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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

One specific characteristic of ovarian cancer cells is their ability to survive in the absence of adhesion. Our previous studies demonstrate that the expression of vitronectin and αvβ3 integrin and the interaction between them are essential for ovarian cancer cell survival in suspension. We thus hypothesize that ovarian cancer malignancies may be suppressed by blocking vitronectin and αvβ3 integrin expression. In the present study, we employed a newly developed technology called RNA interference for inhibiting vitronectin and αvβ3 integrin expression. Four sets of short interfering RNA (siRNA) were designed for vitronectin and β3 integrin subunit mRNA, and these siRNA were expressed in ovarian cancer cells using the pSUPER system. The expression of at least one vitronectin siRNA and one β3 integrin siRNA significantly inhibited cellular vitronectin and β3 integrin expression. Our studies demonstrate that specific and potent siRNA can be developed to suppress vitronectin and αvβ3 integrin expression in ovarian cancer cells.

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Table of Contents

Introduction

This proposal is based on our previous finding that the expression of vitronectin and αvβ3 integrin and their interaction promotes ovarian cancer cell survival in suspension condition. In the studies performed in the first year of the funding period, we demonstrated that the interaction of vitronectin and αvβ3 integrin induces NF-κB activation and the induced NF-κB activity is essential for vitronectin/αvβ3 integrin-mediated ovarian cancer cell survival. In the second year of the funding period, our goal was to develop specific and potent short interfering RNA (siRNA) against vitronectin and β3 integrin subunit. Using a newly developed siRNA expression system (pSUPER), we demonstrate that siRNA can be successfully developed to diminish vitronectin and β3 integrin expression in ovarian cancer cells. Currently, we are developing siRNA-containing adenoviral vectors and will further investigate the importance of vitronectin and αvβ3 integrin in ovarian tumorigenecity in *in vivo* model.

Body

Our previous goal of this project in the second year is to develop potent ribozymes specific for vitronectin and β3 integrin subunit mRNA. However, double strand RNA in the past several years has been demonstrated to effectively suppress gene expression in yeast and fly cells through a process known as RNA interference (1). Especially in last year, the synthetic short (21- to 23-nucleotides) interfering RNA (siRNA) was found to mediate strong and specific suppression of gene expression in mammalian cells (2). In addition, studies have also shown that siRNA is much more potent and stable than the commonly used ribozyme and antisense RNA (3). Considering the superiors of siRNA over ribozyme, we chose to develop siRNAs specific for vitronectin and β3 integrin subunit mRNA instead of ribozymes.

Several strategies have been developed for efficiently delivering siRNA in mammalian cells (4,5,6). Since the goal of this project is to deliver siRNA to ovarian cancer cells with the aid of recombinant adenovirus, we employed a newly developed mammalian expression system that directs the synthesis of siRNA-like transcripts (pSUPER) in target cells for our studies. In this system, the vector contains the polymerase-III H1-RNA gene promoter, and thus allowing the production of a small RNA transcript not only lacking poly-A tail but also containing two 3' overhanging U nucleotides (6). We used previously described strategy (2) for the selection of the potential siRNA of vitronectin and β3 integrin subunit. Briefly, the 19 nucleotide sequence with flanking 5'AA 100 base from the AUG start codon were examined for the GC content and the sequences containing 40-60% GC richness were then selected as potential siRNA sequences. The four potential vitronectin siRNA

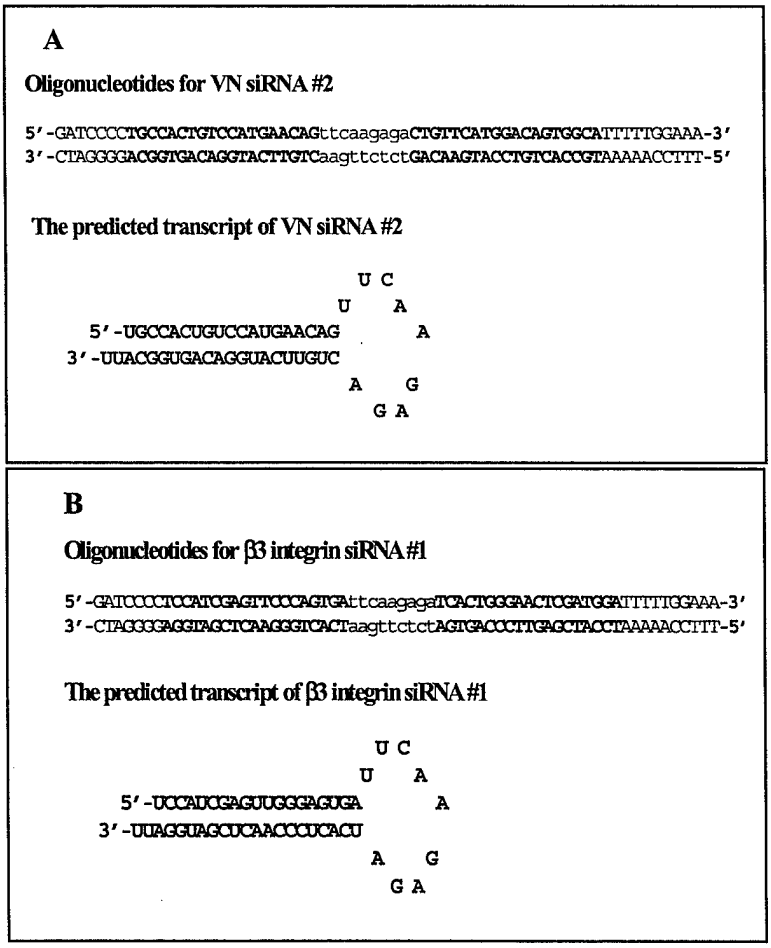

Fig.1. Oligonucleotides for vitronectin siRNA#2 and β3 integrin subunit siRNA and their predicted transcripts.

sequences are at nucleotides +108-126, +258-276, +393-411, and +583-601 relative to AUG translation site in vitronectin mRNA. The four potential β3 integrin siRNA sequences are at nucleotides +233-251, +325-343, +453-471, and +555-573 relative to AUG site in β3 integrin subunit mRNA. All sequences were further analyzed by BLAST and confirmed to be unique for vitronectin or β3 integrin subunit mRNA. To make pSUPER constructs containing these siRNAs, oligonucleotides for the respective siRNA were synthesized and subcloned into Hind III/Bgl II of pSUPER vector. The oligonucleotide sequences encoding vitronectin siRNA #2 (VN-siRNA2) and β3 integrin siRNA #1 (β3-siRNA1) and their predicted RNA transcripts are shown in Figure 1A and B respectively. To determine the effect of these siRNA on vitronectin and β3 integrin expression, the pSUPER containing siRNA sequences were transfected into OVCAR5 cells (expressing both vitronectin and β3 integrin subunit mRNA) with Lipofectamine (Invitrogen). After 72 hrs, the transfected cells were lysed and immunoblotting with respective antibodies was performed to determine the levels of vitronectin or β3 integrin subunit. As shown in Figure 2, at least one vitronectin siRNA (VN-siRNA2) and one β3 integrin siRNA (β3-siRNA1) greatly inhibited vitronectin and β3 integrin expression respectively. These studies demonstrate that siRNA can be successfully used to inhibit both vitronectin and β3 integrin subunit expression.

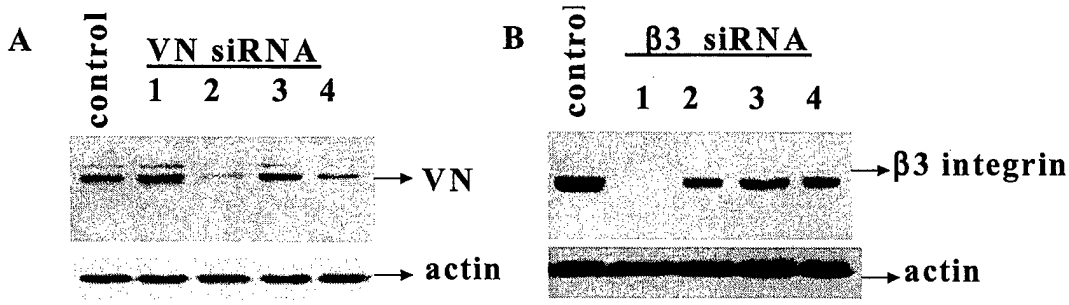

Fig 2. pSUPER-directed siRNAs can efficiently inhibit vitronectin and β3 integrin subunit expression. pSUPER containing various vitronectin siRNAs or β3 integrin siRNAs were transfected into OVCAR5 cells for 72 hrs. Cells were lysed and subjected to immunoblotting with anti-VN or anti-β3 polyclonal antibody to detect vitronectin or β3 integrin subunit expression.

Key Research Accomplishment

- We have generated siRNA targeted against vitronectin and β3 integrin subunit. Introducing siRNAs resulted in dramatic reduction in vitronectin and β3 integrin expression in ovarian cancer cells.

Reportable Outcomes

Two published manuscripts were partially supported by this grant:

1. Chen J, Baskerville C, Han Q, Pan ZK, Huang S. (2001). Alpha(v) integrin, p38 mitogen-activated protein kinase, and urokinase plasminogen activator are functionally linked in invasive breast cancer cells. *J.Biol.Chem.* 276:47901-5.

2. Wang W, Chen JX, Liao R, Deng Q, Zhou JJ, Huang S, Sun P. (2001). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. *Mol.Cell.Biol.* 22:3389-403.

The NCI grant (1R01CA093926) was partially supported by the work generated from this funding.

Conclusions

We have generated potent siRNA for both vitronectin and β3 integrin subunit. This accomplishment will allow us to develop an adenovirus-mediated gene therapy approach for diminishing vitronectin and β3 integrin expression in ovarian cancer cells. Our previous studies have demonstrated that vitronectin and β3 integrin are essential for ovarian cancer cell survival, adenovirus-delivered vitronectin and β3 integrin siRNA will thus be very likely to suppress ovarian cancer malignancies.

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Appendices

Chen J, Baskerville C, Han Q, Pan ZK, Huang S. (2001). Alpha(v) integrin, p38 mitogen-activated protein kinase, and urokinase plasminogen activator are functionally linked in invasive breast cancer cells. *J.Biol.Chem.* 276:47901-5.

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α_v Integrin, p38 Mitogen-activated Protein Kinase, and Urokinase Plasminogen Activator Are Functionally Linked in Invasive Breast Cancer Cells*

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We reported previously that endogenous p38 MAPK activity is elevated in invasive breast cancer cells and that constitutive p38 MAPK activity is important for overproduction of uPA in these cells (Huang, S., New, L., Pan, Z., Han, J., and Nemerow, G. R. (2000) *J. Biol. Chem.* 275, 12266–12272). However, it is unclear how elevated endogenous p38 MAPK activity is maintained in invasive breast cancer cells. In the present study, we found that blocking α_v integrin functionality with a function-blocking monoclonal antibody or down-regulating α_v integrin expression with α_v-specific antisense oligonucleotides significantly decreased the levels of active p38 MAPK and inhibited cell-associated uPA expression in invasive breast cancer MDA-MB-231 cells. These results suggest a function link between α_v integrin, p38 MAPK activity, and uPA expression in invasive tumor cells. We also found that vitronectin/α_v integrin ligation specifically induced p38 MAPK activation and uPA up-regulation in invasive MDA-MB-231 cells but not in non-invasive MCF7 cells. Finally, using a panel of melanoma cells, we demonstrated that the cytoplasmic tail of α_v integrin subunit is required for α_v integrin ligation-induced p38 MAPK activation.

The degradation of extracellular matrix and basement by tumor-associated proteases is an essential process required for cancer cell invasion and metastasis (1, 2). Urokinase plasminogen activator (uPA)[1] is of particular importance because uPA, through interaction with the uPA receptor (uPAR), facilitates the conversion of plasminogen into plasmin and the activation of metalloproteinases (3, 4). These proteases then allow cancer cells to degrade the surrounding matrix proteins and migrate to the distant sites (5). The overexpression of uPA is detected in various malignancies including breast, prostate, and colon cancers (6–8). Recent studies (9–13) have shown further that a high level of uPA in tumors is associated with a rapid disease progression and poor prognosis in breast cancers. In addition, studies performed in experimental models both *in vitro* and *in vivo* demonstrate that the levels of uPA are closely associated with the degree of tumor cell invasion (14–18). Blocking uPA expression or disruption of uPA binding to uPAR has been found to inhibit significantly tumor cell invasion and metastasis in various tumor models (19–22). From these facts, it is apparent that uPA plays a key role in tumor progression and metastasis.

Integrins are heterodimers composed of noncovalently associated α and β subunits. There are at least 14 α and 8 β subunits, forming at least 21 different integrins, which are the major receptors for extracellular matrix proteins (23). The α_v integrins are a major subfamily with restricted tissue/cell distribution (23). They have classical integrin functions such as mediating cell attachment and spreading (24, 25), facilitating cell migration (26), and ligand-receptor internalization (27, 28). They also play an important role in tumor progression and metastasis by mediating angiogenesis (29) and promoting tumor cell survival (30, 31). In addition, ligation of α_v integrins with their ligands has been reported to regulate the expression of several metalloproteinases (MMPs) and uPA/uPAR in various cancer cell types (32, 33). Suppressing α_v integrin expression resulted in the reduction in MMP2, MMP9, and uPAR expression in B-lymphocytes and melanoma cells (32, 34–37). Furthermore, both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins physically interact with uPAR upon uPA ligation, and these interactions are essential for tumor progression (38–40). Therefore, uPA/uPAR and α_v integrins may function in concert to promote tumor metastasis.

The mitogen-activated protein kinases (MAPKs) transduce extracellular signals into cellular responses and play important roles in cell proliferation, apoptosis, differentiation, cell migration, and cytoskeleton remolding (41–43). Mammalian cells express four types of MAPKs, ERKs, p38 MAPKs, c-Jun NH_2-terminal kinases, and big MAPKs. Recent studies have shown that MAPKs including ERK, c-Jun NH_2-terminal kinases, and p38 can be activated by integrin ligation, and the induced MAPK activities are important for many integrin-mediated cellular responses. For example, ligation of β_1 integrin with fibronectin activates ERK1/2, and this β_1 integrin-induced ERK activation is essential for cell cycle progression in NIH-3T3 cells (44). Ligation of $\alpha_2\beta_1$ integrin with collagen induces the activation of p38 MAPK, and the activity of p38 MAPK is essential for collagen-induced collagenase-1 and -3 expression in human fibroblast cells (45, 46), as well as collagen gene transcription in osteosarcoma cells (47) and NmuMg cell migration (48, 49).

