Contribution of Estrogen Receptor α to Oncogenic K-Ras-mediated NIH3T3 Cell Transformation and Its Implication for Escape from Senescence by Modulating the p53 Pathway*

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We previously reported that enhanced transcriptional activation of estrogen receptor α (ERα) contributed to [12Val]K-Ras-mediated NIH3T3 cell transformation. Functional inactivation of ERα by a dominant negative mutant of ERα (DNER) in the presence of activated K-Ras 4B mutant arrested the cell cycle at G0/G1, subsequently provoking replicative cell senescence, finally abrogating tumorigenic potential. p53-dependent up-regulation of p21 was implicated in this cell senescence induction. Alterations in the MDM2 protein in response to DNER accounted for this p21-mediated cell senescence induction. An oncogenic K-Ras 4B mutant significantly increased MDM2 proteins coprecipitated with p53, and suppressed p53 transcriptional activity. In turn, DNER exerted its function to decrease MDM2 proteins coprecipitated with p53, followed by the stimulation of p53 activity in the presence of the oncogenic K-Ras 4B mutant. In addition, overexpression of wild type ERα in NIH3T3 cells resulted in the significant increase in the MDM2 protein level and the resultant suppression of p53 transcriptional activity. Finally, we demonstrated that c-Jun expression overcame the suppression and resultant enhancement of p21 protein level in response to DNER. The data imply that the ERα-AP1 pathway activated by oncogenic K-Ras 4B mutant contributes to the NIH3T3 cells' transformation by modulating p53 transcriptional activity through MDM2.

The prevalence of Ras mutations in human cancer reaches ~30% of all cancers and is thus the most frequently activated oncogene overall (1). Ras is a point of convergence for most mitogenic extracellular stimuli. Ras activation occurs by recruitment to the membrane, followed by recognition of specific tyrosine phosphorylated sites in growth factor receptors. As extracellular signals converge on Ras, a number of intracellular signals are sprayed from it. In this manner, Ras activates and/or branching signal transduction pathways involving the mitogen-activated protein kinase; ERE, estrogen response element; E2, 17β-estradiol; AF, activation function; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyl transferase; SA, senescence-associated; wt, wild type; Ab, antibody; CDK, cyclin dependent kinase; MEF, mouse embryonic fibroblast.

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cative cell senescence. This Ras-induced senescence is accompanied by overexpression of both p16INK4a and p19ARF, and the consequent activation of pRb and p53 (8–11). In turn, oncopgenic Ras has the potential to manifest neoplastic transformation in NIH3T3 cells carrying a homozygous deletion at the INK4a/ARF locus and primary murine fibroblasts genetically deficient in p16INK4a/p19ARF, p19ARF alone, or p53 with no apparent need for additional genetic alterations (8, 12). mdm2 is involved in this neoplastic transformation by oncogenic Ras. A recent report showed how the activation of the Ras/Raf/MEK/MAPK pathway was responsible for induction of mdm2 through its promoter (13). mdm2 induced by the Ras signaling pathway degrades p53 in the absence of p19ARF, facilitating cell proliferation and survival.

In the present study, we investigated the effect of activated Ras expression on NIH3T3 cell transformation in the absence or presence of dominant negative ER that inhibited its AF2 function. The dominant negative ER mutant is the ER mutant containing a frameshift substitution at 554 codon Ser (S554fs ER), which was generated by random chemical mutagenesis (14). The previous report demonstrated that S554fs ER suppressed the activity of wild type ER by 80% when equal amounts of the plasmid encoding the mutant ER and the wild type ER were used (14). Activated K-Ras 4B significantly enhanced the ERα expression level and its transcriptional activity (7). As expected, the DNER expression in the presence of activated K-Ras 4B resulted in the abrogation of transcriptional activity by ER, resulting in G1/G0 arrest followed by cell senescence through p53-dependent up-regulation of p21. Alteration in MDM2 function accounted for this p21 up-regulation. Oncogenic K-Ras 4B expression significantly increased MDM2 proteins coprecipitated with p53 and suppressed p53 transcriptional activity. In turn, DNER decreased MDM2 proteins co-precipitated with p53 sharply, followed by the stimulation of p53 activity in the presence of oncogenic K-Ras 4B. In addition, overexpression of wt ERα in NIH3T3 cells corresponded to a significant increase in mdm2 mRNA and protein, and the resultant suppression of p53 transcriptional activity. Finally, we demonstrated that c-Jun overcame the down-regulation of MDM2 expression by DNER. These results suggested that ERα contributes to oncogenic K-Ras 4B-mediated NIH3T3 cell transformation by p53-independent modulation of MDM2 functions and its suppressive effect on p53 transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**pZIP-Neo SVX1 retrovirus vector and constructs containing cDNA sequences encoding [12Val]K-Ras 4B were gifts from Dr. Channing J. Der. The pSG5 eukaryotic expression vector containing a cDNA sequence encoding human ER was given by Dr. Pierre Chambon (CNRS). The pCAT reporter vector was purchased from Promega Corp. p53-luc was purchased from Invitrogen. pCMV-p53m135 vector was purchased from CLONTECH. pDEB delta vector containing c-jun DNA was used as a c-jun expressing vector as described previously (15). Site-directed Mutagenesis—Dominant negative ER (S554fs ER) DNA was generated by using a Transformer site-directed mutagenesis kit (CLONTECH) according to the manufacturer’s instructions. We used pSG5 human ER as a template DNA and CAT GCG CCC ACT GCC GTC GAG GGG as mutant oligos. The mutation was confirmed by sequencing.

