[CANCER RESEARCH 63, 5454–5461, September 1, 2003]

p38 Kinase Is a Key Signaling Molecule for H-Ras-induced Cell Motility and Invasive Phenotype in Human Breast Epithelial Cells[1]

Mi-Sung Kim,[2] Eun-Jung Lee,[2,3] Hyeong-Reh Choi Kim, and Aree Moon[4]

College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea [M-S. K., E-J. L., A. M.], and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201 [H-R. C. K.]

ABSTRACT

Ras expression has been suggested as a marker for tumor aggressiveness of breast cancer, including the degrees of invasion and tumor recurrence. We showed previously that H-ras, but not N-ras, up-regulates matrix metalloproteinase 2 expression and induces invasive phenotype in MCF10A human breast epithelial cells (A. Moon, *et al.* Int. J. Cancer, *85:* 176–181, 2000). In this study, we show that H-ras also promotes cell motility more effectively than N-ras in MCF10A cells. We have investigated H-ras-specific signaling pathway(s) critical for H-ras-mediated cell motility and invasive phenotype. Whereas neither H-ras nor N-ras activated c-Jun NH_2-terminal kinase 1, both H-ras and N-ras effectively activated extracellular signal-regulated protein kinase (ERK) -1,2. Importantly, prominent activation of p38 mitogen-activated protein kinase was shown only in H-ras-activated cells but not in N-ras-activated MCF10A cells. Functional significance of H-ras-activated p38 in invasiveness and cell motility was evidenced by studies using SB203580, a chemical inhibitor of p38, and a dominant-negative construct of p38. Whereas inhibition of c-Jun NH_2-terminal kinase 1 activity had no effect on H-ras-induced MCF10A cell invasion and motility, the inhibition of the ERK pathway using a chemical inhibitor PD98059 or dominant-negative mutant of mitogen-activated protein/ERK kinase 1, an activator of ERKs, significantly reduced H-ras-induced invasion and migration. We also provide evidence that p38 and, to a lesser degree, ERKs, are critical for H-ras-mediated up-regulation of matrix metalloproteinase 2. Taken together, the present study shows that H-ras activation of both p38 and ERKs induces cell invasion and motility, whereas N-ras activation of ERKs alone is not sufficient. This study reveals the p38 kinase as a key signaling molecule differentially regulated by H-ras and N-ras, leading to H-ras-specific cell invasive and migrative phenotypes in human breast epithelial cells.

INTRODUCTION

Tumor cell invasion and metastasis are complex processes involving ECM[5]-degrading proteinase activity and migration through the ECMs (1). Elevated levels of $p21^{ras}$ expression are associated with tumor aggressiveness in breast cancer, including the degrees of invasion to fat tissues, infiltration into lymphatic vessels, and tumor recurrence (2–4). Of clinical importance, biochemical and molecular approaches, including farnesyl transferase inhibitors and antisense technology, have been used to interrupt *ras*-mediated oncogenic activities (5). Although clinical trials hold promise for the future use, a major drawback of these approaches has been *in vivo* toxicity. Ras is a central signaling molecule that activates several downstream signaling molecules including *raf-1*, *rac*, rho, and phosphatidylinositol-3 kinase, critical for cell survival, proliferation, motility, cytoskeletal organization, and other cellular processes (6). Thus, down-regulation of ras expression or inhibition of general ras activities is detrimental not only to cancer cells, but also to normal cells. A major challenge is to develop drug compounds that target ras-downstream signaling molecules that are required for malignant cancer cell behavior but less critical for normal cell functions.

To unveil *ras* downstream signaling pathways critical for tumor progression, we investigated previously the effects of H-, and N-ras on cancerous phenotypic changes using MCF10A human breast epithelial cell lines in which H- and N-ras are constitutively activated by mutation of gly to asp in the amino acid codon 12 in H- and N-ras, respectively (7). We showed that H-ras, but not N-ras, induced an invasive phenotype in MCF10A human breast epithelial cells, whereas both induced transformed phenotype as determined by anchorage-independent growth and foci formation (7).

Downstream effector molecules of *ras* include members of the MAPK family, composed of JNK, ERK, and stress-activated protein kinase-2 (p38). MAPKs are among the central elements that transduce extracellular signaling into cellular responses (8, 9) and are believed to play pivotal roles in proliferation, apoptosis, differentiation, cytoskeleton remodeling, and cell cycle regulation (10–13). Recent evidences suggest a role for the MAPK members in invasive phenotype and/or cell motility in various cell systems. p38 has been demonstrated to be an important mediator of the cell motility responses in endothelial cells (14, 15) and smooth muscle cells (16). The ERK pathway was shown to be involved in the invasive or migrative behavior of human glioblastoma cells (17), vascular smooth muscle cells (18), and FG carcinoma cells (19), whereas Matsumoto *et al.* (15) reported that ERK, unlike p38, is not a mediator of porcine aortic endothelial cell motility responses elicited by platelet-derived growth factor.

Members of the MMP family play a critical role in tumor invasion and metastasis formation, especially, MMP-2 (M_r 72,000 type IV collagenase, gelatinase A) and MMP-9 (M_r 92,000 type IV collagenase, gelatinase B; Refs. 20, 21). MMP-9 has been suggested to be critical for the induction of an invasive phenotype in rat embryonic fibroblast cells (22, 23). In contrast, our previous study demonstrated that H-ras-induced invasive phenotype of MCF10A cells is associated more closely with the expression of MMP-2 than MMP-9 (7). Whereas the significance of MAPK family members in the regulation of MMP-9 expression has been well documented (24–27), the regulatory mechanisms responsible for the MMP-2 expression are poorly understood at present.

In the present study, we investigated the role of the MAPK family members in H-ras-induced invasive phenotype and motility of MCF10A cells. Here, we report that H-ras-mediated cell migration and invasive phenotype involves activation of both p38 and ERKs, whereas N-ras activated ERKs are insufficient to induce cell migration and invasive phenotype. We also present evidence suggesting that the activation of p38 and, to a lesser degree, ERKs may contribute to

Received 3/19/03; revised 5/22/03; accepted 6/3/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a Korea Health 21 R&D, Ministry of Health and Welfare Grant (02-PJ1-PG10-20801-0001; to A. M.) and by a National Cancer Institute NIH Grant (CA-64139; to H-R. C. K.).

[2] These authors equally contributed to this study.

[3] Present address: Institute of Science and Technology, CJ Corp., Inchonsi, Kyonggi-Do 467-810, Korea.

[4] To whom requests for reprints should be addressed, at College of Pharmacy, Duksung Women's University, 419 Ssangmun-Dong, Tobong-Ku, Seoul 132-714, Korea. Phone: 82-2-901-8394; Fax: 82-2-901-8386; E-mail: armoon@duksung.ac.kr.

[5] The abbreviations used are: ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal protein kinase; ERK, extracellular signal-regulating kinase; MMP, matrix metalloproteinase; DN, dominant-negative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MEK, mitogen-activated protein/extracellular signal-regulating kinase kinase; PI3K, phosphatidylinositol 3′-kinase.

a more invasive phenotype possibly via up-regulation of MMP-2 expression in human breast epithelial cells.

