p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration

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Summary

Transforming growth factor β (TGFβ) contributes to tumor progression by inducing an epithelial to mesenchymal transdifferentiation (EMT) and cell migration. We found that TGFβ-induced EMT was blocked by inhibiting activation of p38 mitogen-activated protein kinase (MAPK) with H-7, a protein kinase C inhibitor, and with SB202190, a direct inhibitor of p38MAPK. Inhibition of the p38MAPK pathway affected TGFβ-mediated phosphorylation of ATF2, but did not inhibit phosphorylation of Smad2. SB202190 impaired TGFβ-mediated changes in cell shape and reorganization of the actin cytoskeleton. Forced expression of dominant-negative (DN) MAPK kinase 3 (MKK3) inhibited TGFβ-mediated activation of p38MAPK and EMT. Expression of DN-p38α impaired TGFβ-induced EMT. Inhibition of p38MAPK blocked TGFβ-induced migration of non-tumor and tumor mammary epithelial cells. TGFβ induced activation of the p38MAPK pathway within 15 minutes. Expression of TGFβ type II (TβRII) and type I (TβRI/Alk5) kinase-inactive receptors blocked EMT and activation of p38MAPK, whereas expression of constitutively active Alk5-T204D resulted in EMT and phosphorylation of MKK3/6 and p38MAPK. Finally, dominant-negative Rac1N17 blocked TGFβ-induced activation of the p38MAPK pathway and EMT, suggesting that Rac1 mediates activation of the p38MAPK pathway. These studies suggest that the p38MAPK pathway is required for TGFβ-mediated EMT and cell migration.

Key words: p38MAPK, TGFβ, Epithelial-mesenchymal transition, Cell migration, Rac1

Introduction

The transforming growth factor β (TGFβ) family of secreted factors regulates various biological processes, including cell proliferation, differentiation and apoptosis (Massague, 1998). TGFβs signal through cell-surface serine-threonine kinase type II and type I receptors. TGFβ binding to TGFβ type II (TβRII) receptor triggers its associaion with the TGFβ type I (TβRI) receptor (Massague, 1998). TβRII phosphorylates and activates TβRI, which, in turn, phosphorylates receptor-associated (RA) Smads (Smad2 and Smad3). RA-Smads bind Smad4 and translocate to the nucleus where they regulate transcription of target genes (Massague, 1998). In addition to Smads, TGFβ can activate Jun N-terminal kinase (JNK) (Atfi et al., 1997; Engel et al., 1999; Frey and Mulder, 1997), extracellular signal-regulated kinase (ERK) (Hartsough and Mulder, 1995), p38 mitogen-activated protein kinase (p38MAPK) (Hanafusa et al., 1999), and Akt (Bakin et al., 2000).

Smad-dependent signaling has been shown to be required for the antiproliferative activity of TGFβ, and components of this pathway are frequently mutated or silenced in several human cancers (de Caestecker et al., 2000). Tumors, however, frequently express high levels of TGFβ and inhibition of TGFβ signaling has been shown to reduce tumor invasiveness and metastasis (Akhurst and Balmain, 1999; Barrack, 1997; Cui et al., 1996; Hojo et al., 1999). A number of studies provide evidence that TGFβ contributes to tumor cell invasion and metastasis by inducing mesenchymal transdifferentiation in epithelial cells (EMT) and stimulating cell migration (Akhurst and Balmain, 1999; Barrack, 1997; Oft et al., 1998). This TGFβ-mediated fibroblastic transdifferentiation is a complex process associated with alterations in epithelial cell junctions, changes in cell morphology, reorganization of the cell cytoskeleton, expression of fibroblastic markers (fibronectin, vimentin), and enhancement of cell migration (Bakin et al., 2000; Miettinen et al., 1994; Piek et al., 1999b).

The molecular mechanisms of TGFβ-mediated EMT and cell migration are not entirely understood. Studies with TGFβ receptors have shown that a truncated TGFβ/bone morphogenic protein (BMP) type I receptor, Alk2, blocks EMT in mouse NMuMG cells (Miettinen et al., 1994). Adenoviral expression of constitutively active human TβRI/Alk5 together with Smad2/3 can induce EMT in these cells (Piek et al., 1999b). Expression of a dominant-negative truncated form of TβRII decreases the formation of invasive spindle tumours (Portella et al., 1998). Adenoviral expression of Smad2/3 induced EMT only in the context of expression of constitutively active Alk5 (Piek et al., 1999b). Overexpression of Smad7, an inhibitor of Smad-dependent signaling, or dominant-negative Smad3 did not affect the transdifferentiation, arguing against

involvement of Smads in EMT (Bhowmick et al., 2001a). Inhibition of JNK with curcumin (Bakin et al., 2000) or by expression of dominant-negative JNK mutant (Bhowmick et al., 2001a) did not affect EMT. TGFβ did not activate the Ras-Raf-ERK1/2 cascade and MEK inhibitors (PD098059 and U0126) did not block EMT in NMuMG cells (Bakin et al., 2000; Piek et al., 1999b). We have recently shown that the phosphatidylinositide 3-kinase (PI3K)-Akt pathway contributes to EMT at the step of tight junction disruption (Bakin et al., 2000). The role of p38MAPK in TGFβ-mediated EMT has not been studied.

The p38MAPK pathway has been implicated in various biological responses to members of the TGFβ superfamily including TGFβ-stimulated migration of smooth muscle cells (Hedges et al., 1999), neuronal differentiation of PC12 cells induced by bone morphogenic protein 2 (BMP-2) (Iwasaki et al., 1999), growth/differentiation factor-5-induced chondrogenesis of ATDC-5 cells (Nakamura et al., 1999), and BMP-mediated cardiomyocyte differentiation (Monzen et al., 1999). Studies in Drosophila have shown that p38MAPKs are required for wing morphogenesis downstream of decapentaplegic (Dpp), a homologue of TGFβ (Adachi-Yamada et al., 1999). The p38MAPK pathway has also been implicated in TGFβ transcriptional responses (Hanafusa et al., 1999; Kucich et al., 2000; Sano et al., 1999).

The molecular mechanism(s) of TGFβ-induced activation of p38MAPK signaling are not defined. Mammalian p38MAPKs are activated by distinct upstream dual specificity MAPK kinases (MKK), MKK3 and MKK6 (Tibbles and Woodgett, 1999). TGFβ-activated kinase 1 (Tak1) phosphorylates MKK3/6 in TGFβ and BMP signaling (Shibuya et al., 1998; Yamaguchi et al., 1995). In addition, other MKK kinases including p21-activating kinase (PAK1) and mixed-lineage kinase (MLK) have been shown to phosphorylate MAPK kinases (MKK3/6) and induce p38MAPKs (Tibbles et al., 1996; Zhang et al., 1995). p38MAPK downstream targets include MAPK-activated protein kinase-2, mitogen- and stress-activated protein kinase-1 (MSK1), and transcription factors ATF2, CHOP, CREB and MEF2C (Tibbles and Woodgett, 1999). Recent studies have found that p38MAPKs are involved in the control of cell cytoskeleton and cell migration via phosphorylation of paxillin and heat shock protein 27 (HSP27) (Hedges et al., 1999).

In these studies we found that H-7, a protein kinase inhibitor, blocks TGFβ-induced EMT and activation of the p38MAPK pathway in NMuMG mouse mammary epithelial cells. The specific p38MAPK inhibitors, SB203580 and SB202190, impaired TGFβ-mediated changes in cell shape, the actin cytoskeleton, and cell migration. H-7 and the p38MAPK inhibitors blocked phosphorylation of ATF2, but did not inhibit TGFβ-mediated phosphorylation of Smad2. Expression of dominant-negative mutants (DN) of MKK3 or p38α inhibited TGFβ-mediated EMT. We also showed that TGFβ activates the MKK3/6-p38MAPK-ATF2 cascade within 15 minutes and expression of DN-MKK3 blocked TGFβ-mediated activation of p38MAPK and EMT. Kinase-inactive TGFβ type II and type I (Alk5) receptors blocked EMT and the activation of p38MAPK. Forced expression of kinase-active Alk5-T204D induced both EMT and phosphorylation of p38MAPK in NMuMG cells. Alk5-T204D-induced EMT was blocked by a p38MAPK inhibitor. Finally, we demonstrated that forced expression of dominant-negative Rac1N17 blocked TGFβ-induced activation of the p38MAPK-ATF2 cascade and EMT.

Materials and Methods

Antibodies and other reagents

TGFβ1 and tumor necrosis factor α (TNFα) were obtained from R&D Systems. Antibodies to fibronectin, Rac1 and Smad2 were from Transduction Laboratories; antibodies to ZO-1 were from Chemicon; the monoclonal antibody to p38MAPK and rabbit polyclonal to haemaglutinin (HA) epitope were from Santa Cruz Biotechnology, Phalloidin-FITC, phalloidin-Texas Red, and Hoechst 3342 were from Molecular Probes. The β-tubulin-Cy3 antibody was from Sigma. Antibodies to phospho-Ser473 Akt, total Akt, phospho-MKK3/6, phospho-p38MAPK, and phospho-ATF2 were from New England BioLabs, and to C-terminal phospho-Smad2 from Upstate Biotechnology. LY294002, H-7, SB203580, and SB202190 were from Calbiochem. GDP and GTPγS were purchased from Sigma. The GST-ATF2 fusion protein was from New England Biolabs. TBS buffer contained 20 mM Tris-HCl, pH 7.6, 150 mM NaCl. TBST was TBS supplemented with 0.1% Tween 20 (*v/v*).

Cell culture

NMuMG mouse mammary epithelial cells, SiHa human cervical carcinoma cells, MDA-MB-231 human breast cancer cells and HEK293T human kidney cells were purchased from American Tissue Culture Collection (ATCC). Cells were cultured as recommended by ATCC. 4T1 tumor cells were provided by F. Miller (Karmanos Cancer Center, Detroit, MI) and cultured in 10% FBS-DMEM.