Cell Culture—NIH3T3 cells were cotransfected with the pZIP-Neo SVX1 retrovirus vector, which contained a neo marker for selection and a pSG5 eukaryotic expression vector to produce various reconstituted cells using LipofectAMINE Plus (Invitrogen). Thus, control mock cells contained two empty vectors, NIH3T3 cells transfected with wt type ER or dominant negative ER were established by cotransfection with an empty pZIP-Neo SVX1 vector and pSG5-human ER (ER cells) or human DNER (S554fs) cDNA (DNER cells). NIH3T3 cells harboring mutant versions of [12Val]K-Ras 4B were established by transfection with pZIP-Neo SVX1 containing a cDNA sequence encoding [12Val]K-Ras 4B and empty pSG5 (K12V cells) or pSG5-human DNER (S554fs) (K12V DNER cells). Stably transfected cells were selected and isolated in growth medium containing G418 at 400 μg/ml (Geneticin, Invitrogen). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Seihika) supplemented with 10% calf serum (HyClone).

**Retroviral Gene Transfer and Expression—**Ecopack2–293 cells were purchased from CLONTECH and used as packaging cells. Retroviral gene transfer and expression were performed as described previously (16). To rescue recombinant SVXV virus, 5 × 103 Ecopack2–293 cells were transfected with 10 μg of pZIPneoSVX(V) vector containing Kras12V or empty vector using LipofectAMINE Plus (Invitrogen). 2 days after transfection, viral media was collected and filtered through a 0.45-μm Nalgene filter. To infect cells, 1 ml of the supernatant was added to 3 × 105 mock or DNER cells in a 60-mm dish and incubated for 24 h at 37°C in the presence of 8 μg/ml Polybrene (Sigma Chemical Co.). Complete media was added, the cultures were incubated for 2–3 days, and cells were used for analysis.

**Western Blot—**To detect each protein expression, subconfluent cells were lysed with ice-cold radiomune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40) containing freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin). For analysis of phosphorylated proteins, cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 10% glycerol, 157 mM NaCl, 1.5 mM MgCl2, 50 mM NaF, 1 mM sodium vanadate, containing freshly added protease inhibitor. After centrifugation at 13,000 × g for 10 min to remove debris, 100 μg of the proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in a semidry transfer cell (Bio-Rad). The blots were incubated with diluted primary antibodies. Primary antibodies, including anti-p21 polyclonal antibody (C-19), anti-p16 polyclonal antibody (M-156), and anti-MDM2 monoclonal antibody (SMP-14) were obtained from Santa Cruz Biotechnology. The anti-p53 monoclonal antibody (Ab-1 or Ab-3) and anti-MAPK monoclonal antibody (Ab-1) were obtained from Calbiochem. Anti-ER monoclonal antibody (AER314) was purchased from Neomarkers. Anti-phosphospecific MAPK antibody and p53 (Ser20) antibody were from Promega and Cell signaling, respectively. After incubation with each primary antibody, blots were incubated with horseradish peroxidase-linked, anti-mouse, or anti-rabbit antibodies and analyzed with an ECL system (Amersham Biosciences, Inc.). The amount of each protein was quantitated using NIH3TIS3 image software.

**Analysis of Interaction between p53 and MDM2—**Interaction between p53 and MDM2 was analyzed by immunoprecipitation with an anti-p53 Ab followed by Western blotting with an anti-MDM2 antibody. The precipitated proteins were immunoprecipitated with p53 monoclonal antibody (Ab-1, Calbiochem). The precipitates were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and then analyzed by Western blot with an anti-MDM2 monoclonal antibody (SMP-14, Santa Cruz Biotechnology) or an anti-p53 monoclonal antibody (Ab-3, Calbiochem).

