p38 Mitogen-Activated Protein Kinase Pathway Is Involved in Protein Kinase Cα–Regulated Invasion in Human Hepatocellular Carcinoma Cells

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Abstract

Protein kinase Cα (PKCα) has been suggested to play an important role in tumorigenesis, invasion, and metastasis. In this study, we investigated the signal pathways selectively activated by PKCα in human hepatocellular carcinoma (HCC) cells to determine the role of mitogen-activated protein kinases (MAPK) in PKCα-mediated HCC migration and invasion. A stable SK-Hep-1 cell clone (siPKCα-SK) expressing DNA-based small interfering RNA (siRNA) PKCα was established and was then characterized by cell growth, migration, and invasion. The expression of PKCα was decreased in siPKCα-SK, and cell growth, migration, and invasion were reduced. These changes were associated with the decrease in p38 MAPK phosphorylation level, but not in c-jun-NH_2-kinase-1/2 (JNK-1/2) and extracellular signal-regulated kinase-1/2 (ERK-1/2). This phenomenon was confirmed in the SK-Hep-1 cells treated with antisense PKCα oligonucleotide. The p38 MAPK inhibitor SB203580 or dominant negative p38 mutant plasmid (DN-p38) was used to evaluate the dependency of p38 MAPK in PKCα-regulated migration and invasion. Attenuation of cell migration and invasion was revealed in the SK-Hep-1 cells treated with the SB203580 or DN-p38, but not with ERK-1/2 inhibitor PD98059 or JNK-1/2 inhibitor SP600125. Overexpression of constitutively active MKK6 or PKCα may restore the inactivation of p38 and the attenuation of cell migration and invasion in siPKCα-SK. Similar findings were observed in the stable HA22T/VGH cell clone expressing siRNA PKCα. This study provides new insight into the role of p38 MAPK in PKCα-mediated malignant phenotypes, especially in PKCα-mediated cancer cell invasion, which may have valuable implications for developing new therapies for some PKCα-overexpressing cancers. [Cancer Res 2007;67(9):4320–7]

Introduction

The α-isoform of protein kinase C (PKCα) is widely expressed in tissues regulating apoptosis, proliferation, differentiation, migration, and adhesion (1). However, this isoform has been suggested to play an important role in tumorigenesis, invasion, and metastasis (2–9). Thus, inhibition of general PKCα activities may be detrimental to cancer cells. Rational therapies targeting PKCα downstream signaling molecules essential to malignant cancer cell behavior but less critical to normal cell functions should have a potential impact.

The mitogen-activated protein kinase (MAPK) pathway is relevant to human carcinogenesis (10–12). There are three subfamilies of mammalian MAPKs: extracellular signal-regulated kinases (ERK), Jun NH_2-terminal kinases (JNK), and p38 kinases. They are major players in mediating a variety of signals for cellular functions (13). In addition, activation of ERK has been observed in a number of tumors (14). Although activation of c-jun-NH_2-kinase and p38 has been found in prostate carcinoma (15) and breast cancinoma (16), deactivation of these kinases has been reported in colon cancer (17, 18). Moreover, high levels of p38 have also been observed in thyroid neoplasms (19). PKCα is known to associate with the activation of the MAPK family (20). However, the role of MAPKs in the malignant behavior of PKCα-overexpressing cancer cells has not been clearly identified.

In addition to participating in cell transformation and tumorigenesis, p38 MAPK activation has an association with the invasive or metastatic potential in some human cancer cells (21, 22). PKCα has been reported to activate p38 MAPK and to induce the expression of tumor necrosis factor α in lipopolysaccharide-stimulated microglia (23). These changes lead to the assumption that PKCα also causes its activation in PKCα-overexpressing cancer cells. In our previous study, PKCα has been found to be highly expressed in the poorly differentiated human hepatocellular carcinoma (HCC) cell lines (SK-Hep-1 and HA22T/VGH) as compared with the well-differentiated ones (PLC/PRF/5, Hep3B, and HepG2; ref. 24). To determine the role of MAPKs in PKCα-mediated HCC migration and invasion, we investigated the signal pathways selectively activated by PKCα in the SK-Hep-1 and HA22T/VGH cells. We found that the DNA-based small interfering RNA (siRNA) not only decreases PKCα expression but also attenuates cell migration and invasion in these cell lines through the suppression of p38 MAPK signaling pathway. The p38 MAPK inhibitor SB203580 and dominant negative p38 mutant plasmid (DN-p38) reveals the inactivation of p38 and the attenuation of cell migration and invasion in the SK-Hep-1 and HA22T/VGH cells. Moreover, overexpression of constitutively active MKK6 or PKCα may restore the inactivation of p38 and the attenuation of cell migration and invasion in the stable siRNA-PKCα–transfected clones.

Materials and Methods

Materials. Anti-PKCα, β, δ, ε, ζ, η, and ι were purchased from BD Biosciences. Anti-p44/p42 MAPK, anti-p44/p42 MAPK (Thr^{202}/Tyr^{204}),

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anti-p38 MAPK, anti–phospho-p38 MAPK (Thr^{180}/Tyr^{182}), anti-JNK, and anti–phospho-JNK (Thr^{183}/Tyr^{185}) polyclonal antibodies were purchased from Cell Signaling, anti-flag from Sigma, and α-tubulin polyclonal antibodies from Santa Cruz Biotechnology, Inc. Horseradish peroxidase (HRP)–labeled anti-mouse and anti-rabbit secondary antibody was obtained from Promega. Antisense PKCα oligonucleotide was provided by MDBio, Inc. The p38 MAPK inhibitor SB203580, ERK-1/2 inhibitor PD98059, and JNK-1/2 inhibitor SP600125 were obtained from Calbiochem. Flag-tagged dominant negative p38 mutant plasmid was kindly donated by Dr. M.L. Kuo (Institute of Toxicology, College of Medicine, Taiwan National University, Taipei, Taiwan), flag-tagged constitutively active MKK3, MKK4, and MKK6 were donated by Dr. J.J. Yang (School of Dentistry, Chung Shan Medical University, Taichung, Taiwan), and the constitutive PKCα mutant plasmid, encoding only the catalytic domain of PKCα, was donated by Dr. W.J. Wu (Institute of Toxicology, College of Medicine, Chung Shan Medical University, Taichung, Taiwan).