Plasmids and retroviral constructs

The retroviral vectors pGabe and pGabe-TβRII-K277R were provided by Martin Oft (UCSF, San Francisco, CA) and have been described previously (Oft et al., 1998). The TβRII-K277R construct contains an HA-tag at the N-terminus. Human wild-type Alk5, dominant-negative Alk5-K232R, and constitutively active Alk5-T204D constructs were provided by Masahiro Kawabata (The Cancer Institute, Tokyo, Japan). To generate pBMN-Alk5 constructs, the *Eco*RI/*Sal*I fragments of Alk5 and Alk5-K232R including the C-terminal HA-tag were cloned in the retroviral pBMN-IRES-EGFP vector provided by Garry Nolan (Stanford University). The pBMN-Rac1N17 was engineered by cloning a *Bam*HI-*Xho*I fragment encoding Rac1N17 from pCDNA3-Rac1N17 (a gift of Richard Cerione, Cornell University, Ithaca, NY) at the *Bam*HI-*Sal*I site of the retroviral pBMN-IRES-GFP vector. RhoAN19 and RhoAQ63L were previously described (Bakin et al., 2000). The pBMN-MKK3AL and pBMN-MKK6AL plasmids were generated by cloning *Sal*I-*Not*I fragment of MKK6AL or *Xho*I-*Not*I fragment of MKK3AL from pCDNA3 vector into the retroviral pBMN-IRES-GFP vector. pCDNA3-MKK3AL and pCDNA3-MKK6AL plasmids were a gift of James Woodgett (The Ontario Cancer Institute, Toronto, Ontario). pBMN-p38AGF encoding a dominant-negative mutant of p38α and containing N-terminal Flag epithope was generated by cloning a *Hind*III-*Xba*I fragment of p38AGF from pcDNA3-p38AGF at the *Xho*I site of pBMN-IRES-GFP. pcDNA3-p38AGF was a gift of Roger Davies (University of Massachusetts, Worcester, MA). Plasmids phCMV-VSVG, encoding vesicular stomatitis virus glycoprotein (VSV-G), and pCMVgag-pol, containing the Moloney murine leukemia virus (MoMLV) gag and pol genes, were provided by Jane Burns (University of California at San Diego).

Retroviral infection of cells

Retroviruses were prepared by transfection of HEK293T cells with 15

μg DNA/100 mm dish of three plasmids encoding gag/pol, VSV-G, and the target construct, ratio 4:3:8. Supernatants from cells were collected for 3 days and combined, filtered through 0.4 μm filters, and stored in aliquots at –80°C. NMuMG cells were infected with supernatant containing retroviruses in the presence of 6 μg/ml Polybrene (Sigma) as described previously (Yee et al., 1994). Three days later, GFP-positive cells were selected by flow cytometry. Under these conditions more than 95% of selected cells expressed GFP at the time of experiments.

Immunoblot analysis

Cells were incubated in serum-free medium for 4 hours prior to treatment with TGFβ1. Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Protein concentrations in cell lysates were determined by the Bradford method. Protein extracts (50 μg/lane) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (100 mA, 2.5 hours). Membranes were blocked with 5% milk in TBST for 1 hour at room temperature (RT) and then incubated with primary antibodies in TBST plus 1% milk for 16 hours at 4°C, followed by incubation with secondary antibodies for 1 hour at RT. Membranes were washed three times in TBST and immunoreactive bands visualized by ECL (Pierce).

p38MAPK in vitro kinase assay

p38MAPK was precipitated from protein extracts (200 μg) with a p38MAPK monoclonal antibody (Santa Cruz Biotechnology) for 2 hours at 4°C. An in vitro kinase reaction was performed in a 40-μl volume by adding to the immune complexes 1 μg GST-ATF2 and 10 μCi [γ-^{32}P]ATP (specific activity 3000 Ci/mmol, New England Nuclear) for 20 minutes at 30°C in the presence of 10 μM PKA peptide inhibitor (Calbiochem). Reactions were terminated by the addition of Laemmli loading buffer and heating, followed by 15% SDS-PAGE and transfer to nitrocellulose (NC) membranes. Quantitative analysis of [γ-^{32}P]-labeled bands was performed using a

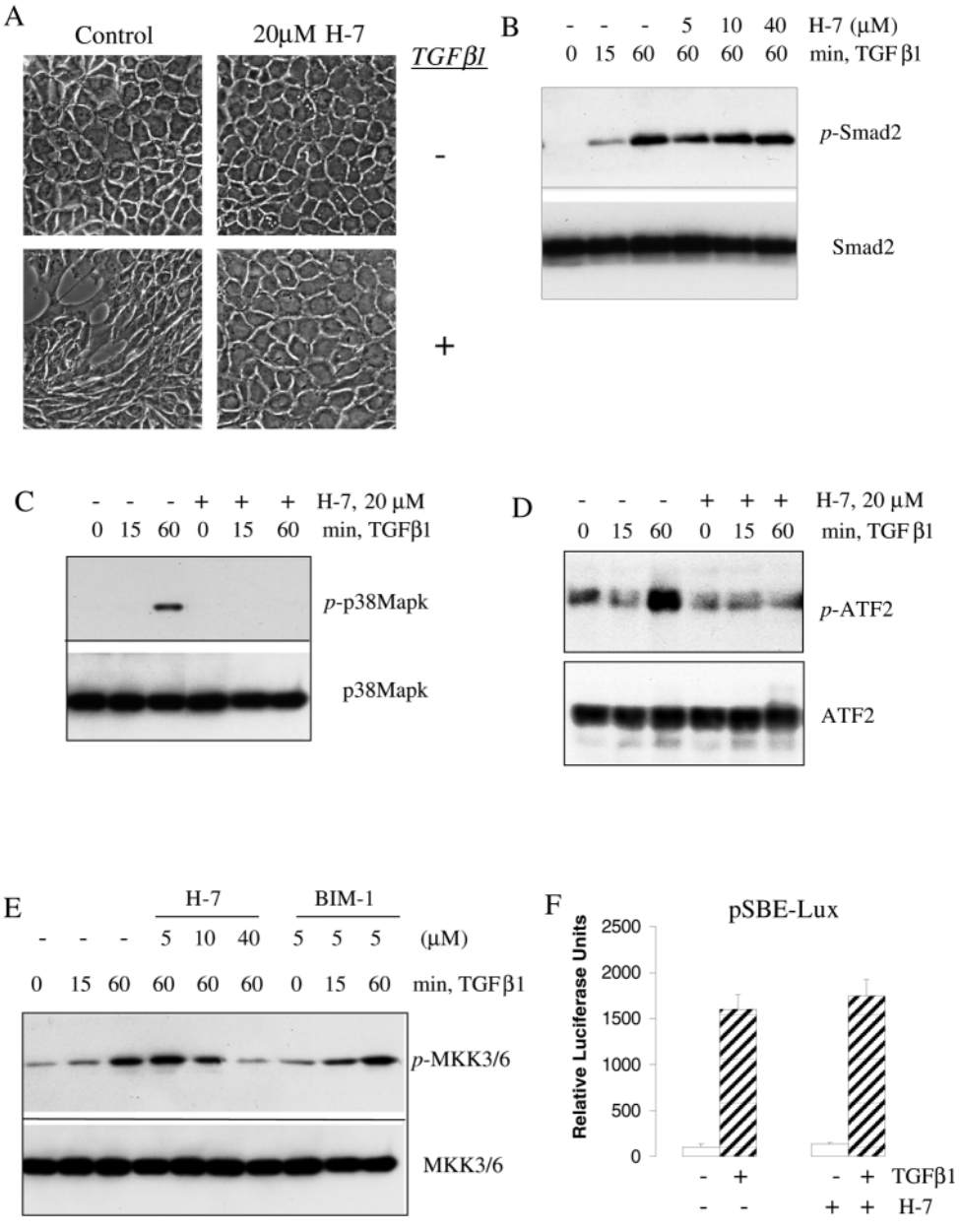

Fig. 1. Inhibition of TGFβ-mediated EMT and p38MAPK activation by H-7 kinase inhibitor. (A) NMuMG mammary epithelial cells were grown on glass coverslips for 24 hours and treated (bottom row) or not (top row) with 2 ng/ml TGFβ1 for 24 hours. Where indicated, cells were co-incubated with 20 μM H-7. Phase contrast images were taken at 200× magnification. (B-E) Immunoblot analysis of whole-cell extracts from NMuMG cells treated with 2 ng/ml TGFβ1 for the indicated times. Kinase inhibitors were added 60 minutes before TGFβ treatment. (B) Immunoblot detection of phospho-Smad2 and total Smad2. (C) Detection of phospho-p38MAPK total p38MAPK. (D) Inhibition of TGFβ-induced ATF2 phosphorylation by H-7. Immunoblots with antisera to phospho-ATF2 and total ATF2. (E) TGFβ-induced phosphorylation of MKK3/6 in cells co-treated with various concentrations of H-7 or 5 μM BIM-I, a PKC inhibitor. (F) Luciferase activity in NMuMG transfected with Smad-dependent reporter pSBE-Lux and pCMV-Rl vectors and treated with 1 ng/ml TGFβ1 for 16 hours in the absence or presence of 20 μM H-7. Each bar represents the mean±s.d. of three wells.

PhosphorImager (Molecular Dynamics). The same NC-membranes were probed with a monoclonal antibody to p38MAPK.