**Immunohistochemistry and Immunofluorescent Stain—**ER expression or K-Ras expression was analyzed by immunohistochemistry using an avidin-biotinylated immunoperoxidase technique (Vector Laboratories, Burlingame, CA). Cells were plated at ~1 × 104 cells per two-well chamber (Nunc, Roskilde, Denmark). After 2 days, cells were permeabilized and fixed by treatment with 10% formalin and incubated with a mouse monoclonal anti-ER antibody (AER314, Neomarkers) or anti-K-Ras antibody (P234, Santa Cruz Biotechnology) overnight at 4°C. Bound antibodies were detected with a biotinylated anti-mouse IgG secondary antibody and an avidin-biotin complex linked to horseradish peroxidase followed by incubation with diaminobenzidine tetrahydrochloride as the substrate. In immunofluorescence stain, bound ER antibodies were visualized with green fluorescence from secondary antibody staining with anti-mouse IgG conjugated to Alexa 488 (Molecular Probes). The cells were counterstained with 4',6-diamidino-2-phenylindole to visualize the nuclei (Vectashield, Vector).

**Immunoprecipitation Analysis of Ras—**Cells were labeled overnight in growth medium supplemented with [35S]-methionine/cysteine (Trans-35S-label, 400 μCi/ml, ICN, Irvine, CA). Ras proteins were immunoprecipitated using the Y13–259 rat anti-Ras monoclonal antibody (Calbiochem), resolved by SDS-PAGE, and subjected to fluorography and cells harboring or lacking Ras expressed or not expressed. Luciferase Assay—Transient transfection of 1 μg of a luciferase reporter (p53-Luc) plasmid was performed using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. After transfection, the cells were incubated with DME containing 10% calf serum. Luciferase assays were performed using a Luciferase assay kit (Stratagene, La Jolla, CA). Transfection efficiency was normalized by β-ga.
CAT Assay—Vitellogenin A2 ERE (5'-GATCTAGGTCACGTGAGCTA-3') were inserted into the PCAT-Enhancer Vector, which contained an SV40 enhancer element (Promega). Each cell was transfected with the PCAT vector containing vitellogenin A2 ERE. After transfection, the cells were incubated for 72 h with phenol-red free DME containing 10% charcoal-dextran-treated calf serum. The medium was then replaced overnight with phenol red-free DME containing 1% charcoal-dextran-treated calf serum in the presence or absence of 10^{-7} M 17β-estradiol (Sigma). After washing in phosphate-buffered saline, the cells were suspended in 0.25 M Tris-Cl, pH 7.8 and lysed by five cycles of freezing and thawing. A CAT assay of 10 μg of cell extract was performed as described previously (17).

Senescence-associated β-Galactosidase (SA-β-gal) Staining—Cells cultured on chamber slides (Nalge, Nunc International) were fixed in 2% formaldehyde/0.2% glutaraldehyde. SA-β-galactosidase staining was performed as described before (18).

Semiquantitative RT-PCR—Total cellular RNA was isolated using ISOGEN (Nippon Gene). One microgram of total RNA was reverse-transcribed into cDNA by reverse transcriptase and amplification of mouse mdm2 and a control glyceraldehyde-3-phosphate dehydrogenase gene sequence. The primers used for PCR were as follows: 5'-CTGTGCTGGCGACCGGAGAC-3' (mdm2 exon 2 sense); 5'-GCCAGGCAATCCAGGACTG-3' (mdm2 exon 3 antisense); 5'-TGAAAGCTGGAGTCACAG-GATTGGG-3' (glyceraldehyde-3-phosphate dehydrogenase sense); 5'-CATGTCGGCCATGAGGTCCACCAC-3' (glyceraldehyde-3-phosphat dehydrogenase antisense).

The conditions for PCR reactions of mdm2 were denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s.

The PCR exponential phase was determined on 20–30 cycles to allow comparison among cDNAs developed from identical reactions. After electrophoresis in 1.0% agarose gel and ethidium bromide staining, the photographs were analyzed by FAS II (TOYOBO) and Digital Image File DF-20 (FUJI Film). The amount of each PCR product was quantified using IMAGE software (National Institutes of Health). The optimal number of PCR cycles was found to be 25.