Our previous studies (50) showed that endogenous p38 MAPK activity is elevated in cultured invasive breast cancer cells and that the higher p38 MAPK activity is important for

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[1] The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, urokinase-specific surface receptor; MAPK, mitogen-activated protein kinases; mAb, monoclonal antibody; Vn, vitronectin; ERK, extracellular signal-regulated kinase; MMPs, metalloproteinases.

breast cancer invasiveness by stabilizing uPA mRNA. However, it is unclear what maintains the elevated endogenous p38 MAPK activity in invasive tumor cells. In this paper, we investigated the role of integrins in the constitutive p38 MAPK activity and uPA expression. In the present study, we demonstrate that blocking α_v integrin ligation or down-regulating α_v integrin expression decreases endogenous p38 MAPK activity and inhibits uPA expression. Plating cells on vitronectin (Vn)-coated surface activates p38 MAPK and increases uPA expression in invasive MDA-MB-231 cells but not in non-invasive MCF7 cells. These findings suggest that α_v integrin ligation specifically activates p38 MAPK and up-regulates uPA expression in invasive cancer cells. In addition, we provided evidence that only the cytoplasmic tail of α_v integrin subunit is important for α_v integrin-mediated p38 MAPK activation.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Polyclonal antibodies to phospho-p38 and p38 were obtained from Cell Signaling (Beverly, MA). The polyclonal antibody to uPA was obtained from American Diagonostica (Greenwich, CT). Function-blocking monoclonal antibodies (mAbs) to β_1 (P4C10), $\alpha_6\beta_4$ (ASC-3), $\alpha_v\beta_5$ (P1F6), and α_v (AV-1) and the non-function-blocking mAb to α_v (LM142) were purchased from Chemicon (Temecula, CA). MDA-MB-231 and MCF7 cell line were obtained from ATCC (Manassas, VA). M21-L4, M21-L12, CS1, CS1/β_3, and CS1/β_5 cell lines were provided by Dr. David Cheresh (The Scripps Research Institute). All cells were maintained in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2.

Analyzing the Effect of Function-blocking Integrin Antibodies on p38 MAPK Activity and uPA Expression—MDA-MB-231 cells were cultured in a 6-well plate for 24 h, and 20 μg/ml function-blocking mAbs (P4C10, ASC-3, P1F6 or AV-1) was then added to the cells for 8 h. Cells were lysed in RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, and protease inhibitor mixture). Cell lysates were boiled in nonreducing SDS sample buffer, electrophoresed on 10% polyacrylamide SDS gel, and transferred to a nitrocellulose membrane. The p38 MAPK and activated p38 MAPK were detected with anti-p38 and anti-phospho-p38 MAPK polyclonal antibodies, respectively. The amount of uPA was detected with an anti-uPA polyclonal antibody.

Analyzing the Effect of α_v Integrin Subunit-specific Antisense Oligonucleotide on p38 MAPK Activity and uPA Expression—We previously developed two efficient α_v-specific antisense oligonucleotides (AS1 and AS2) and a non-functional control oligonucleotide (AS3) (37). The sequences for AS1, AS2, and AS3 are GGGACGCCAAGCCGGGAG (nucleotide −16 to −33 relative to AUG translational start codon), AAAAGCCATCGCCGAAAG (nucleotide 9 to −8 relative to AUG translational start codon), and GGAGGCCGCGGGGACCGA (nucleotide 49 to 32 relative to AUG translational start codon), respectively. To down-regulate α_v integrin expression on MDA-MB-231 cells, we added antisense oligonucleotides, AS1, AS2, or AS3 (2 nmol), into overnight MDA-MB-231 culture with 10 μl of LipofectAMINE2000 (Life Technologies, Inc.) in a total volume 1 ml for 36 h. Cells were lysed, and the cell lysates were subjected to immunoblotting to detect the levels of activated p38 MAPK and uPA with the respective antibodies.

Analysis of the Effect of α_v Integrin Ligation on the Activities of MAPKs and uPA Expression—To determine the effect of α_v integrin ligation on the activities of MAPKs, cell lines MDA-MB-231 or MCF7 were starved in serum-free medium for 24 h, and 3-cm culture dishes were coated with 1 μg/ml of Vn solution at 4 °C. Cells were detached with 10 mM EDTA, kept in suspension in serum-free medium for 30 min, and then replated onto Vn-coated culture dishes. At varying times, cells were lysed in ice-cold RIPA buffer, and cell lysates were then subjected to immunoblotting to detect the levels of activated p38 MAPK.

To determine the effect of α_v integrin ligation on uPA expression, MDA-MB-231 or MCF7 cells were plated on Vn-coated dishes and cultured for 1 or 2 days. Cells were lysed, and cell lysates were subjected to immunoblotting to detect the levels of uPA.

Construction and Transfection of Cytoplasmic Tail-deleted α_v, β_3, and β_5 Integrin Subunit Expression Vectors—To prepare cells expressing the cytoplasmic tail-deleted α_v integrin subunit, the expression vector pcDNA1/$\alpha_v\Delta995$ was transfected into M21L12 cells with LipofectAMINE according to manufacturer's protocol. The pcDNA1/$\alpha_v\Delta995$ contains the α_v integrin subunit with carboxyl-terminal deletion up to GFFKR sequence (provided by Zhuohua Zhang, Burnham Institute). Cells were selected with G418 (0.8 mg/ml) for 3 weeks, and the resistant cells were plated on a Vn-coated surface for 1 h. The attached cells were collected and selected by Vn adhesion a second time. The cells were then analyzed using flow cytometry with an α_v-specific mAb (LM142, Chemicon) to confirm cell surface α_v integrin expression. The established cell line was designated as M21/$\alpha_v\Delta995$.

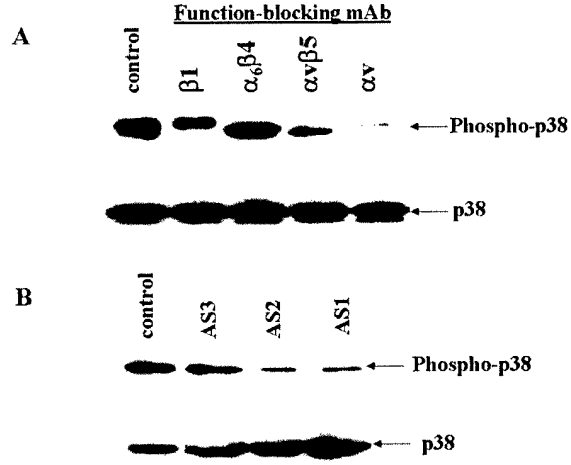

FIG. 1. **The functionality of α_v integrins is associated with constitutive p38 MAPK activity in MDA-MB-231 cells.** *A*, MDA-MB-231 cells were treated with 20 μg/ml function-blocking mAbs to β_1 (P4C10), $\alpha_6\beta_4$ (ASC-3), $\alpha_v\beta_5$ (P1F6), or α_v (AV-1) for 8 h. Cells were then lysed, and active p38 MAPK was detected by immunoblotting. The membrane was stripped and reprobed for total p38 MAPK to ensure equal protein loading. *B*, MDA-MB-231 cells were treated with 2 nmol of α_v-specific antisense oligonucleotides (AS1 and AS2) or the control oligonucleotide (AS3) for 36 h. Cells were then lysed, and active p38 MAPK was detected by immunoblotting. The membrane was stripped and reprobed for p38 MAPK to ensure equal protein loading.

To prepare cells expressing cytoplasmic tail-deleted β_3 or β_5 integrin subunit, pCI/$\beta_3\Delta716$ or pCI/$\beta_5\Delta720$ were transfected in CS1 cells as described above. Plasmid pCI/$\beta_3\Delta716$ contains the β_3 integrin subunit with a carboxyl-terminal deletion ending with the sequence ALLIW, and pCI/$\beta_5\Delta720$ contains the β_5 integrin subunit with a carboxyl-terminal deletion ending with LLAIW (provided by Glen Nemerow, Scripps Research Institute). The transfected cells were selected with G418, and the resistant cells were further selected by two rounds of Vn adhesion. The expression of cell surface $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins was confirmed by flow cytometry using $\alpha_v\beta_3$-specific mAb (LM609, Chemicon) or $\alpha_v\beta_5$-specific mAb (P1F6, Chemicon), respectively. The new lines were designated as CS1/$\beta_3\Delta716$ and CS1/$\beta_5\Delta720$.

RESULTS

α_v Integrin Function / Expression Is Essential for Endogenous p38 MAPK Activity in Invasive Breast Cancer Cells—Extracellular matrix proteins including Vn, osteopontin, fibronectin, and laminin have been reported to up-regulate uPA expression in various cell types (51–56). Integrin ligation has also been shown to activate p38 MAPK in mast cells and fibroblasts (45, 46, 57). We thus hypothesized that integrin ligation may contribute to the elevated endogenous p38 MAPK activity and up-regulated uPA expression in invasive breast cancer cells. To test this hypothesis, we treated the invasive MDA-MB-231 cells with function-blocking mAbs to β_1, α_v, $\alpha_v\beta_5$, and $\alpha_6\beta_4$ integrins. Immunoblotting with an anti-phospho-p38 antibody showed that the levels of activated p38 MAPK were moderately inhibited by function-blocking mAb to β_1 integrin subunit (P1F6) and not affected by function-blocking mAbs to $\alpha_6\beta_4$ integrin (ASC-3) (Fig. 1*A*). In contrast, function-blocking mAb to α_v integrin (AV-1) and $\alpha_v\beta_5$ integrin (P1F6) significantly inhibited p38 MAPK phosphorylation (Fig. 1*A*). Because MDA-MB-231 cells expressed significant levels of each of these integrins (data not shown), these results suggest that the functionality of α_v integrins is important for the sustained endogenous

p38 MAPK activity in MDA-MB-231 cells.

To examine further the functional correlation between α_v integrins and p38 MAPK activity, we also treated MDA-MB-231 cells with two previously developed α_v-specific antisense oligonucleotides (AS1 and AS2) (37) to down-regulate α_v integrin expression. As determined by flow cytometry, AS1 and AS2 at 2 μM reduced over 80% of cell surface α_v integrin expression (data not shown). Treatment of cells with AS1 or AS2 significantly inhibited the levels of endogenous p38 MAPK activity (Fig. 1*B*); in contrast, MDA-MB-231 cells treated with the non-functional AS3 displayed a similar level of p38 MAPK phosphorylation as the untreated cells (Fig. 1*B*). These results suggest that α_v integrins are essential for elevated endogenous p38 MAPK activity in invasive breast cancer cells.

α_v Integrin Function / Expression Is Essential for uPA Expression in Invasive Breast Cancer Cells—We next examined the role of α_v integrin on cell-associated uPA expression. Various amounts of α_v integrin function-blocking mAb (AV-1) were added to cultures of attached MDA-MB-231 cells for 8 h. After treatment, the cells were lysed, and the levels of uPA were analyzed by immunoblotting with an anti-uPA antibody. The amount of uPA was reduced by mAb AV-1 in a dose-dependent manner. At least 80% of cell-associated uPA expression was blocked with 20 μg of AV-1 (Fig. 2*A*). In contrast, a non-function-blocking mAb to α_v integrins (LM142) showed no inhibitory effect on the levels of cell-associated uPA (Fig. 2*A*).

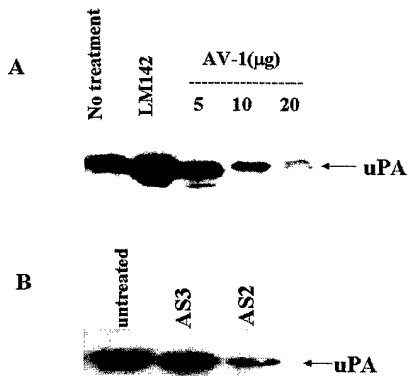

FIG. 2. **The functionality of α_v integrins is required for uPA expression in MDA-MB-231 cells.** *A*, MDA-MB-231 cells were treated with 20 μg/ml of non-function mAb (LM142) or various concentrations of function-blocking mAb (AV-1) for 8 h. Cell lysates were subjected to immunoblotting to detect cell-associated uPA. *B*, MDA-MB-231 cells were treated with 2 nmol of α_v-specific antisense oligonucleotide (*AS2*) or a control oligonucleotide (*AS3*) for 36 h. Cells were then lysed and subjected to immunoblotting to detect cell-associated uPA expression.

These data suggest that functional α_v integrins are required for uPA expression in MDA-MB-231 cells. In a parallel study, we also treated MDA-MB-231 cells with the α_v-specific antisense oligonucleotide, AS2, or control oligonucleotide, AS3, and followed the effect on uPA expression by immunoblotting. The amount of cell-associated uPA was significantly decreased in AS2-treated cells, whereas AS3 showed no inhibitory effect on uPA levels (Fig. 2*B*). Taken together, these studies suggest a functional link between α_v integrins, p38 MAPK activity, and uPA expression in invasive breast cancer cells.

α_v Integrin Ligation Activates p38 MAPK and Induces uPA Expression in Invasive Breast Cancer Cells—We next examined whether α_v integrin ligation was capable of activating p38 MAPK in both invasive MDA-MB-231 and non-invasive MCF7 breast cancer cells. Cells were first starved overnight and then suspended in serum-free medium for 30 min to reduce endogenous p38 MAPK activity. Subsequently, we plated cells on Vn-coated surfaces for varying times (10 min to 2 h). Immunoblotting with an antibody specific to phospho-p38 MAPK showed that α_v integrin ligation with Vn induced dramatic p38 MAPK phosphorylation as early as 10 min and reached maximal p38 MAPK activity at 20 min in MDA-MB-231 cells (Fig. 3*A*). Interestingly, α_v integrin ligation did not activate p38 MAPK in non-invasive MCF7 cells (Fig. 3*B*). These results suggest that α_v integrin ligation may specifically induce p38 MAPK activation in invasive breast cancer cells.

To examine the effect of α_v integrin ligation on uPA expression, MDA-MB-231 or MCF7 cells were plated on Vn-coated plates for 1–2 days. Cells were then lysed, and the amount of uPA protein was quantitated by immunoblotting. MDA-MB-231 cells cultured on a Vn-coated surface exhibited a much higher amount of cell-associated uPA protein than those cultured on an uncoated surface (Fig. 4). In contrast, no difference in the levels of uPA expression could be detected between MCF-7 cells cultured on Vn-coated and uncoated surface (Fig. 4). These results suggest that Vn may specifically induce uPA expression in invasive breast cancer cells.