Immunofluorescence microscopy

NMuMG cells (10^5cells/well) were grown in DMEM containing 5% FBS on glass coverslips (22×22 mm) for 24 hours before treatment with 2 ng/ml TGFβ1. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at RT and then permeabilized with 0.05% Triton X-100 for 10 minutes. Cells were washed three times in PBS after each treatment. Cells were blocked with 3% milk in PBS for 30 minutes at RT, incubated for 60 minutes with primary antibodies diluted in 1% milk/PBS (1/300 for ZO-1, 1/500 for Smad2, 1/250 for fibronectin), and then with fluorescent secondary antibodies (1/500) for 45 minutes at RT. Microtubules were stained for 30 minutes at RT with β-tubulin-Cy3 diluted 1/250 in 1% milk/PBS. Actin was stained with phalloidin-FITC (4 units/ml) or phalloidin-Texas Red (2 units/ml). Cell nuclei were stained with 1 μg/ml Hoechst for 10 minutes at RT. Coverslips were mounted on 25×75 mm microslides (VWR Scientific) using AquaPolyMount (Polysciences). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

Transcriptional assays

NMuMG cells (3×10^4) were seeded in 24-well plates and transfected with 0.16 μg/ml pSBE-Lux containing 12 repeats of Smad binding sequence (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) with 0.002 μg/ml pCMV-Rl (Promega, Madison, WI) using FuGENE6 reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were incubated for 8 hours in 0.5% FBS-DMEM prior to treatment with 1 ng/ml TGFβ1 for 16 hours. Firefly luciferase (Luc) and *Renilla reniformis* luciferase (RlLuc) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luc activity was normalized to RlLuc activity and presented as Relative Luciferase Units. All assays were done in triplicate wells and each experiment was repeated at least twice.

Affinity precipitation of Rac using GST-PBD

A fusion protein containing the GTPase-binding domain from human PAK1 (PBD) and glutathione S-transferase (GST) was expressed in *Escherichia coli* using pGEX-4T3-GST-PBD as described (Benard et al., 1999). pGEX-4T3-GST-PBD was kindly provided by Gary Bokoch (Scripps Research Institute). NMuMG cells (2×10^7/assay) were treated with 2 ng/ml TGFβ1 for 15 minutes followed by cell lysis in 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 5% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin in the presence of 8 μg GT-PBD. Cell lysates were clarified by low speed centrifugation at 4°C. HEK293T cells transfected with Rac1N17 or Alk5 mutants were lysed in the same buffer. After clarification, cell lysates (350 μg/assay) were incubated with 8 μg GST-PBD. To prepare cytosolic Rac1 loaded with GDP or GTPγS, cell lysates (equivalent of 2×10^6 cells) were incubated for 15 minutes at 30°C in the presence of 10 mM EDTA and 100 mM GTPγS or 1 mM GDP to facilitate nucleotide exchange (Benard et al., 1999). The loading reaction was terminated by addition of 60 mM $MgCl_2$. Affinity precipitation was performed using 15 μl of glutathione-Sepharose 4B beads (Pharmacia) for 1 hour at 4°C. The bead pellets were washed three times with 20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% NP-40 and 2 times in PBS. The bead pellet was finally suspended in 40 μl of Laemmli sample buffer. Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an antibody to Rac1 (Transduction Laboratories).

Migration assays

NMuMG or MDA-MB-231 cells (1×10^5/well) were plated in DMEM/0.5%FBS in the upper chamber of 5 μm pore (24-well) transwells (Costar, High Wycombe, UK) and incubated alone or with 2 ng/ml TGFβ1 in the absence or presence of SB202190. After 16

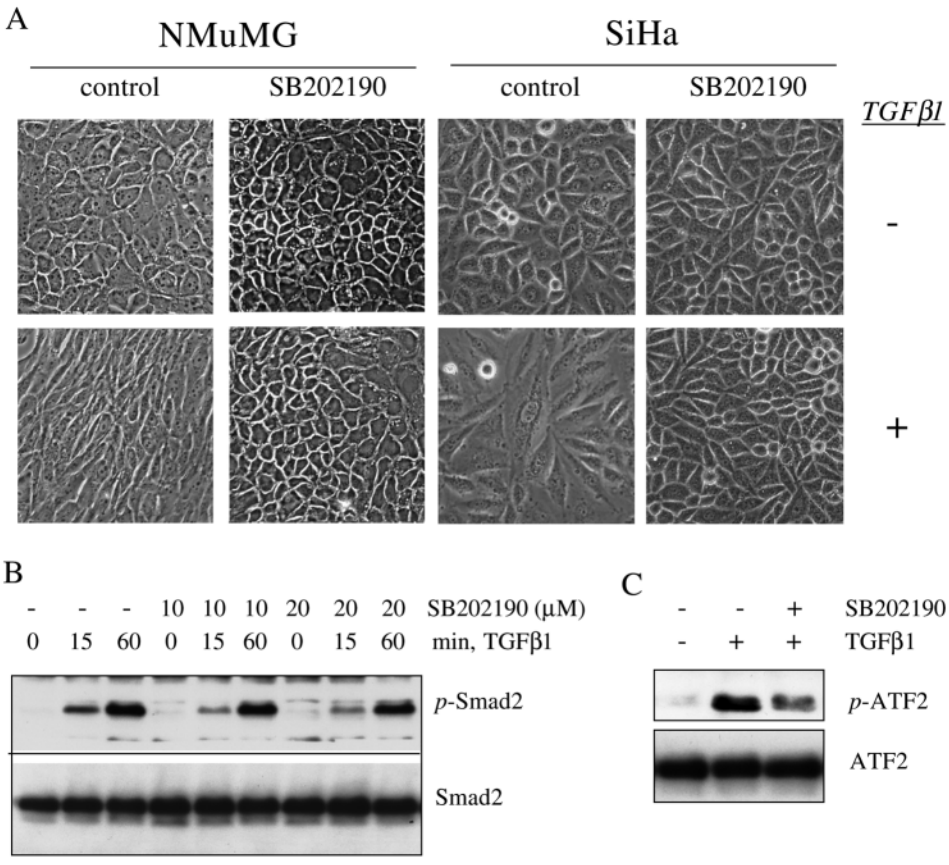

Fig. 2. Blockade of TGFβ-induced EMT by SB202190. (A) NMuMG cells grown on glass coverslips were treated (bottom row) or not (top row) with 2 ng/ml TGFβ1 for 24 hours in the absence or presence of 10 μM SB202190. Phase contrast images were taken at 200× magnification. (B) phospho-Smad2 and total Smad2 immunoblot analysis of whole-cell extracts from cells treated with 2 ng/ml TGFβ1 in the absence or presence of SB202190. (C) Immunoblots with antisera to phospho-ATF2 and total ATF2. SB202190 inhibits TGFβ-induced phosphorylation of ATF2.

hours, cells were fixed in 100% methanol and cells remaining at the top of the polycarbonate membrane were removed with cotton swabs. The cells that had migrated through pores to the lower surface were stained with Diff-quick stain (VWR Scientific). Membranes were mounted on 25×75 mm microslides. Four random images were recorded at 200× magnification and cells were counted. Experiments were performed in duplicate.

Wound closure assay

MDA-MB-231 and 4T1 cells ($1\text{-}2\times10^5$/well) were seeded in 12-well plates. Cells were incubated in serum-free medium for 32 hours prior to wounding. The wounds were made by scraping with plastic tip across the cell monolayer. Cells were treated with kinase inhibitors 60 minutes before wounding. The wounded cells were treated or untreated with 2 ng/ml TGFβ1. Phase contrast images were recorded at the time of wounding (0 hours) and 16 hours thereafter. The wound closure was estimated as the ratio of the remaining wound area relative to the initial wounded area. Experiments were repeated at least three times.

Results

H-7 inhibits TGFβ-mediated activation of p38MAPK and EMT

We investigated TGFβ-mediated EMT in NMuMG mouse mammary epithelial cells. These mammary epithelial cells have cuboidal cell shape and form tight and adherens junctions. Treatment with 2 ng/ml TGFβ for 24 hours induced changes in the cell morphology from cuboidal to an elongated spindle-like shape (Fig. 1A). Consistent with previous studies (Miettinen et al., 1994), TGFβ-mediated EMT was blocked in the presence of 20 μM H-7 (Fig. 1A). The inhibitors were added 60 minutes prior to addition of TGFβ and were present during a complete duration of the experiment. Although H-7 has been introduced as a protein kinase C (PKC) inhibitor, it can inhibit other kinases including PKA and PKG (Quick et al., 1992). Therefore, we investigated the effect of H-7 on the signaling pathways induced by TGFβ. We found that TGFβ1-induced phosphorylation of Smad2 was not affected by the presence of H-7 at the concentration that blocks EMT (Fig. 1C). Inhibition of JNK and ERK1/2 did not affect EMT (Bakin et al., 2000; Bhowmick et al., 2001a). Therefore, we tested whether H-7 affects TGFβ-mediated activation of the p38MAPK pathway using polyclonal antibodies to phosphorylated (active) MKK3/6 and p38MAPK. TGFβ-mediated phosphorylation of p38MAPK was blocked in the presence of 20 μM H-7 (Fig. 1C). H-7 also inhibited TGFβ-induced phosphorylation of ATF2, a substrate of p38MAPK (Fig. 1D).