MATERIALS AND METHODS

Cell Lines. Development and characterization of MCF10A, H-ras MCF10A, and N-ras MCF10A cells have been described previously (7). Briefly, retroviral vectors containing a mutant H-ras (pBW1423) and a mutant N-ras (pBW1775; provided by Dr. Douglas Lowy, NCI, Bethesda, MD) were transfected into the amphotropic package cells line GP+envAm12 (Genix Pharmaceuticals, Tarrytown, NY). MCF10A cells were infected with the viral medium and selected in the presence of 400 μg/ml G418. More than 100 NeoR colonies were pooled together and named H-ras MCF10A and N-ras MCF10A, respectively. The cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml EGF, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine, and 0.5 μg/ml fungizone.

Transfection. Transfection was performed using Lipofectamine reagent (Life Technologies, Inc., Rockville, MD) following the manufacturer's instruction. The DN construct of p38 (28) was kindly provided by Dr. Young-Joon Surh (College of Pharmacy, Seoul National University, Seoul, Korea). DN MEK-1 (29) and DN JNK-1 (30) were kindly provided by Dr. Yong J. Lee (Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA).

Immunoblot Analysis. Equal amounts of protein extracts in SDS-lysis buffer were subjected to 12% SDS-PAGE analysis and electrophoretically transferred to nitrocellulose membrane. Anti-JNK-1, anti-phosphorylated JNK, anti-ERK-1,2, antiphosphorylated ERK-1,2, and antiphosphorylated p38 antibodies were purchased from New England Biolabs (Beverly, MA). Anti-p38 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Enhanced chemiluminescence (Amersham-Pharmacia, Buckinghamshire, United Kingdom) system was used for detection. Relative band intensities were determined by quantitation of each band with an Image Analyzer (Vilber Lourmet, Marne-la-Vallee Cedex 1, France).

Gelatin Zymography. Cells were cultured in serum-free DMEM/F12 medium for 48 h. Conditioned medium was collected and centrifuged at 3000 rpm for 10 min to remove cell debris. The protein concentration was measured using BCA protein assay reagents (Pierce, Rockford, IL). Equal amounts of protein of conditioned media were mixed with 2× Laemmli nonreducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 three times for 30 min, rinsed for 15 min with 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $CaCl_2$, 0,02% Brij-35, and 0.2% sodium azide, and incubated overnight at 37°C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 solution containing 10% acetic acid and 20% methanol for 30 min and destained with 10% acetic acid solution. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

MTT Assay. Cells (1×10^4) cultured in a 96-well plate were treated with SB203580 or PD98059. MTT solution purchased from Promega Corporation (Madison, WI; 5 mg/ml) was added to the medium, and the cells were additionally incubated for 4 h. After 100 μl of supernatant was replaced with 100 μl of DMSO, absorbance of each well was measured at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA). Percentage of cell survival was determined as the relative absorbance of treated *versus* untreated cells.

***In Vitro* Invasion Assay.** *In vitro* invasion assay was performed using 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) as described previously (7). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Lower compartment was filled with serum-free medium containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, incubated for 17 h, fixed with methanol, and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400. Thirteen fields were counted for each filter, and each sample was assayed in triplicate.

***In Vitro* Migration Assay Using Transwell.** *In vitro* migration assay was performed using a 24-well Transwell unit with polycarbonate filters. Experimental procedures are the same as the *in vitro* invasion assay described above except that the filter was not coated with Matrigel for the migration assay.

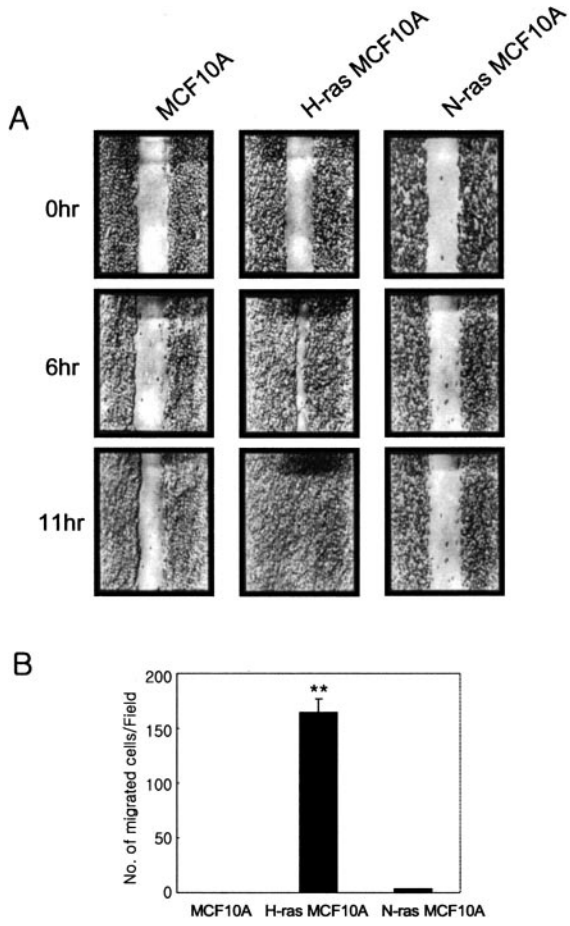

Fig. 1. H-ras, but not N-ras, induces MCF10A cell motility. *A*, wound migration assay: after pretreatment of cells with mitomycin C (25 μg/ml) for 30 min, injury line was made on the confluent monolayer of cells. Cell motility was examined with light microscope (×40) at indicated time points. *B*, cell migration assay using a transwell: numbers of migrated cells were counted in 13 microscopic fields (×400). The results presented were means of triplicates; bars, ±SE. **, statistically different from MCF10A cells at $P < 0.01$.

Wound Migration Assay. Cells were pretreated with mitomycin C (25 μg/ml) for 30 min before injury line was made. The injury line was made with a tip with 2 mm in width on the cells plated in culture dishes at 90% confluency. After being rinsed with PBS, cells were allowed to migrate in complete medium, and photographs were taken (×40) at indicated time points.

RESULTS

H-ras, but not N-ras, Induces MCF10A Cell Motility. Because migrative capacity is a prerequisite for cell invasion through the basement membrane, we asked whether H-ras promotes not only invasive phenotype but also cell motility more effectively than N-ras in MCF10A cells. As evidenced by migration through wound (Fig. 1*A*) and Transwell chamber (Fig. 1*B*), motility of MCF10A cells was greatly induced by H-ras, but not by N-ras. It should be mentioned that both H-ras and N-ras increased cell proliferation rates and anchorage-independent cell growth at comparable levels (7),[6] suggesting that H-ras-mediated migrative property of MCF10A cells is unlikely to result from increased cell proliferation rates. To ensure this, the wound migration assay was performed using cells pretreated with mitomycin C, a cell cycle blocker at the S phase (Ref. 31; Fig. 1*A*).

[6] Unpublished observations.