Cell culture. The human SK-Hep-1 (American Type Culture Collection) and HA22T/VGH (Food Industry Research and Development Institute, Hsinchu, Taiwan) cells were cultured in DMEM supplemented with 10% FCS (Hyclone), 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL) and grown at 37°C in 5% CO_2.

siRNA-PKCα plasmid construction. We constructed the siPKCα-expressing plasmid vector using the pcDNA-HU6 vector (denoted by Dr. J. Tsai Chang, Institute of Toxicology, College of Medicine, Chung Shan Medical University, Taichung, Taiwan) as the vector backbone according to Tsai Chang (25). The sequence of the 19-nucleotide siRNA-PKCα duplex from the human PKCα gene (GenBank accession no. NM_002737) corresponding to the coding regions 492 to 510 (Fig. 1*A, underline*) was designed using the BLOCK-iT RNAi Design.[5] The sequences designed to produce hairpin RNAs identical to the oligonucleotide siRNA duplex sequences are follows: sense: 5′-GATCCGC GTC CTG TTG TAT GAA ATT TCA AGA GAA-3 and antisense: 5′-AGCTTAAA AAG CGT CCT GTT GTA TGA AAT TCT CTT GAA-3. To generate siRNA duplex, sense and antisense oligonucleotides (40 μmol/L) were annealed by incubating the mixed oligonucleotides in the PCR thermocycler using the following profile: 37°C for 30 min and 65°C for 15 min. The completed siRNA duplex was then cloned into the pcDNA-HU6 vector in frame of the *Bam*HI and *Hin*dIII sites. The insert was screened by PCR with HU6 primer and confirmed by sequencing with HU6 primer.

Transient transfection. Transfections were done using lipofectin. Cells seeded at 60-mm dish were cultured in DMEM supplemented with 10% FCS at 37°C for 24 h. After incubation, the cells were rinsed with serum-free MEM before adding 1 mL MEM containing 15 μg/mL LipofectAMINE 2000 Transfection Reagent (Invitrogen) and 2 or 5 μg of the indicated plasmid. The cells were then incubated at 37°C for 6 h before adding 1 mL MEM supplemented with 20% FCS to the medium. After incubation at 37°C for 18 h, the medium was replaced with fresh 10% FCS-DMEM, and the cells were incubated at 37°C for 24 h. The cells were then lysed for Western blotting.

Western blotting. The cultured cells were washed twice with PBS and then lysed with a lysing buffer containing 50 mmol/L Tris-HCl (pH, 7.4), 2 mmol/L EDTA, 2 mmol/L EGTA, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40, and 0.3% sodium deoxycholate. The cell lysates were centrifuged at 100,000 × *g* and 4°C for 30 min. The supernatant was collected, and the protein concentration was determined by the Bradford method. Equal amounts of protein extracts (50 μg) were subjected to 12.5% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore). After blocking, the membrane was incubated with the specific anti-PKCα, β, δ, ε, ζ, η, or ι antibody (1:500), anti–ERK-1/2, anti-phosphorylated ERK-1/2, anti-p38, anti-phosphorylated p38, anti–JNK-1/2, anti-phosphorylated JNK-1/2 (1:500), anti-flag (1:4,000), or α-tubulin antibody (1:2,000). The blots were then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:3,000) at room temperature for 2 h. Proteins were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Gene transfection and establishment of siRNA-PKCα–transfected stable clones. Low-passage cells were seeded at a density of 3×10^5 cells in 60-mm tissue culture dishes and then transfected with siRNA-PKCα plasmid (5 μg) using the LipofectAMINE 2000 Transfection Reagent (Invitrogen). After transfecting for 5 h, the cells were washed thrice in serum-free MEM and allowed to recover for 24 h in fresh medium. Stable clones were selected by growing the cells at 1:10 to 1:15 (vol/vol) in DMEM medium supplemented with geneticin (G418; 600 μg/mL) at 37°C for 5 weeks. Individual clones were then transferred to 96-well plates and grown until confluent. After transferring to flasks, the cells were cultivated until confluent, harvested, and frozen in liquid nitrogen for further experiments.

Cell proliferation assay. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (26). The cells were seeded in 24-well plates at 1×10^4 cells per well and cultured in DMEM containing 10% serum at 37°C overnight. These cells were treated with and without various plasmids or p38 MAPK inhibitor and incubated for 24 or 48 h. After incubation, the medium was replaced with a fresh one, and the cells were incubated with 5 mg/mL MTT for 4 h before dissolving in 1 mL isopropanol for 10 min. The absorbance at 570 nm was then measured using a spectrophotometer. Cells at the log phase were used to calculate the doubling time according to the equation doubling time (h) = [log 2 × (24 × number of days)]/[log $density_{final}$ − log $density_{initial}$].

Migration assay. Migration assay was done using the 48-well Boyden chamber (Neuro Probe) plated with the 8-μm pore size polycarbonate membrane filters (Neuro Probe; ref. 27). The lower compartment was filled with DMEM containing 20% FCS. The HA22T/VGH and SK-Hep-1 cells were placed in the upper part of the Boyden chamber and incubated for 6 and 12 h, respectively. After incubation, the cells were fixed with methanol and stained with 0.05% Giemsa for 1 h. The cells on the upper surface of the filter were removed with a cotton swab. The filters were then rinsed in distilled water until no additional stain leached. The cells were then air-dried for 20 min. The migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at 200× magnification. The fourth fields were counted for each filter, and each sample was assayed in triplicate.

Invasion assay. Invasion assay was done using a 48-well Boyden chamber with polycarbonate filters. The upper side was precoated with 10 μg/mL Matrigel (Collaborative Biomedical Products). The HA22T/VGH and SK-Hep-1 cells were placed in the upper part of the Boyden chamber and incubated at 37°C for 12 and 24 h, respectively. The experimental procedures were the same as that in migration assay.