Next, we checked whether H-7 inhibits activation of MKK3/6. We found that TGFβ-induced phosphorylation of MKK3/6 was inhibited by H-7 in a dose-dependent manner (Fig. 1E), suggesting that H-7 inhibits a kinase upstream of MKK3/6. This kinase is downstream of TGFβ receptors as incubation with 5-40 μM H-7 did not block phosphorylation of Smad2 (Fig. 1B). Consistent with this result, H-7 did not block TGFβ-mediated activity of Smad-dependent luciferase reporter (Fig. 1F). Since H-7 can inhibit PKC, we examined activation of p38MAPK in the presence of another PKC

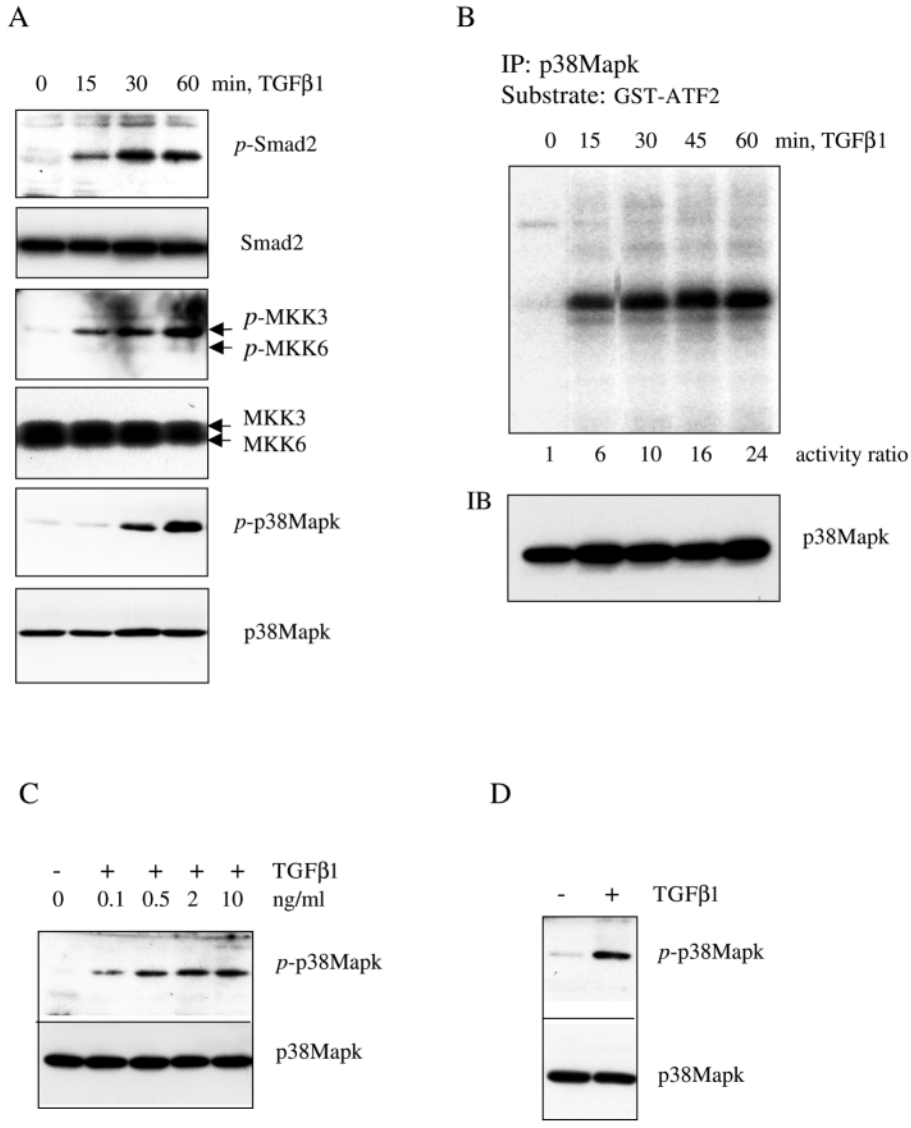

Fig. 3. Activation of the p38MAPK pathway in response to TGFβ. NMuMG cells were incubated in serum-free medium for 4 hours before addition of TGFβ1. (A) Immunoblot analyses with antibodies to phospho-Smad2, phospho-MKK3/6 and phospho-p38MAPK, and total Smad2, MKK3/6 and p38MAPK. (B) Detection of p38MAPK kinase activity in whole-cell extracts from NMuMG cells treated with 2 ng/ml TGFβ1 using GST-ATF2 as substrate. The products were separated by SDS-PAGE and transferred onto nitrocellulose-membrane. γ-^{32}P incorporation into ATF2 was quantitated using PhosphorImager. The membrane was probed with antibody to p38MAPK. (C) Immunoblot detection of TGFβ1 dose-dependent effect on p38MAPK phosphorylation at 60 minutes in NMuMG cells. (D) Induction of p38MAPK phosphorylation by 2 ng/ml TGFβ1 at 60 minutes in SiHa cells.

inhibitor, bisindolylmaleimide-I (BIM-I) (Davies et al., 2000). Treatment of cells with doses of BIM-I (1-5 μM) that block typical PKCs (Davies et al., 2000) did not affect phosphorylation of MKK3/6 in response to TGFβ (Fig. 1D). These results suggest that H-7 impairs TGFβ signaling by inhibiting activation of the p38MAPK pathway downstream of TGFβ receptors, and not through its effect on PKCs.

p38MAPK is involved in TGFβ-mediated EMT

To test whether p38MAPK is involved in EMT, we used specific inhibitors of p38MAPK, SB202190 and SB203580 that do not affect JNK, MEK1/2 and ERK1/2 (Davies et al., 2000). Microscopic examination showed that cell elongation induced by TGFβ in NMuMG cells was blocked by co-treatment with 10 μM SB202190 (Fig. 2A). Similarly, the p38MAPK inhibitor blocked TGFβ-induced cell elongation in cervical cancer epithelial SiHa cells (Fig. 2A). Previous studies have shown that these p38MAPK inhibitors may affect the kinase activity of TGFβ receptors (Eyers et al., 1998). Therefore, we examined their effect on TGFβ-receptor-dependent phosphorylation of Smad2. Treatment of cells with TGFβ in the presence of SB202190 did not significantly affect the expression and TGFβ-induced phosphorylation of Smad2 (Fig. 2B), whereas it

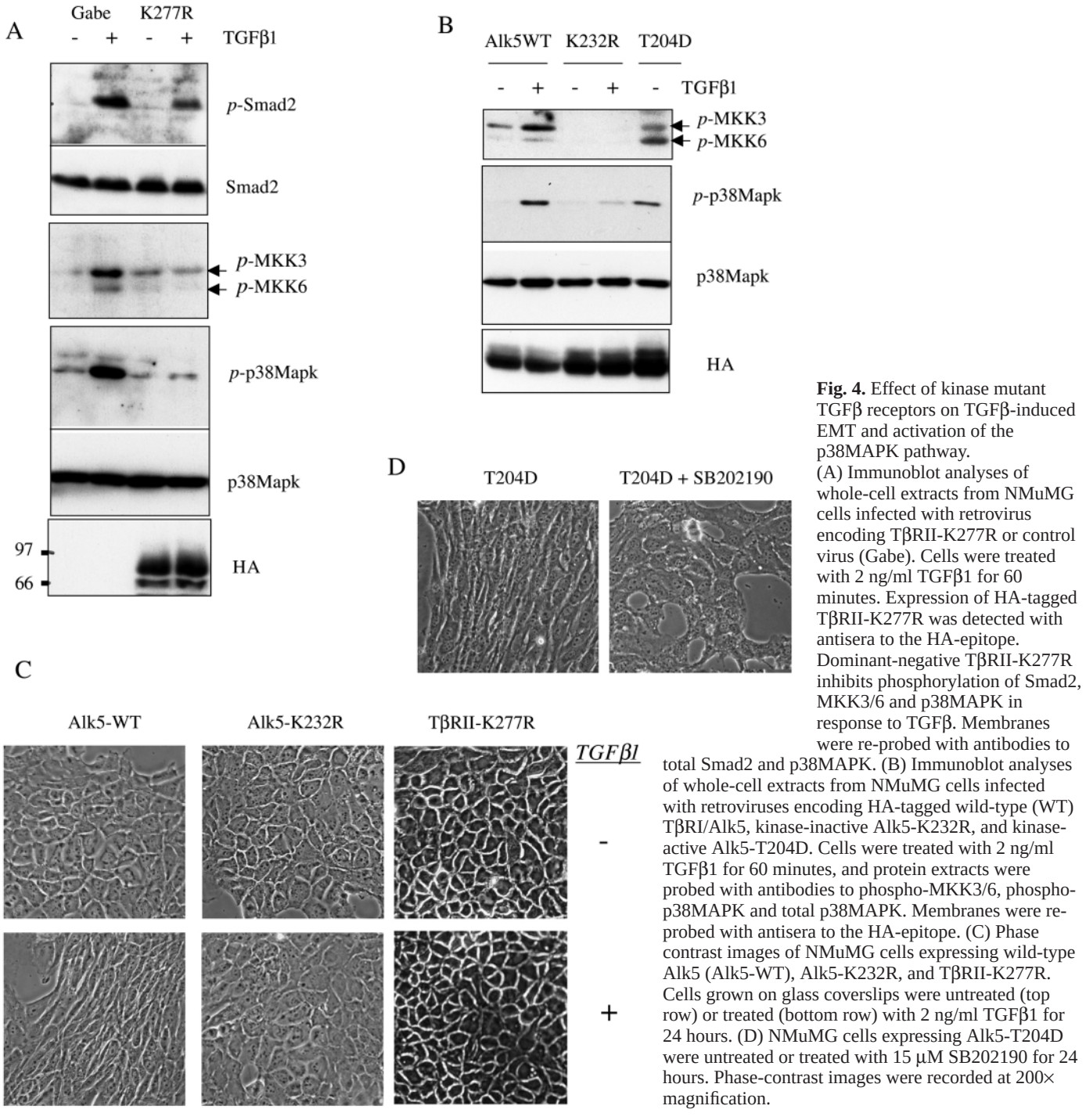

Fig. 4. Effect of kinase mutant TGFβ receptors on TGFβ-induced EMT and activation of the p38MAPK pathway. (A) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retrovirus encoding TβRII-K277R or control virus (Gabe). Cells were treated with 2 ng/ml TGFβ1 for 60 minutes. Expression of HA-tagged TβRII-K277R was detected with antisera to the HA-epitope. Dominant-negative TβRII-K277R inhibits phosphorylation of Smad2, MKK3/6 and p38MAPK in response to TGFβ. Membranes were re-probed with antibodies to total Smad2 and p38MAPK. (B) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retroviruses encoding HA-tagged wild-type (WT) TβRI/Alk5, kinase-inactive Alk5-K232R, and kinase-active Alk5-T204D. Cells were treated with 2 ng/ml TGFβ1 for 60 minutes, and protein extracts were probed with antibodies to phospho-MKK3/6, phospho-p38MAPK and total p38MAPK. Membranes were re-probed with antisera to the HA-epitope. (C) Phase contrast images of NMuMG cells expressing wild-type Alk5 (Alk5-WT), Alk5-K232R, and TβRII-K277R. Cells grown on glass coverslips were untreated (top row) or treated (bottom row) with 2 ng/ml TGFβ1 for 24 hours. (D) NMuMG cells expressing Alk5-T204D were untreated or treated with 15 μM SB202190 for 24 hours. Phase-contrast images were recorded at 200× magnification.

reduced phosphorylation of ATF2 (Fig. 2C). Similar results were obtained with SB203580 (data not shown).