The α_v Integrin Subunit Cytoplasmic Tail Is Essential for α_v Integrin Ligation-induced p38 MAPK and ERK Activation—We also investigated the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in Vn-induced p38 MAPK activation using human melanoma M21 and CS1 cell systems. In the M21 cell system, the M21-L12 line does not express the α_v integrin subunit, thus no α_v integrin is present at the cell surface; the M21-L4 line was established by the stable transfection of the α_v integrin subunit in M21-L12 cells, and this line expresses both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (58). In the CS1 cell system, the CS1 line expresses neither β_3 nor β_5 integrin subunit; the CS1/β_3 and CS1/β_5 lines were created by

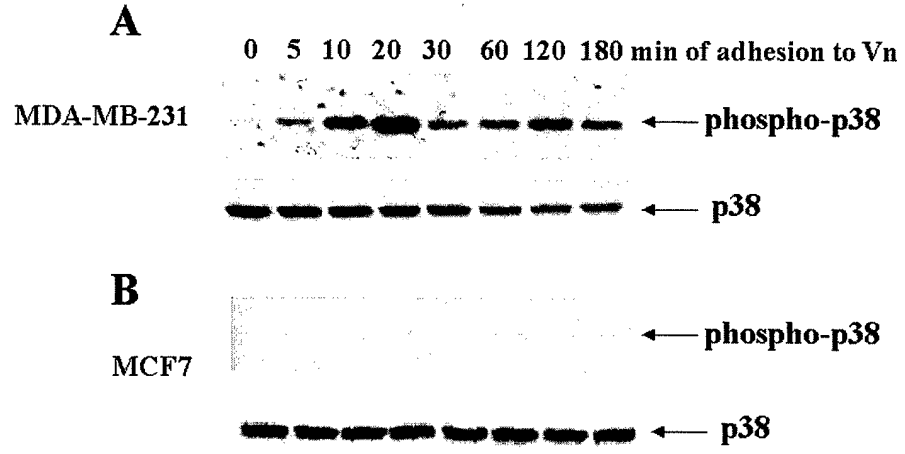

FIG. 3. **Engaging α_v integrin with immobilized Vn specifically activates p38 MAPK in invasive breast cancer cells.** Invasive MDA-MB-231 (*A*) or non-invasive MCF7 (*B*) cells were starved overnight and detached with EDTA. Cells were then washed and kept in suspension in serum-free medium for 30 min. Cells were plated on a Vn-coated surface for various times and then lysed and subjected to immunoblotting to detect active p38 MAPK. The membranes were stripped and reprobed for p38 MAPK to ensure equal protein loading.

the stable transfection of β_3 subunit or β_5 subunit in CS1 cells, thus these lines express either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin, respectively (59, 60). Allowing M21-L4, CS1/β_3, or CS1/β_5 cells to attach on Vn-coated surface for 30 min resulted in a significant increase in p38 MAPK phosphorylation (Fig. 5, *A* and *B*). However, plating M21-L12 or CS1 cell on Vn did not lead to p38 MAPK activation (Fig. 5, *A* and *B*). These results suggest that both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are capable of mediating p38 MAPK activation.

To determine which integrin subunit is important for p38 MAPK activation, we examined the effect of Vn on p38 MAPK activation in M21-L12 cells transfected with α_v integrin subunit lacking its intracellular domain ($\alpha_v\Delta995$). Although both this line and M21-L4 line adhered equally well to Vn (data not shown), the Vn-induced p38 MAPK activation was completely abolished in M21/$\alpha_v\Delta995$ cells (Fig. 5*A*). These results suggest that the cytoplasmic tail of α_v subunit is required for α_v integrin ligation-induced p38 MAPK activation. Subsequently, we also examined Vn-induced p38 MAPK activation in CS1 cells transfected with intracellular domain-lacking β_3 ($\beta_3\Delta716$) or β_5 subunit ($\beta_5\Delta720$) plasmids, and we found that Vn-induced p38 MAPK activation was not disabled in these two lines (Fig. 5*B*). These results strongly suggest that the intracellular domain (the cytoplasmic tail) of the α_v subunit, rather than the intracellular domain of the β_3 or β_5 subunit, is essential for α_v integrin-mediated p38 MAPK activation.

DISCUSSION

Our previous studies (50) have demonstrated that the endogenous p38 MAPK activity is elevated in invasive breast cancer cells and that constitutive p38 MAPK activity is essential for uPA/uPAR expression and matrix invasion by breast cancer cells. However, it is not clear how elevated p38 MAPK activity is maintained in invasive breast cancer cells. Early studies (51–56) have shown that extracellular matrix proteins including vitronectin, osteopontin, fibronectin, and laminin can up-regulate uPA expression in various cell types including melanoma, macrophage, and breast cancer cells. Several recent studies (45, 57) have also demonstrated that engaging integrins with their ligands can activate p38 MAPK in mast and fibroblast cells. These findings prompted us to investigate whether integrin ligation contributes to the elevated p38 MAPK activity in invasive breast cancer cells. We found that treatment of highly invasive MDA-MB-231 cells with function-blocking mAb to α_v integrins greatly reduced the level of p38 MAPK activity and uPA expression (Figs. 1*A* and 2*A*). Similarly, down-regulating α_v integrin expression with antisense oligonucleotides also significantly inhibited p38 MAPK activity and uPA expression in MDA-MB-231 cells (Figs. 1*B* and 2*B*). These finding suggest a function link between α_v integrin, p38 MAPK, and uPA expression in invasive breast cancer cells.

Whereas α_v integrins are expressed in both invasive and noninvasive breast cancer cells, we detected elevated p38 MAPK activity and uPA overproduction in most of invasive breast cancer cells but not in non-invasive cells (50). We thus compared the extent of α_v integrin ligation-induced p38 MAPK activation and uPA up-regulation in both invasive and non-invasive cells. We have shown that plating invasive MDA-MB-231 cells on Vn-coated surface induces dramatic p38 MAPK phosphorylation (Fig. 3*A*) and up-regulates uPA expression (Fig. 4*A*); in contrast, engaging α_v integrins with immobilized Vn neither activated p38 MAPK (Fig. 3*B*) nor induced uPA expression in non-invasive MCF7 cells (Fig. 4*B*). A recent study reported that α_v integrins can be present in two functional states in breast cancer cells, and only the activated state of α_v integrins is expressed in metastatic cells (61). Functional studies further showed that only the activated α_v integrins were able to promote breast cancer cell invasion and metastasis (61, 62). Therefore, we consider the possibility that only the activated state of α_v integrins is capable of mediating p38 MAPK activation and uPA up-regulation.

Both integrin subunits are capable of mediating signaling events. In the present study, we have shown that only the cytoplasmic tail of α_v integrin subunit is essential for Vn-induced p38 MAPK activation. A recent study (47) has shown that engaging the $\alpha_2\beta_1$ integrin with a three-dimensional col-

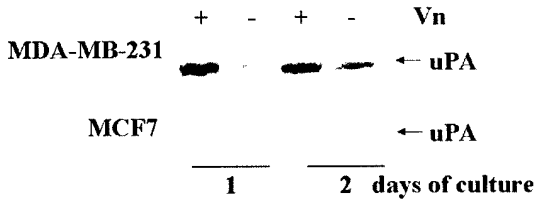

FIG. 4. **Adherence to Vn specifically induces uPA expression in invasive breast cancer cells.** Invasive MDA-MB-231 and non-invasive MCF-7 cells were plated on Vn-coated or noncoated surfaces for 1 or 2 days. Cells were then lysed, and immunoblotting was performed to detect cell-associated uPA.

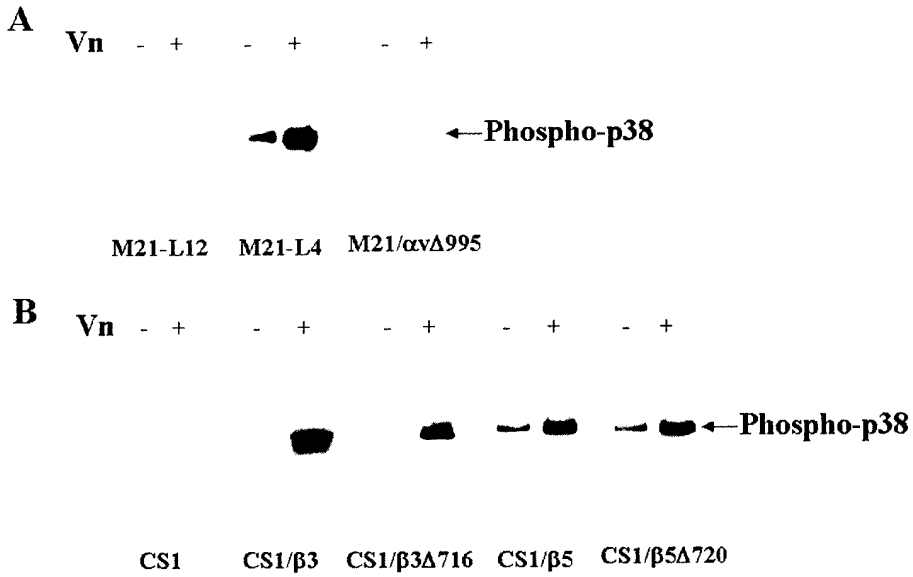

FIG. 5. **The cytoplasmic tail of the α_v integrin subunit is essential for α_v integrin ligation-induced p38 MAPK activation.** Cells were starved overnight and kept in suspension for 30 min. Cells were then plated on Vn-coated surface for 30 min and then lysed, and active p38 MAPK was detected by immunoblotting. *A*, M21-L12, M21-L4, and M21/$\alpha_v\Delta995$. *B*, CS1, CS1/β_3, CS1/$\beta_3\Delta716$, CS1/β_5, and CS1/$\beta_5\Delta720$.

lagen gel activates p38 MAPK. In another study (49), the cytoplasmic tail of α_2 integrin subunit was found to be specific for collagen I-stimulated p38 MAPK activation in NmuMg cells. Furthermore, the amino acid residues essential for p38 MAPK activation are distinct from those required for ERK activation (48). We currently are investigating the role of the α_v integrin cytoplasmic tail in uPA up-regulation.

Substantial evidence has been generated which indicates that uPA plays an important role in tumor invasion and metastasis (3, 4). Experimental models have also shown that inhibiting uPA function can significantly block tumor progression in animals (19–21). However, broad inhibitors of uPA (serpin) have not been considered as therapeutic agents because of their general effect on the fibrinolytic system (4). In the present study, we demonstrate that α_v integrin expression/function is important for uPA up-regulation in invasive breast cancer cells. These findings implicate that therapeutic approaches for breast cancer may be developed by blocking α_v integrin expression or function.

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Sequential Activation of the MEK–Extracellular Signal-Regulated Kinase and MKK3/6–p38 Mitogen-Activated Protein Kinase Pathways Mediates Oncogenic *ras*-Induced Premature Senescence†

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In primary mammalian cells, oncogenic *ras* induces premature senescence, depending on an active MEK–extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway. It has been unclear how activation of the mitogenic MEK-ERK pathway by *ras* can confer growth inhibition. In this study, we have found that the stress-activated MAPK, p38, is also activated during the onset of *ras*-induced senescence in primary human fibroblasts. Constitutive activation of p38 by active MKK3 or MKK6 induces senescence. Oncogenic *ras* fails to provoke senescence when p38 activity is inhibited, suggesting that p38 activation is essential for *ras*-induced senescence. Furthermore, we have demonstrated that p38 activity is stimulated by *ras* as a result of an activated MEK-ERK pathway. Following activation of MEK and ERK, expression of oncogenic *ras* leads to the accumulation of active MKK3/6 and p38 activation in a MEK-dependent fashion and subsequently induces senescence. Active MEK1 induces the same set of changes and provokes senescence relying on active p38. Therefore, oncogenic *ras* provokes premature senescence by sequentially activating the MEK-ERK and MKK3/6-p38 pathways in normal, primary cells. These studies have defined the molecular events within the *ras* signaling cascade that lead to premature senescence and, thus, have provided new insights into how *ras* confers oncogenic transformation in primary cells.

The *ras* proto-oncogene family encodes small GTP binding proteins that transduce growth signals from cell surface receptors in response to extracellular stimuli (1, 6, 37). Previous studies have suggested that aberrant activation of *ras* is a crucial step during tumorigenesis. Constitutive activation of *ras* genes is found associated with a wide variety of human tumors at high frequency (3, 4). In both cell culture models and animal models, activated *ras* cooperates with other oncogenic genetic alterations to induce transformation (13, 19, 25, 49, 57, 61).

The transforming activity of activated *ras* depends on at least three downstream effectors, including Raf-1/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and Ral-GDS (29, 48, 53, 56), which mediate different aspects of oncogenic transformation. It is believed that activation of the MAPK pathway provides cells with constitutive mitogenic signals independent of extracellular stimuli (7). Interaction between Ras and Raf-1 leads to the sequential activation of the MAP kinase kinases (MAPKKs) MEK1 and MEK2, and the MAPKs extracellular signal-regulated kinase 1 (ERK1) and ERK2. Activated ERK1 and ERK2 promote cell proliferation. For example, it has been demonstrated that active ERK stimulates DNA synthesis (18), inactivates cell cycle inhibitor kinase MYT1 (45), and enhances the activity of AP-1 transcription factor, which induces the expression of growth-promoting genes such as that for cyclin D1 (33, 55).

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In contrast to its mitogenic activity, expression of oncogenic *ras* in normal primary cells induces premature senescence, a permanent growth arrest that is morphologically indistinguishable from replicative senescence observed in aged primary cells (51). This senescence-like growth arrest induced by *ras* is associated with accumulation of growth inhibitors such as p53 and $p16^{INK4A}$ (51). Interestingly, the ability of oncogenic *ras* to induce premature senescence depends on the Raf-MEK-ERK pathway that mediates cell proliferation (36). Constitutive activation of this pathway induces p53, p16, and p21 and leads to premature senescence. In addition, *ras* fails to induce senescence when the activation of the MEK-ERK pathway is specifically inhibited. It remains unclear how activation of the mitogenic Raf-MEK-ERK pathway by *ras* can induce premature senescence and how this negative growth impact of *ras* is bypassed in tumors.

Besides the Raf-MEK-ERK cascade, oncogenic *ras* also activates the Jun amino-terminal kinase (JNK) and p38 MAPK pathways in several different cell lines (8, 31, 38, 62). Like ERK, JNK also enhances the activity of AP-1 and promotes cyclin D1 transcription when activated by its upstream kinases, MKK4 and MKK7, and thus is likely to be involved in the ability of *ras* to regulate cell proliferation (7, 30, 31, 44). The p38 MAPK is phosphorylated and activated by its upstream MAPKKs MKK4, MKK3, and MKK6, usually in response to nonmitogenic signals such as proinflammatory cytokines and environmental stress (43). However, the biological significance of p38 activation by oncogenic *ras* remains unclear. It has been reported that under certain biological conditions p38 can negatively regulate cell growth. Microinjection of a p38-encoding plasmid into NIH 3T3 fibroblasts led to down-regulation of cyclin D1 expression and cell cycle arrest at G_1 (40). Ectopic

expression of MEKK3, a MAPKKK that activates p38, induced G_1 arrest and reversed *ras*-mediated transformation in NIH 3T3 cells, again through down-regulation of cyclin D1 (14). In addition, the MKK6-p38γ cascade mediated γ-irradiation-induced G_2 cell cycle arrest (58).

The negative role of p38 in proliferation led us to investigate the involvement of this pathway in oncogenic *ras*-induced premature senescence. In the present study, we have demonstrated that oncogenic *ras* induces premature senescence through sequential activation of the MEK-ERK pathway and the MKK3/6-p38 pathway in primary human fibroblasts. The MEK-ERK pathway, when activated by *ras*, stimulates the activity of p38, which in turn leads to senescence. Therefore, besides transducing mitogenic signals, the *ras*-activated MEK-ERK pathway has an additional biological consequence, induction of premature senescence through the p38 pathway. These results have important implications for our understanding of the mechanisms by which *ras* transforms cells.

MATERIALS AND METHODS

Cell culture. BJ human foreskin fibroblasts were obtained from J. Smith (Baylor College of Medicine) maintained in minimum essential medium supplemented with 10% fetal calf serum, nonessential amino acids, glutamine, and antibiotics. LinX-A retroviral packaging cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, and antibiotics.