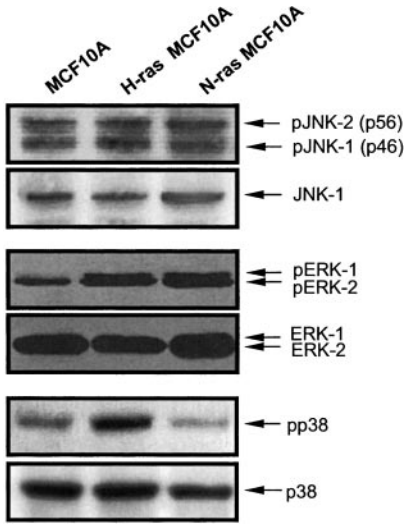

Fig. 2. p38 is prominently activated by H-ras, but not by N-ras. The levels of activated JNK-1,2, ERK-1,2, and p38 in MCF10A, H-ras MCF10A, and N-ras MCF10A cells were determined by immunoblot analysis of whole cell lysates using phosphospecific anti-JNK-1,2, anti-ERK-1,2 and anti-p38 antibodies (pJNK-1,2, pERK-1,2, and pp38, respectively), and anti-JNK-1, ERK-1,2, and p38 antibodies.

These results demonstrate that H-ras, but not N-ras, enhances cell motility in MCF10A cells.

p38 is Prominently Activated by H-ras, but not by N-ras. To investigate the molecular mechanism by which H-ras selectively induces invasive phenotype and motility in MCF10A cells, we examined whether the ras effector pathways are differentially regulated by H-ras and N-ras. Shown in Fig. 2 is the activation of MAPK family members in the parental, H-ras, and N-ras MCF10A cells. Whereas JNK-1,2 were activated neither by H-ras nor by N-ras, ERK-1,2 were effectively activated by both H-ras and N-ras. Interestingly, prominent activation of p38 was shown only in H-ras-activated cells but not in N-ras MCF10A cells, suggesting the possible involvement of p38 in H-ras-induced invasiveness and/or motility.

H-ras-induced Invasion and Motility Are p38-dependent. To assess the functional significance of H-ras-activated p38 in invasion and motility, we asked if interfering with p38 activation by SB203580, a specific p38 inhibitor, diminishes H-ras-mediated *in vitro* invasiveness and motility. We first examined the levels of the active MAPK family members in H-ras MCF10A cells treated with 50 μM SB203580 for 30 min to ensure the selective inhibition of p38 by this compound. As shown in Fig. 3*A,* SB203580 specifically inhibited the activation of p38 with little effects on the phosphorylated forms of JNK-1 or ERK-1,2. To evaluate whether p38 is critical for H-ras-induced invasion, we examined the effect of SB203580 on the invasive phenotype of H-ras MCF10A cells by performing *in vitro* invasion assay. Treatment of 50 μM SB203580 significantly reduced the number of invaded cells by ~43% (Fig. 3*B*). Next we examined the role of p38 in the migratory property of H-ras MCF10A cells. Width of injury line was measured and plotted as percentage of control for quantification of the inhibitory effect of SB203580 on cell migration. H-ras MCF10A cell migration was significantly inhibited (0% *versus*

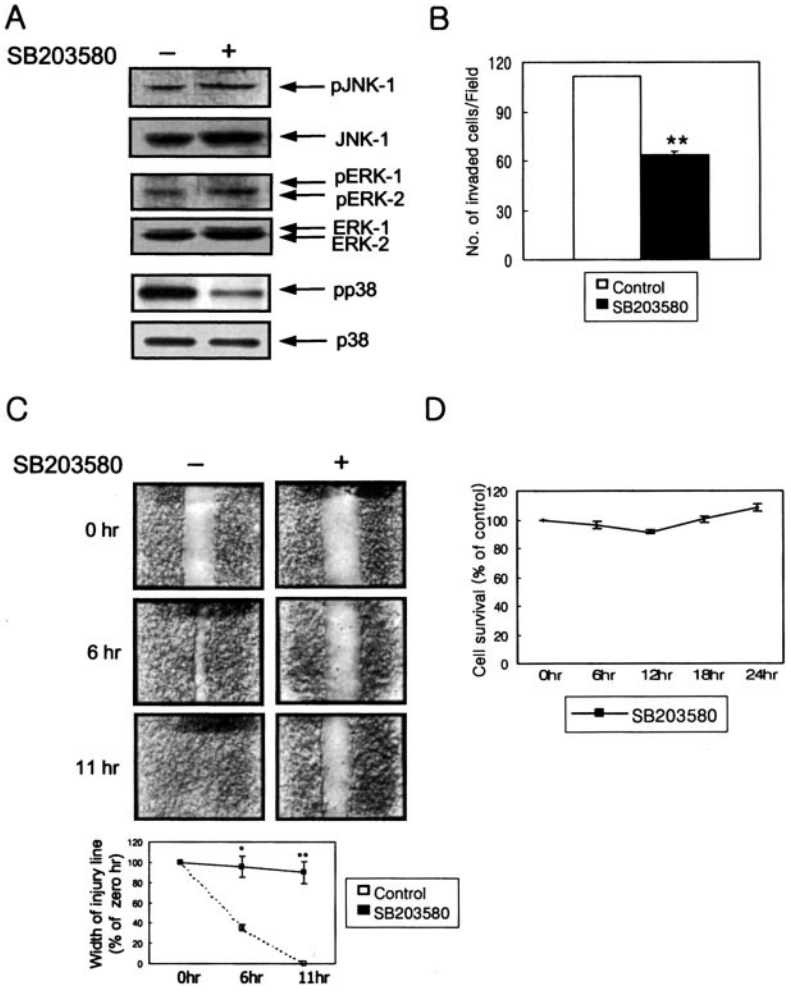

Fig. 3. SB203580 inhibits H-ras-induced invasion and motility. *A*, whole cell lysates prepared from the H-ras MCF10A cells with or without 50 μM SB203580 treatment for 30 min were analyzed for expression of phosphorylated and total forms MAPKs by immunoblot analysis. *B*, H-ras MCF10A cells pretreated with 50 μM SB203580 for 30 min were subjected to *in vitro* invasion assay for 17 h in the presence of the compound. The number of invaded cells per field was counted (×400) in 13 fields. The results represent means of triplicates; *bars,* ±SE. **, statistically different from control at $P < 0.01$. *C*, H-ras MCF10A cells were preincubated with 25 μg/ml mitomycin C and 50 μM SB203580 for 30 min. Injury line was made on the confluent monolayer of H-ras MCF10A cells, and the cells were incubated in complete medium containing 50 μM SB203580. Cellular migration was observed with light microscope (×40) at indicated time points. Width of injury line from three independent experiments was measured and plotted. The results presented were means of triplicates; *bars,* ±SE. * and **, statistically different from control at $P < 0.05$ and at $P < 0.01$, respectively. *D*, H-ras MCF10A cells (1×10^5) in a 48-well plate were treated with 50 μM SB203580. Cell survival was determined by MTT assay. The results presented were means of triplicates; *bars,* ±SE.

