a positive control, extracts were also prepared from NIH 3T3 cells treated with IFN (1,000 U/ml, 36 h) (lane 2) or, as a negative control, left untreated (lane 1). Extracts were subjected to immunoblotting using the indicated specific antibodies. **D**: Control cells and transformed cells were transfected with the 202-luc-reporter plasmid (2.5 μg) along with pRL-TK (0.25 μg) plasmid as described in the procedure. The activity of reporter was determined after 48 h of transfections. The activity of reporter in control cells is indicated as 1.

assays, primarily contained c-Jun (Fig. 3F, see lanes 1 and 5). Interestingly, low levels of JunD were also detected in AP-1 complex, which bound to the 202AP-1CS1 or 202AP-1CS2

Fig. 3. Transformed NIH 3T3 cells contain increased levels of AP-1 DNA binding activity, which selectively binds to the two potential AP-1 DNA binding sites present in the 5′-regulatory region of the *202* gene. **A**: Control cells and transformed cells were transfected with the AP-1-luc reporter plasmid (2.5 μg) along with pRL-TK (0.25 μg) plasmid as described in the procedure. The activity of reporter was determined after 44–48 h of transfections. The activity of reporter in control cells is indicated as 1. **B**: Schematic presentation of the 5′-regulatory region of the *202* gene with potential DNA binding sites for AP-1 transcription factor (indicated as CS1, CS2, CS3, and CS4). The numbers at the 5′-end and at the 3′-end indicate the nucleotides in the 5′-regulatory region of the *202* gene. The lower panel indicates the DNA binding consensus sequence for transcription factor AP-1 and its comparison with the potential AP-1 DNA binding sites in the *202* gene. **C**: Nuclear extracts containing equal amounts of proteins prepared from control (**lanes 2–4**) and transformed (**lane 5**) cells were subjected to electrophoretic mobility shift assays using a labeled oligonucleotide containing the AP-1 DNA binding consensus sequence. Nuclear extracts from control cells without any further treatment (lanes 2 and 4), after incubation with 20-fold excess of unlabeled AP-1 consensus oligonucleotide (**lane 3**). Extracts from transformed cells (lane 5). As a control, labeled AP-1-specific oligonucleotide was loaded in **lane 1**. An arrow indicates AP-1-specific band. The free-labeled oligonucleotide is indicated at the bottom of the gel. **D**: Nuclear extracts containing equal amounts of proteins prepared from control (lanes 2, 5, and **8**) and transformed (lanes 3, **6**, and **9**) cells were subjected to electrophoretic mobility shift assays using a labeled oligonucleotide containing the AP-1 DNA binding consensus sequence (lanes 1–3), the 202AP-1CS1 (lanes 4–6) or the 202AP-1CS2 (**lanes 7**–9) sequence. As a control, labeled AP-1-specific oligonucleotide (lane 1), the 202AP-1CS1 oligonucleotide (lane 4), and the 202AP-1CS2 oligonucleotide (lane 7) were also run. An arrow indicates AP-1-specific band. The star indicates a non-specific band. The free-labeled oligonucleotides are indicated at the bottom of the gels. **E**: Nuclear extracts containing equal amounts of proteins prepared from control cells (lanes 1–**10**) were subjected to electrophoretic mobility shift assays using a labeled oligonucleotide containing the AP-1 DNA binding consensus sequence. Nuclear extracts from control cells without any further treatment (lane 1), after incubation with 20-fold (lanes 2, 5, and 8), 50-fold (lanes 3, 6, and 9), and 100-fold (lanes 4, 7, and 10) excess of unlabeled oligonucleotide containing the indicated AP-1 DNA binding consensus sequence. An arrow indicates the AP-1-specific band. The free-labeled oligonucleotide is indicated at the bottom of the gel. **F**: Nuclear extracts containing equal amounts of proteins prepared from transformed cells (lanes 1–8) were subjected to electrophoretic mobility shift assays using a labeled oligonucleotide containing the 202AP-1CS1 (lanes 1–4) or the 202AP-1CS2 (lanes 5–8) sequence. Nuclear extracts were incubated with the specific antibodies to c-Jun (lanes 1 and 5), JunD (lanes 2 and 6), JunB (lanes 3 and 7), and, as a negative control, an isotype anti-p53 antibodies (lanes 4 and 8) before incubation with labeled oligonucleotide. An arrow indicates the AP-1-specific band. A white arrow indicates the location of supershifted band. The free-labeled oligonucleotide is indicated at the bottom of the gel.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AP-1
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Fig. 3.