RESULTS

Establishment and Characterization of Reconstituted Cells Expressing DNER Generated by the Cotransfection System.—We established four kinds of reconstituted NIH3T3 cells that expressed dominant negative ER (DNER) and/or constitutively activated [12Val]K-Ras 4B proteins: mock cell cotransfection of an empty pZIP-NeoX/X1 retrovirus vector and an empty pSG5 eukaryotic expression vector, DNER cell cotransfection of an empty pZIP vector and a pSG5 vector containing ER cDNA with a frameshift substitution (SS54fs), K12V cell cotransfection of a pZIP vector containing [12Val]K-Ras 4B cDNA and an empty pSG5 vector, and K12V DNER cell cotransfection of a pZIP containing [12Val]K-Ras 4B and pSG5 vector containing ER cDNA with an SS54fs mutation, respectively. SS54fs DNER cDNA was generated by site-directed mutagenesis.

We determined the ER protein levels in these reconstituted cells by Western blots using ER monoclonal antibodies that failed to recognize a DNER form differentially from endogenous ER protein. As shown in previous reports (7), an ~2-fold increase in ER protein levels was observed in K12V cells compared with that in mock cells (Fig. 1A). This protein level of ER was almost equal to that in DNER and K12V DNER cells. Immunohistochemical and immunofluorescent staining showed the preferential localization of ER in nuclei of K12V cells, in contrast with the cytoplasmic localization in DNER cells (Fig. 1A). ER was colocalized both in the cytoplasmic and nuclear compartments in K12V DNER cells. Similar levels of exogenous K-Ras 4B protein and phosphorylated MAPK protein were detectable both in K12V and K12V DNER cells (Fig. 1B).

To investigate the alterations in transcriptional activity by ERα in these reconstituted cells, we transiently transfected them with pCAT-enhancer vector, which contained an SV40 enhancer element and vitellogenin A2 ERE (Fig. 1C). The transfection efficiency of each cell line was normalized by β-galactosidase assays. The values are the means obtained from four to six repeated experiments.
S554fs ER was powerful and were compatible with a previous report (14).

Cell Senescence Induction by DNER in the Presence of Activated K-Ras 4B in NIH3T3 Cells—Although mock cells displayed spindle-shaped morphology, K12V cells grew as rounded, refractile cells and had the potential to form tumors in nude mice. In response to DNER expression in K12V cells, we observed profound alterations in both cell proliferation and morphology, whereas none of these alterations was detectable by DNER expression in mock cells (Fig. 2A). To characterize these effects, cells were seeded at 2.5 × 10^4 cells per well, and cell numbers were obtained as a function of time. As shown in Fig. 2B, K12V cell proliferation was markedly inhibited by DNER expression, despite no alterations in mock cell proliferation. In addition, DNER expression inhibited the colony-forming ability of K12V cells in soft agar (data not shown). There were also dramatic alterations in cell morphology. DNER expression caused cells to exhibit increased size and flattened morphology, as well as enlarged nuclei in all three individual clones from K12V DNER cells. A significant increase in the proportion of enlarged cells with multinuclei was detectable in these cells (6-fold) compared with those in mock, DNER, and K12V cells (Fig. 2A).

We therefore analyzed K12V cells for changes in cell cycle progression in the absence or presence of DNER by FACS with propidium iodide staining. DNER expression exhibited a marked reduction in the S-phase population declining from 13.2% to 5.0% in K12V cells. Conversely, the percentage of cells in the G0/G1 phase population increased from 65% to 81%, indicating the correlation of DNER expression with the arrest of K12V cells in the G0/G1 phase (Fig. 2C). Of note, there was no evidence indicating the accumulation of a sub-G1 population as seen in apoptosis. Senescent, but not presenescent, quiescent cells express a senescence-associated β-galactosidase activity, which can be detected by incubating cells at pH 6.0 (18). Because coexpressing DNER and [12Val]K-Ras proteins exhibited a striking feature characteristic of senescent cells, we examined whether these cells expressed this senescence-specific marker (Fig. 2D). SA-β-galactosidase-positive mock, DNER, and K12V cells were undetectable. In contrast, almost 30–50% of K12V DNER cells were SA-β-galactosidase-positive. Very strong SA-β-galactosidase activities were shown in enlarged K12V DNER cells with multinuclei. These results indicated that DNER expression was able to induce replicative cell senescence in NIH3T3 cells transformed by the activated K-Ras 4B, but not in untransformed mock cells. To validate the DNER effect on K12V cells by using the [12Val]K-Ras retroviral construct as a retrovirus, mock cells or DNER cells were infected with the virus expressing either empty vector or [12Val]K-Ras, respectively. Vector or [12Val]K-Ras-infected mock cells and vector-infected DNER cells (Fig. 2E, panels a, b, d) displayed no remarkable change in morphology. In contrast, in response to [12Val]K-Ras infection, a significant proportion of enlarged cells with multinuclei were observed in DNER cells (Fig. 2E, panel e). These enlarged cells were SA-β-galactosidase-positive (data not shown). The proportion of senescent cells was higher in [12Val]K-Ras-infected DNER cells (70–80%) than that in the stable K12VDNER transfectants (30–50%). These results demonstrated that coexpression of activated K-Ras and DNER induced senescence in NIH3T3 cells.