Antisense knock-out assay. The antisense knock-out assay was done according to Shen et al. (28), and the following PKCα antisense and sense (as a control) sequences were used: antisense 5′-GTT CTC GCT GGT GAG TTT CA-3′, sense 5′-GGT TTT ACC ATC GGT TCT GG-3′ (28). These sequences were formed for targeting the AUG region and had no more than four contiguous intrastrand base pairs or four contiguous G:C pairs. Before antisense oligonucleotide treatment, cells were plated at 70% density and 37°C for 24 h. The cells were then washed in triplicate with serum-free DMEM and incubated with 5 μmol/L sense or antisense oligonucleotide in serum-free DMEM containing 10 μg/mL lipofectin (Invitrogen) at 37°C. The medium was changed to 10% FCS-DMEM 6 h later before culturing at 37°C for 24 or 48 h.

Statistical analysis. Data were expressed as mean ± SE and analyzed by ANOVA. Student's *t* test was used in two-group comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Generation of stable siRNA-PKCα expressing SK-Hep-1 and HA22T/VGH clones. To identify an efficient RNA target sequence for generation of stable vector-based siRNA clones, two chemically synthesized siRNA constructs were designed based

[5] Available at http://www.invitrogen.com.

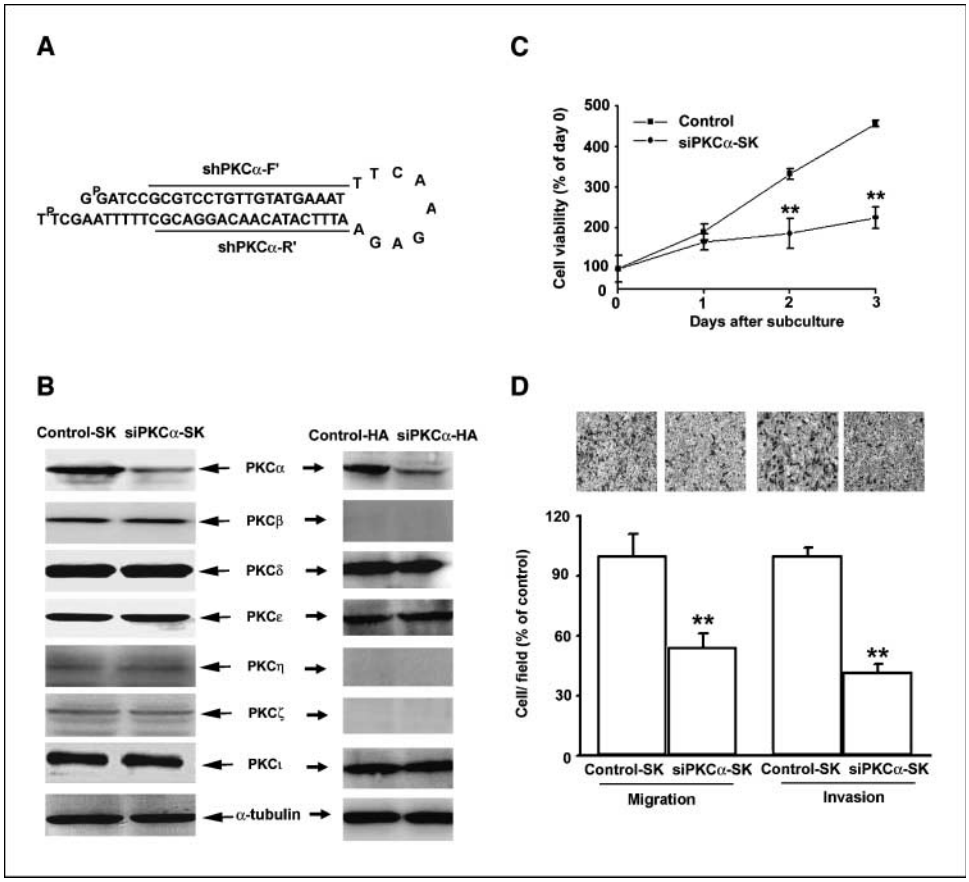

Figure 1. Silencing of PKCα expression in SK-Hep-1 (siPKCα-SK) and HA22T/VGH (siPKCα-HA) cells and inhibition of cell proliferation, migration and invasion. *A,* the sequence of 19-nucleotide siRNA duplex that were used to target on PKCα. *B,* Western blot analysis of PKC isoforms expression in cell lysates of stable siRNA-PKCα–transfected clones. Western blotting was done using antibodies specific for the PKC isoforms, and α-tubulin was simultaneously immunodetected to verify the loading of similar amounts of cell lysates. *C,* proliferation of stable siRNA-PKCα–transfected clones. Cells (2×10^4) were seeded in 24-well dishes with DMEM medium, and their viabilities were assessed by the MTT assay. *D,* migration and invasion assays on stable siRNA-PKCα–transfected clones. Cells (3×10^5) were seeded in the Boyden chamber for migration assay for 12 h or Matrigel invasion assay for 24 h. The cells were then stained, counted, and photographed. Percentages of migration and invasion were quantified as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SE. SK-Hep-1 (*Control-SK*) and HA22T/VGH (*Control-HA*) cells transfected with pcDNA-HU6 were used as controls themselves. **, $P < 0.01$ versus control-SK.

on the PKCα mRNA. These constructs were tested by transient transfection for their ability to down-regulate the expression levels of PKCα protein in SK-Hep-1 and HA22T/VGH cells. Figure 1*A* shows one representative siRNA-PKCα. Compared with the cells without transfection, this construct was found to be able to reduce the level of PKCα by 9% in 1-μg transient siRNA-PKCα–transfected SK-Hep-1 cells, 23% in 2-μg transient transfected cells, and 96% in 5-μg transient transfected cells (data not shown).

To establish stable siRNA-PKCα clones, SK-Hep-1 cells were transfected with the control pcDNA-HU6 or pcDNA-HU6-siRNA-PKCα plasmids. After geneticin selection, 1 (siPKCα-SK) of 12 clones was picked, spread, and collected. HA22T/VGH cells were also transfected with the same plasmids and generated a stable cone (siPKCα-HA). By Western blotting, the expression of PKCα in this siRNA transfectant was determined to be decreased by 82% and 78% in siPKCα-SK and siPKCα-HA, respectively (Fig. 1*B*). However, the expression of other PKC isoforms remained unchanged in the two stable clones.