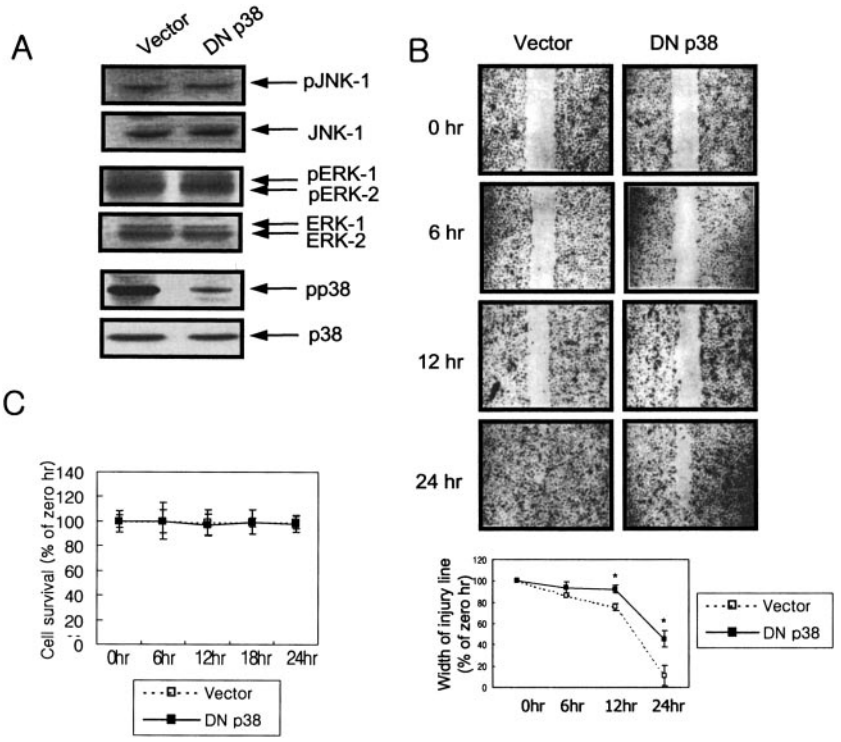

Fig. 4. Expression of a DN p38 mutant inhibits H-ras-induced invasion and motility. *A*, H-ras MCF10A cells were transiently transfected with either the control vector or DN p38 construct. Whole cell lysates were analyzed for expression of phosphorylated and total forms MAPKs by immunoblot analysis. *B*, migratory abilities of the H-ras MCF10A cell transfectants were determined by wound migration assay. Width of injury line from three independent experiments was measured and plotted. The results presented were means of triplicates; *bars,* ±SE. ∗, statistically different from control at $P < 0.05$. *C*, cell survival of H-ras MCF10A cell transfectants (1×10^5) was determined by a trypan blue exclusion assay. Number of viable cells was counted for indicated time points. The results presented were means of triplicates; *bars,* ±SE.

~80%) in the presence of 50 μM SB203580 after 11 h as shown by wound migration assay (Fig. 3*C*).

Because *in vitro* invasiveness and motility depend on the number of viable cells, we tested the viability of the SB203580-treated cells to exclude the possibility that the observed inhibition of H-ras-induced invasion/motility was because of a cytotoxic effect. Survival of the treated cells for up to 24 h was comparable with that of cells at 0 h as determined by MTT assay (Fig. 3*D*), indicating that the inhibition of invasion and motility was not because of a cytotoxic effect of SB203580. These findings indicate a critical role for p38 in H-ras-induced invasive phenotype and migration in human breast epithelial cells.

To additionally investigate the role for p38 in cellular invasion and migration, we transfected H-ras MCF10A cells with DN p38 in which the dual phosphorylated motif, Thr-Gly-Tyr, was mutated to Ala-Gly-Phe (28). Specific inactivation of p38 in DN p38-transfected cells was confirmed by immunoblot analysis (Fig. 4*A*). Phosphorylation of p38 was inhibited prominently in DN p38 transfectants, whereas phosphorylated levels of JNK-1 or ERK-1,2 were not altered by DN p38 transfection. To additionally test a notion that the p38 pathway plays a critical role for H-ras-mediated cell migration, we performed wound migration assay using DN p38 transfectants. Cell migration was clearly reduced in DN p38 transfectants as shown in Fig. 4*B*. The migration end point was 24 h instead of 11 h (as in Fig. 1*A* and Fig. 3*C*), because the transfected cells migrated slower than nontransfectants. Invasive phenotype was also inhibited by transfection with DN p38 construct (data not shown). As shown in Fig. 4*C,* DN p38 transfection did not lead to a significant reduction in viability, showing that DN p38-mediated inhibition of H-ras MCF10A cell invasion/motility was not because of a cytotoxic effect. The results demonstrate that activation of p38 pathway by H-ras is critical for ras-induced breast epithelial cell migration and invasion.

Activation of ERK-1,2 Is Not Sufficient but Required for H-ras-induced Invasion and Motility. Because studies have shown that the MEK/ERK pathway is critical for cell motility in some cell types (16), we asked if ERK-1,2 also play a role in ras-induced human breast epithelial cell invasion and/or migration. When the H-ras MCF10A cells were treated with 25 μM PD98059 for 30 min, activation of ERK-1,2 was markedly inhibited without significant changes in the phosphorylation of other MAPKs (Fig. 5*A*). Treatment of PD98059 resulted in inhibition of both invasiveness (Fig. 5*B*) and cell migration (Fig. 5*C*) without affecting cell survival (Fig. 5*D*).

We additionally investigated the functional role of the ERK pathway using a DN MEK-1 construct in which both of the phosphorylated motifs, Ser (218) and Ser (222), are mutated to Ala (29). As shown in Fig. 6*A,* ERK-1,2 were specifically inactivated in DN MEK-1 transfectants, whereas the levels of phosphorylated JNK-1 and p38 were not affected. Selective inhibition of the ERK pathway by DN MEK-1 transfection significantly inhibited invasive phenotype (Fig. 6*B*) and motility (Fig. 6*C*), demonstrating the significance of the ERK pathway in H-ras-induced invasion and migration in MCF10A cells. Given that ERK-1,2 were activated not only in H-ras MCF10A cells but also in noninvasive/nonmigrative N-ras MCF10A cells (Fig. 2), these results suggest that the activation of ERK-1,2 is not sufficient but required for H-ras-induced invasion and motility.

H-ras-induced Invasion and Motility Are Independent of JNK-1 Activation. We then examined the role for JNK-1 in H-ras-induced MCF10A cell motility and invasive phenotype using a DN mutant of JNK-1 where Thr (183) and Tyr (185) were mutated to Ala and Phe, respectively (30). Introduction of a DN JNK-1 mutant into H-ras MCF10A cells effectively reduced the phosphorylated JNK-1 level in H-ras MCF10A cells without affecting activation of ERK-1,2 or p38 (Fig. 7*A*). Interfering with JNK-1 activation by DN JNK-1 transfection did not affect H-ras MCF10A cell invasiveness (data not shown) and migration (Fig. 7*B*), indicating that the H-ras-induced invasion and motility are not dependent on JNK-1 pathway.