Induction of the p21 CDK Inhibitor through a p53-dependent Pathway in Senescent K12V DNER Cells—During the replicative senescence of normal diploid human fibroblasts, the expression of both p21 and p16 CDK inhibitors increases dramatically. The increase is sequential; p21 expression increases when the majority of cells have lost their growth potential, whereas p16 expression increases in the terminal stages of senescence when all cells have lost their growth potential (19). NIH3T3 cells sustain a homozygous deletion at the INK4a/ARF locus, which encodes p16INK4a and p19ARF with different reading frames (20, 21). In agreement with the homozygous deletion, either of p16 or p19 expression was undetectable in the reconstituted NIH3T3 cells, whereas A431 cells and MEF expressed detectable levels of these proteins (Fig. 3A). As expected, the DNER expression increased p21 expression level 3.5– to 4.2-fold in K12V cells but not mock cells (Fig. 3A).

It is well known that p21 is under the transcriptional control of p53, and p53-independent modulation of p21 is also known. To address whether or not p21 induction in senescent K12V DNER cells was p53-dependent, we investigated the alteration in p53 transcriptional activity by a luciferase assay using the luciferase reporter containing p53-responsive consensus sequences in the promoter. Expression of the activated [12Val]K-Ras 4B in NIH3T3 cells resulted in significant reduction of p53 transcriptional activity (42% of that in mock cells) (Fig. 3B). In turn, DNER expression in the presence of activated [12Val]K-Ras 4B corresponded to the marked enhancement of p53 transcriptional activities in individual K12V DNER clones C1 and C2 (3.4- to 3.7-fold). To further demonstrate the p53-dependent p21 induction in K12V DNER cells, we transiently transfected each reconstituted NIH3T3 cell with a pCMV-p53 mt 135 vector, which expressed a dominant negative p53 protein and analyzed the p53-dependent luciferase activity at 72 h after transfection. Dominant negative p53 completely inhibited the luciferase activity in mock and K12V cells and suppressed the activity partially (10–20% of that in the individual K12V DNER clones) in K12V DNER cells (Fig. 3C). This reflected significant induction of p53 activity in K12V DNER cells, compared with that in mock and K12V cells. In accordance with the suppression of p53 activities, expression of a dominant negative p53 corresponded to undetectable p21 in mock and K12V cells and detectable but significantly lower levels of p21 that were less than a half of that in K12V DNER cells. Significant reduction in p21 levels owing to a dominant negative p53 resulted in the decrease in senescent cell populations in K12V DNER cells (Fig. 3C). These results indicated that activated [12Val]K-Ras 4B had the potential to suppress p53-transcriptional activity and DNER expression in the presence of activated K-Ras 4B-induced replicative senescence by up-regulating p21, at least in part via a p53-dependent pathway, in NIH3T3 cells.

Contribution of ERα to the p53-independent Up-regulation of MDM2—MDM2 acts as a major regulator of p53 by targeting its destruction. It has been shown recently that the mdm2 gene is up-regulated by the Ras-driven Raf/MEK/MAPK pathway in a p53-independent manner (13). The Ras-driven pathway also increases the EBox steady-state level, functioning as a transcription factor in NIH3T3 cells (7). According to this line of reasoning, EBox is predicted to contribute to the p53-independent up-regulation of MDM2 downstream of Ras. We addressed whether the level of MDM2 protein involved in the p53 binding complex was altered in response to the activated [12Val]K-Ras 4B and/or DNER. At first, we carried out semiquantitative RT-PCR to evaluate the level of mdm2 mRNA in each cell line. We obtained the PCR exponential phase and determined the optimal number of PCR cycles, which was 25 cycles (Fig. 4A). As reported previously, the activated [12Val]K-Ras 4B induced the expression of mdm2 mRNA in NIH3T3 cells (3.1-fold) (Fig. 4B). In turn, DNER expression in K12V cells erased this stimulatory effect (1.3-fold) (Fig. 4B, lane 3). By using immunoprecipitation with an anti-p53 antibody followed by Western blots with an anti-MDM2 antibody, we detected a significant in-
crease in MDM2 protein in the p53 precipitates in the presence of activated [12Val]K-Ras 4B and the erasure of this effect by DNER expression in a NIH3T3 cell system (Fig. 4). The phosphorylation levels of p53 protein at Ser-20 (Fig. 4).