site. However, JunB was not detected in AP-1 complexes bound to either the 202AP-1CS1 site or 202AP-1CS2 site.

c-Jun/AP-1 DNA Binding Sites Contribute to the Regulation of the *202* Gene

To determine the relative contributions of the two AP-1 DNA binding sites (the 202AP-1CS1 and 202AP-1CS2) present in the 5′-regulatory region of the *202* gene in the regulation of the 202 gene expression during Ras-mediated transformation of NIH 3T3 cells, we utilized reporter plasmids in which we mutated either the 202AP-1CS1 site or 202AP-1CS2 site or both sites. As seen in Figure 4A, mutation in the CS1 site resulted in significant decreases in the activity of the 202-luc-reporter in vector transfected as well as Ras transformed cells. In contrast, a mutation in the CS2 site in two independent experiments resulted in significant increases in the activity of 202-luc-reporter in Ras transformed cells as compared to vector-transfected cells. Furthermore, mutations in both CS1 and CS2 sites did not result in any additional decreases in the activity of the 202-luc-reporter. These observations, thus, are consistent with the possibility that the 202AP-1CS1 site in the *202* gene is needed for the basal transcription of the *202* gene in NIH 3T3 cells. However, the 202AP-2CS2 site in the *202* gene is responsive to the activated Ras in NIH 3T3 cells. Because both of these AP-1 sites that are next to each other (Fig. 3B) and bound to c-Jun/AP-1 in gel mobility shift assays (Fig. 3D and F), it is likely that the binding of AP-1 to one of these sites facilitates the binding to the other site [Shaulian and Karin, 2002].

Because in nuclear extracts prepared from Ras transformed NIH 3T3 cells c-Jun and JunD appear to be part of the AP-1 DNA binding activity, which bound to the two 202 AP-1 DNA binding sites in gel mobility shift assays (Fig. 3F), we tested whether ectopic expression of c-Jun or JunD could regulate the activity of the 202-luc-reporter. As shown in Figure 4B, the expression of JunD or c-Jun in NIH 3T3 cells in two independent experiments resulted in a moderate increase in the activity of the 202-luc. More importantly, mutations in the 202AP-1CS1 site resulted in a significant reduction in JunD and c-Jun-mediated increases in the activity of the 202-luc-reporter. Collectively, these observations support the notion that the members of the Jun/AP-1 family positively regulate