Effects of siRNA-PKCα transfection on cell proliferation, migration, and invasion. Because PKCα plays an important role in cell proliferation, migration, and invasion, we examined the potential activities of the stable siRNA-PKCα clone in these aspects. Growth curves indicate that the control cells had rapid growth rates, whereas the growth rate was markedly reduced in siPKCα-SK (Fig. 1*C*). In siPKCα-SK cells, the doubling time was increased by 206.2%, and the potentials of cell migration and invasion were decreased by 45% and 51%, respectively (Fig. 1*D*). The corresponding figures in the siPKCα-HA cells were 255.4%, 56%, and 46%, respectively. These findings indicate that transient siRNA-PKCα transfection has significant inhibitory effects on cell proliferation, migration, and invasion.

p38 MAPK is prominently inhibited by PKCα antisense oligonucleotide and siRNA-PKCα transfection. In addition to siRNA, antisense oligonucleotide has also been successfully used in many human cell models to inhibit protein synthesis of specific PKC isoforms (29). Our previous study had found that the reduction of PKCα expression by PKCα antisense ODN inhibited the migratory and invasive potential of the SK-Hep-1 cells lines.[6] To investigate the molecular mechanism by which PKCα selectively induces invasive phenotype and motility, we examined the phosphorylation levels of MAPK family members both in a stable siRNA-PKCα clone and in antisense PKCα oligonucleotide-transfected cells. The phosphorylation level of p38 was decreased by 85% in siPKCα-SK (Fig. 2*A*). Moreover, transfection with antisense PKCα oligonucleotide also reduced the phosphorylation level of p38 MAPK, whereas no changes were observed in the sense oligonucleotide-treated group (Fig. 2*B*). However, the phosphorylation levels of ERK and JNK remained unchanged in siPKCα-SK or antisense PKCα oligonucleotide-transfected cells. Similar findings were also observed in HA22T/VGH cells (data not shown). These changes suggested that PKCα may be involved in the activation of p38 MAPK in HCC cells.

[6] T-T. Wu, Y-H. Hsieh, Y-S. Hsieh, and J-Y. Liu, submitted for publication.

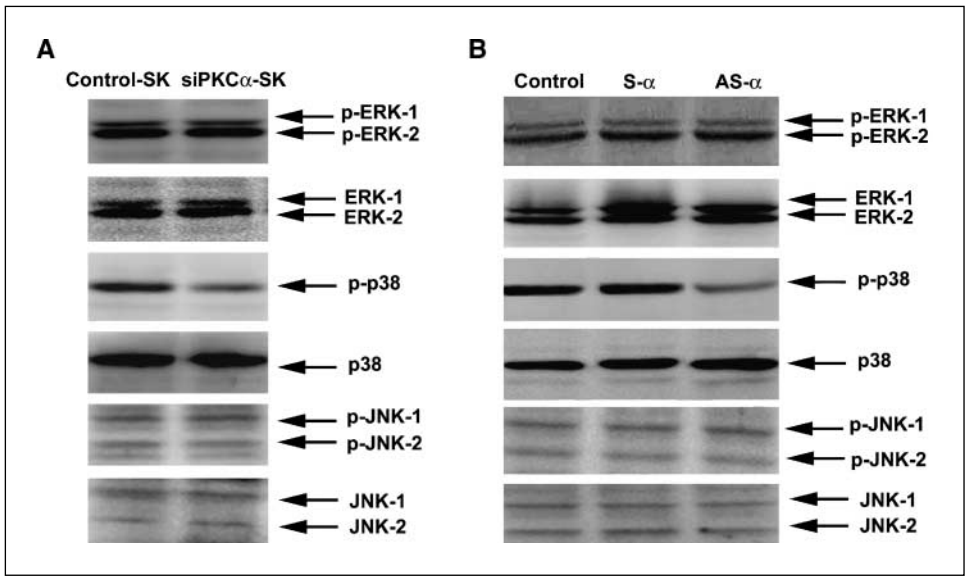

Figure 2. p38 MAPK is prominently silenced by siRNA-PKCα transfection and PKCα antisense oligonucleotide in SK-Hep-1 cells. *A,* levels of activated ERK-1/2, p38 MAPK, and JNK-1/2 in a stable siRNA-PKCα–transfected clone (*siPKCα-SK*). Cells transfected with pcDNA-HU6 were designed as control-SK. *B,* levels of activated ERK-1/2, p38 MAPK, and JNK-1/2 in SK-Hep-1 cells transfected with 5 μmol/L PKCα antisense oligonucleotide (*AS-α*) or PKCα sense oligonucleotide (*S-α*). Untreated cells were designed as the control. The MAPK levels were determined by Western blotting of whole cell lysates using specific antibodies and phosphorylated antibodies. Data are representatives of three independent experiments with similar results.

PKCα-regulated migration and invasion is p38 MAPK–dependent. We first determined the specific effect of the p38 MAPK inhibitor SB203580. After treating SK-Hep-1 cells with SB203580 (50 μmol/L) for 24 h, the activity of p38 MAPK was specifically inhibited (Fig. 3*A*), and no changes were observed in the levels of the phosphorylated forms of ERK-1/2 or JNK-1/2. We then examined the effect of SB203580 on the migratory/invasive phenotype of SK-Hep-1 cells by migration and invasion assay.