FIG. 2. Induction of senescence by coexpression of [12Val]K-Ras and dominant negative ER. A, morphology of each reconstituted cell line (magnification ×200). K12V DNER cells acquired flat and enlarged morphology. B, anchorage-dependent cell growth was investigated in each cell line. Cells were plated at 2.5 × 10⁴ cells per well of 24-well plates in DMEM supplemented with 10% fetal calf serum. Viable cells were counted on days 2, 4, 6, and 9. Marked suppression of K12V cell growth was noticeable in response to DNER. C, FACS with propidium iodide staining was analyzed. DNER expression exhibited a significant reduction in the S-phase population declining from 13.2% to 5.0% in K12V cells. The percentage of cells in the G₀/G₁ phase increased from 65% to 81%. D, the senescence-associated ß-galactosidase activity at pH 6.0 was investigated in each cell line. SA-ß-galactosidase activities are shown in the K12VDNER cells. Very strong SA-ß-galactosidase activities in enlarged K12VDNER cells with multinuclei are shown. E, mock cells or DNER cells were infected with the virus expressing either empty vector or [12Val]K-Ras, respectively. Vector or [12Val]K-Ras-infected mock cells (a, d) and vector-infected DNER (b) cells displayed no remarkable change in morphology. Significant population of enlarged cells with multinuclei were observed in only [12Val]K-Ras-infected DNER cells (e). K-Ras protein expression in [12Val]K-Ras-infected DNER cells was determined by immunohistochemistry using anti-K-Ras monoclonal antibody (F234) (f).

FIG. 3. Cell senescence induction in K12V cells in response to DNER via p53-dependent p21 up-regulation. A, Western blots were performed using each anti-body (p21, p53, and p16). The marked induction of p21 proteins was noticeable in K12VDNER cells. No significant difference in p53 expression was observed in each cell line. Expression of p16 proteins was not detected in the reconstituted NIH3T3 cell lines. MEF cells and A431 cells were used as a positive control for detection of p16 protein. Relative ratio of p21 expression level to that in mock cells is shown. B, transient transfections of each cell line with a p53 luciferase reporter gene were used to monitor p53 activity in the steady state. 72 h after transfection, cells were lysed for analysis. p53 activity was decreased in K-Ras-transformed cells (42%) and increased in senescent K12VDNER cells (3.7-fold and 3.4-fold in C1 and C2, respectively) compared with that in mock cells. C, transient cotransfections of each cell line with the dominant negative p53 expression vector and p53 luciferase gene were performed. 72 h after transfection, cells were lysed for Western blot and stained with SA-ß-galactosidase. Expression of dominant negative p53 proteins markedly inhibited the luciferase activity in mock, K12V, and K12VDNER cells. Inhibition of the levels of p21 expression and a decrease in the number of senescent cells were observed by the introduction of a dominant negative p53 gene into K12VDNER cells. The relative ratio of p21 expression level in each cell line in the absence or the presence of DN p53 is shown.
Ser-20 codon of p53 was detectable between K12V cells and K12VDNER cells. No significant difference in phosphorylation at the Ser-15 (data not shown) seemed to be analogous between K12V cells and K12VDNER cells. These results implied that the effects of Ras on the suppression of AP-1 activity by DNER involved in the p53 immunoprecipitate resulted in the suppression of transcriptional activity by p53 (20%) in ER cells (data not shown). The up-regulation of MDM2 by K-Ras12V or by ERα overexpression of constitutively activated c-Jun, which are known as downstream effector of Ras, leads to a marked induction of mdm2 gene, whereas DNER erased this effect of activated K-Ras 4B in NIH3T3 cells that sustained a homozygous deletion at the INK4a/ARF locus. To address whether the ERα was a downstream effector of K-Ras 4B, we established ERα overexpressing cells by transfecting NIH3T3 cells with a pSG5 vector containing wt ER cDNA. These ERα cells did not exhibit any transformed phenotypes or tumorigenic potentials.