TGFβ activates the p38MAPK pathway in NMuMG and SiHa cells

We next examined activation of the p38MAPK pathway in response to TGFβ. Protein extracts were prepared from cells starved in serum-free medium for 4 hours and treated with TGFβ1. Phosphorylation of MKK3/6 was detected after 15 minutes of TGFβ treatment reaching a maximum at 60 minutes, whereas an increase in p38MAPK phosphorylation at Thr180/Tyr182 was observed at 30 minutes and reached a plateau at 60 minutes (Fig. 3A). To confirm the immunoblot data, we tested p38MAPK-specific activity using an in vitro kinase assay with GST-ATF2 fusion protein as substrate. Treatment with TGFβ increased γ-^{32}P incorporation into GST-ATF2 in a time-dependent fashion, sixfold at 15 minutes and reaching a maximal stimulation of 24-fold above control by 60 minutes (Fig. 3B). This increase in p38MAPK kinase activity at 15 minutes may reflect a higher sensitivity of the in vitro kinase assay compared with detection of phosphorylated p38MAPK by immunoblot. TGFβ-induced activation of p38MAPK was dose-dependent with 0.1 ng/ml being sufficient to induce phosphorylation of p38MAPK with a maximal effect observed between 0.5 and 2 ng/ml (Fig. 3C). Treatment of SiHa human cervical carcinoma cells with TGFβ1 for 60 minutes resulted in phosphorylation of p38MAPK (Fig. 3D), suggesting activation of p38MAPK signaling in response to TGFβ1 in these cells.

Kinase activities of TGFβ receptors are required for TGFβ-induced p38MAPK activation

To confirm the role of TGFβ receptors in activation of p38MAPK, we engineered cells expressing TβRII-K277R, a kinase-inactive mutant of TGFβ type II receptor (Wrana et al., 1994). NMuMG cells were infected with retrovirus encoding TβRII-K277R and enhanced green fluorescent protein (EGFP) or with control retrovirus encoding EGFP only (Gabe). Fluorescent cells were selected by flow cytometry and expression of the HA-tagged mutant receptor was confirmed by immunoblot analysis (Fig. 4A). TGFβ-mediated phosphorylation of Smad2, MKK3/6, and p38MAPK was inhibited in TβRII-K277R cells compared with control Gabe cells (Fig. 4B). TβRII-K277R also blocked EMT (Fig. 4D) and cell migration (Fig. 8A), indicating that TβRII kinase activity is required for these TGFβ responses.

To determine whether the activation of p38MAPK was TGFβ-specific, we expressed wild-type TβRI/Alk5 (Alk5-WT), kinase-inactive Alk5-K232R, or kinase active Alk5-

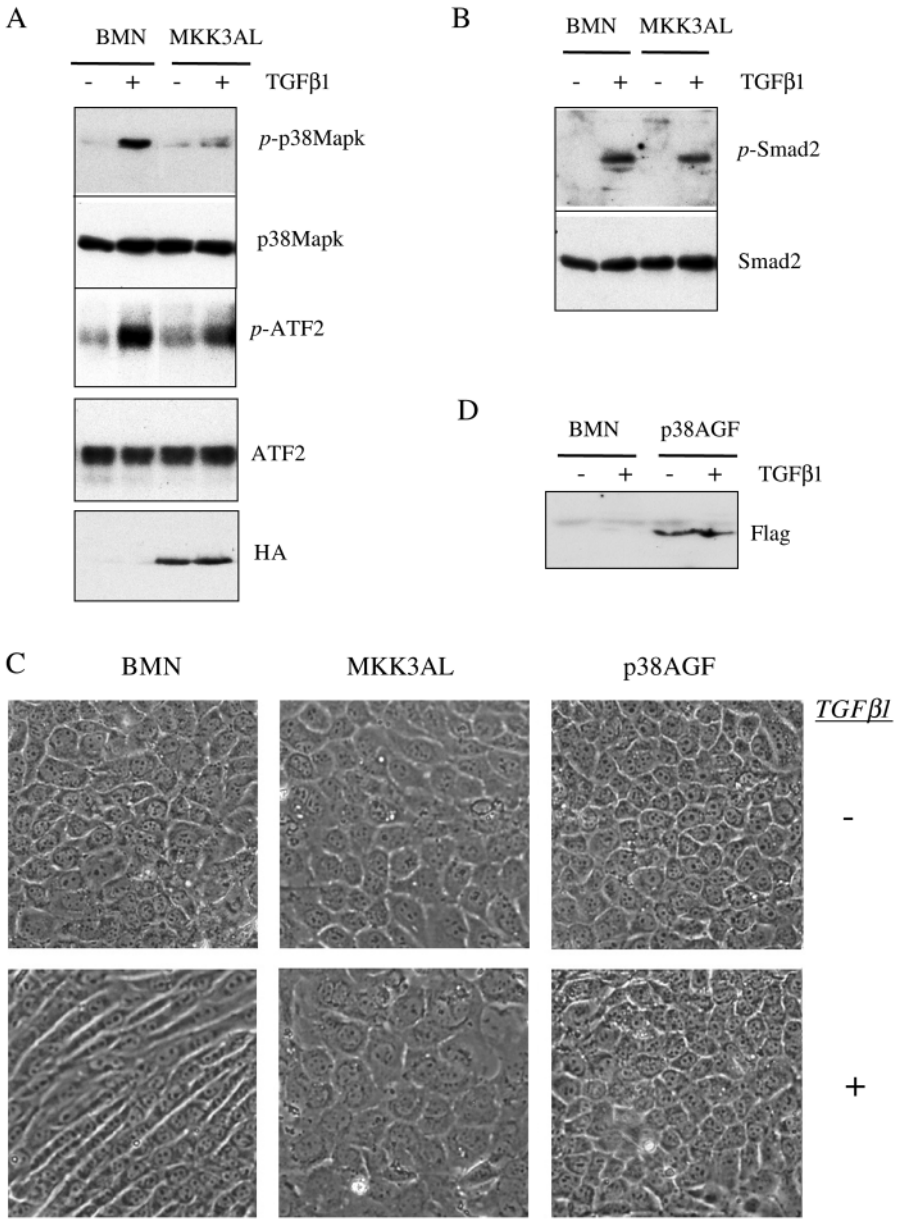

Fig. 5. Effect of dominant-negative MKK3AL and p38AGF on TGFβ-mediated EMT. (A) Immunoblot analysis of p38MAPK and ATF2 phosphorylation in NMuMG cells transfected with empty vector (BMN) or plasmid encoding HA-tagged MKK3AL. Thirty-six hours after transfection cells were treated with 2 ng/ml TGFβ1 for 60 minutes. Whole-cell extracts were probed with phospho-specific antisera, and re-probed with antisera to total protein. Expression of MKK3AL was detected with antisera to the HA-epitope. (B) Immunoblot detection of Smad2 phosphorylation in MKK3AL-expressing cells. (C) Phase-contrast images of NMuMG cells infected with control (BMN) retrovirus or retroviruses encoding dominant-negative MKK3 (MKK3AL) or p38α (p38AGF). Cells were untreated (top row) or treated with 2 ng/ml TGFβ1 for 24 hours. Images were recorded at 200× magnification. (D) Immunoblot detection of Flag-tagged p38AGF in NMuMG cells infected with p38AGF encoding retrovirus compared with control retrovirus (BMN).

T204D (Kawabata et al., 1995) in NMuMG cells. Alk5 mutants were expressed using a bi-cistronic retroviral vector encoding EGFP. GFP-positive cells were selected by flow cytometry and expression of mutants was confirmed by immunoblot analysis (Fig. 4B). Kinase-inactive Alk5-K232R significantly reduced TGFβ-induced phosphorylation of MKK3/6 and p38MAPK, whereas kinase active Alk5-T204D induced phosphorylation of MKK3/6 and p38MAPK in the absence of added ligand (Fig. 4B). Microscopic studies showed that TGFβ-induced EMT was impaired in cells expressing kinase-inactive Alk5-K232R. Cells expressing Alk5-T204D exhibited a fibroblastic morphology similar to Alk5-WT cells treated with TGFβ for 24 hours (Fig. 4C). Treatment of cells expressing Alk5-T204D with the p38MAPK inhibitor SB202190 reversed these morphological changes.