H-ras Up-Regulation of MMP-2 Involves p38 and ERK. Invasive phenotype of cancer cells is often associated with increased expression of MMP-2 and/or MMP-9, which can degrade type IV collagen, the major structural collagen of the basement membrane

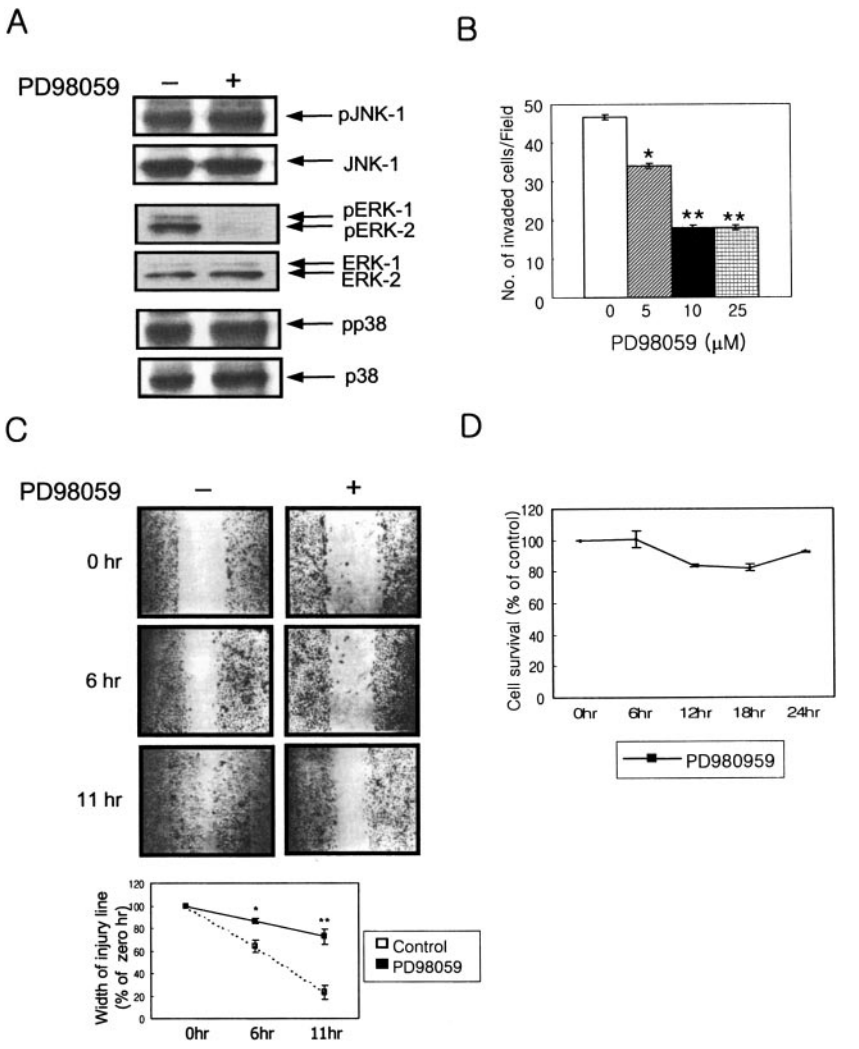

Fig. 5. PD98059 inhibits H-ras-induced invasion and motility. *A*, immunoblot analysis was performed to determine the effect of PD98059 on the activation of MAPKs. Whole cell lysates prepared from the H-ras MCF10A cells with or without 25 μM PD98059 treatment for 30 min were analyzed by immunoblot analysis. *B*, H-ras MCF10A cells pretreated with PD98059 at various concentrations for 30 min were subjected to *in vitro* invasion assay for 17 h in the presence of PD98059. The number of invaded cells per field was counted (×400) in 13 fields. The results presented were means of triplicates; *bars,* ±SE. * and **, statistically different from control at $P < 0.05$ and at $P < 0.01$, respectively. Cell migration (*C*) and survival (*D*) were determined as described in the legend for Fig. 3, *C* and *D,* respectively, except that the cells were treated with 25 μM PD98059 instead of SB203580. * and **, statistically different from control at $P < 0.05$ and at $P < 0.01$, respectively.

(32). We showed previously that the H-ras-induced invasive phenotype is associated more closely with the levels of MMP-2 expression than the MMP-9 level in human breast epithelial cells (7). Because the present study showed that ERKs and especially p38 were critical for H-ras-mediated invasion, we asked whether p38 and/or ERKs mediate H-ras induction of MMP-2 and/or MMP-9 expression. When p38 activation is inhibited with 50 μM SB203580, the level of MMP-2, but not MMP-9, was decreased by ~80% (Fig. 8*A*). Interestingly, the MMP-9 expression levels were slightly increased after the treatment with low concentrations of SB203580 (1–10 μM). When ERK activation was inhibited by 25 μM PD98059, the MMP-9 and MMP-2 expression levels were decreased by ~80% and ~40%, respectively (Fig. 8*B*). Of note, whereas PD98059 is a specific inhibitor of ERKs at lower concentrations (up to 25 μM), it also inhibited other signaling molecules such as JNK and p38 at 50 μM concentration (data not shown). These results suggest that activation of p38 and, to a lesser degree ERKs, is required for enhanced MMP-2 expression. However, ERK activation is more critical than p38 activation for MMP-9 induction. Taken together, we propose that H-ras activation of both p38 and ERK induces MMP-2 and MMP-9 expression, resulting in phenotypic conversion of noninvasive MCF10A cells to an invasive phenotype (Fig. 8*C*). In contrast, N-ras-mediated ERK activation alone leading to MMP-9 induction is not sufficient for invasive phenotype of MCF10A cells (7).

DISCUSSION

Mutations or overexpression of the *ras* oncogene are among the most frequent genetic alterations in human tumors. One member of the *ras* family appears to be the preferred "target gene" in particular tumors. The single point mutation at amino acid residue 12 (gly to asp) of H-ras is more often found in mammary carcinoma, whereas the same mutation of N-ras is detected in teratocarcinoma and leukemia (33). The present study revealed differential regulation of ras signaling pathways by H-ras and N-ras with a single mutation at the codon 12, providing an insight into the prospect of developing targeted therapy for mammary carcinoma.