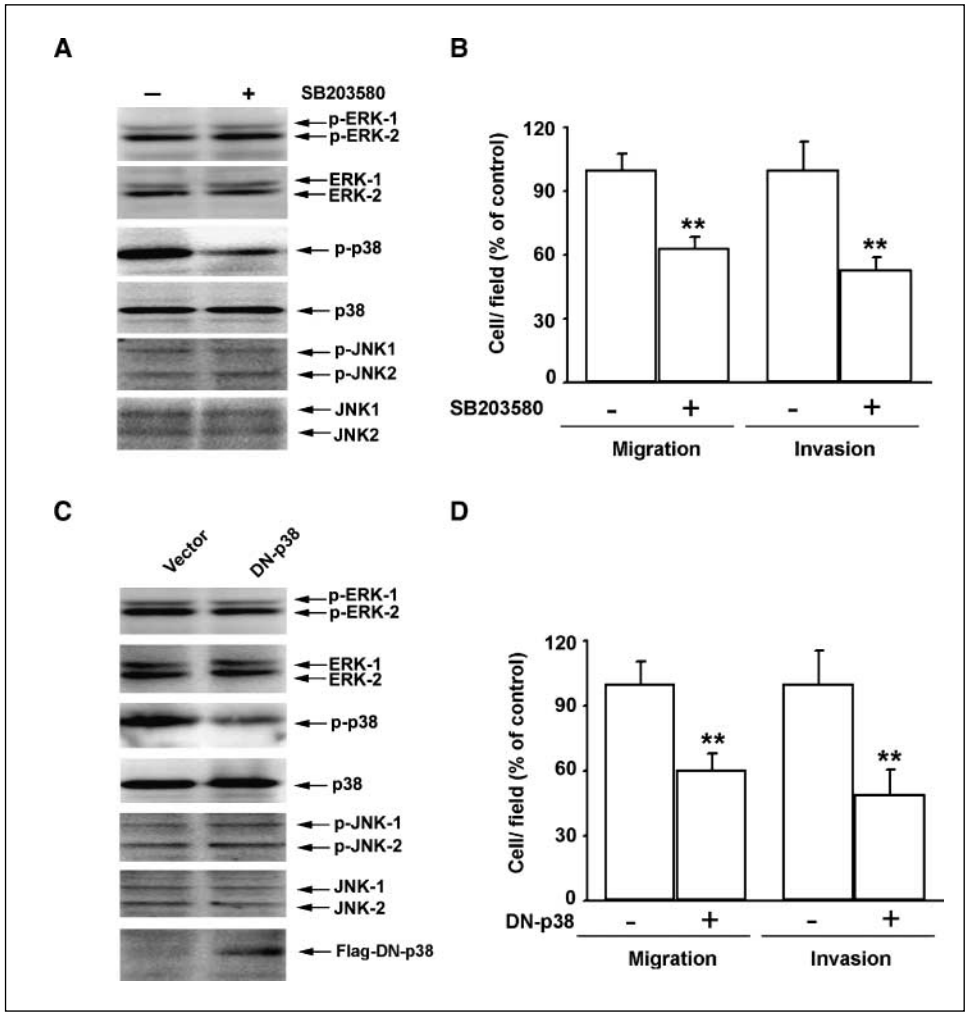

Figure 3. Inactivation of p38 MAPK inhibits migration and invasion of SK-Hep-1 cells. *A,* inhibitory effect of the p38 MAPK inhibitor SB203580. Whole cell lysates prepared from the SK-Hep-1 cells with or without SB203580 (50 μmol/L) treatment for 24 h were analyzed for expression of phosphorylated and total MAPKs by Western blotting. *B,* migratory and invasive potentials of SK-Hep-1 cells. The cells pretreated with SB203580 (50 μmol/L) for 24 h were subjected to migration assay for 12 h or Matrigel invasion assay for 24 h in the presence of the compound. *C,* inhibitory effect of transfection with DN-p38 construct. SK-Hep-1 cells were transiently transfected with either DN-p38 construct or the vector. Whole cell lysates were analyzed for expression of phosphorylated and total MAPKs and flag-tagged DN-p38 by Western blotting. *D,* migratory and invasive potentials in cells with DN-p38 transfection. SK-Hep-1 transfectants were determined by migration and invasion assays. *Columns,* mean of three independent experiments; *bars,* SE. **, $P < 0.01$ versus control.