Plasmids and reagents. BabeHygro-hTERT, BabePuro-*Ha-rasV12*, and BabePuro-MEK1Q56P were obtained from R. A. Weinberg, D. Beach, and S. Lowe, respectively. BabePuro-MKK3E, -MKK6E, -MKK7D, and -p38αWT were constructed by cloning the respective cDNA fragment from an adenovirus vector (23, 59) into BabePuro vector. BabeHygro-MKK3A and -MKK6A were constructed by cloning the respective cDNA fragment from an adenovirus vector (23) into BabeHygro vector. Dominant-negative mutant alleles of p38α, p38β, p38γ, and p38δ were obtained from Jiahui Han and were excised from pcDNA3 vectors (20, 26, 27, 35) and subcloned into the BabeHygro vector. SB203580 and U0126 were purchased from Calbiochem. Tumor necrosis factor alpha (TNF-α) was purchased from Chemicon International, Inc.

Retroviral gene transduction. Retroviral gene transduction was carried out as previously described, using an amphotropic packaging cell line (LinX-A) (54). Treatments with SB203580 (8 μM), U0126 (5 μM), or vehicle control usually started at the time of initial retroviral infection. Cells transduced with retroviruses were purified with 100 μg of hygromycin B/ml, 400 μg of G418/ml, and/or 1 μg of puromycin/ml, starting 1 to 2 days after infection. We typically achieve 30 to 50% infection rates in BJ cells before selection and 90 to 100% after selection.

Analysis of senescence. Cells infected with appropriate retroviruses were selected with puromycin for 4 days to eliminate the uninfected cells, after which assays were performed to analyze premature senescence. The day when drug selection was completed (day 5 after infection) was defined as day 0.

For growth curves, 10^4 cells were plated into each well in 12-well plates in duplicates or triplicates. Every 3 to 4 days, cells were trypsinized from plates and cell numbers were counted. At each split, 10^4 cells were reseeded to each well in fresh plates and allowed to grown until the next split. Population doublings (PD) were calculated with the formula PD = log(*n*2/*n*1)/log2, where *n*1 is the number of cells seeded and *n*2 is the number of cells recovered (52). Cells with senescence-associated β-galactosidase (SA-β-gal) activity were detected as previously described (51). At least 200 cells were counted in randomly chosen fields from each culture well.

Western blotting. Lysates for Western blot analysis were normally prepared 8 to 10 days after the initial retroviral gene transduction. Cells at 30 to 50% confluence were lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% NP-40, and Complete protease inhibitors [Roche]) or RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM Na_3VO_4, and Complete protease inhibitors [Roche]). After the lysates were cleared by centrifugation, protein concentrations were determined by Bradford assays. Twenty to 80 μg of proteins was separated on an SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Trans-Blot nitrocellulose membranes (Bio-Rad). The primary antibodies used were for Ha-Ras (C-20; Santa Cruz), phospho-ERK (Thr202/Tyr204; Cell Signaling), ERK (Cell Signaling), ERK2 (C-14; Santa Cruz), actin (Sigma), $p16^{INK4A}$ (DCS-50; Novocastra), p53 (CM1 from Novocastra and FL-393 from Santa Cruz), phospho-p38 (Thr180/Tyr182; Cell Signaling), p38 (Cell Signaling), phospho-MEK1/2 (Ser217/221; Cell Signaling), phospho-MKK3/6 (Ser189/207; Cell Signaling), MKK3 (C-19; Santa Cruz), MKK6 (V-20; Santa Cruz), phospho-MKK4 (Thr261; Cell Signaling), MKK4 (C-20; Santa Cruz), phospho-JNK (Thr183/Tyr185; Cell Signaling), JNK (Cell Signaling), and JNK1 (C-17; Santa Cruz). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Santa Cruz) or goat anti-mouse IgG (Jackson Laboratories) antibodies were used as secondary antibodies. Reactive proteins were visualized using enhanced chemiluminescence (SuperSignal; Pierce).

Immunoprecipitation and protein kinase assays. Immunoprecipitation of p38 protein and subsequent protein kinase assays using [γ-^{32}P]ATP were carried out as described previously (23, 28). Cells at 30 to 50% confluence were lysed in RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, and Complete protease inhibitors [Roche]). p38 protein was purified from equal amounts of lysates using an antibody against p38 (Cell Signaling) (23) or actin (Sigma) and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). Protein kinase assays were performed at 37°C for 30 min in a 50-μl volume with 7 μg of glutathione *S*-transferase (GST)–ATF2 (residues 1 to 109), 10 μM ATP, 10 μCi of [γ-^{32}P]ATP, 20 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol. The reactions were terminated with Laemmli sample buffer, and the products were separated on SDS-PAGE and visualized and quantitated with a phosphorimager. Nonradioactive p38 kinase assays were carried out using the p38 MAP Kinase Assay Kit from Cell Signaling, following the manufacturer's instructions.

ERK and JNK kinase activities were measured using the p44/42 MAP Kinase Assay Kit and the SAPK/JNK MAP Kinase Assay Kit from Cell Signaling, following the manufacturer's instructions.

Cell cycle analysis. After 2 days of selection with puromycin (3 days postinfection), cells transduced with Ha-*rasV12* or a vector control in the presence of SB203580 or vehicle control were grown in medium containing 0.2% fetal bovine serum for 21 h before being labeled with 30 μM bromodeoxyuridine (BrdU) (Sigma) for 3 h. Cells were removed from plates with trypsin and fixed in 70% ethanol at 4°C overnight. Cells were then treated with 2 N HCl–0.5% Triton X-100 for 30 min at room temperature, followed by neutralization with 0.1 M $Na_2B_4O_7$ and subsequent incubation with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson) for 1 h at room temperature. Finally, cells were washed with PBS–1% bovine serum albumin–0.5% Tween 20, resuspended in PBS containing 5 μg of propidium iodide/ml, and analyzed by two-dimensional flow cytometry to detect both fluorescein and propidium iodide.

Northern blotting. Total RNA was isolated from cells using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. Eight micrograms of RNA was separated on a 1% agarose gel containing 3.7% formaldehyde in 1× morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, 5 mM NaOAc, and 1 mM EDTA; pH 7.0), transferred to Hybond N+ nylon membranes in 10× SSC (1.5 M NaCl and 150 mM sodium citrate; pH 7.0), and hybridized at 65°C in Church-Gilbert buffer (1% bovine serum albumin, 400 mM $NaPO_4$ [pH 7.0], 15% formamide, 1 mM EDTA, and 7% SDS) to an 800-bp human p16 cDNA probe labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP by random priming. After extensive washing with 0.2× SSC–0.1% SDS buffer at 65°C, the signals were visualized and quantitated with a phosphorimager.

RESULTS

p38 is activated during *ras*-induced premature senescence. Activation of p38 by oncogenic *ras* was previously observed in immortalized murine NIH 3T3 fibroblasts (8, 38, 62). While oncogenic *ras* induces premature senescence in primary cells, immortalized murine cells are refractory to premature senescence (51). To explore the possible involvement of the p38 pathway in oncogenic *ras*-induced senescence, we examined whether Ha-*rasV12*, an oncogenic *ras* mutant allele found in human tumors, activated p38 during the onset of senescence in BJ primary human foreskin fibroblasts. Ha-*RasV12* was transduced into BJ cells via retroviral infection. Consistent with previous findings in other primary human and murine fibroblasts, Ha-RasV12 induced premature senescence in early-

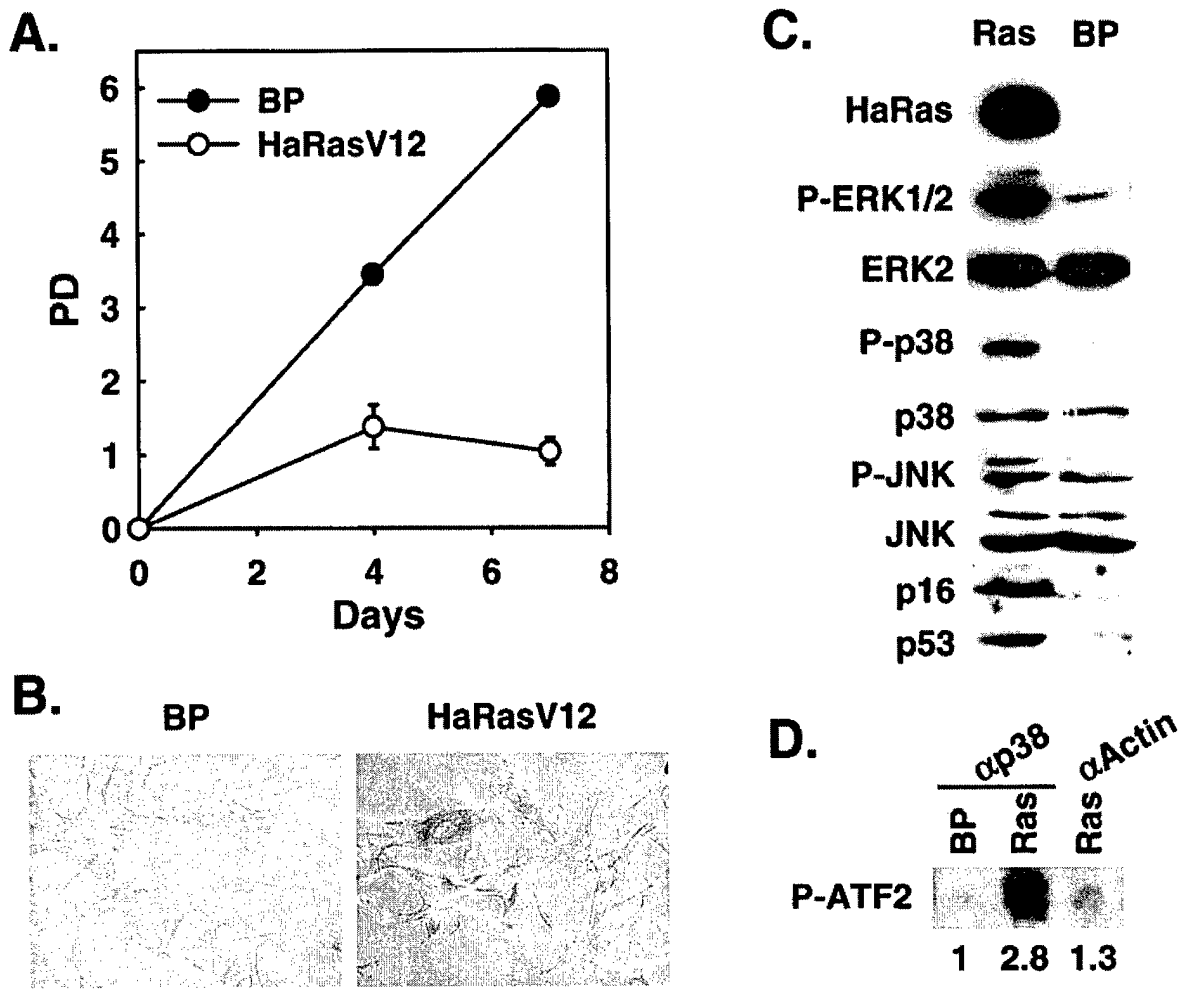

FIG. 1. Induction of premature senescence by oncogenic *ras* correlates with p38 activation. (A) PD of BJ cells transduced at PD 20 with Ha-RasV12 or a vector control. Values are means ± standard deviations for duplicates. (B) Morphology of BJ cell populations transduced with Ha-RasV12 or a vector control, after staining for SA-β-gal (pH 6.0) at day 7 postselection. (C) Western blot analysis of BJ cells transduced with Ha-RasV12 (Ras) or a vector control (BP), showing the levels of Ha-Ras, phospho-ERK (P-ERK1/2), ERK2, phospho-p38 (P-p38), p38, phospho-JNK (P-JNK), JNK, $p16^{INK4A}$, and p53. (D) p38 protein kinase activity in BJ cells transduced with Ha-RasV12 (Ras) or a vector control (BP), determined at day 4 postselection in a kinase assay by using [γ-^{32}P]ATP and GST-ATF2 as substrates, following immunoprecipitation of p38 with an anti-p38 antibody (αp38) or an antiactin antibody (αActin). Shown are the relative amounts of phosphorylated ATF2 substrate after normalization to background. The signals were visualized and quantitated with a phosphorimager.

passaged BJ cells (Fig. 1). Cells stopped proliferating 7 to 10 days after the initial transduction of Ha-*RasV12* (Fig. 1A). The growth-arrested cells acquired the enlarged and flattened morphology that was characteristic of cellular senescence. In addition, these cells accumulated SA-β-gal (Fig. 1B), a biomarker for senescent cells (12).

In BJ cells undergoing premature senescence, we detected increased phosphorylation of p38 on Thr180 and Tyr182 in Western blot analysis by using an antibody that specifically recognizes p38 phosphorylated on these sites (Fig. 1C). The phosphorylation of p38 on Thr180 and Tyr182 is known to lead to the activation of p38 (10, 21, 22, 46, 47). To confirm the activation of p38, p38 protein was isolated from Ha-*RasV12*-expressing cells or control cells by immunoprecipitation using an anti-p38 antibody, and it was tested for its ability to phosphorylate a downstream substrate, ATF2 (10, 46). Indeed, p38 purified from *ras*-expressing cells had an increased (nearly threefold higher) activity in phosphorylation of ATF2, compared to that from cells expressing a vector control (Fig. 1D). The immune complex precipitated from *ras*-expressing cells by using a control antibody (antiactin) displayed no kinase activity. Therefore, in BJ primary human fibroblasts, oncogenic *ras*-induced premature senescence correlated with p38 activation. In contrast, although we observed a slight increase in the level of phospho-JNK when Ha-*rasV12* was expressed in BJ fibroblasts (Fig. 1C), the JNK activity was not detected from the *ras*-expressing cells in an in vitro kinase assay using c-Jun as substrate (Fig. 4B).