MKK3/6 kinases mediate activation of p38MAPK and EMT in response to TGFβ

Dual-specificity MKK3 and MKK6 kinases have been implicated in activation of p38MAPK (Raingeaud et al., 1996). Phosphorylation of both kinases is induced by TGFβ or by expression of active Alk5-T204D in NMuMG cells (Fig. 4B). Therefore, we tested the effect of dominant-negative MKK3AL (Huang et al., 1997; Zanke et al., 1996) on TGFβ-mediated activation of p38MAPK and EMT in NMuMG cells. Expression of HA-tagged MKK3AL reduced phosphorylation of endogenous p38MAPK and ATF2 (Fig. 5A), whereas expression and phosphorylation of Smad2 were not affected (Fig. 5B). Similar results were obtained with dominant-negative MKK6AL (data not shown). Next, we examined the effect of MKK3AL on EMT. TGFβ induced EMT in NMuMG cells infected with control retrovirus encoding EGFP only (BMN), whereas EMT was inhibited in MKK3AL-expressing cells (Fig. 5C). SB202190, a p38MAPK inhibitor, blocks activity of p38α and p38β but does not inhibit p38γ and p38δ (Davies et al., 2000). Since, SB202190 blocked EMT (Fig. 2A), we tested the effect of p38AGF, a dominant-negative mutant of p38α, on TGFβ-mediated EMT. TGFβ-induced morphological transformation in NMuMG infected with retroviruses encoding p38AGF was impaired compared with cells infected with control BMN virus (Fig. 5C). These findings suggest that MKK3/6 kinases mediate TGFβ-induced activation of p38MAPK and EMT in NMuMG cells.

p38MAPK is involved in TGFβ-induced reorganization of the actin cytoskeleton

We characterized the effect of p38MAPK inhibitors on reorganization of the actin cytoskeleton in response to TGFβ. Microscopic examination of F-actin by staining with phalloidin-fluorescein showed a cortical arrangement of actin at the cell-cell junctions without significant stress fibers (Fig. 6A). Treatment with TGFβ1 for 24 hours induced formation of actin stress fibers arranged along the largest cell axis. SB202190 did not significantly change the actin organization in TGFβ-untreated cells, but impaired TGFβ-induced formation of actin stress fibers (Fig. 6A). Similar blockade of stress fiber formation was observed in cells pretreated with H-7 (data not shown). Examination of the actin cytoskeleton in MKK3AL cells showed that MKK3AL did not affect the cortical arrangement of actin in untreated cells, but inhibited TGFβ-induced actin stress fiber formation (Fig. 6B). These

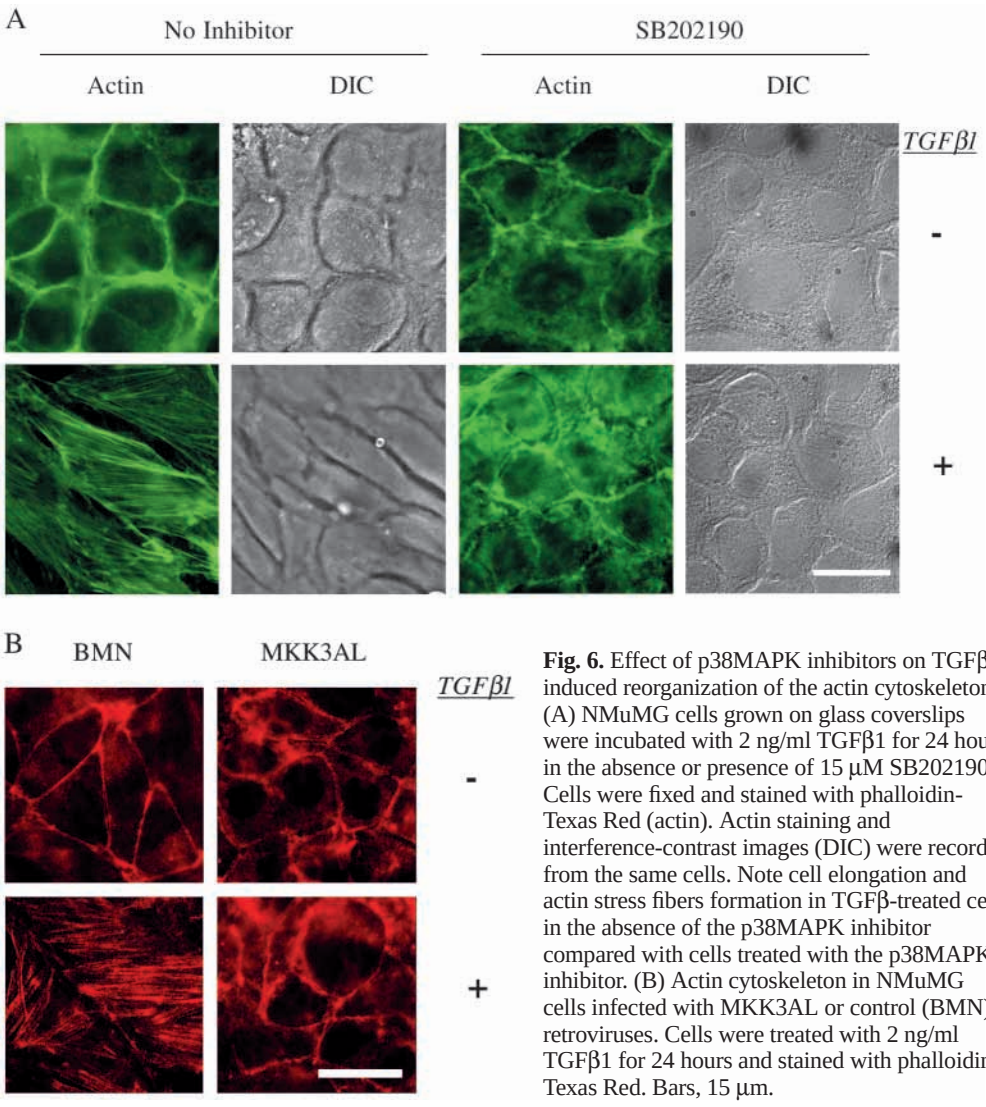

Fig. 6. Effect of p38MAPK inhibitors on TGFβ-induced reorganization of the actin cytoskeleton. (A) NMuMG cells grown on glass coverslips were incubated with 2 ng/ml TGFβ1 for 24 hours in the absence or presence of 15 μM SB202190. Cells were fixed and stained with phalloidin-Texas Red (actin). Actin staining and interference-contrast images (DIC) were recorded from the same cells. Note cell elongation and actin stress fibers formation in TGFβ-treated cells in the absence of the p38MAPK inhibitor compared with cells treated with the p38MAPK inhibitor. (B) Actin cytoskeleton in NMuMG cells infected with MKK3AL or control (BMN) retroviruses. Cells were treated with 2 ng/ml TGFβ1 for 24 hours and stained with phalloidin-Texas Red. Bars, 15 μm.

data suggest that p38MAPK contributes to the reorganization of the actin cytoskeleton induced by TGFβ during EMT.

Rac GTP-binding protein is involved in TGFβ-induced activation of p38MAPK and EMT

There is evidence that small GTP-binding proteins are involved in TGFβ signaling (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). Rac1 and CDC42 have been implicated in the activation of the MKK3/6-p38MAPK cascade in several systems (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). To test whether Rac1 or RhoA are involved in p38MAPK activation in response to TGFβ, we transfected dominant-negative RhoAN19 or Rac1N17 in NMuMG cells. Rac1N17 inhibited TGFβ1-induced phosphorylation of p38MAPK and its downstream substrate ATF2, whereas neither dominant-negative RhoAN19 nor constitutively active RhoAQ63L did not affect p38MAPK phosphorylation (Fig. 7A,B). These data suggest that Rac1 mediates p38MAPK activation in response to TGFβ.

To examine whether Rac1 activity is induced by TGFβ, we performed affinity precipitation assays using a fusion protein of the GTPase-binding domain (amino acids 67-152) from human PAK1 (PBD) and GST. The GST-PBD fusion protein has been shown to specifically bind active Rac1 loaded with GTP (Benard et al., 1999). Treatment of NMuMG cells for 15 minutes with TGFβ resulted in the increase in Rac1 binding to purified GST-PBD (Fig. 7C). GST-PBD effectively interacted with the active GTPγS-bound form of Rac1 but did not bind to the inactive GDP-bound form of Rac1 (Fig. 7C, left inset). To confirm that TGFβ receptors can mediate activation of Rac1, we expressed mutants of Alk5-TβRI in HEK293T cells. Kinase-inactive Alk5K232R reduced the level of active Rac1, whereas kinase-active Alk5T204D increased the amount of Rac1 bound to GST-PBD. Expression of dominant-negative Rac1N17 reduced the amount of Rac1 recovered from GST-PBD beads (Fig. 7D). Since active Rac1