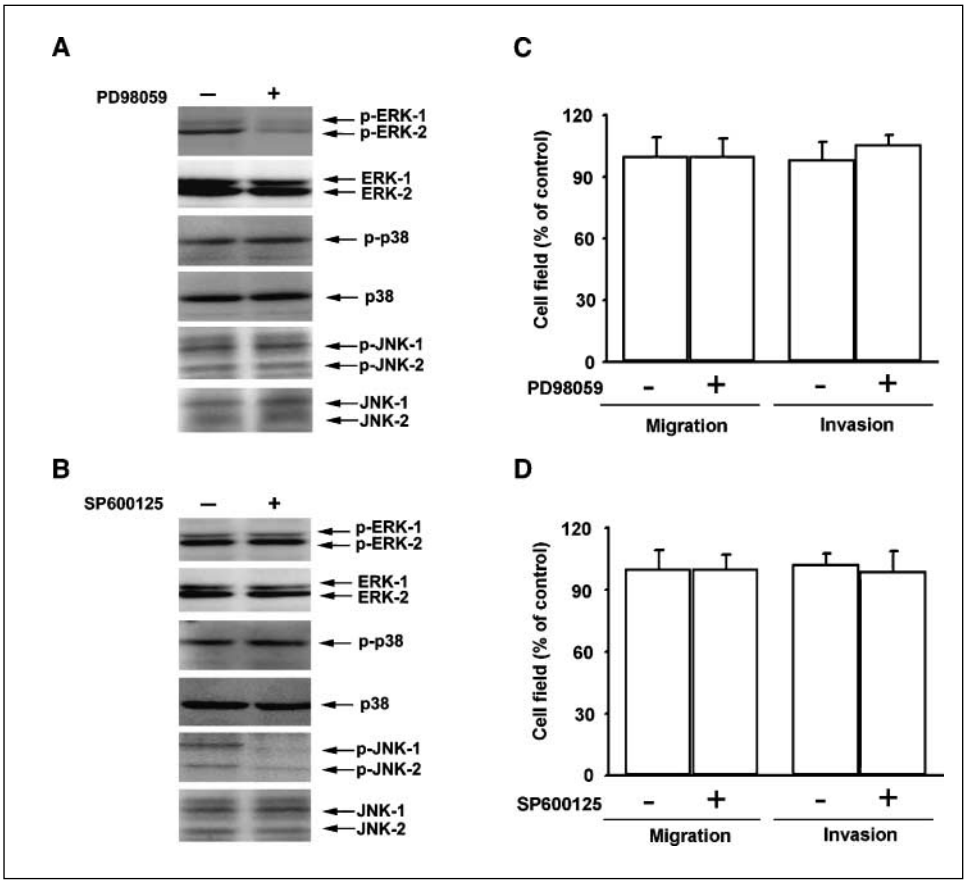

Figure 4. ERK-1/2 inhibitor PD98059, and JNK-1/2 inhibitor SP600125 did not inhibit migration and invasion in SK-Hep-1 cells. *A* and *B,* inhibitory effects of PD98059 and SP600125. Whole cell lysates prepared from the SK-Hep-1 cells with or without PD98059 (50 μmol/L) treatment for 24 h were analyzed by Western blotting. *C* and *D,* migratory and invasive potentials of the cells treated with the inhibitors. SK-Hep-1 cells pretreated with or without PD98059 (50 μmol/L) or SP600125 (50 μmol/L) for 24 h were subjected to migration assay for 12 h or Matrigel invasion assay for 24 h in the presence of PD98059 or SP600125. *Columns,* mean of three independent experiments; *bars,* SE.

Treatment of SB203580 (50 μmol/L) significantly reduced the number of cells with migratory and invasive potentials by 40% and 55%, respectively (Fig. 3*B*). In HA22T/VGH cells, SB203580 significantly reduced the migratory and invasive potentials by 42% and 48%, respectively. Moreover, the treated cells survived up to day 2, indicating that the inhibitory changes were not due to the cytotoxic effect of SB203580. These findings suggest a critical role for p38 MAPK in PKCα-regulated migration and invasion in SK-Hep-1 and HA22T/VGH cells.

In addition to the treatment with p38 MAPK inhibitor, we also transfected the SK-Hep-1 cells with dominant negative p38 mutant plasmid (DN-p38), in which the dual phosphorylated motif Thr-Gly-Tyr was mutated to Ala-Gly-Phe (30). Specific inactivation of p38 in DN-p38–transfected cells was confirmed by Western blotting (Fig. 3*C*). Expression of flag-tagged DN-p38 was shown in DN-p38–transfected cells, and phosphorylation of p38-MAPK was significantly inhibited, whereas the levels of phosphorylated ERK-1/2 or JNK-1/2 were not altered by DN-p38 transfection. We then did migration and invasion assay using DN-p38 transfectants. Cell migration was significantly reduced in DN-p38–transfected cells (Fig. 3*D*). Invasive phenotype was also significantly inhibited by DN-p38 transfectants (Fig. 3*D*). In HA22T/VGH cells, DN-p38 transfection reduced the number of cells with migratory and invasive potentials by 49% and 67%, respectively. DN-p38 transfection did not lead to a significant reduction in cell viability. These findings suggest that DN-p38–mediated inhibition of SK-Hep-1 and HA22T/VGH cell migration/invasion was not due to a cytotoxic effect.

ERK-1/2 and JNK-1/2 may not be required for cell migration and invasion. Because the ERK-1/2 or JNK-1/2 pathway is critical for cell migration and invasion in some cell types (31), we determined whether ERK-1/2 or JNK-1/2 also play a role in human SK-Hep-1 cell migration and invasion. After treating the SK-Hep-1 cells with the ERK-1/2 inhibitor PD98059 (50 μmol/L; Fig. 4*A*) or the JNK-1/2 inhibitor SP600125 (50 μmol/L; Fig. 4*B*) for 24 h, activation of ERK-1/2 or JNK-1/2 were specifically inhibited. However, both PD98059 and SP600125 did not affect cell migration and invasion in SK-Hep-1 cells (Fig. 4*C* and *D*). These phenomena were also observed in HA22T/VGH cells (data not shown).

MKK6 restored p38 MAPK activation and cell migration and invasion in stable PKCα-siRNA–transfected clones. Because MAPK kinases (MAPKK) MKK3, MKK4, and MKK6 have been reported to be capable of activating p38 MAPK (32), we determined whether these MAPKKs were able to activate p38 MAPK and induce cell migration/invasion in siPKCα-SK. Compared with the untreated control, constitutively active MKK6 increased the phosphorylation of p38 MAPK (Fig. 5*A*) and significantly increased the migratory and invasive potentials (Fig. 5*B*). Although MKK3 and MKK4 revealed a slight increase in p38 MAPK activity, they exhibited no stimulatory effect in the cell migratory/invasive phenotype. To confirm the constitutively active MKK6-induced p38 MAPK activation required for the cell migratory and invasive phenotypes, siPKCα-1 with transfection of MKK3 or MKK6 was treated with the p38 MAPK inhibitor SB203580. SB203580 almost completely abrogated the elevated level of the phosphorylation of p38 MAPK by constitutively active

MKK3 or MKK6 (Fig. 5*C*) and also inhibited the constitutively active MKK6-induced migration/invasion (Fig. 5*D*). The expression of flag-tagged MAPKKs was also shown in MAPKK-transfected cells (Fig. 5*A* and *C*). Moreover, constitutively active MKK6 also elevated phosphorylation of p38 MAPK and significantly increased the migratory and invasive potentials in siPKCα-HA cells (data not shown). These findings showed that MKK6 rather than the other MAPKKs, mediated through p38 MAPK activation, induced cell migration/invasion in siPKCα-SK and siPKCα-HA, suggesting that activation of p38 MAPK pathway by PKCα is critical in inducing SK-Hep-1 and HA22T/VGH cell migration and invasion.