The present study demonstrated that constitutive activation of the H-ras pathway, but not the N-ras pathway, was sufficient to convert the nonmigrative, noninvasive phenotype of MCF10A breast epithelial cells to a motile and invasive phenotype. Although its molecular action is unclear at present, only H-ras, but not N-ras, effectively activated the p38 pathway in MCF10A cells, whereas both activated the ERK pathway. N-ras activation of the ERKs was insufficient to induce a migrative and invasive phenotype (7). However, ERKs activation was required for H-ras-mediated cell migration and invasive phenotype in MCF10A cells. Unlike p38 and ERKs, involvement of JNK in cell invasiveness or migration has not been reported extensively. JNK-dependent signaling molecules were shown recently to regulate invasive behavior and MMP-9 production of human glio-

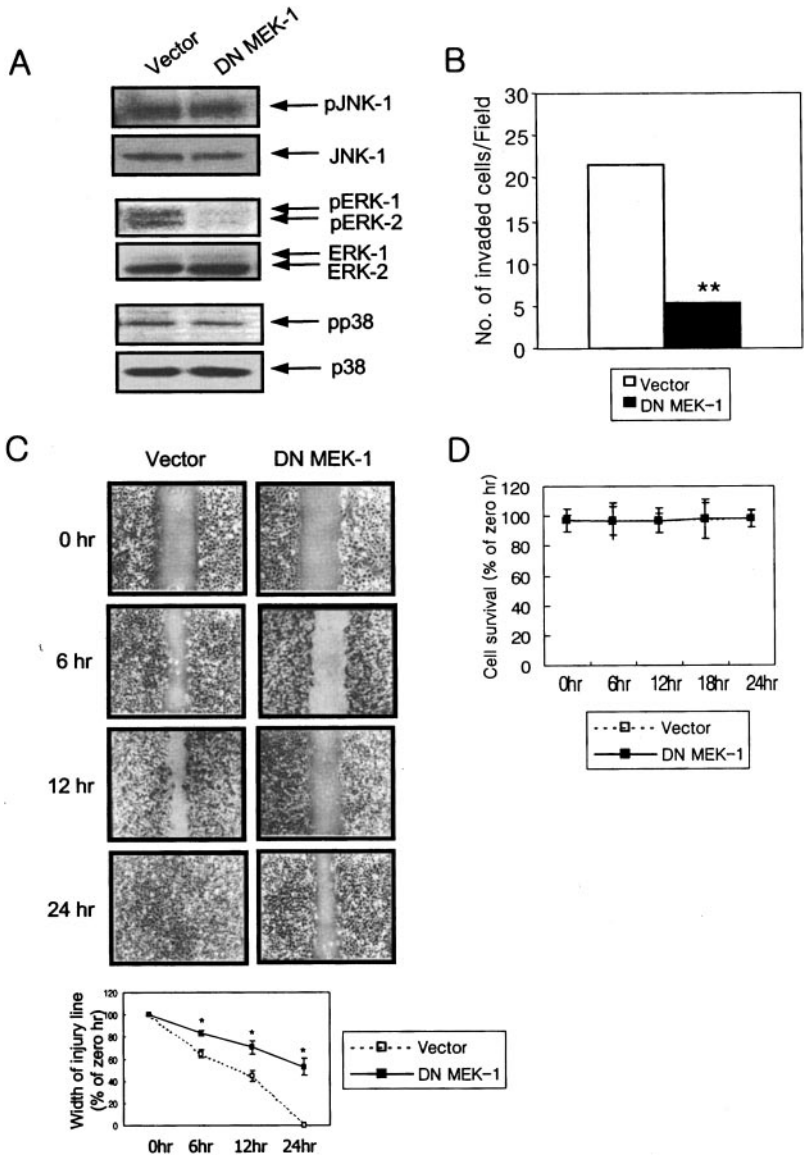

Fig. 6. DN MEK-1 inhibits H-ras-induced invasion and motility. *A*, H-ras MCF10A cells were transiently transfected with either the control vector or DN MEK-1 construct. Whole cell lysates were analyzed for the levels of phosphorylated and total forms MAPKs by immunoblot analysis. *B*, transfected cells were subjected to *in vitro* invasion assay for 17 h as described in "Materials and Methods." The number of invaded cells per field was counted (×400) in 13 fields. The results presented were means of triplicates; *bars,* ±SE. **, statistically different from control at $P < 0.01$. Cell migration (*C*) and survival (*D*) of H-ras MCF10A cells transfected with control vector or DN MEK1 construct were determined as described in the legend for Fig. 4, *B* and *C,* respectively.

blastoma cells (17). However, JNK-1 played no significant role in ras-mediated migrative and invasive behavior of H-ras MCF10A cells. Consistently, inhibition of JNK pathway in ras-transformed NIH3T3 cells had no effect on the ability to invade Matrigel (34).

Previous studies have shown that p38 lies downstream of the Ras-related GTP-binding proteins Rac and Cdc42, and is directly activated by MKK3, MKK6, and MKK4 (35–38). The relationship between the two MAPK pathways, p38 and ERK, has been elucidated recently in Ras-induced cellular responses. The existence of a Ras/p38 pathway independent of the classical Ras/ERK pathway has been suggested in platelet-derived growth factor-induced cell motility (15). However, Chen *et al.* (39) have reported that Ras-activation of MEK/ERK is a prerequisite for p38 pathway, suggesting ERK as an upstream regulator of p38. In MCF10A cells, however, the DN MEK-1 mutant prevented ERK activation without any effect on p38 activation (Fig. 6*A*), ruling out the possibility that ERK is an upstream regulator of the p38 pathway. It appears that the p38-mediated signaling pathway is independent of the ERK-mediated pathway, and both pathways cooperate in H-ras-mediated migrative and invasive responses in MCF10A cells.

An essential part of invasion and metastasis includes degradation of the basement membrane and the stromal ECM by members of the MMP family (32). Our previous study suggested the involvement of MMP-2 in H-ras-induced invasive phenotype of MCF10A cells (7). The present study suggests that activation of p38 may contribute to a more invasive phenotype *via* the secretion of MMP-2 in H-ras MCF10A cells. Consistently, a recent report (40) shows that the inhibition of p38 by SB203580 results in MMP-2 down-regulation in malignant melanoma cells. In contrast, ERK and p38 signaling pathways had little effect on MMP-2 expression (41) or were shown to inhibit MMP-2 expression (42) in different cell systems. Deletion and site-directed mutagenesis analyses of the MMP-2 promoter demonstrated that the transcription factors SP1, Sp3, and AP-2, rather than AP-1, were functionally important for MMP-2 gene expression in astroglioma cells (43). It would be of interest to determine the p38-responsive promoter sequences of the MMP-2 gene. Additional studies to delineate MMP-2 regulation by the p38 pathway remain to be performed.