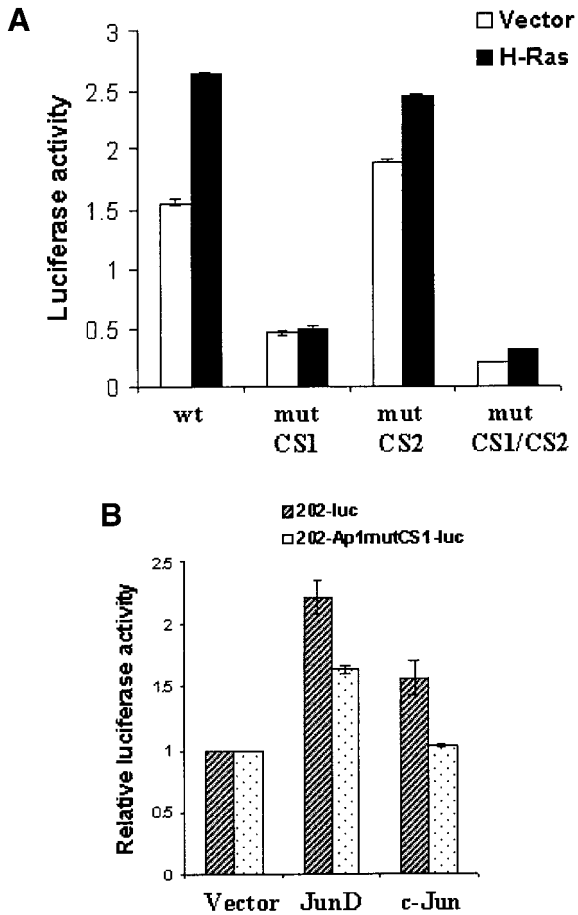

Fig. 4. Transcriptional activation of the 202-luc-reporter depends on potential AP-1 DNA binding sites. **A**: Control (vector) and transformed (H-Ras) cells were transfected with equal amounts of the 202-luc-reporter plasmid (wt), the 202AP-1mutCS1-luc reporter plasmid (the 202AP-1CS1 site mutated) (mutCS1), the 202AP-1mutCS2-luc reporter plasmid (the 202AP-1CS2 site mutated) (mutCS2), or the 202AP-1mutCS1CS2-luc reporter plasmid (both CS1 and CS2 sites mutated) (mutCS1/CS2) as described in the procedure. The activity of reporter was determined after 44–48 h of transfections. The activity of luciferase reporter is shown. **B**: NIH 3T3 cells were transfected with the 202-luc-reporter plasmid or the 202AP-1mutCS1-luc-reporter plasmid along with empty vector or plasmid encoding mouse JunD or plasmid encoding c-Jun. The activity of reporters was determined after 48 h of transfections. The activity of reporter in control cells transfected with vector is indicated as 1.

the expression of the *202* gene through the AP-1 DNA binding sites in untransformed as well as in Ras-transformed NIH 3T3 cells.

Treatment of Cells With the Specific Inhibitor of MEK Results in a Decrease in p202 Levels

To elucidate the Ras signaling pathways involved in the regulation of the *202* gene during

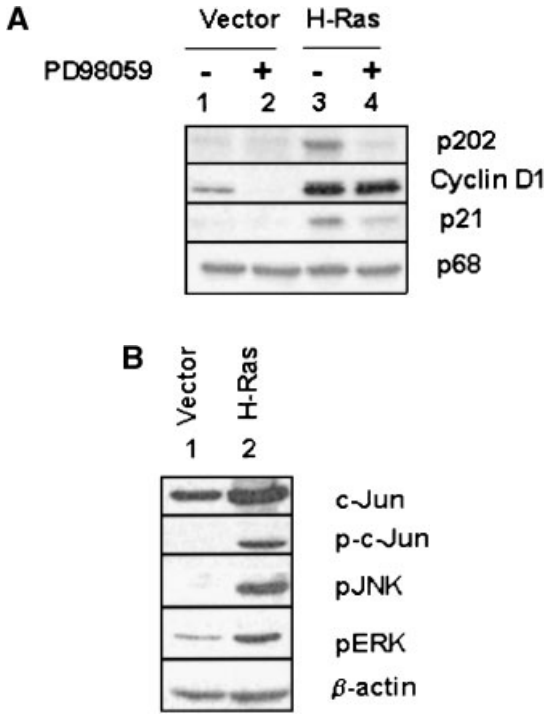

Fig. 5. Treatment of transformed cells with the specific inhibitor of MEK results in decreases in the steady-state levels of p202. **A**: Control cells and transformed cells were either treated with the specific inhibitor of MEK (PD98059) for 24 h (**lanes 2** and **4**) or, as a control, cells were treated with DMSO (**lanes 1** and **3**). Total cell extracts were analyzed by immunoblotting using the indicated antibodies. **B**: Extracts from control (lane 1) and transformed (lane 2) cells were analyzed by immunoblotting using the indicated antibodies specific to c-Jun, phospho c-Jun (Ser-63), phospho JNK, phospho ERK, and beta-actin.