Fig. 5. Enhancement of the levels of MDM2 expression by overexpression of ERα in NIH3T3 cells. A, the levels of mdm2 mRNA and protein were investigated in ER overexpressing cells (ERα cells). RT-PCR on 25 cycles and Western blots were performed as described in Fig. 4. The levels of mRNA and protein expression were increased compared with those in mock cells (5.2- and 2.0-fold, respectively). B, interaction of MDM2 and p53 was also enhanced (1.8-fold). The association between MDM2 protein and p53 protein was examined, as described in Fig. 4, C; the levels of MDM2 protein expression in the presence or absence of E2 or 10% calf serum were investigated. Cells were incubated with phenol red and serum-free DMEM overnight and followed by the treatment with 10% calf serum or 10^{-7} M 17β-estradiol for 18 h. The expression level of MDM2 was very low in the absence of serum in all three cell lines. E2 treatment enhanced the level of MDM2 proteins in K12V cells but not in ERα cells. Expression of MAPK is shown as an internal control. The relative ratio of MDM2 protein level in the presence of E2 or serum to that without serum is shown in each cell line.

MDM2 expression is modulated by the Ras-MAPK pathway through activation of Ets and AP-1 sites in the mdm2 p2 promoter, and then, overexpression of constitutively activated forms of c-Ets1−2 and c-Jun, which are known as downstream targets of Ras, leads to a marked induction of mdm2 p2 promoter activity. ERα also enhances the transcription of genes that contain AP-1 sites, the cognate binding sites for the Jun-Fos complex (22). Previous reports showed that transactivation functions of ERα through AP-1 sites required the ligand binding domain and AF-2 of ERα. The AF-2 function was eliminated in DNER (S554fs) that we used in this study. To further investigate whether the suppression of AP-1 activity by DNER in K12V cells resulted in a decrease in MDM2 levels, we transfected with pcDEB delta c-jun cDNA into K12V DNER cells transiently (15). Transient transfection of pcDEB delta c-jun cDNA in K12V DNER cells resulted in an increase in the c-Jun
in this transformation process. Functional inactivation of ER 
the signaling route that involves Ras/ER 
constitutively stimulated MAPK in the presence of DNER (Fig. 

However, the activated K-Ras 4B mutant 
in NIH3T3 cells transiently. Consistent with the increase in c-Jun levels, MDM2 protein levels were elevated and p21 protein levels were decreased, resulting in a decrease in the senescent cell number. The relative ratio of c-Jun, MDM2, and p21 protein expression level to that in the absence of c-Jun is shown.

protein level (2.0- to 3.2-fold) (Fig. 6). Consistent with the increase in c-Jun, MDM2 protein levels were elevated, followed by a subsequent decrease in p21 protein level despite the presence of DNER. As a result, c-Jun expression resulted in the escape of NIH3T3 cells from senescence. These results demonstrate that ERα functions downstream of Ras and the MDM2 modulation by ERα were regulated by AP-1 activity, indicating the signaling route that involves Ras/ERα/AP-1/MDM2/p53 in the regulation of NIH3T3 cell senescence.

**DISCUSSION**

The present study examined the role of the Ras-ERα signal transduction pathway in cell transformation mediated by the activated [12Val]K-Ras 4B. The tumorigenic effects of this oncogenic mutant are well established in some murine cell lines with homozygous deletion at the INK4a/ARF or with functionally inactivated pRB and/or p53. Enhanced steady-state levels of ERα that functioned as a transcription factor were involved in this transformation process. Functional inactivation of ERα by expression of a powerful DNER, S554fs, in the presence of an activated K-Ras 4B mutant completely inhibited tumorigenic potentials in NIH3T3 cells. Of note in response to DNER, the reconstituted NIH3T3 cells stopped proliferating and arrested the cell cycle at G0/G1, subsequently provoking replicative cell senescence. However, the activated K-Ras 4B mutant constitutively stimulated MAPK in the presence of DNER (Fig. 1B). This strengthens the implication of Ras-ERα signaling in cell proliferation in addition to various transformed phenotypes in NIH3T3 cell transformation mediated by the K-Ras 4B mutant. The activated Ras mutant by itself may be insufficient to transform NIH3T3 cells and requires enhanced ERα activity. Previous observations using neonatal rats exposed to carcinogens are compatible with the view that ER contributes to cell transformation mediated by mutant Ras (23). The activated ras oncogene remains latent within the mammary gland until exposure to E2.

We examined p21 expression levels to address the molecular mechanism responsible for senescence induction in K12V cells in response to DNER, p53-dependent p21 up-regulation mainly accounted for cell cycle arrest and the subsequent replicative cell senescence. DNER expression did not elicit detectable levels of p21 up-regulation in mock cells, indicating the specific effect of DNER on NIH3T3 cells transformed by the activated K-Ras 4B. A p53 luciferase assay also demonstrated the marked stimulation of p53 transcriptional activities in response to DNER in the presence of activated K-Ras 4B mutant in NIH3T3 cells. The data implied that DNER in the presence of an activated [12Val]K-ras 4B mutant provoked p53-dependent cell senescence.