We showed previously that N-ras induced MMP-9 expression more effectively than H-ras (7). Interestingly, MMP-9 expression is enhanced on down-regulation of the p38 pathway by low concentrations of SB203580 in H-ras-activated MCF10A cells (Fig. 8*A*). This is

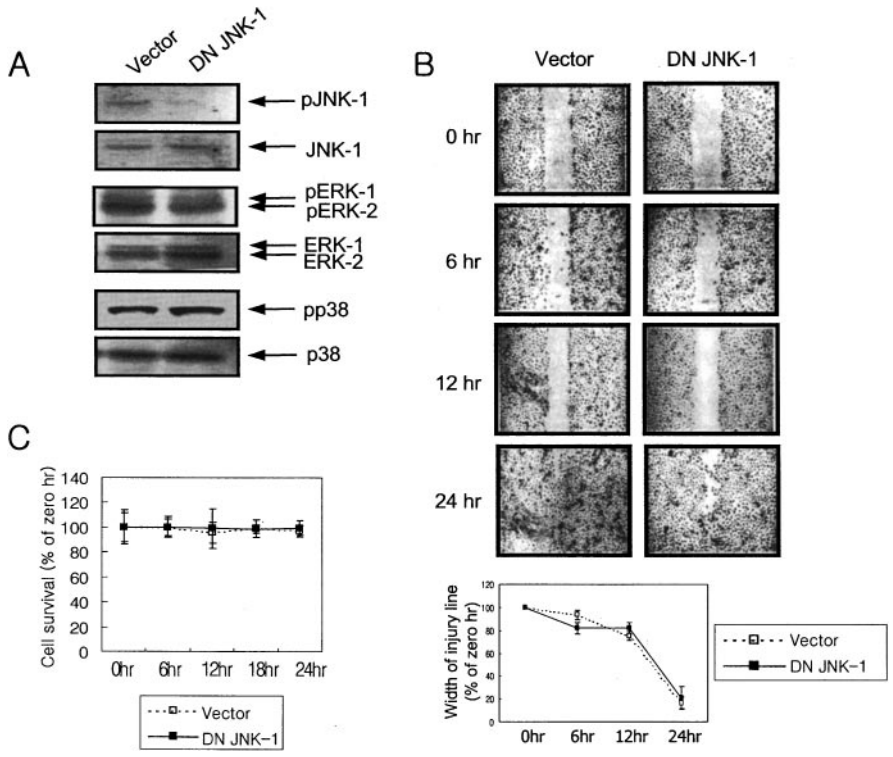

Fig. 7. Expression of a DN JNK-1 mutant does not affect H-ras-induced invasion and motility. H-ras MCF10A cells were transiently transfected with either the control vector or DN JNK-1 construct. Immunoblot analysis (*A*), wound migration assay (*B*), and survival assay (*C*) were performed using control vector and DN-JNK-1-transfected H-ras MCF10A cells as described in the legend for Fig. 4.

consistent with the observation that N-ras activation of ERK without p38 activation induces MMP-9 expression more effectively than H-ras activation of both ERKs and p38. N-ras-mediated ERK activation and MMP-9 induction were insufficient for induction of invasive phenotype (7). Consistently, when MMP-2 expression was down-regulated by inhibition of the p38 pathway, MMP-9 expression alone was insufficient for induction of cell motility and invasive phenotype of H-ras MCF10A cells (Figs. 3 and 8). However, inhibition of H-ras MCF10A cell invasion was more drastic when both MMP-2 and MMP-9 were down-regulated on inhibition of the ERK pathway by 25 μM PD98059 (~60% loss, Fig. 5*B*; Fig. 8*B*) compared with the inhibition of MCF10A cell invasiveness when MMP-2 expression alone was inhibited using antisense MMP-2 oligodeoxyribonucleotide (~30% loss; Ref. 7). Thus, for completion of H-ras-induced invasion of human breast epithelial cells, not only MMP-2 but also other gene products such as MMP-9 may be required, although MMP-9 alone is not sufficient.

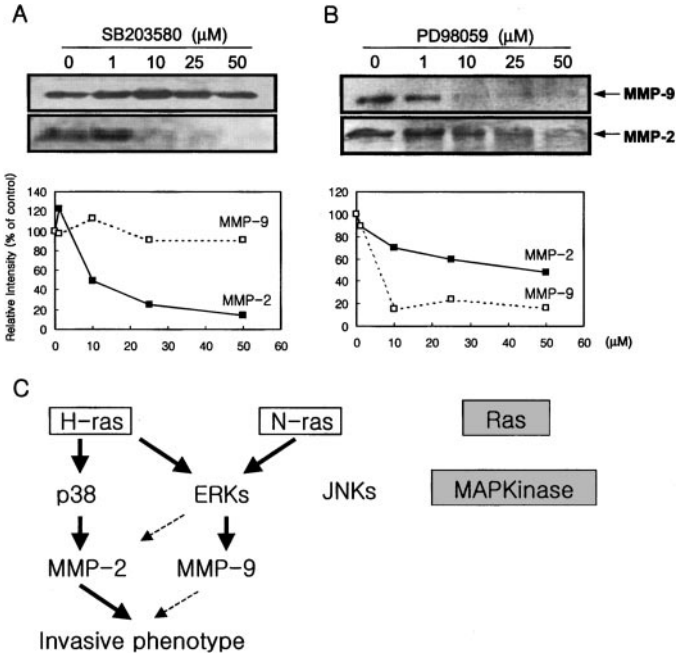

Fig. 8. SB203580 effectively inhibits MMP-2 expression. *A* and *B*, H-ras MCF10A cells were treated with various concentrations of SB203580 (*A*) or PD98059 (*B*) for 48 h. Levels of secreted MMP-2 (M_r 72,000) and MMP-9 (M_r 92,000) were determined by immunoblot analysis. Relative levels of MMP-2 and MMP-9 were quantitated and plotted (*bottom panel*). *C*, a working model for regulation of invasive phenotype by Ras signaling molecules in MCF10A cells. Strong and weak activations were represented as *bold* and *dotted arrows,* respectively.

Many studies have identified the PI3K as a key regulator for cell growth and survival. Involvement of PI3K activity in other aspects of tumor progression including invasion and metastasis has been suggested recently (44). We are currently investigating the functional role of PI3K pathway in H-ras-induced invasive phenotype and motility of MCF10A cells.

In this report, we show that p38 and ERK signaling can regulate H-ras MCF10A cell migration and invasion, suggesting overlapping signaling pathways for regulation of cell motility and ECM degrading activities. Our studies establish an experimental system in which *ras*-mediated overlapping and unique signaling pathways for cell motility and ECM-degrading activities can be additionally analyzed at the molecular levels.

ACKNOWLEDGMENTS

We thank Dr. Xuwen Liu (Department of Pathology, Wayne State University) for helping with preparation of this article.

REFERENCES

1. Fidler, I. J. Critical factors in the biology of human cancer metastasis. Cancer Res., *50:* 6130–6138, 1990.
2. Clair, T., Miller, W., and Cho-Chung, Y. Prognostic significance of the expression of the ras protein with a molecular weight of 21, 000 by human breast cancer. Cancer Res., *49:* 5290–5293, 1987.