Constitutively active PKCα restored p38 MAPK activation and cell migration and invasion in stable PKCα-siRNA–transfected clones. To confirm the role of PKCα in cell migration and invasion, siPKCα-SK and siPKCα-HA were transfected with the active PKCα mutant (with kinase domain only) to determine whether the level of p38 MAPK phosphorylation is restored. By Western blotting, both active PKCα (50 kDa) and wild-type PKCα (80 kDa) were shown to be expressed in siPKCα-SK with transfection of active PKCα plasmid (5 μg) or vector. However, the level of active PKCα (50 kDa) was notably higher in siPKCα-SK with the transfection of active PKCα plasmid than that with the vector (Fig. 6*A*). Compared with cells transfected with the vector, phosphorylation of p38 MAPK was increased in siPKCα-SK transfected with active PKCα plasmid by 2.5-fold (Fig. 6*A*). In contrast, changes in phosphorylation of ERK and JNK were not apparent. Active PKCα enhanced cell proliferation in siPKCα-SK and decreased the cell doubling time (data not shown). Moreover, constitutive PKCα respectively increased cell migration and invasion by 38% and 60% in siPKCα-SK cells, compared with the cell transfected with the vector (Fig. 6*B*). In siPKCα-HA cells, constitutive PKCα also increased phosphorylation of p38 MAPK and the potentials of cell migration and invasion (data not shown). These findings suggest that PKCα is sufficient for activation of p38 MAPK and induce cell migration/invasion.

Discussion

In this study, we showed that PKCα-specific siRNA and antisense oligonucleotide reduced the spontaneous activation of p38 MAPK, and the cell migration and invasion were also decreased by the antisense (data not shown) and siRNA. The same phenomenon was also observed in cell treatment with the p38 MAPK inhibitor SB203580 and DN-p38. Moreover, the reduction of activation of p38 MAPK cell migration/invasion and activation in both siPKCα-SK and siPKCα-HA was restored by the treatment with MKK6 or constitutively active PKCα. Although ERK- or JNK-dependent signaling molecules have been shown to be involved in the invasive behavior of human HCC cells (33, 34), ERK and JNK pathways were not observed to play a significant role in PKCα-mediated migratory and invasive behavior of SK-Hep-1 and HA22T/VGH cells. These results suggest that PKCα may activate p38 MAPK in malignant progression of HCC, and p38 MAPK is an important downstream signaling molecule that plays an essential role in mediating PKCα-induced cell invasion.

It has been reported that p38 MAPK is directly activated by MKK3, MKK6, and MKK4 (32). Therefore, one possible mechanism of the activation of p38 MAPK by PKCα is that the PKCα phosphorylate or its downstream signaling molecules interact with these MAPKKs and lead to p38MAPK activation. However, we have revealed that only MKK6 may induce a high activation of p38 MAPK and then promote cell migration/invasion in siPKCα-SK and siPKCα-HA cells. These results are consistent with the findings that

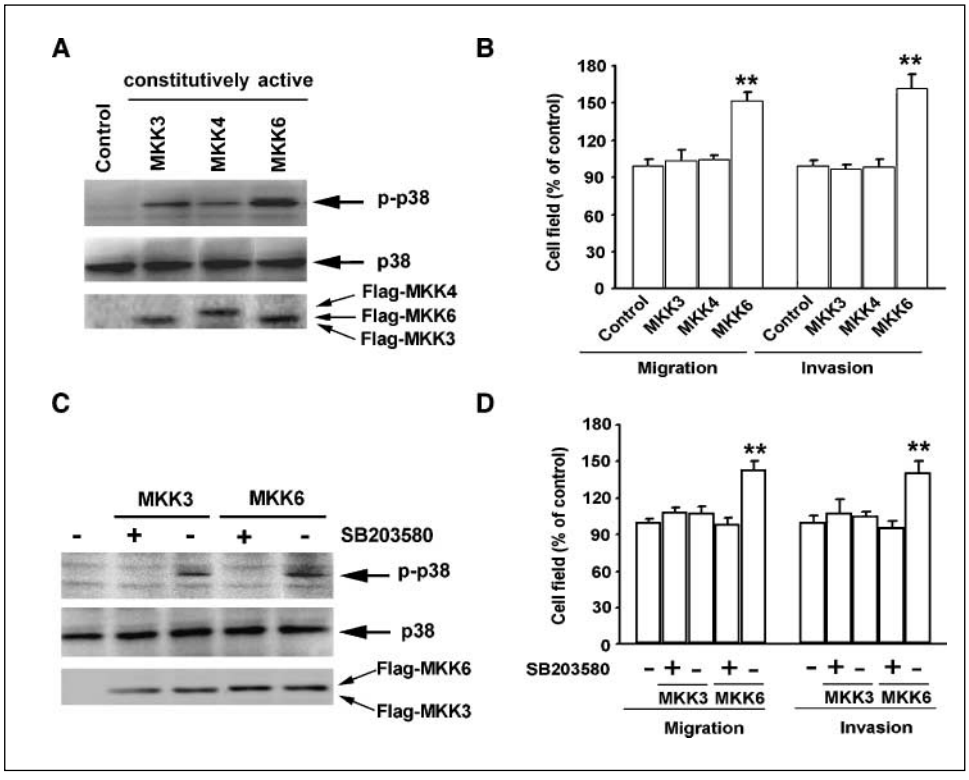

Figure 5. Constitutively active MKK6 renders siPKCα-SK cell migratory and invasive phenotypes. *A,* effects of transfection with MAPKKs. siPKCα-SK was transfected with constitutively active MKK3, MKK4, and MKK6 vector for 48 h. The cells were lysed, and Western blotting was done to detect phosphorylated p38 MAPK, p38 MAPK, and flag-tagged MAPKKs. *B,* migratory and invasive potentials in cells transfected with MAPKKs. Migration and invasion assays were done on the siPKCα-SK cells transfected with constitutively active MKK3, MKK4, and MKK6 vector and vector. **, $P < 0.01$ versus control. *C,* effects of p38 MAPK inhibitor SB203580 on cells transfected with MAPKKs. siPKCα-SK was transfected with constitutively active MKK3 or MKK6 vector for 48 h. SB203580 (50 μmol/L) was then added to the cells for 24 h and subsequently lysed for Western blotting to detect phosphorylated p38 MAPK, p38 MAPK, and flag-tagged MAPKKs. *D,* migratory and invasive potentials in cells transfected with MAPKKs and treated with SB203580. Migration and invasion assays were done on the siPKCα-SK transfected with constitutively active MKK3, MKK6, or vector, and SB203580 (50 μmol/L) was then incubated with the cells for 24 h. **, $P < 0.01$ versus control.