Constitutively active MKK3 and MKK6 induce premature senescence. p38 MAPK can be activated through phosphorylation by two upstream MAPKKs, MKK3 and MKK6, in response to extracellular stimuli (10, 21, 22, 46, 47). MKK3 and

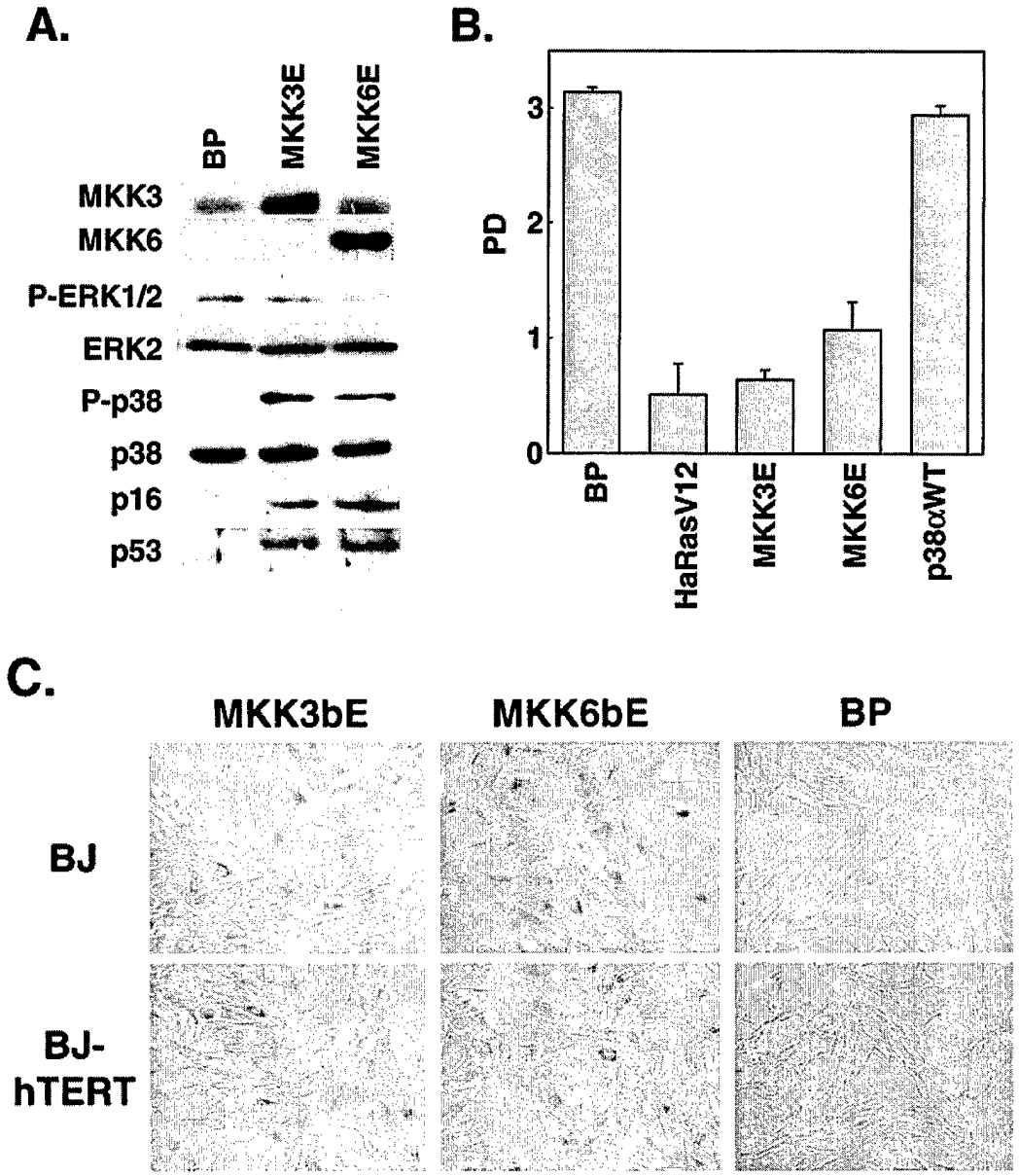

FIG. 2. Activation of p38 by active MKK3 or MKK6 induces premature senescence. (A) Western blot analysis of BJ cells transduced at PD 18 with a vector control (BP) or constitutively active MKK3 (MKK3E) or MKK6 (MKK6E). Levels of MKK3, MKK6, phospho-ERK (P-ERK1/2), ERK2, phospho-p38 (P-p38), p38, $p16^{INK4A}$, and p53 were determined 10 days postinfection. (B) PD of BJ cells between day 7 and day 11 after infection at PD 18 with a vector control (BP), Ha-RasV12 (HaRasV12), a constitutively active MKK3 (MKK3E) or MKK6 (MKK6E), or wild-type p38α (p38αWT). Values are means ± standard deviations (SD) for triplicates. (C) Morphology of BJ cells (BJ) or BJ cells immortalized with hTERT (BJ-hTERT) that had been transduced with a vector control (BP) or constitutively active MKK3 (MKK3E) or MKK6 (MKK6E), after staining for SA-β-gal (pH 6.0) at day 12 postinfection. (D) Quantification of percentages of SA-β-gal-positive cells within cell populations shown in panel C. Values are means ± SD for two separate wells. At least 200 cells were counted for each sample.

MKK6 are activated by dual phosphorylation on Ser207/Thr211 and Ser189/Thr193, respectively. Substitution of these serine/threonine residues with glutamic acid (E) results in constitutively active forms of MKK3 and MKK6 (MKK3E and MKK6E) that can phosphorylate and activate p38 independently of upstream signals (22, 47). We therefore examined whether activation of p38 by these two mutant MKKs would lead to senescence. MKK3E and MKK6E were expressed in early-passaged BJ fibroblasts via retroviral gene transduction (Fig. 2A). While Ha-RasV12 led to the phosphorylation of both p38 and ERK, MKK3E and MKK6E only increased activating phosphorylation of p38 but not that of ERK (Fig. 2A). MKK3E and MKK6E also stimulated p38 kinase activity (see Fig. 8C). BJ cells transduced with a vector control (BP) or a wild-type p38α gene that was not activated displayed normal growth rates (Fig. 2B). In contrast, expression of MKK3E or MKK6E led to growth arrest in BJ cells (Fig. 2B). The arrested cells acquired a senescence-like morphology (Fig. 2C). In ad-

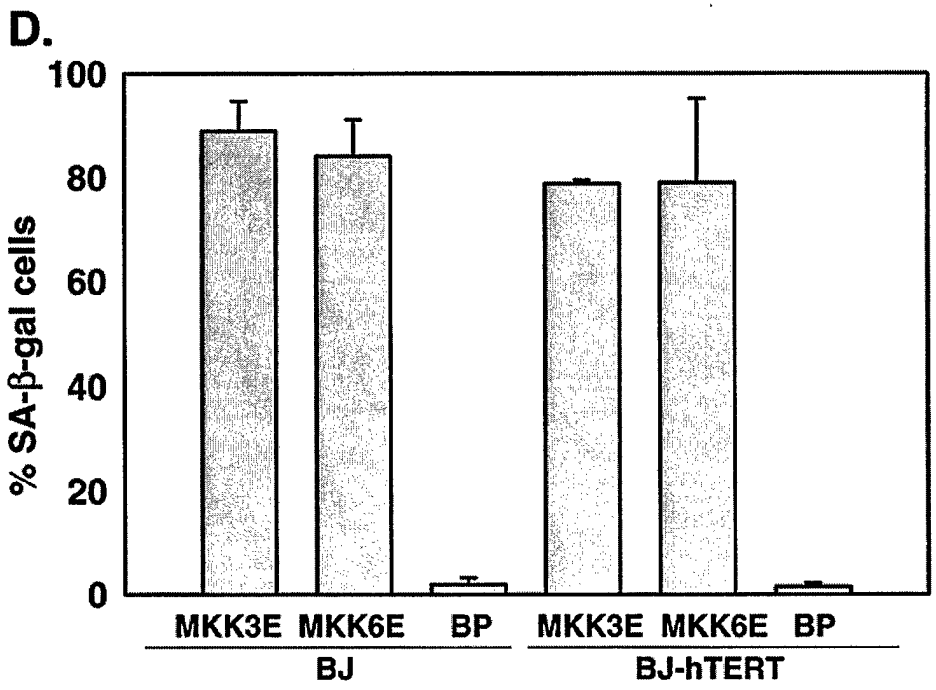

FIG. 2.—*Continued.*

dition, we estimated that more than 80% of MKK3E- or MKK6E-expressing cells had accumulated SA-β-gal, while very few of the vector control cells displayed this senescence marker (Fig. 2C and D). These results indicated that constitutive activation of p38 could induce premature senescence.

Oncogenic *ras*-induced premature senescence differs from replicative senescence in that it is telomere independent. Although the human telomerase catalytic subunit hTERT prevents replicative senescence when ectopically expressed (2), it does not rescue oncogenic *ras*-induced premature senescence (60). MKK3E and MKK6E also induced senescence in BJ cells that had been immortalized with hTERT (Fig. 2C and D). When not transduced with MKK3/6, these hTERT-expressing cells proliferated well beyond PD 150, while the control BJ cells underwent replicative senescence at PD 80 to 90 (data not shown). Thus, senescence induced by p38 activation was not rescued by forced expression of hTERT either, suggesting that both oncogenic *ras* and activated p38 induced senescence via a pathway that was distinct from that mediating replicative senescence.

Like Ha-RasV12, MKK3E and MKK6E induced the expression p53 and $p16^{INK4A}$ (Fig. 2A). While the role of p53 in premature senescence is still controversial, $p16^{INK4A}$ was previously shown to be involved in *ras*-induced senescence (64) and thus may also mediate senescence induced by activated p38 in our study. Therefore, we further explored the mechanism of $p16^{INK4A}$ accumulation following p38 activation. $p16^{INK4A}$ mRNA was up-regulated by three- to fourfold following p38 activation by active MKK3 or MKK6, as determined in a Northern blotting analysis using a $p16^{INK4A}$ cDNA as probe (Fig. 3). Therefore, although we cannot rule out that MKK3/6 may also regulate p16 protein levels, at least part of p16 accumulation is due to the up-regulation of its mRNA.

TNF-α-induced senescence in BJ fibroblasts. Because activation of p38 by oncogenic *ras* or active MKK3 and MKK6 can lead to premature senescence in primary BJ fibroblasts, we explored the possibility that other p38 activators may also induce senescence in these cells. TNF-α is an inflammatory cytokine that has been shown to activate p38 (32, 50). In BJ

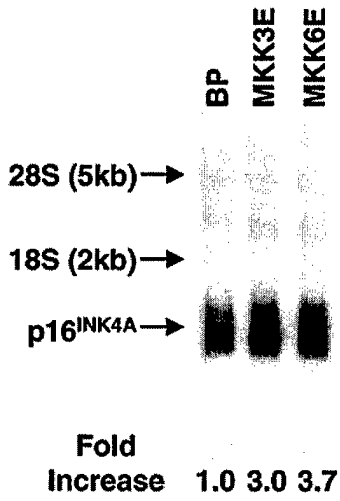

FIG. 3. Active MKK3 and MKK6 up-regulate 16^{INK4A} mRNA levels. Total RNA was isolated from BJ cells 8 days after being transduced with a vector control (BP) or retroviruses encoding active MKK3 (MKK3E) or MKK6 (MKK6E). Eight micrograms of RNA was separated on an agarose gel, transferred to nylon membrane, and hybridized to a $p16^{INK4A}$ cDNA probe labeled by random priming. The signals were visualized and quantitated with a phosphorimager. The numbers represent the relative intensities of $p16^{INK4A}$ signals after normalization to those of background and GAPDH signals (data not shown).

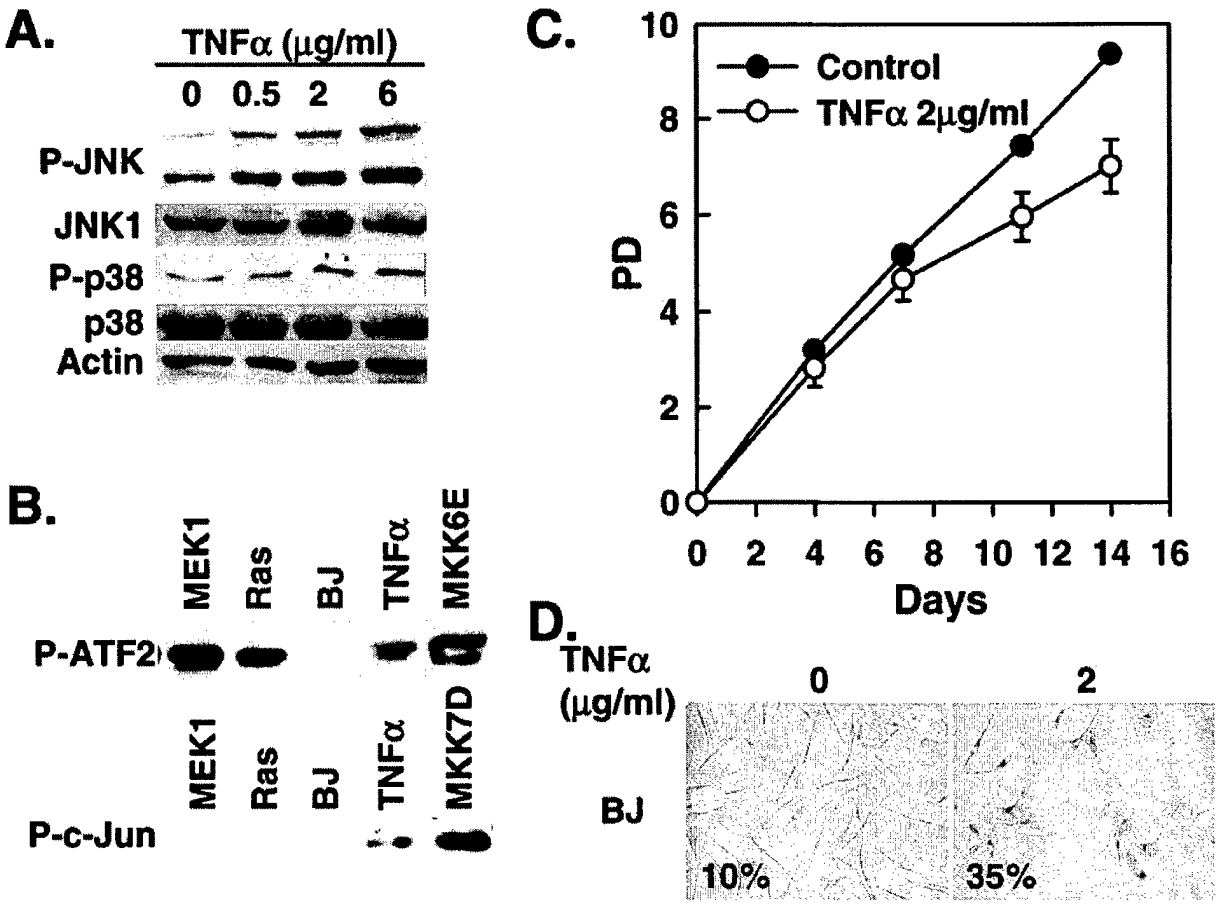

FIG. 4. TNF-α induces senescence in BJ cells. (A) Western blot analysis of BJ cells (PD 24) treated with the indicated concentrations of TNF-α, showing levels of phospho-JNK (P-JNK), JNK1, phospho-p38 (P-p38), p38, and actin. (B) p38 (P-ATF2) and JNK (P-c-Jun) kinase activities were determined in BJ cells 8 days after being transduced at PD 32 with MEK1Q56P (MEK1), Ha-RasV12 (Ras), a vector control (BP), or MKK6E or after being treated with 2 μg of TNF-α/ml. p38 and JNK were isolated and assayed from 200 μg (p38) or 250 μg (JNK) of cell lysates by using the p38 MAP Kinase Assay Kit and the SAPK/JNK MAP Kinase Assay Kit (Cell Signaling), respectively. The phosphorylated substrates, ATF2 for p38 and c-Jun for JNK, were visualized by Western blot analysis by using antibodies against phospho-ATF2 or phospho-c-Jun. (C) PD of BJ cells (PD 29) treated with 2 μg of TNF-α/ml or vehicle control. Values are means ± standard deviations for triplicates. (D) Morphology of BJ cells treated with 2 μg of TNF-α/ml (2) or vehicle control (0), after staining for SA-β-gal (pH 6.0) at day 11 of the treatment. Numbers represent percentages of cells positive for SA-β-gal in each population.

fibroblasts, TNF-α treatment induced the accumulation of phospho-p38 (Fig. 4A) and an increase in p38 activity (Fig. 4B). When treated continuously with TNF-α, BJ cells displayed a slower growth rate than the control cells (Fig. 4C). More than 30% of the cells within the TNF-α-treated population were positive for SA-β-gal (Fig. 4D), indicating that TNF-α-induced growth inhibition was at least partially due to premature senescence. Compared with Ha-RasV12- or MEK1Q56P-induced premature senescence, the senescent phenotypes induced by TNF-α were less severe. This might be attributed to the fact that Ha-RasV12 or MEK1Q56P led to stronger activation of p38 than did TNF-α (Fig. 4B). Overall, these data suggest that activation of p38 by TNF-α can also lead to premature senescence in primary BJ fibroblasts.