3. Clark, G. J., and Der, C. J. Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res. Treat., *35:* 133–144, 1995.
4. Watson, D. M., Elton, R. A., Jack, W. J., Dixon, J. M., Chetty, U., and Miller, W. R. The H-ras oncogene product p21 and prognosis in human breast cancer. Breast Cancer Res. Treat., *17:* 161–169, 1991.
5. Barbacid, M. ras genes. Annu. Rev. Biochem., *56:* 779–827, 1987.
6. Boguski, M. S., and McCormick, F. Proteins regulating Ras and its relatives. Nature (Lond.), *663:* 643–654, 1993.
7. Moon, A., Kim, M. S., Kim, T. G., Kim, S. H., Kim, H. E., Chen, Y. Q., and Kim, H. R. H-ras, but not N-ras, induces an invasive phenotype in human breast epithelial cells: a role for MMP-2 in the H-ras-induced invasive phenotype. Int. J. Cancer, *85:* 176–181, 2000.
8. Davis, R. J. Transcriptional regulation by MAP kinases. Mol. Reprod. Dev., *42:* 459–467, 1995.
9. Seger, R., and Krebs, E. G. The MAPK signaling cascade. FASEB J., *9:* 726–735, 1995.
10. Brunet, A., and Pouyssegur, J. Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science (Wash. DC), *272:* 1652–1655, 1996.
11. Foltz, I. N., Lee, J. C., Young, P. R., and Schrader, J. W. Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. J. Biol. Chem., *272:* 3296–3301, 1997.
12. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science (Wash. DC), *275:* 90–94, 1997.
13. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science (Wash. DC), *270:* 1326–1331, 1995.
14. Rousseau, S., Houle, F., Landry, J., and Han, J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene, *15:* 2169–2177, 1997.
15. Matsumoto, T., Yokote, K., Tamura, K., Takemoto, M., Ueno, H., Saito, Y., and Mori, S. Platelet-derived growth factor activates p38 mitogen-activated protein kinase through a Ras-dependent pathway that is important for actin reorganization and cell migration. J. Biol. Chem., *274:* 13954–13960, 1999.
16. Hedges, J. C., Dechert, M. A., Yamboliev, I. A., Martin, J. L., Hickey, E., Weber, L. A., and Gerthoffer, W. T. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. J. Biol. Chem., *274:* 24211–24219, 1999.
17. Lakka, S. S., Jasti, S. L., Kyritsis, A. P., Yung, W. K., Ali-Osman, F., Nicolson, G. L., and Rao, J. S. Regulation of MMP-9 (type IV collagenase) production and invasiveness in gliomas by the extracellular signal-regulated kinase and jun amino-terminal kinase signaling cascades. Clin. Exp. Metastasis, *18:* 245–252, 2000.
18. Graf, K., Xi, X. P., Yang, D., Fleck, E., Hsueh, W. A., and Law, R. E. Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension, *29:* 334–339, 1997.
19. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., Lanerolle, P., and Cheresh, D. A. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol., *137:* 481–492, 1997.
20. Tryggvason, K. Type IV collagenase in invasive tumors. Breast Cancer Res., *24:* 209–218, 1993.
21. Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T., and Liotta, L. A. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem., *265:* 13933–13938, 1990.
22. Bernhard, E. J., Muschel, R. J., and Hughes, E. N. Mr 92, 000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells. Cancer Res., *50:* 3872–3877, 1990.
23. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. Direct evidence linking expression of matrix metalloproteinase 9 (92 kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc. Natl. Acad. Sci. USA, *91:* 4293–4297, 1994.
24. Simon, C., Juarez, J., Nicolson, G. L., and Boyd, D. Effect of PD 098059, a specific inhibitor of mitogen-activated protein kinase kinase, on urokinase expression and *in vitro* invasion. Cancer Res., *56:* 5369–5374, 1996.
25. Gum, R., Wang, H., Lengyel, E., Juarez, J., and Boyd, D. Regulation of 92 kDa type IV collagenase expression by the jun aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. Oncogene, *14:* 1481–1493, 1997.
26. Simon, C., Goepfert, H., and Boyd, D. Inhibition of the p38 mitogen-activated protein kinase by SB 203580 blocks PMA-induced Mr 92, 000 type IV collagenase secretion and *in vitro* invasion. Cancer Res., *58:* 1135–1139, 1998.
27. Simon, C., Simon, M., Vucelic, G., Hicks, M. J., Plinkert, P. K., Koitschev, A., and Zenner, H. P. The p38 SAPK pathway regulates the expression of the MMP-9 collagenase via AP-1-dependent promoter activation. Exp. Cell Res., *271:* 344–355, 2001.
28. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem., *270:* 7420–7426, 1995.
29. Sugimoto, T., Stewart, S., Han, M., and Guan, K. L. The kinase supressor of Ras (KSR) modulates growth factor and Ras signaling by uncoupling Elk-1 phosphorylation from MAPkinase activation. EMBO J., *17:* 1717–1727, 1998.
30. Gupta, S., Campbell, D. D., Derijard, B., and Davis, P. J. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science, *267:* 389–393, 1995.
31. Jozaki, K., Marucha, P. T., Despins, A. W., and Kreutzer, D. L. An *in vitro* model of cell migration: evaluation of vascular endothelial cell migration. Anal. Biochem., *190:* 39–47, 1990.
32. Ura, H., Bonfil, R. D., Reich, R., Reddel, R., Pfeifer, A., Harris, C. C., and Klein-Szanto, A. J. P. Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells. Cancer Res., *49:* 4615–4621, 1989.
33. Franks, L. M., and Teich, N. M. Cellular and Molecular Biology of Cancer. Oxford University Press, 1997.
34. Janulis, M., Silberman, S., Ambegaokar, A., Gutkind, S., and Schultz, R. M. Role of mitogen-activated protein kinases and c-Jun/AP-1 *trans*-activating activity in the regulation of protease mRNAs and the malignant phenotype in NIH 3T3 fibroblasts. J. Biol. Chem., *274:* 801–813, 1999.
35. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol., *16:* 1247–1255, 1996.
36. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem., *270:* 27995–27998, 1995.
37. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science (Wash. DC), *267:* 682–685, 1995.
38. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem., *270:* 23934–23936, 1995.
39. Chen, G., Masahiro, H., Jiahuai, H., and Dennis, W. S. The p38 pathway provides negative feedback for Ras proliferative signaling. J. Biol. Chem., *275:* 38973–38980, 2000.
40. Denkert, C., Siegert, A., Leclere, A., Turzynski, A., and Hauptmann, S. An inhibitor of stress-activated MAP-kinases reduces invasion and MMP-2 expression of malignant melanoma cells. Clin. Exp. Metastasis, *19:* 79–85, 2002.
41. Smolian, H., Aurer, A., Sittinger, M., Zacher, J., Bernimolin, J. P., Burmester, G. R., and Kolkenbrock, H. Secretion of gelatinases and activation of gelatinase A (MMP-2) by human rheumatoid synovial fibroblasts. Biol. Chem., *382:* 1491–1499, 2001.
42. Esparza, J., Vilardell, C., Calvo, J., Juan, M., Vives, J., Urbano-Marquez, A., Yague, J., and Cid, M. C. Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways. Blood, *94:* 2754–2766, 1999.
43. Qin, H., Sun, Y., and Benveniste, E. N. The transcription factors Sp1, Sp3, and AP-2 are required for constitutive matrix metalloproteinase-2 gene expression in astroglioma cells. J. Biol. Chem., *274:* 29130–29137, 1999.
44. Ellerbroek, S. M., Halbleib, J. M., Benavidez, M., Warmka, J. K., Wattenberg, E. V., Stack, S., and Hudson, L. G. Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association. Cancer Res., *61:* 1855–1861, 2001.