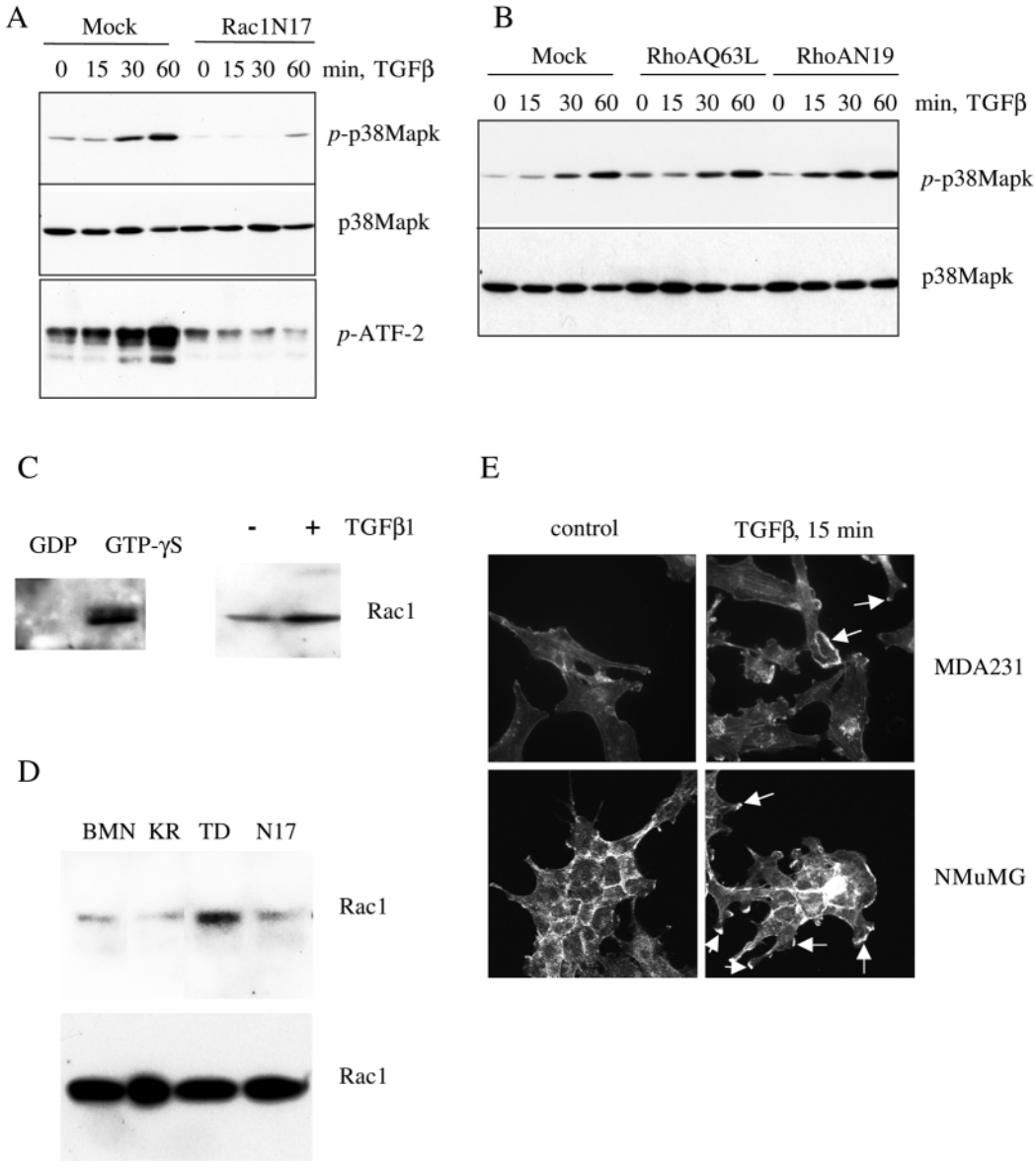

Fig. 7. Rac1 is involved in TGFβ-mediated activation of p38MAPK. (A) Immunoblot analysis of p38MAPK and ATF2 phosphorylation in cells expressing Rac1N17 and treated with 2 ng/ml TGFβ1. (B) p38MAPK phosphorylation in cells expressing RhoAN19 or RhoAQ63L. (C) NMuMG cells were treated with 2 ng/ml TGFβ1 for 15 minutes. Cell lysates were clarified and used for affinity precipitation with 8 μg of GST-PBD. Proteins bound to GST-PBD were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with antibody to Rac1. The inset at the top-left shows the total signal detected using cell lysate pre-exchanged with either GTPγS or GDP as described in Materials and Methods. (D) 293T cells were transfected with control plasmid (BMN), kinase-inactive Alk5K232R, kinase-active Alk5T204D or dominant-negative Rac1N17. Cells were lysed 48 hours after transfection. Cell lysates were clarified and used for affinity precipitation with 8 μg of GST-PBD as described above. The bottom inset shows the Rac1 signal detected in total cell lysates. (E) Confocal images of F-actin in NMuMG cells treated with 2 ng/ml TGFβ1 for 15 minutes and stained with phalloidin-Texas Red. Arrows indicate the spots of actin polymerization at the cell edges.

mediates actin ruffling and lamellipodia formation (Hall, 1998), we examined F-actin in NMuMG and MDA-MB-231 cells treated with 2 ng/ml of TGFβ1 for 15 minutes. Confocal microscopy of cells stained with phalloidin-Texas Red showed that TGFβ induced actin ruffles, a phenotype associated with active Rac (Fig. 7E).

In order to examine the role of Rac1 in EMT, NMuMG cells were infected with a retrovirus encoding dominant-negative Rac1N17 and Green Fluorescent Protein (GFP). Immunoblot analysis showed at least twofold higher levels of Rac1 in cells infected with Rac1N17 retrovirus compared with cells infected with control BMN virus encoding GFP only (Fig. 8A). TGFβ induced phosphorylation of MKK3/6 and p38MAPK in cells infected with control retrovirus whereas, in Rac1N17 cells, this induction was significantly reduced (Fig. 8B). Rac1N17 did not significantly affect TGFβ-dependent phosphorylation of Smad2 (Fig. 8C). Microscopic examination showed that TGFβ1 induced cell elongation and the formation of actin stress fibers in control BMN cells, whereas these effects were impaired in cells expressing Rac1N17 (Fig. 8D). These findings suggest that Rac1 is involved in TGFβ-induced EMT and activation of p38MAPK.

p38MAPK inhibitors block TGFβ-mediated cell motility

TGFβ stimulates chemotaxis and migration of tumor and nontumor cells (Ashcroft et al., 1999; Postlethwaite et al., 1987). Recent studies implicated p38MAPK in TGFβ-induced chemotaxis of human neutrophils (Hannigan et al., 1998). We next tested the effect of p38MAPK inhibitors on TGFβ-mediated migration of NMuMG (nontumor) and MDA-MB-231 (tumor) cells. TGFβ stimulated approximately threefold the chemotactic migration of NMuMG cells through polycarbonate filters (Fig. 9A). Migration of NMuMG cells was significantly inhibited by SB202190, as were NMuMG cells infected with kinase-inactive TGFβ type II receptor (TβRII-K277R) compared with those infected with control Gabe retrovirus (Fig. 9A). TGFβ stimulated approximately sixfold migration of breast cancer MDA-MB-231 cells. This was also blocked by SB202190 (Fig. 9B).

To investigate further the role of p38MAPK in TGFβ-mediated cell migration, wounds were made in confluent cultures of MDA-MB-231 and 4T1 breast cancer cells. These cells are not growth inhibited by TGFβ1. Addition of TGFβ1 to serum-free medium accelerated the wound closure in both cell lines, whereas in the presence of the p38MAPK inhibitor

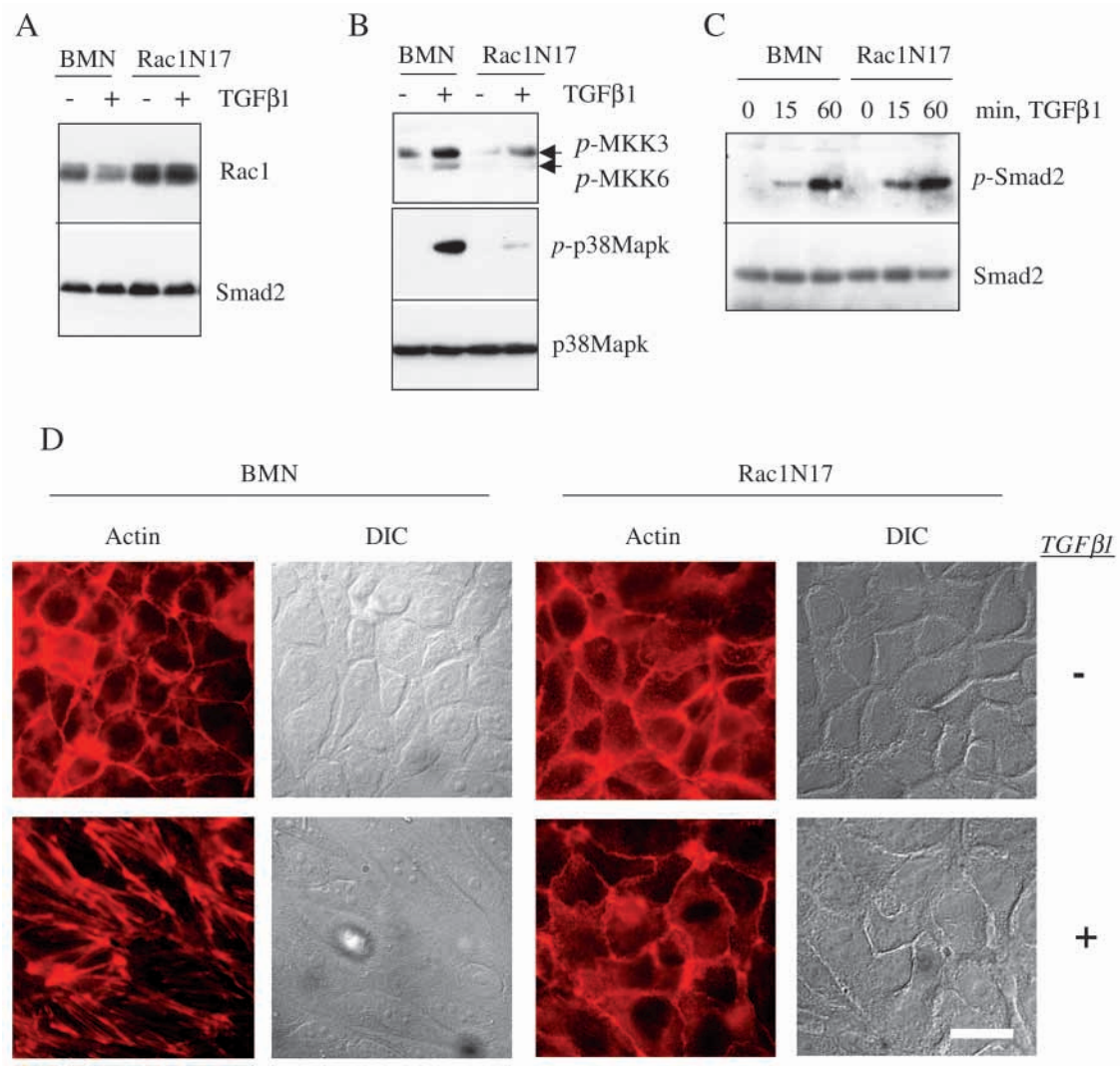

Fig. 8. Dominant-negative Rac1N17 blocks TGFβ-mediated activation of p38MAPK and EMT. NMuMG cells were infected with retrovirus encoding Rac1N17 or control virus (BMN) and treated with 2 ng/ml TGFβ1. (A) Detection of Rac1N17 expression with antisera to Rac1. Cells infected with Rac1N17 show a higher level of Rac1 expression. (B) Immunoblot detection of MKK3/6 and p38MAPK phosphorylation in control (BMN) and Rac1N17 expressing cells. (C) Immunoblot with antisera to phospho-Smad2 and total Smad2. (D) Microscopic images from NMuMG cells infected with control retrovirus (BMN) or retrovirus encoding Rac1N17. Cells grown on glass coverslips were treated with 2 ng/ml TGFβ1 for 24 hours. Cells were stained with phalloidin-Texas Red (actin). Actin and interference-contrast images (DIC) were recorded from the same cells. Bar, 15 μm.