Ras-induced transformation of NIH 3T3 cells, we treated control and Ras transformed cells with an inhibitor of MEK (PD 98059). As shown in Figure 5A, the treatment of cells with PD 98059 resulted in a significant decrease in the levels of p202 (compare lane 4 with lane 3). However, the levels of cyclin D1 did not change significantly and a moderate decrease in the levels of $p21^{WAF1/CIP1}$ was seen. Furthermore, consistent with the above observations, in extracts prepared from Ras transformed cells we could detect increases in the levels of c-Jun and the phosphorylation of c-Jun, JNK, and ERK (Fig. 5B). Collectively, these observations are consistent with the possibility that Ras/Raf/MEK/JNK and ERK pathways contribute to the transcriptional activation of the *202* gene during Ras-induced transformation of NIH 3T3 cells.

Transfection of a Plasmid Encoding p202 in Ras Transformed Cells Reduces Colony Formation and Decreases in the Levels of p202 Sensitize Ras Transformed Cells to Apoptosis

To investigate the functional consequences of increases in the levels of p202 in Ras-transformed NIH 3T3 cells, we chose to transfect a p202 encoding plasmid in a stable cell line generated from pUSE plasmid transfected pooled cell clones (G418 resistant) and pUSE-H-Ras transfected and transformed NIH 3T3 pooled cell clones. Consistent with earlier reports [Choubey, 2000; Hertel et al., 2000], transfection of pCDNA3.1-202 plasmid (encoding p202; Zeocin resistant) in pUSE vector transfected cells resulted in 13% fewer G418 and Zeocin-resistant colonies than the pCDNA3.1 vector transfected cells (Fig. 6, compare top right panel with top left panel). Interestingly, transfection of a pCDNA3.1-202 plasmid in Ras transformed cells (in two experiments) resulted in a significant (about 50–53%) decrease in G418 and Zeocin-resistant colony formation as compared to pCDNA3.1 transfected Ras transformed cells (Fig. 6, compare bottom right panel with bottom

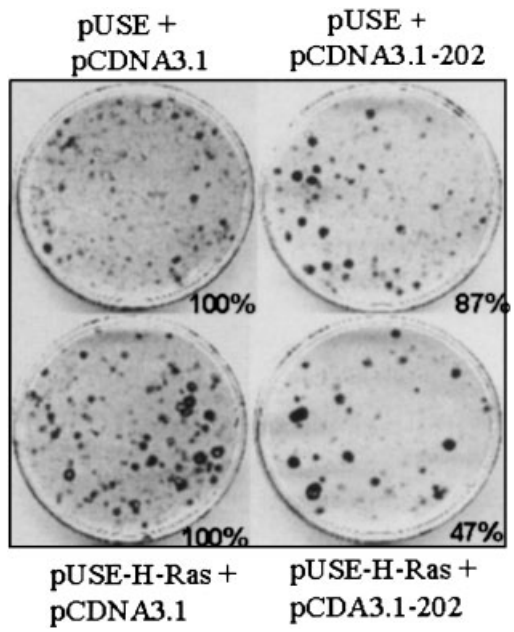

Fig. 6. Overexpression of p202 in H-Ras transformed cells results in inhibition of colony formation. Control (transfected with pUSE vector) and transformed (transfected with plasmid encoding activated H-Ras) cells were transfected with equal amounts of vector (pCDNA3.1) or a plasmid encoding p202 (pCDNA3.1-202). Transfected cells were selected in G418 and Zeocin for two weeks as described in Materials and Methods.

left panel). These observations provide support to the idea that in NIH 3T3 cells oncogenic Ras-mediated increases in the levels of p202 reduce the extent of colony formation by oncogenic H-Ras.