While the JNK activity was undetectable in cells expressing oncogenic *ras* or active MEK1 (Fig. 4B), TNF-α led to increases in the phosphorylation of JNK in a dose-dependent manner (Fig. 4A) and induced JNK activity toward phosphorylation of c-Jun (Fig. 4A and B). TNF-α did not induce the activation of ERK in BJ cells (data not shown).

Activation of JNK by active MKK7 does not induce senescence. Overexpression of oncogenic *ras* led to an increase in the levels of phospho-JNK (Fig. 1C). In a kinase assay in which the JNK activity induced by TNF-α or active MKK7 was well detected, the JNK kinase activity was undetectable from *ras*- or MEK1-expressing cells (Fig. 4B). However, it was possible that *ras* still stimulated JNK activity, but only to a level that was imperceptible by the kinase assay. In addition, TNF-α, which induced senescence in BJ cells, also activated JNK (Fig. 4A and B). Therefore, we further investigated the role of JNK activation in premature senescence. Early-passaged BJ cells were infected with a recombinant retrovirus encoding a constitutively active mutant of MKK7 (MKK7D), in which Ser271 and Thr275 had been mutated to Asp (41, 59). MKK7D led to a marked increase in the phosphorylation of JNK1 and JNK2 without affecting the phosphorylation status of p38 (Fig. 5A)

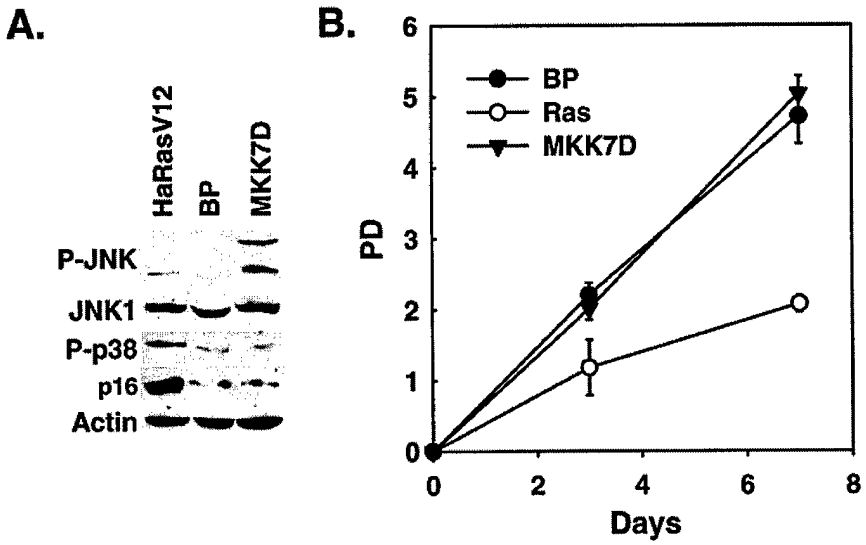

FIG. 5. Activation of JNK by active MKK7 does not induce senescence. (A) Levels of phospho-JNK (P-JNK), JNK1, phospho-p38 (P-p38), $p16^{INK4A}$, and actin were determined by Western blot analysis 10 days after transduction of BJ cells at PD 24 with Ha-RasV12 (HaRasV12), constitutively active MKK7 (MKK7D), or a vector control (BP). (B) PD of BJ cells transduced at PD 24 with Ha-RasV12 (Ras), constitutively active MKK7 (MKK7D), or a vector control (BP). Day 0 represents the fifth day postinfection. Values are means ± standard deviations for triplicates.

and greatly stimulated the ability of JNK to phosphorylate c-Jun (Fig. 4B). However, BJ cells expressing MKK7D did not undergo premature senescence. These cells proliferated continuously, as did the cells infected with a vector control (Fig. 5B), and they showed no senescent phenotypes. Active MKK7 did not induce the accumulation of $p16^{INK4A}$, either (Fig. 5A). Therefore, activation of the JNK pathway is not sufficient to cause premature senescence. Taken together with the observation that Ha-RasV12 did not significantly induce JNK activation, these data suggest that activation of JNK is unlikely to be the major mediator of premature senescence induced by oncogenic *ras*.

p38 activation is required for *ras*-induced senescence. Although activation of p38 by active MKK3 or MKK6 is sufficient to cause premature senescence by itself, the possibility still exists that *ras*-induced senescence is not mediated by p38. As a result, p38 activation may not be required for the ability of *ras* to induce senescence. Alternatively, activation of p38 may be an essential step in the pathway leading from *ras* activation to premature senescence. To differentiate these possibilities, we took advantage of SB203580, a pyridinyl imidazole derivative that could specifically inhibit the activation of p38 but not that of the other MAPK pathways. SB203580 blocks the ability of active p38α and p38β to phosphorylate and activate their downstream targets (9, 15, 16). Treatment of *ras*-expressing cells with SB203580 reduced the ability of p38 to phosphorylate ATF2 (see Fig. 8C), while having no effect on the phosphorylation of ERK1 and ERK2 induced by *ras* (Fig. 6C, top panels). In addition, treatment with SB203580 did not alter the *ras*-induced activity of ERK toward phosphorylation of one of its natural substrates, Elk1, as determined in an in vitro kinase assay following the isolation of ERK from cells by immunoprecipitation (Fig. 6C, bottom panel). As a control, U0126, a specific inhibitor of the MAPKKs MEK1 and MEK2, was used to block the activation of the MEK-ERK pathway by oncogenic *ras* (11, 17). In BJ cells, U0126 inhibited the *ras*-induced phosphorylation of ERK1 and ERK2 (Fig. 4C, upper panels) and reduced the activity of ERK toward phosphorylation of Elk1 in an in vitro kinase assay (Fig. 6C, bottom panel).

When Ha-*RasV12* was transduced into BJ cells in the presence of SB203580, premature senescence was prevented compared to when the vehicle control was present (Fig. 6A). Treatment with U0126 also rescued premature senescence in BJ cells expressing Ha-RasV12 (Fig. 6A), confirming the essential role of MEK-ERK activation in *ras*-induced senescence (36, 64). In addition, SB203580 and U0126 also significantly reduced the percentage of Ha-RasV12-expressing cells that were positive for SA-β-gal activity from more than 80% to 10 to 30% (Fig. 6B), and these compounds blocked the induction of $p16^{INK4A}$ by *ras* (Fig. 6C), suggesting that the onset of the senescence process was blocked when activation of the p38 or MEK-ERK pathway by *ras* was prevented in these cells. These results demonstrated that the activation of p38 is essential for oncogenic *ras* to induce premature senescence in BJ primary fibroblasts. When the ability of *ras* to activate p38 was specifically blocked, premature senescence did not occur, even though the MEK-ERK pathway was still active.

To confirm the essential role of p38, *ras*-induced premature senescence was analyzed in early-passaged BJ cells that had been transduced with dominant-negative mutants of different p38 isoforms, p38α(FA), p38β(FA), p38γ(FA), and p38δ(KM). These mutant alleles of p38 harbor mutations within the dual phosphorylation sites (TGY to FGA for α, β, and γ, or to KGM for δ) and have been shown to inhibit the activities of endogenous p38 in cells in a dominant-negative fashion (20, 26, 27, 35). Expression of p38β(FA), p38γ(FA), or p38δ(KM) partially rescued the growth inhibition provoked by oncogenic *ras* (Fig. 6D), indicating that these three isoforms of p38 were involved in *ras*-induced senescence. Unlike the other p38 isoforms, p38α(FA) had no apparent effect on the negative

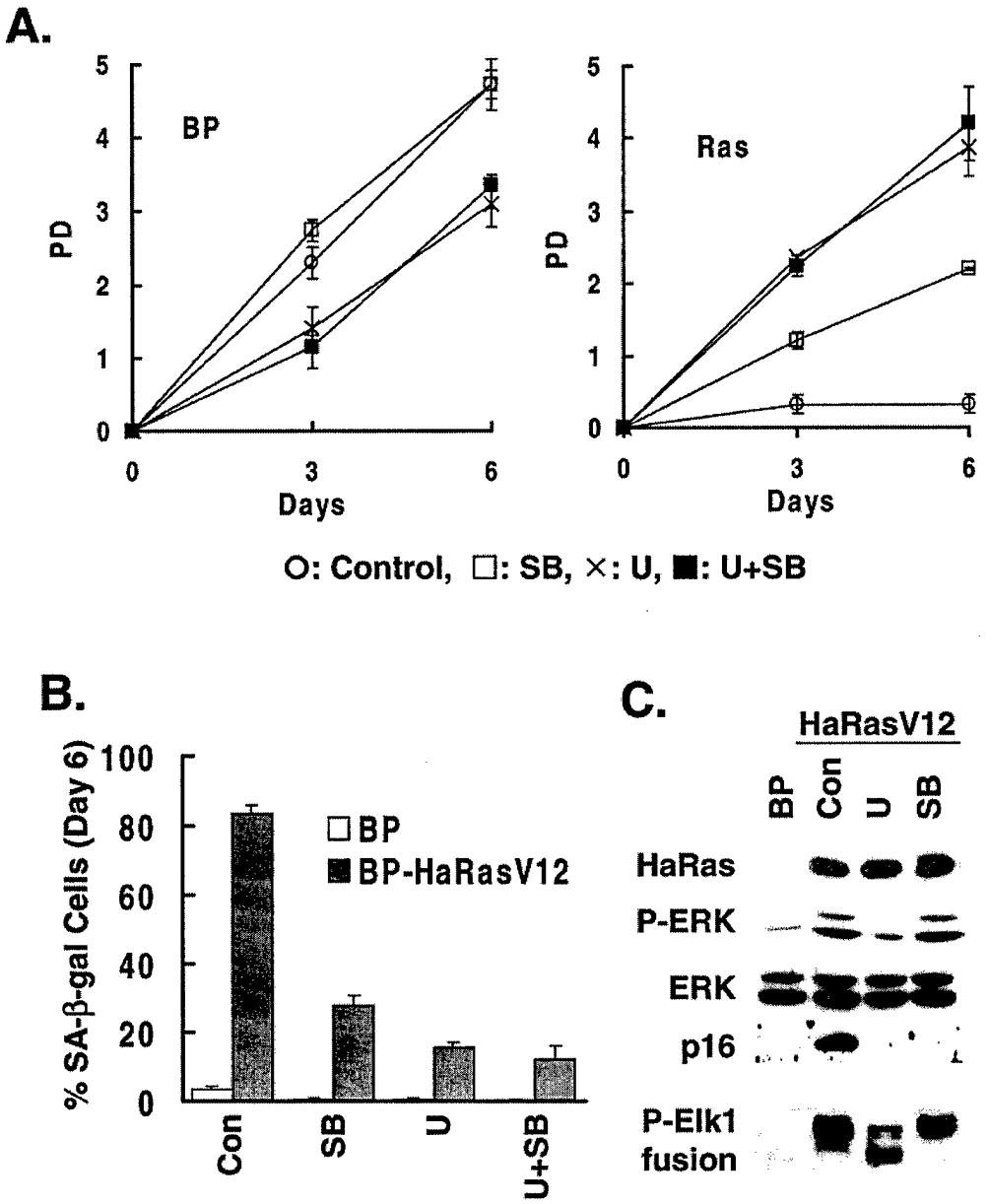

FIG. 6. Specific inhibition of p38 activation by *ras* rescues *ras*-induced premature senescence. (A) PD of BJ cells transduced at PD 18 with a vector control (BP) or Ha-RasV12 (Ras) in the presence of a vehicle control, SB203580, U0126, or U0126 and SB203580. Values are means ± standard deviations (SD) for triplicates. (B) Percentage of SA-β-gal-positive cells within cell populations transduced at PD 18 with a vector control or Ha-RasV12 in the presence of a vehicle control (Con), SB203580 (SB), U0126 (U), or U0126 and SB203580 (U+SB). Values are means ± SD for triplicates. (C) Western blot analysis of BJ cells transduced at PD 18 with a vector control (BP) or Ha-RasV12 (HaRasV12) in the presence of a vehicle control (Con), U0126 (U), or SB203580 (SB), showing the levels of Ras, phospho-ERK (P-ERK), ERK, and $p16^{INK4A}$. The bottom panel (P-Elk1 fusion) shows the kinase activity of ERK immunoprecipitated from the same cells, as determined in an in vitro kinase assay using Elk1 as substrate. The phosphorylated substrate was visualized by Western blot analysis with an antibody against phospho-Elk1. (D) Dominant-negative alleles of p38β, p38γ, and p38δ rescued *ras*-induced senescence. BJ cells expressing a dominant-negative allele of p38β [p38β(AF)], p38γ [p38γ(AF)], p38δ [p38δ(AF)], or a vector control (BH) were transduced with Ha-RasV12 (Ras; open symbols) or a vector control (BP; filled symbols) at PD 34. The PD of these cells were followed starting from day 5 postinfection (designated as day 0). Values are means ± SD for triplicates.

growth regulation by *ras* (data not shown). These results with dominant interfering mutant alleles of p38 further confirmed that p38 activity was essential for oncogenic *ras* to induce premature senescence in BJ fibroblasts.

p38 is not required for the mitogenic activity of *ras*. Since oncogenic *ras* induces both proliferation and premature senescence in normal diploid human fibroblasts, we further investigated whether p38 was also essential for the mitogenic activity

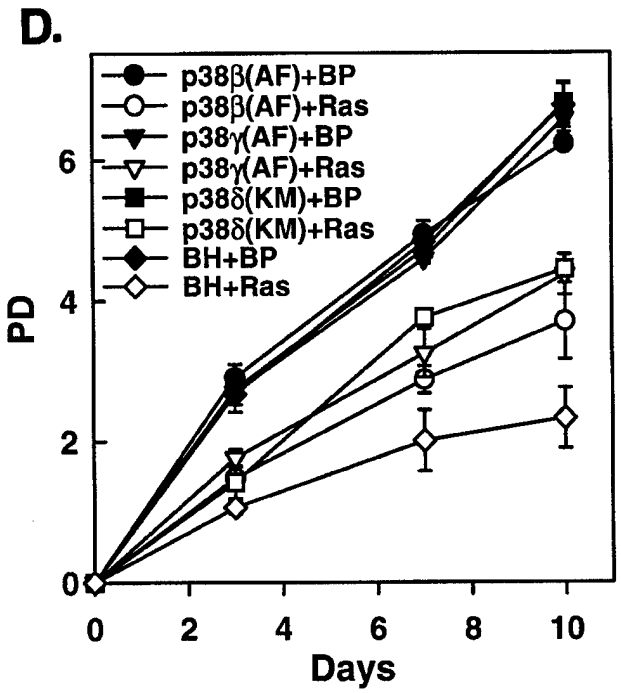

FIG. 6—*Continued.*

of *ras*. In primary fibroblasts, premature senescence is a late response to oncogenic *ras* expression, while the immediate consequence of *ras* activation is growth stimulation (36). Indeed, the growth arrest was not obvious until 7 to 10 days after the initial transduction of the Ha-*rasV12* gene in BJ cells (day 1 to 4 in growth curve assays; see Materials and Methods) (Fig. 1A and data not shown).