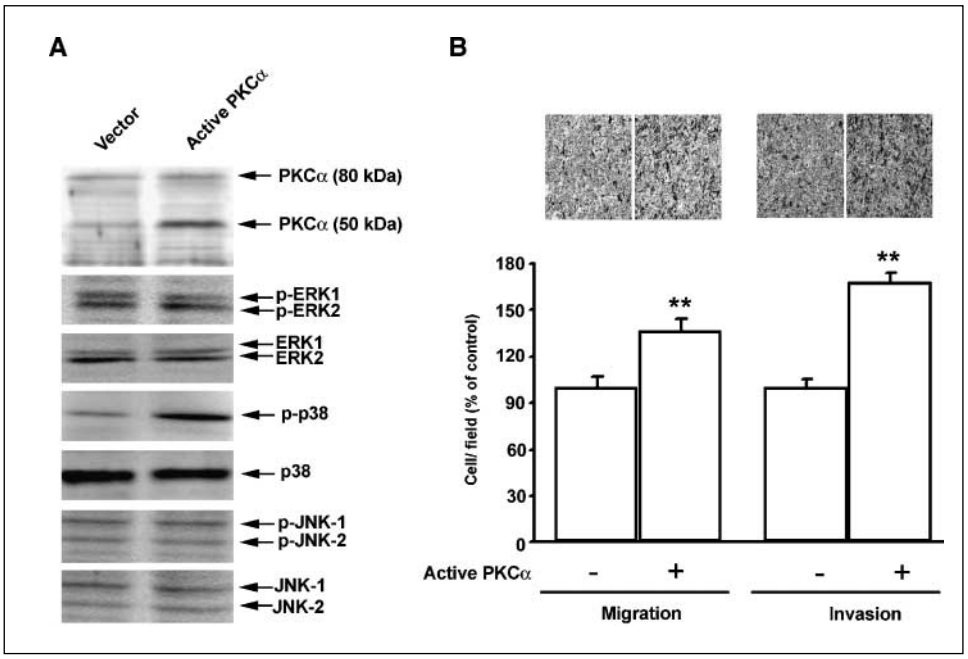

Figure 6. Constitutively active expression of PKCα enhanced phosphorylation of p38 MAPK and promoter cell migration and invasion in siPKCα-SK cells. *A,* effects of active PKCα transfection. siPKCα-SK was transfected with 5 μg of vector (*Vector*) or active PKCα plasmid (*Active PKCα*) for 48 h and subsequently lysed for Western blotting to detect the expression of PKCα and phosphorylated and total MAPKs. *B,* effects of active PKCα transfection on migratory and invasive potentials. Migration and invasion assays were done on the siPKCα-SK cells transfected with 5 μg of vector or active PKCα plasmid. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ versus control.

p38 MAPK activation by MKK6 transfection is sufficient to induce invasive and migratory phenotypes in MCF10A cells (22). Moreover, at least four isoforms of p38 MAPK have been identified and characterized (35), and MKK6 is a common activator of the isoforms, whereas MKK3 only activates some of them (36). Our findings also suggest that PKCα-mediated cell malignant progression may require the activation of all isoforms of p38 MAPK. However, this suggestion needs further investigations.

The p38 MAPK signaling pathway has also been reported to be important for matrix metalloproteinase (MMP) expression and *in vitro* invasiveness in many cell types. Transforming growth factor β (TGF-β)–mediated activation of p38 MAPK may increase the invasive potential and MMP-2 level in human prostate cancer cells (21). In addition, the MMP-2 is also induced by the Rac-MKK3/6-p38 MAPK pathway, which is a unique signaling pathway in H-Ras–activated MCF10A human breast epithelial cell lines, leading to the migratory or invasive phenotype (22). In SK-Hep-1 cells, a significant down-regulation of MMP-2 or MMP-9 expression is associated with the decrease in cell invasion and migration (37). In our preliminary study, the reduction in the expressions of MMP-2 or MMP-9 have been observed in SK-Hep-1 cells treated with siRNA-PKCα, antisense PKCα oligonucleotide, the p38 MAPK inhibitor SB203580, or DN-p38 (data not shown). Based on these findings, we suggest that p38 MAPK is necessary for PKCα-mediated increases in MMPs and the migratory or invasive potential in human HCC cells.

We have observed that the constitutive expression of active PKCα results in the activation of p38 MAPK and cell migration and invasion in siPKCα-SK and siPKCα-HA cells, and there is no change in the level of ERK phosphorylation (Fig. 6). These findings were surprising because it has been shown that constitutively active PKCα can induce ERK activation accompanied by the induction of growth inhibition of HepG2 cells (38). In human rhabdomyosarcoma cells, all three MAPKs including ERK can be activated by the constitutively active PKCα associated with the regulation of sarcomeric myosin expression (39). It confirmed that alterations of cell responses induced by PKCα are complex and depend on the types and sometimes conditions of cells (1).

Contrary to our findings, interfering with the p38 MAPK pathway by the p38 MAPK inhibitor SB203580 markedly decreases TGF-β1–induced caspase-3 activation and cell apoptosis in Hep3B cells (40). Moreover, constitutively active MKK6 transfection to the HepG2 cells increases p38 MAPK activity, cytochrome *c* release from the mitochondria to the cytosol, and caspase-3 activity, accompanied by apoptosis. In addition, p38 MAPK activities in these cells are significantly lower than those in the adjacent uninvolved liver tissue (41). Thus, p38 MAPK may contribute to the suppression of HCC development. In this study, we showed that this kinase may directly affect the invasiveness of SK-Hep-1 and HA22T/VGH cells mediated by PKCα activation. The phenomenon is further complicated by reports indicating that the level of active phospho-ERK coupled to the ERK/p38 activity ratio has been shown to be predictive of *in vivo* behavior in ~90% of cancer cell lines tested (42), suggesting that a low ERK/p38 ratio favors tumor growth, whereas a high value predisposes to growth arrest. Although there is no real evidences of ERK/p38 ratio derived from HCC cells, interactions between MAPKs need to be considered in assessing the full kinase contribution to liver carcinogenesis. Understanding the p38 MAPK downstream signaling molecules may have valuable implications for developing new therapies for some PKCα-overexpressing cancers.

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