Because under reduced serum conditions increases in the cellular levels of p202 also correlate with the inhibition of apoptosis [Wang et al., 2000; Xin et al., 2001], to further investigate the functional consequences of increases in the levels of p202 in Ras transformed NIH 3T3 cells, we tested whether the decreases in the levels of p202 affect the sensitivity of Ras transformed NIH 3T3 cells to apoptosis. As reported previously [Downward, 1998], Ras transformed NIH 3T3 cells exhibited resistance to apoptosis under reduced serum conditions (Fig. 7A). However, decreases in the levels of p202 in pools of Ras transformed NIH 3T3 cells (Fig. 7B) resulted in increased susceptibility to apoptosis under reduced serum conditions (Fig. 7C). Importantly, a moderate increase in the levels of p202 in Ras transformed NIH 3T3 cells (Fig. 7B, compare lane 2 with lane 1) did not result in additional increase in cell survival under reduced serum conditions, indicating that the basal levels of p202 in NIH 3T3 cells are sufficient for cell survival. Together, these observations are consistent with the possibility that continued expression of p202 may be necessary to prevent apoptosis of H-Ras transformed NIH 3T3 cells under reduced serum conditions.

DISCUSSION

We identified the protein, p202, as an IFN-inducible protein with a potential to mediate the biological activities of IFNs, including the cell growth inhibition [Choubey et al., 1989; Choubey and Lengyel, 1993; Choubey, 2000; Choubey and Kotzin, 2002]. Consistent with this prediction, ectopic expression of p202 in a variety of cultured cells (both normal and cancer cells) results in retardation of cell proliferation and decreases tumor formation in animal models [Gutterman and Choubey, 1999; Choubey, 2000; Wen et al., 2000; Choubey and Kotzin, 2002]. Curiously, the following recent observations have raised the possibility that in addition to IFN action p202 can also participate in the regulation of cell growth in other physiological settings. Firstly, under reduced serum conditions, the levels of p202 increase significantly (sixfold) [Geng et al., 2000] and the

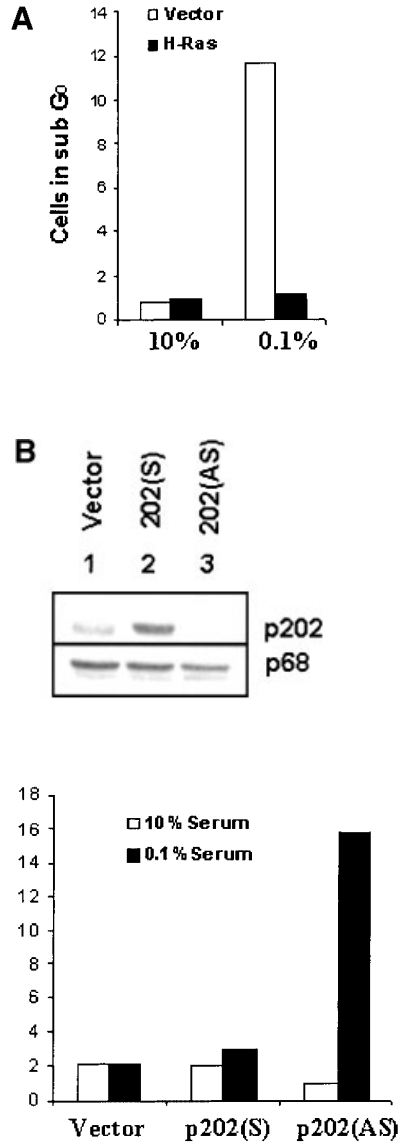

Fig. 7. Decreases in the levels of p202 increase the susceptibility of H-Ras transformed NIH 3T3 cells to apoptosis induced by reduced serum conditions. **A**: Control and transformed cells (pooled from more than 100 colonies) were incubated in growth medium supplemented either with either 10% serum or 0.1% serum for two days. After incubation, cells (both adherent and floating) were subjected to flow-cytometry to determine the presence of sub G_0 (indicative of cell death) cells as described in the procedure. **B**: Extracts prepared from pools of H-Ras transformed NIH 3T3 colonies transfected with vector (**lane 1**), pCDNA3.1-202(S) (**lane 2**) or pCDNA3.1-202(AS) (**lane 3**) were subjected to immunoblotting using antibodies to p202. The same blot was also probed with antibodies to p68 protein. (p68 is a protein detected by antiserum to p202. The levels of p68 do not change under the experimental conditions tested so far; Choubey and Lengyel, 1993). **C**: The pools of H-Ras transformed NIH 3T3 cells transfected with vector, pCDNA3.1-202(S) or pCDNA3.1-202(AS) were incubated in medium either supplemented with 10% serum or 0.1% serum for two days. After the incubation cells (both adherent and floating) were subjected to flow-cytometry to determine the percent of sub-G_0 cells.