To examine the mitogenic activity of *ras*, BrdU incorporation was measured in BJ cell populations shortly after *ras* transduction. Four days postinfection and immediately after the infected cells had been purified with puromycin, oncogenic *ras* expression resulted in a two- to threefold increase in BrdU incorporation when the cells were grown in a low serum concentration (Fig. 7), confirming the previous findings in IMR90 human fibroblasts (36). This assay revealed the role of oncogenic *ras* in mitogenic stimulation before premature senescence occurred in BJ cells. The *ras*-induced increase in BrdU incorporation was not prevented when p38 was inhibited by SB203580 (Fig. 7), indicating that the mitogenic activity of oncogenic *ras* does not rely on active p38. These results suggest that while p38 activation is essential for *ras*-induced premature senescence, the mitogenic activity of *ras* seems to be independent of p38.

p38 is activated as a result of sustained MEK-ERK activation by *ras*. Results from this study and previous studies have demonstrated that both the MEK-ERK and MKK3/6-p38 MAPK pathways are necessary for oncogenic *ras*-induced senescence. In addition, activation of ERK or p38 alone by their respective upstream kinases induced premature senescence. These observations suggest that MEK-ERK and MKK3/6-p38 may act in a linear pathway in mediating *ras*-induced senescence. Supporting this hypothesis, inhibition of both MEK-ERK and MKK3/6-p38 pathways simultaneously by U0126 and SB203580 did not have additive effects in preventing *ras*-induced senescence (Fig. 6A and B). Although MEK-ERK and MKK3/6-p38 constitute two separate MAPK pathways that mediate responses to different extracellular signals, our results suggest a possibility that sustained stimulation of one pathway by *ras* expression may lead to the activation of the other.

Since activation of p38 by MKK3 or MKK6 did not increase ERK phosphorylation (Fig. 2A), we examined the effects of an active MEK-ERK pathway on the activity of p38. To activate the ERK pathway, a constitutively active mutant of MEK1 (MEK1Q56P) (5) was transduced into BJ cells. The active MEK1 increased the activating phosphorylation of ERK (Fig. 8A) and led to premature senescence in primary BJ fibroblasts (see Fig. 11), consistent with previous findings in other primary fibroblasts (36, 64). Expression of the constitutively active MEK1 also induced the phosphorylation of p38 on Thr180 and Tyr182 (Fig. 8A) and increased the kinase activity of p38 toward phosphorylation of ATF2 (Fig. 8C). Therefore, constitutive activation of the MEK-ERK pathway led to activation of p38.

Results from previous studies and those presented here (Fig.

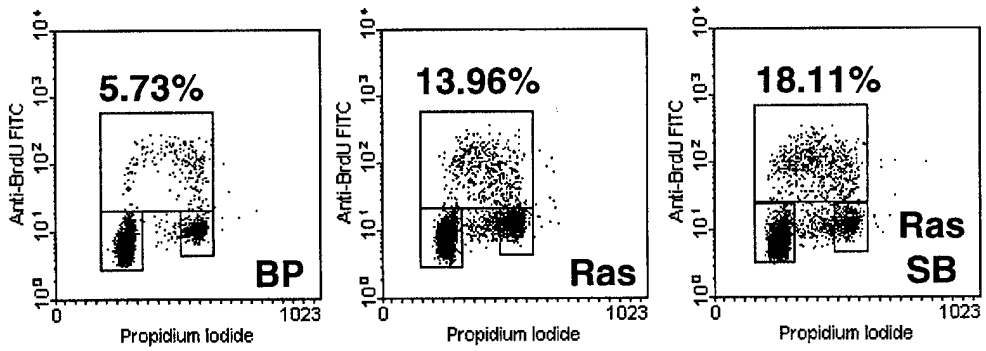

FIG. 7. p38 is not required for the mitogenic activity of *ras*. Three days postinfection, early-passaged BJ cells transduced at PD 18 with a vector control (BP) or Ha-RasV12 (Ras) in the presence of vehicle control or SB203580 (SB) were placed in low-serum medium for 20 h and labeled with BrdU for 3 h. Cells were harvested, stained with FITC-conjugated anti-BrdU antibody and propidium iodide, and analyzed for BrdU incorporation and DNA content by two-color flow cytometry. The upper box represents cells incorporating BrdU (in S phase), the lower-left box represents G_1 cells, and the lower-right box represents G_2/M cells. The percentage of BrdU-positive cells is indicated for each cell population.

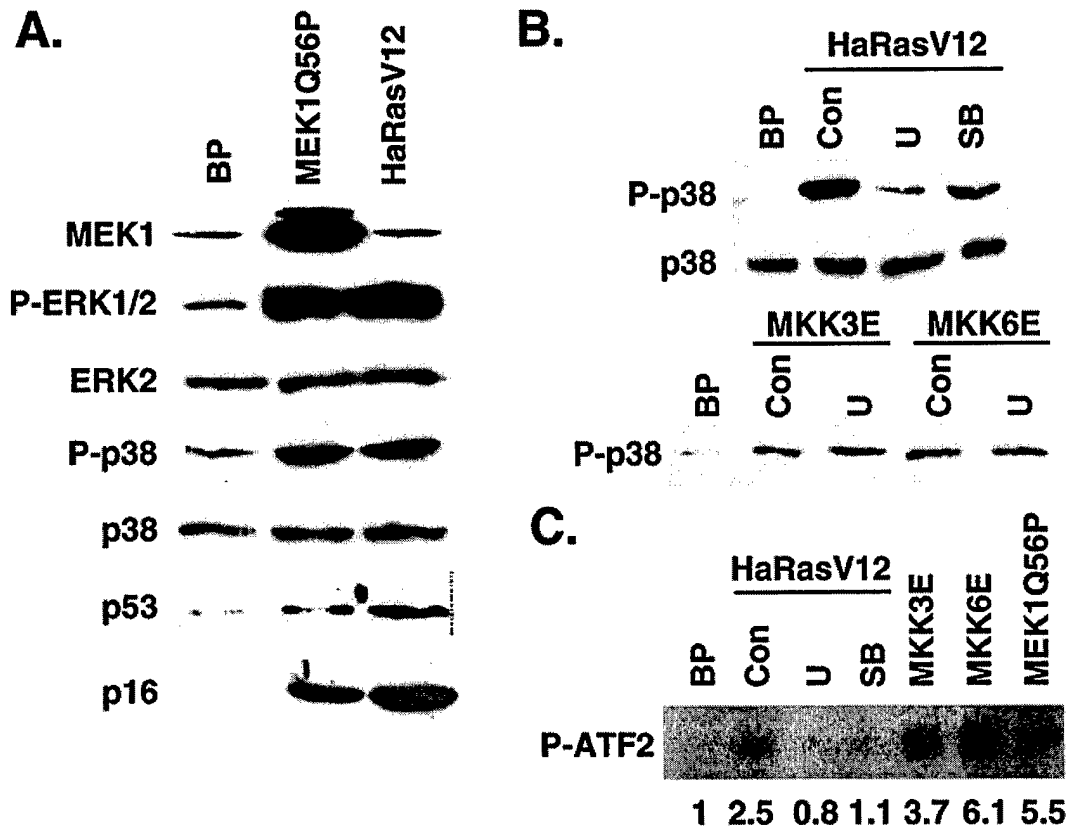

FIG. 8. p38 activation by *ras* is mediated by an active MEK-ERK pathway. (A) Constitutively active MEK1 increases p38 phosphorylation. Shown are the results of Western blot analysis of BJ cells transduced with a vector control (BP), Ha-RasV12 (HaRasV12), or MEK1Q56P (MEK1Q56P) at day 6 postselection, indicating the protein levels of MEK1 (MEK1), phospho-ERK (P-ERK1/2), ERK2 (ERK2), phospho-p38 (P-p38), p38 (p38), p53 (p53), and $p16^{INK4A}$ (p16). (B) Inhibition of MEK-ERK activity prevented *ras*-induced p38 phosphorylation. Shown are the results of Western blot analysis of BJ cells transduced with a vector control (BP), Ha-RasV12 (HaRasV12), MKK3E (MKK3E), or MKK6E (MKK6E) in the presence of vehicle control (Con), U0126 (U), or SB203580 (SB) at day 10 postinfection, with the levels of phospho-p38 and p38 indicated. (C) Protein kinase activity of p38 immunoprecipitated from BJ cells transduced at PD 30 with a vector control (BP), Ha-RasV12 (HaRasV12) in the presence of vehicle control (Con), U0126 (U), or SB203580 (SB), constitutively active MKK3 (MKK3E) or MKK6 (MKK6E), or active MEK1 (MEK1Q56P) at day 10 postinfection. [γ-^{32}P]ATP and GST-ATF2 were used as substrates. The numbers represent the relative amounts of phosphorylated ATF2 substrate after normalization to background. The signals were visualized and quantitated with a phosphorimager.

1C) have demonstrated that oncogenic *ras* activates both ERK and p38 pathways. Our observation that activated MEK1 could lead to p38 activation raised a possibility that *ras* might stimulate p38 activity through activation of the MEK-ERK pathway. Indeed, inhibition of MEK activity by U0126 treatment greatly reduced the ability of Ha-RasV12 to induce p38 phosphorylation (Fig. 8B) and p38 activity toward phosphorylation of ATF2 (Fig. 8C). U0126 did not reduce the p38 phosphorylation induced by active MKK3 or MKK6 (Fig. 8B), demonstrating that U0126 blocked *ras*-induced p38 activation by inhibiting MEK activity rather than by inhibiting MKK3/6 directly. Thus, the activation of p38 by oncogenic *ras* requires a functional MEK-ERK pathway. This finding suggests that in primary human fibroblasts p38 is not activated by oncogenic *ras* directly, but rather as a result of the *ras*-induced activation of the MEK-ERK pathway.

MKK3/6 mediates p38 activation by oncogenic *ras* and active MEK1. We have demonstrated the stimulation of p38 activity as a result of MEK-ERK activation by oncogenic *ras*. p38 is activated through phosphorylation by its upstream kinases, MKK4, MKK3, and MKK6 (10, 21, 22, 32, 46, 47). We therefore investigated whether MKK4, MKK3, and MKK6 were involved in the activation of p38 by oncogenic *ras* and active MEK1.

In early-passaged BJ cells, MEK1Q56P and Ha-RasV12 induced the activating phosphorylation of MKK3/6 (on Ser189/Thr193 of MKK3 and on Ser207/Thr211 of MKK6, respectively) (21, 46, 47) (Fig. 9A). On the other hand, while treatment of BJ cells with NaCl led to strong activation of MKK4, Ha-RasV12 and MEK1Q56P did not stimulate the activating phosphorylation of MKK4 (Fig. 9A). The *ras*-induced increases in the levels of active MKK3/6 depended on active MEK, because inhibition of MEK activity with U0126 abolished the ability of Ha-RasV12 to induce the accumulation of phospho-MKK3/6 (Fig. 9B). Consistent with the role of MEK in MKK3/6 activation, U0126 also blocked MKK3/6 phosphorylation induced by MEK1Q56P (Fig. 9B). Therefore, MKK3/6 can be activated by *ras* in a MEK-dependent fashion during the onset of premature senescence. Our data suggested that MKK3/6 could be important in mediating p38 activation in

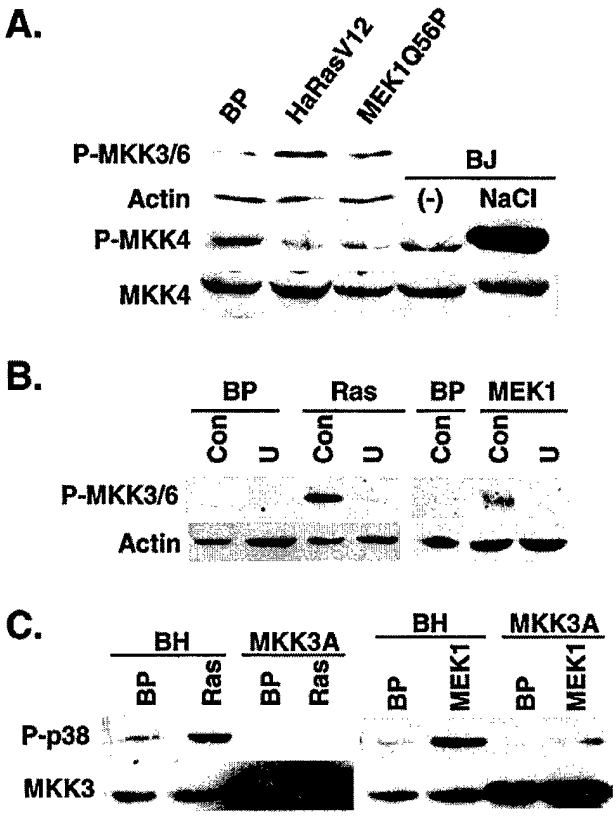

FIG. 9. MKK3/6 mediates the activation of p38 by oncogenic *ras* and active MEK1. (A) Ha-RasV12 and MEK1Q56P induced the accumulation of active MKK3/6 but had no effect on the levels of active MKK4. The levels of phospho-MKK3/6 (P-MKK3/6), actin, phospho-MKK4 (P-MKK4), and MKK4 were determined by Western blotting 10 days after the transduction of BJ cells at PD 33 with a vector control (BP), oncogenic Ras (HaRasV12), or constitutively active MEK1 (MEK1Q56P). The levels of phospho-MKK4 (P-MKK4) and MKK4 were also determined in BJ cells (PD 26) treated with 700 mM NaCl for 15 min (NaCl) or left untreated (−). (B) Up-regulation of phospho-MKK3/6 by Ha-RasV12 and MEK1Q56P relied on MEK activity. The levels of phospho-MKK3/6 (P-MKK3/6) and actin were determined by Western blotting 7 days after the transduction of BJ cells at PD 39 with a vector control (BP), Ha-RasV12 (Ras), or MEK1Q56P (MEK1) in the presence of vehicle control (Con) or U0126 (U). (C) MKK3 is required for p38 activation by oncogenic *ras* and active MEK1. The levels of phospho-p38 (P-p38) and MKK3 were determined by Western blot analysis in BJ cells expressing a dominant-negative allele of MKK3 (MKK3A) or a vector control (BH) 8 days after transduction at PD 33 with Ha-RasV12 (Ras), MEK1Q56P (MEK1), or a vector control.

response to oncogenic *ras* and active MEK and that MKK4 was probably not involved in this pathway.