Activation of p53 protein is limited by a short feedback loop involving the mdm2 gene product, p53 stimulates transcription of the mdm2 gene, and MDM2 protein binds to activated p53 protein. This interaction inhibits p53 transcriptional activity and promotes its export to the cytoplasm for proteasome-mediated degradation. Thus, we examined the interaction of MDM2 protein with p53 in response to activated K-Ras 4B and/or DNER. As reported previously (13), the activated K-Ras 4B up-regulated the transcription of the mdm2 gene. In accordance with this up-regulation, increases in MDM2 proteins coprecipitated with p53 accounted for the suppression of p53 transcriptional activity. Thus, MDM2 induced by this Ras mutant is functionally active. Most importantly, DNER completely erased the enhanced transcription of mdm2 in response to an activated K-Ras 4B mutant, followed by the absence of detectable MDM2 protein coprecipitated with p53. Decreases in mdm2 mRNA and protein in response to DNER accounted for a 3.4- to 3.7-fold stimulation of p53 transcriptional activity, compared with that in mock cells. Thus, DNER had the potential to erase the functional activation of MDM2 in response to the K-Ras 4B mutant. These results suggest that ERα is an important downstream effector of Ras, modulating p53 transcriptional activity through MDM2. However, the effects of MDM2 on p53 are also regulated by p19ARF in normal cells. The Ras-mediated pathway induces the expression of p19ARF (11, 24), which inhibits MDM2 activity (25, 26). ERα is specifically involved in Ras-mediated MDM2 activation but not in the induction of p19ARF in NIH3T3 cells.

In about 30% of human cancers, Ras is constitutively activated by mutation (1). Activated Ras elicits ERα activation. In addition, the events provoking deregulation of signals transmitted by ERα are linked to the development of hormone-dependent and -independent cancers. This suggests the prevalence of functional ERα activation in various human cancers. ERα-induced MDM2 may block the p53 function that induces growth arrest and senescence of cancer cells, allowing progression of cancer cells with wt p53 to those with more invasive characters. The low incidence of p53 mutations that are detectable in poorly differentiated breast and endometrial cancers with worse prognosis is compatible with this assumption. However, repression of p19ARF may be required for ERα-mediated down-regulation of p53 functions. Strong selection pressure for disrupting the p19ARF-MDM2-p53 pathway may be involved in the development of hormone-dependent cancers by compromising p53 functions. Although many of the deletions that affect p16INK4a also encompass p19ARF, only a minority of the tumor-associated mutations in INK4a locus affects the sequence of p19ARF (in exon 2), and no mutations have yet been recorded in exon 1α (27). The p19ARF protein encoded by exon 1α alone is sufficient to induce cell cycle arrest. However, it is known that Tbx2, a gene that immortalizes MEF by down-regulating primarily p19ARF concentrations, is amplified in a subset of primary human breast cancers. Thus, the events disrupting the p19ARF-MDM2-p53 pathway are predicted to be associated with the development of some cancer types.

Constitutively activated forms of c-Ets1–2 and c-Jun are able
to up-regulate mdm2 expression (13). The oncogenic K-Ras 4B mutant in the presence of DNER putatively activates c-Ets1–2 and c-Jun, which are known as downstream targets of MAPK, because activated MAPK is detectable in K12V DNER cells. Nonetheless, the present study demonstrated that DNER suppressed mdm2 transcription and MDM2 protein coprecipitated with p53 in the presence of an activated K-Ras 4B mutant. The results suggest that ERα is a point of convergence for these mitogenic signals. Thus, we investigated the alteration in mdm2 expression levels in K12 DNER cells by transient transfection of c-jun cDNA. c-Jun expression restored the MDM2 expression level partially, followed by the subsequent suppression of p21 expression. As a result, K12V DNER cells escaped from premature senescence through the recovery of AP-1 activity. Thus, it is possible that liganded ERα elicits mdm2 up-regulation through activating the AP-1 site in the mdm2 promoter by stimulating the AP-1 activity.

The ligand-independent interaction of ERα with p53 was also described recently. ERα and HDM2, mdm2 human homologues, and p53 proteins form a ternary complex, and ERα protects p53 from being inactivated by hdm2 (28). The other and represses the E2-activated transcriptional activity of ER (29). In the present study, we demonstrated the ERα-mediated suppression of p53 transcriptional activity through mdm2 up-regulation in NIH3T3 cells. Although the genetic backgrounds of the cells used were different, the mechanism corresponding to the different interaction of ERα with p53 remains unknown. Detailed functional analyses using various types of cells is required to establish the biological significance of ERαp53 interactions in normal and cancer cells.

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