increased levels of p202 inhibit apoptosis induced by c-Myc [Wang et al., 2000] and, as expected, the decreased levels of p202 increase the susceptibility to apoptosis [Koul et al., 1998]. Secondly, the levels of p202 decrease after increases in the levels of wild type p53 in response to DNA damage (UV-treatment) and the increased levels of p202 delay c-Myc-p53-induced apoptosis [D'Souza et al., 2001]. Thirdly, the levels of p202 increase during differentiation of C2C12 myoblasts in vitro [Datta et al., 1998]. Together, these observations support the overall hypothesis that p202 regulates cell proliferation and cell survival.

In our previous study [Geng et al., 2000], we noted that in murine fibroblasts (AKR-2B and NIH 3T3) the levels of p202 increase under reduced serum conditions and the presence of serum growth factors (for example, PDGF, bFGF, and TGF-β1) abrogated the increase. These observations, thus, raised the possibility that the signaling pathways activated by cell growth factors and growth factor receptors may negatively regulate the expression of the *202* gene. Because Ras functions as a relay switch that is positioned downstream of cell surface receptor tyrosine kinases and upstream of a cytoplasmic cascade of kinases that include the mitogen-activated protein kinases (MAPKs) [Schlessinger and Bar-Sagi, 1994; Campbell et al., 1998; Malumbres and Pellicer, 1998], we tested whether the expression of activated H-Ras (Q61L) regulates the expression of the *202* gene. To our surprise, the expression of oncogenic H-Ras (Q61L) in NIH 3T3 cells, which resulted in cell transformation, accompanied increases in the levels of p202. The increase was evident both at the RNA and protein levels (Fig. 2).

To elucidate the molecular mechanism, by which transformation of NIH 3T3 cells by oncogenic H-Ras results in the transcriptional activation of the *202* gene, we searched the 5′-regulatory region of the *202* gene for the presence of Ras-responsive cis-elements. The search revealed that the 5′-regulatory region (about 800 bp from the start of transcriptional initiation sites; see in Choubey et al., 1989) of the *202* gene contains potential cis-elements shown to be responsive to the Ras signaling. These cis-elements include at least four potential AP-1 DNA binding sites (Fig. 3B). The gel mobility shift assays using oligonucleotides containing potential AP-1 DNA binding sites (the 202AP-1CS1, 202AP-1CS2, or 202AP-1CS3), coupled with promoter-reporter assays, indicated that in control as well as in transformed NIH 3T3 cells the transcription of the 202-luc-reporter was dependent on the transcriptional activity of AP-1. Furthermore, increases in the activity of the 202-luc in transformed cells correlated with increases in the activity of AP-1. Consistent with this observation, in transformed NIH 3T3 cells the transcription of the *202* gene was in part depended on an AP-1 DNA binding sites (the 202AP-1CS1 and 202AP-1CS2) present in the 5′-regulatory region of the *202* gene because mutagenesis of this site resulted in significant decreases in the activity of the 202-luc-reporter (Fig. 4A).