The involvement of MKK3/6 in *ras*- and MEK-mediated p38 activation was further demonstrated using dominant-negative mutants of MKK3 and MKK6. These inactive mutants (MKK3A and MKK6A) were constructed by substitution of the two serine/threonine residues in the dual phosphorylation sites with alanine (23). In BJ cells expressing the dominant-negative allele of MKK3, MKK3A, the ability of Ha-RasV12 and MEK1Q56P to induce the activating phosphorylation of p38 was significantly reduced (Fig. 9C). Similar results were obtained in BJ cells transduced with MKK6A (data not shown). These studies confirmed the essential roles of MKK3 and MKK6 in mediating *ras*- and MEK-induced activation of p38. Taken together, our findings argue that MEK, when turned on by oncogenic Ras, may stimulate p38 activity by activating MKK3/MKK6 in BJ human fibroblasts.

Activation of MEK-ERK by *ras* precedes the activation of MKK3/6-p38 and senescence. The finding that the MKK3/6-p38 pathway is activated as a result of the activation of the MEK-ERK pathway by oncogenic *ras* suggests that activation of the MEK-ERK pathway may precede that of the MKK3/6-p38 pathway following the overexpression of *ras*. Such a temporal difference will be more obvious if the activation of the MKK3/6-p38 pathway by MEK is not achieved through direct signaling but instead involves indirect, slow routes such as transcriptional regulation or other mechanisms that require novel protein synthesis. To determine the timing of the activation of the MEK-ERK and MKK3/6-p38 pathways, we followed the time course of the status of these two pathways, starting from day 3 through day 9 after the initial transduction of BJ cells with Ha-*RasV12* (Fig. 10). By day 3, MEK and ERK had already been strongly activated. Since it minimally took 3 days to obtain a pure cell population transduced with *ras*, we were unable to monitor the status of MEK and ERK before day 3. However, because phospho-MEK and phospho-ERK were already at their maximum levels by day 3, it was likely that the MEK-ERK pathway was activated by *ras* well before day 3, which would be consistent with the direct activation of MEK-ERK by *ras*. On the contrary, phosphorylation of MKK3/6 and that of p38 were not induced by *ras* until day 4. We did not observe any time gap between the induction of phospho-

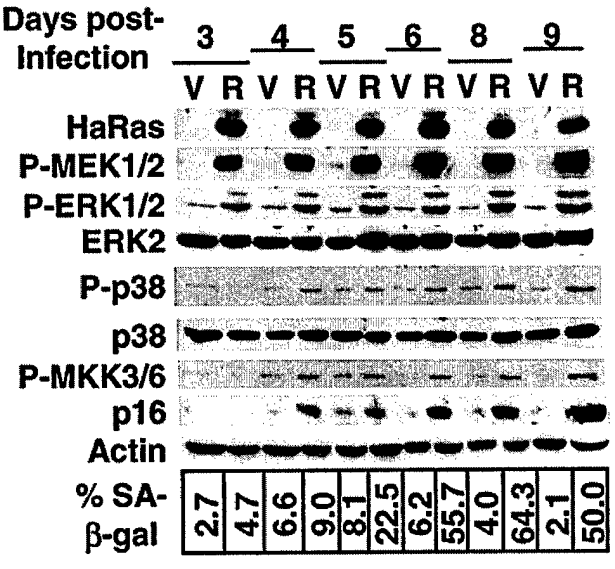

FIG. 10. Activation of MEK-ERK precedes the activation of MKK3/6-p38 and premature senescence. The levels of Ha-Ras, phospho-MEK1 and 2 (P-MEK1/2), phospho-ERK1 and 2 (P-ERK1/2), ERK2, phospho-p38 (P-p38), p38, phospho-MKK3 and 6 (P-MKK3/6), $p16^{INK4A}$, and actin were determined by Western blotting after different days (days 3 to 9) following the transduction of BJ cells at PD 35 with a vector control (V) or Ha-RasV12 (R). The percentages of cells positive for SA-β-gal within these same cell populations are shown at the bottom.

MKK3/6 and that of phospho-p38, suggesting that p38 was activated by MKK3/6 directly. The p16 protein was induced moderately at day 4 and then to higher levels after day 6. Cells displaying the SA-β-gal senescence marker started to accumulate at day 5 and reached the maximum percentage within the population at day 6.

These data demonstrated that the MEK-ERK pathway was activated earlier by *ras* than the MKK3/6-p38 pathway was and that the accumulation of p16 and the appearance of the senescence phenotype occurred even later than the activation of p38. The results support our conclusion that p38, when activated by *ras* as a result of sustained MEK-ERK activation, triggers premature senescence.

Senescence induced by *ras* and MEK1 both require active p38. The dependence of p38 activation by *ras* on active MEK-ERK, together with the finding that active p38 leads to senescence, suggests that the sequential activation of MEK-ERK and MKK3/6-p38 by *ras* may be responsible for the ability of oncogenic *ras* to induce premature senescence in primary cells. To explore this possibility, we analyzed premature senescence induced by Ha-RasV12, MEK1Q56P, MKK3E, or MKK6E under conditions when MEK or p38 was specifically blocked. Specific inhibition of the p38 activity with SB203580 prevented premature senescence induced by Ha-RasV12, MEK1Q56P, MKK3E, and MKK6E. In contrast, treatment with U0126, which blocks activation of the MEK-ERK pathway, only rescued senescence caused by expression of Ha-RasV12 and MEK1Q56P but not that by constitutively active MKK3 or MKK6 (Fig. 11). Therefore, both oncogenic *ras*-induced and active MEK1-induced senescence relies on an active p38 pathway, while constitutively activated p38 can cause premature senescence without the activity of MEK-ERK.

These results have placed MKK3/6-p38 downstream of MEK-ERK concerning the execution of senescence elicited by *ras*, and thus they have defined a pathway leading from oncogenic *ras* expression to premature senescence. Oncogenic *ras* activates the MEK-ERK pathway, which in turn induces the phosphorylation and activation of MKK3/6 and p38. Activated p38 then provokes premature senescence by inducing the accumulation of growth inhibitors such as $p16^{INK4A}$. Confirming this notion, senescence induced by active MKK3 and MKK6 consistently occurred earlier than that induced by Ha-RasV12 or active MEK1. In growth curve assays, Ras- or MEK1-expressing cells retained substantial levels of growth during the first 3 days (day 5 to 8 postinfection) compared to control cells, and they underwent massive growth arrest afterwards (Fig. 1A and 11). In contrast, cells transduced with MKK3E or MKK6E almost completely ceased to proliferate within the first 3 days of the assays. In fact, the growth inhibition was already noticeable in MKK3E- and MKK6E-expressing cells during puromycin selection (1 to 2 day postinfection), even before the growth curve assays began. The direct activation of p38 by exogenously expressed, active MMK3 or MKK6 might have bypassed the need for the activation of endogenous MKK3/6 proteins by MEK or *ras*.

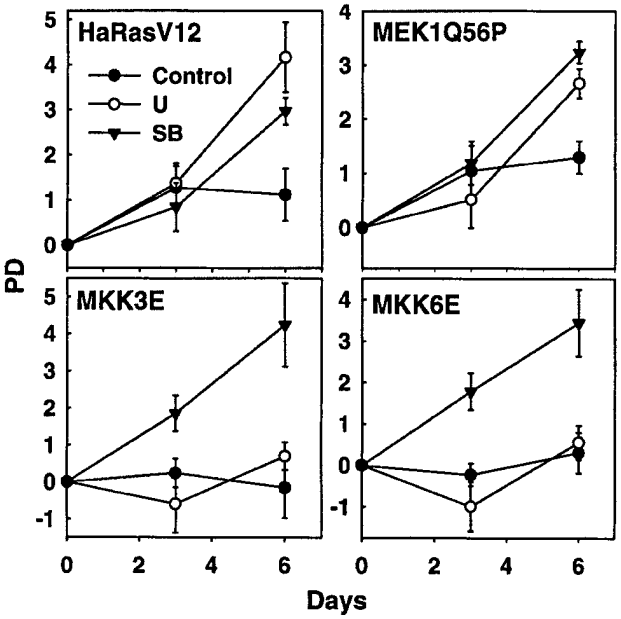

FIG. 11. Both Ha-RasV12- and MEK1Q56P-induced senescence requires active p38. Shown are the growth curves of BJ cells transduced at PD 17 with Ha-*rasV12* (Ha-RasV12) or constitutively active MEK1 (MEKQ56P), MKK3 (MKK3E), or MKK6 (MKK6E) in the presence of vehicle control, U0126 (U), or SB203580 (SB). Values are means ± standard deviations as determined for triplicates at the indicated days postselection.

DISCUSSION

Oncogenic *ras*-induced activation of the MEK-ERK pathway, which usually confers growth stimulation, leads to premature senescence that serves as a tumor-suppressing response in normal primary cells (36). In tumor cells or cell lines where premature senescence has been compromised, oncogenic *ras* or a constitutively active MEK-ERK pathway only promotes cell growth and leads to transformation (36). It has been unclear how activation of the mitogenic MEK-ERK pathway by oncogenic *ras* can lead to senescence. In this study, we have demonstrated that activation of the MEK-ERK pathway by *ras* leads to the activation of another MAPK, p38, and that activated p38 in turn induces the accumulation of growth inhibitors and premature senescence. Thus, while the *ras*-activated MEK-ERK cascade may transduce mitogenic signals through transcription factor AP-1 (31), it provokes premature senescence by activating the p38 pathway in normal, primary cells (Fig. 12). These findings have identified within the *ras* signaling cascade a branch that mediates premature senescence, and thus they have provided an explanation to how *ras* achieves both positive and negative growth regulation. Our data also suggest that one possible way for a tumor to bypass *ras*-induced premature senescence and achieve oncogenic transformation is to acquire genetic mutations that nullify the activation of the p38 pathway.

The p38 pathway mediates responses to environmental stresses, including DNA-damaging agents such as UV and γ-irradiation (43, 46, 58). A recent study reported that Ha-RasV12 induced premature senescence by increasing the intracellular levels of reactive oxygen species (34). Since reactive oxygen species induce DNA damage, it is possible that in primary BJ fibroblasts p38 is activated as a result of DNA damage due to prolonged oxidative stress induced by oncogenic *ras*. If this

notion is confirmed, premature senescence may serve as a DNA damage check point that is enforced by the activated p38 pathway in primary fibroblasts.

MEK-ERK and MKK3/6-p38 constitute two independent MAPK pathways that mediate responses to different extracellular stimuli (6). Our results have revealed an interaction between these two pathways, concerning the effects of oncogenic *ras* in primary cells. Like oncogenic Ras, constitutively active MEK1 induced the accumulation of active MKK3/6, the activation of p38 and, subsequently, the occurrence of premature senescence. Activation of MKK3/6 and p38 by Ha-*rasV12* requires an activated MEK-ERK pathway. In addition, the ability of active MEK1 to mediate *ras*-induced premature senescence relies on the activation of p38. These observations have defined a linear pathway involving the sequential activation of MEK-ERK and MKK3/6-p38 by *ras*, which mediates premature senescence in primary human fibroblasts.

Several lines of evidence suggest that oncogenic *ras* and active MEK-ERK do not activate the MKK3/6-p38 pathway through direct signaling. First of all, the activation of MKK3/6 and p38 occurred after 4 days following oncogenic *ras* overexpression, lagging that of MEK and ERK by at least 1 to 2 days (Fig. 10). This observation is inconsistent with a mechanism of direct activation, whose effects would be achieved within hours if not minutes after the initiation of the signal. Instead, MKK3/6 and p38 may be activated as a result of the sustained presence of an active MEK-ERK cascade. Confirming the direct activation of p38 by MKK3/6, increases in the phosphorylation of MKK3/6 and p38 were observed simultaneously following *ras* gene transduction. In addition, premature senescence is a late response of normal cells to oncogenic activation of *ras*. Growth arrest induced by Ha-*rasV12* or active MEK1 was not apparent until 7 to 10 days after the initial transduction of these genes (Fig. 1A and 11). This agrees with a model in which the trigger of *ras*- or MEK-induced senescence is activated through an indirect, slow route. Also supporting the indirect activation model, active MKK3 or MKK6 induced senescence much more rapidly than oncogenic *ras* or active MEK1 (Fig. 11). Growth inhibition was already noticeable 1 to 2 days after the transduction of active MKK3 or MKK6. The ectopic expression of active MKK3 or MKK6 might have bypassed the slow route that was required for MEK to activate MKK3/6 and thus may have speeded up the senescence process.

A similar reliance of p38 activation by *ras* on MEK-ERK has been observed in immortalized NIH 3T3 fibroblasts with another oncogenic *ras* gene, Ha-*rasL61*, although oncogenic *ras* does not induce senescence in these cells (8). The expression of an active MEK also activated p38 in PC12 cells, where p38 activation is required for neuronal differentiation induced by nerve growth factor (42). Therefore, the sequential activation of the MEK-ERK and MKK3/6-p38 pathways may also operate in other cell types and may mediate cellular processes other than premature senescence.

Premature senescence is accompanied, and thus may be mediated by, the accumulation of growth inhibitors such as $p16^{INK4A}$ and p53. We found that activation of p38 is sufficient to induce increases in the protein levels of these genes. In the case of $p16^{INK4A}$, activation of p38 by MKK3 or MKK6 led to increases in its mRNA levels. Activated p38 is known to regulate gene expression by increasing mRNA stability (24, 39, 63). Therefore, it is conceivable that activation of p38 by *ras* may result in the stabilization of $p16^{INK4A}$ and p53 mRNA, leading to the accumulation of $p16^{INK4A}$ and p53 proteins and, eventually, premature senescence.

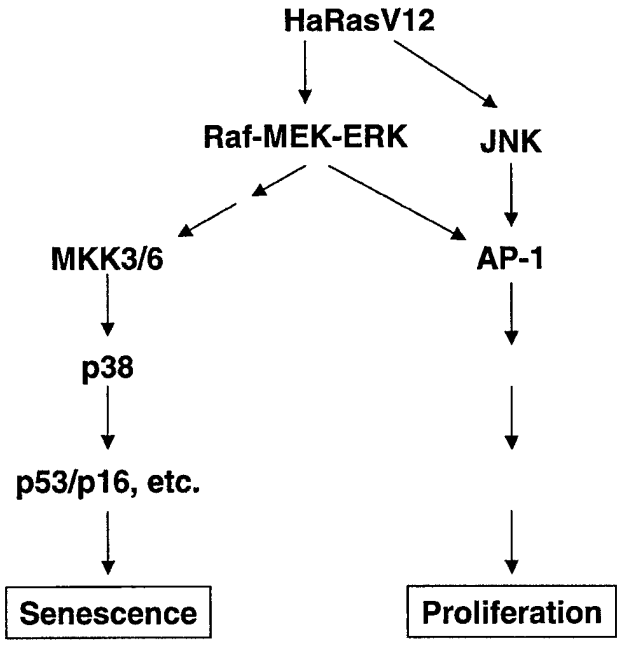

FIG. 12. Schematic representation of a model indicating the role of p38 in oncogenic *ras*-induced premature senescence in primary fibroblasts. Activation of the Raf-MEK-ERK pathway by oncogenic *ras* confers cell proliferation through transcription factor AP-1, as suggested in previously published literature, and concurrently leads to premature senescence by stimulating the activity of the p38 pathway, whose activation is sufficient to induce the accumulation of p53 and $p16^{INK4A}$.

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