Depending on the cell type and experimental setting (transient vs. stable Ras activation), the activation of Ras is known to result in the activation of multiple signaling pathways [Malumbres and Pellicer, 1998]. In agreement with previous reports [Liu et al., 1995; Pumiglia and Decker, 1997], we noted that sustained expression of activated Ras resulted in increases in the steady-state levels of cyclin D1. Similarly, consistent with previous reports [Lloyd et al., 1997; Pruitt et al., 2000], we also found that sustained expression of activated Ras resulted in increases in the levels of $p21^{WAF1/CIP1}$ and the levels of p27 did not change significantly (Fig. 1D). In the previous studies it has been suggested that in NIH 3T3 cells sustained expression of activated Ras activates Raf and activation of Raf is sufficient to upregulate cyclin D1 and $p21^{WAF1/CIP1}$ [Pumiglia and Decker, 1997; Pruitt et al., 2000]. Because in NIH 3T3 cells Raf is shown to activate expression of cyclin D1 and $p21^{WAF1/CIP1}$ through MEK [Pruitt et al., 2000], we tested whether the inhibition of MEK activity in transformed cells with the specific and potent inhibitor (PD 98059) results in inhibition of p202 expression. These experiments revealed that the inhibition of MEK activity by PD98059 resulted in significant decreases in the levels of p202 (Fig. 5A). As expected, the levels of p21 also decreased. However, the levels of cyclin D1 did not decrease significantly. Because the regulation of cyclin D1 levels by Ras is very complex [Amanatullah et al., 2001], further work will be needed to determine why inhibition of MEK pathway in our cells did not result in significant decreases in cyclin D1 levels. Interestingly, we also noted that, under our experimental conditions, transformation of

NIH 3T3 cells by oncogenic H-Ras (Q61L) was associated with increases in the steady-state levels of c-Jun and the phosphorylation (indicative of activation) of c-Jun, JNK, and ERK (Fig. 5B). Together, these observations are consistent with the possibility that in NIH 3T3 cells sustained expression of activated Ras (Q61L) resulted in activation of both families of MAP kinase: JNKs and ERKs. The activation of JNK and ERK MAP kinases is shown to result in increases in the activity of transcription factor AP-1 [Karin, 1995]. Moreover, our observation that mutations in the 202AP-1CS1 site resulted in significant reduction in the basal as well as Ras-mediated increases in the activity of the 202-luc-reporter supports the hypothesis that Ras/Raf/MEK/JNK/c-Jun pathway contributes to the transcriptional activation of the *202* gene. Further work is in progress to determine whether the activation of Raf, MEK, or JNK alone is sufficient to activate the transcription of the *202* gene.

It is of note that under reduced serum conditions the increased levels of JunD/AP-1 in murine fibroblasts were correlated with the increased levels of p202 and overexpression of JunD in promoter-reporter assays resulted in increases in the activity of the 202-luc-reporter [Geng et al., 2000]. Additionally, we noted that the JunD/AP-1 complexes bound to the 202AP-1CS1 site in gel mobility shift assays. In H-Ras transformed NIH 3T3 cells the increased levels of c-Jun correlated with the increased levels of p202 and overexpression of both c-Jun and JunD resulted in stimulation of the 202-luc-reporter activity (Fig. 4B). Moreover, the c-Jun/AP-1 complexes bound the two potential AP-1 DNA binding sites (the 202AP-1CS1 and 202AP-1CS2) in the *202* gene (Fig. 3F). Together, these observations support the idea that the two members of the Jun family (JunD and c-Jun) positively regulate the expression of the *202* gene. Further work is in progress to determine whether other signaling pathways, which activate the activity of AP-1, also regulate the expression of the *202* gene.

Ectopic expression of p202 in murine L929 cells results in inhibition of basal as well as induced transcriptional activity of c-Fos and c-Jun complexes, which correlates with the binding of p202 to c-Fos and c-Jun. Therefore, transcriptional activation of the *202* gene by the members of AP-1 family in transformed NIH 3T3 cells in our studies raises the possibility that transcriptional activation of the *202* gene by AP-1 may be part of a regulatory loop, which may be important in the regulation of cell survival. Because the regulation of AP-1 activity is complex [Shaulian and Karin, 2002] and appears to depend on cell type, further work will be needed to determine whether p202 in transformed cells binds to various AP-1 family members and whether the lack of p202 binding to the AP-1 family members contributes to increased cell survival.

Another growth-inhibitory molecule whose expression is regulated by Ras is p21 $^{WAF1/CIP1}$ CDK inhibitor [Lloyd, 1998]. The levels of p21 $^{WAF1/CIP1}$ are low during quiescence and tend to increase in response to mitogenic stimuli, particularly those that activate the Raf/MEK/ERK pathway [Lloyd et al., 1997; Lloyd, 1998]. However, majority of observations suggest that p21 antagonizes Ras-mediated growth stimulation [Lloyd et al., 1997; Lloyd, 1998; Pruitt et al., 2000]. Surprisingly, our experiments indicated that tranfection of a plasmid encoding p202 in NIH 3T3 cells reduces colony formation by oncogenic H-Ras (Fig. 6). Thus, raising the possibility that the increased levels of p202 by antagonizing Ras-induced cell growth stimulation limit deregulation of cell cycle progression. Together, these observations are consistent with the recent report that oncogenic Ras can transform NIH 3T3 cells without deregulation of cell cycle [Jacobsen et al., 2002].

Decreases in the levels of p202 under reduced serum conditions sensitize H-Ras transformed NIH 3T3 cells to apoptosis (Fig. 7). These observations make it conceivable that continued expression of p202 in transformed cells under reduced serum conditions is needed to maintain cell survival. Consistent with this hypothesis, it has been shown that the increased levels of p202 in transfected cells inhibit apoptosis [Wang et al., 2000; D'Souza et al., 2001; Xin et al., 2001] and the decreases in the levels of p202 increase the susceptibility of murine fibroblasts to apoptosis [Koul et al., 1998].

Studies have indicated that the IFN treatment of Ras transformed NIH 3T3 cells result in reversion of transformed phenotype [Samid et al., 1987; Rimoldi et al., 1988]. Our observation that p202 in NIH 3T3 cells reduces colony formation by oncogenic H-Ras (Fig. 6) raises the possibility that IFN-induced levels of p202 inhibit growth of Ras transformed cells. It is of

note that overexpression of p202 in human prostate cancer cell lines (PC-3, DU-145) resulted in retardation of cell proliferation and reduced ability to form colonies in soft agar (indicative of reversion of transformed phenotype) [Yan et al., 1999]. Additionally, overexpression of p202 in human breast cancer cell line (MCF-7) resulted in decreases in the ability to form tumors in nude mice [Wen et al., 2000]. Although it remains to be seen how ectopic expression of p202 reduces colony formation in Ras transformed NIH 3T3 cells or human cancer cell lines, the ability of p202 to upregulate the expression of $p21^{WAF1/CIP1}$ (independent of p53) [Gutterman and Choubey, 1999] and to inhibit the transcriptional activity of several factors (for example, c-myc, E2F, and AP-1) [Choubey, 2000], known to promote cell proliferation, makes it possible that p202 inhibits growth of Ras transformed cells (and perhaps other cancer cells), in part, by inhibiting the transcriptional activities of these factors.

Activated H-Ras is known to inhibit adenovirus-encoded E1A protein induced apoptosis in cells with wild type p53 [Lin et al., 1995]. Additionally, activated H-Ras cooperates with E1A to overcome p53-mediated growth arrest [Sakamuro et al., 1997]. Because the increased levels of p202 inhibit p53-mediated transcription [Datta et al., 1996], our observation that sustained expression of oncogenic H-Ras results in increases in the levels of p202 may also provide a molecular basis by which H-Ras inhibits E1A-induced p53-dependent cell growth arrest and apoptosis.

Based on our novel observations, the following conclusions can be drawn: (1) transformation of NIH 3T3 cells by oncogenic Ras (Q61L) results in the transcriptional activation of the *202* gene, resulting in increases in the steady-state levels of p202; (2) the increase in the levels of p202 correlates with increases in the activity of AP-1 in transformed cells and the increase, in part, depends potential AP-1 DNA binding sites in the 5′-regulatory region of the *202* gene; (3) the transcriptional activation of the *202* gene in Ras-transformed NIH 3T3 cells might involve Ras/Raf/MEK/JNK/c-Jun pathway; and (4) continued expression of p202 in Ras transformed cells may be necessary for cell survival under reduced serum conditions. Our observations described herein will serve as molecular basis for further studies involving the elucidation of the functional role of p202 in cell survival.

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