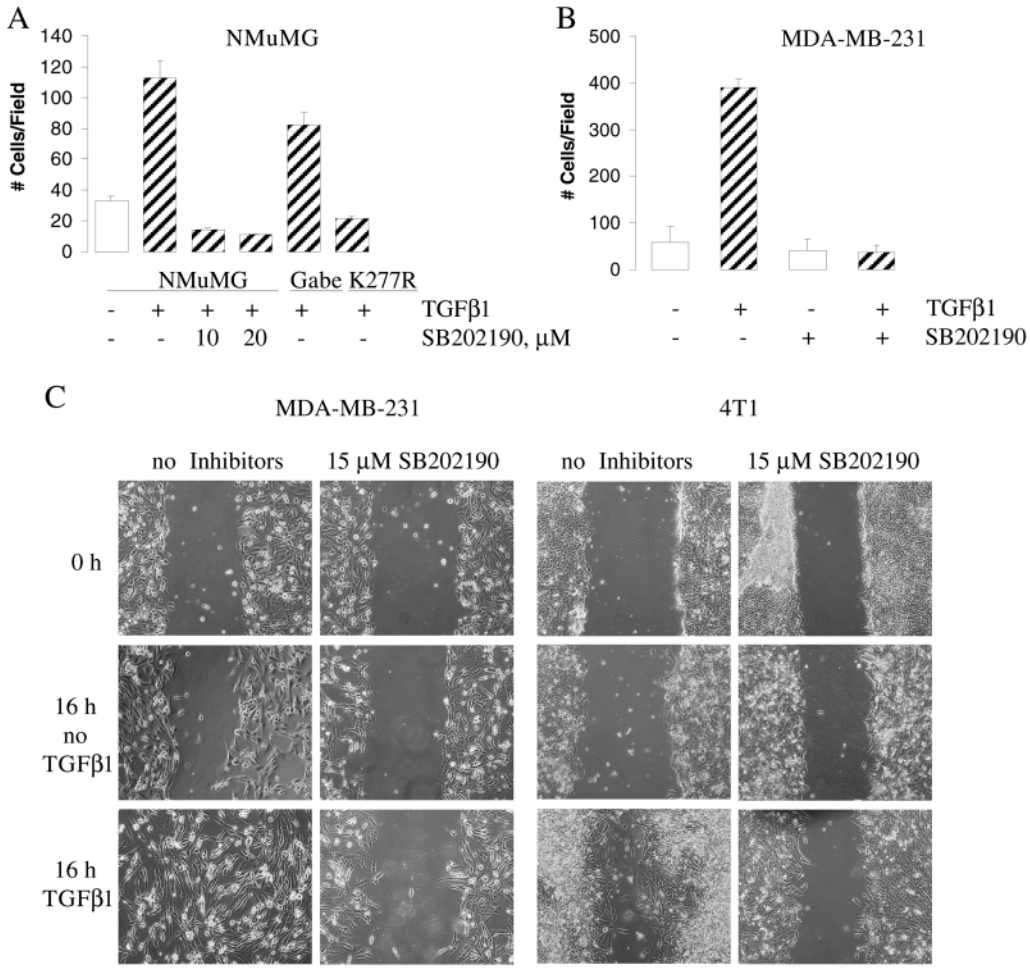

Fig. 9. Involvement of p38MAPK in TGFβ-mediated cell migration. (A,B) NMuMG or MDA-MB-231 cells (1×10^5/well) were seeded in the upper chamber of 5 μm pore transwells and 2 ng/ml TGFβ1 was added to the lower chamber. Cells were incubated for 16 hours in the absence or presence of SB202190, a p38MAPK inhibitor. Cells migrating through pores were stained and counted from four random fields. Experiments were performed in duplicates. Values are the mean ±s.d. of cells per field. Migration of NMuMG cells expressing kinase-inactive TβRII-K277R was compared with cells infected with control Gabe virus. (B) Blockade of MDA-MB-231 cell migration with 10 μM SB202190. (C) Wound closure in monolayers of MDA-MB-231 and 4T1 cells following 16 hours of treatment with 2 ng/ml TGFβ1 in the absence or presence of 10 μM SB202190. Phase contrast images were recorded at 100× magnification. Similar results were obtained three times.

the wounds stayed opened (Fig. 9D). These data suggest that p38MAPK is involved in TGFβ-induced cell migration.

Discussion

TGFβ can induce mesenchymal transdifferentiation in epithelial and endothelial cells (Boyer et al., 1999; Brown et al., 1999; Miettinen et al., 1994). Early studies have shown that protein kinase inhibitor H-7 blocks TGFβ-induced EMT but a particular signaling cascade affected by H-7 was not identified (Miettinen et al., 1994). We found that H-7 inhibited TGFβ-induced phosphorylation of MKK3/6 kinases, but did not affect phosphorylation of Smad2 and Smad-dependent transcriptional responses. These results suggest that H-7 affects a kinase that mediates signaling downstream of TGFβ receptors, but upstream of MKK3/6 kinases. This kinase is distinct from typical PKCs, since BIM-I, an inhibitor of typical PKCs, did not block TGFβ-induced EMT and phosphorylation of MKK3/6 and p38MAPK. The candidate kinases include atypical PKCs and kinase(s) implicated in activation of MKK3/6 such as PAK1 (Zhang et al., 1995), TAK1 (Yamaguchi et al., 1995), and MLK3 (Tibbles et al., 1996).

The H-7 studies suggested a critical role for the p38MAPK pathway in EMT. This hypothesis was further tested using the p38MAPK specific inhibitors, SB202190 and SB203580, which do not inhibit JNK, MEK1/2 and ERK1/2 kinases (Davies et al., 2000). SB202190 and SB203580 blocked TGFβ-induced cell morphological changes in NMuMG mouse mammary epithelial cells and SiHa human cervical carcinoma cells. The p38MAPK inhibitors blocked TGFβ-induced phosphorylation of ATF2, a p38MAPK substrate, without effect on Smad2 phosphorylation, implying that under these experimental conditions the blockade of p38MAPK did not affect TGFβ receptor kinase activity.

To test whether activation of p38MAPK by TGFβ is a direct event, we investigated the kinetics of activating phosphorylation of MKK3/6 and p38MAPK. TGFβ induced phosphorylation of Smad2 and MKK3/6 kinases with similar kinetics (15 minutes). Phosphorylation of p38MAPK was delayed (30 minutes) suggesting that this event requires activation of MKK3/6. We further showed that dominant-negative mutants of MKK3 and MKK6 interfering with p38MAPK activation (Raingeaud et al., 1996) impaired TGFβ-induced phosphorylation of p38MAPK and ATF2, indicating that the MKK3/6-p38MAPK module mediates TGFβ signaling in NMuMG cells. The dose-dependent increase in p38MAPK activity was confirmed by in vitro kinase assay and by phosphorylation of ATF2.

To confirm the specificity of TGFβ signaling to p38MAPK we performed studies with TGFβ receptor mutants. Kinase-inactive type II receptor blocked EMT and phosphorylation of Smad2 as well as MKK3/6 and p38MAPK, indicating that

kinase function of TβRII is required for activation of p38MAPK and EMT. Kinase-inactive TβRI/Alk5-K232R also blocked TGFβ-induced activation of the p38MAPK pathway, whereas expression of kinase active Alk5-T204D resulted in phosphorylation of MKK3/6 and p38MAPK and EMT in the absence of added TGFβ1. Thus, kinase activities of both TGFβ receptors are required for TGFβ-induced activation of the p38MAPK pathway, and Alk5-T204D can signal to p38MAPK in the absence of added ligand. Alk5-T204D-induced EMT was inhibited by SB202190, a p38MAPK inhibitor, suggesting that p38MAPK mediates EMT induced by Alk5-T204D.

Activation of p38MAPK is mediated by Rac1/CDC42 GTP-binding proteins (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). Small GTP-binding proteins are also involved in TGFβ responses (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). We found that dominant-negative Rac1N17 impaired activation of the p38MAPK pathway in NMuMG cells, whereas RhoAN19 did not block this event. Expression of Rac1N17 did not affect phosphorylation of Smad2. These data suggest that Rac1 mediates TGFβ-induced p38MAPK activation independently of Smad activation. The mechanism of downstream signaling events is unclear. Previous studies showed that PAK1 mediates p38MAPK activation downstream of Rac1 and CDC42 (Zhang et al., 1995). Furthermore, TGFβ-activated kinase 1 (TAK1), has been implicated in p38MAPK activation in response to BMP and TGFβ in several cell systems (Yamaguchi et al., 1995).

Expression of dominant-negative Rac1N17 in NMuMG cells inhibited TGFβ1-induced changes in cell shape and the actin cytoskeleton suggesting involvement of Rac1 in TGFβ-induced EMT. This result is consistent with other reports. For example, both D-Rac and D-p38 have been reported to contribute to Dpp signaling during wing morphogenesis in *Drosophila* (Adachi-Yamada et al., 1999; Eaton et al., 1995). There is also evidence that Rac1 is required for EMT induced by hepatocyte growth factor (HGF) in MDCK cells (Ridley et al., 1995; Royal et al., 2000). Dominant-negative Rac/CDC42 mutants inhibit oncogenic Ras-induced cell transformation (Qiu et al., 1997; Qiu et al., 1995), and Ras has been shown to cooperate with TGFβ in the induction of EMT (Oft et al., 1996). In addition, Rho/Rac/CDC42 proteins are involved in morphogenesis by regulating the actin cytoskeleton (Hall, 1998). Therefore, Rac1 may contribute to TGFβ-induced EMT via its effects on the cell cytoskeleton and/or via activation of the p38MAPK pathway. In NMuMG cells, TGFβ1 induced actin ruffles and activation of Rac1 within 15 minutes (Fig. 7C,E). Expression of kinase-inactive Alk5K232R reduced, whereas constitutively active Alk5-T204D increased, Rac1 loading with GTP (Fig. 7D) and induced the formation of strong actin ruffles (data not shown). These results suggest that Rac activation and actin ruffling induced by TGFβ may precede the formation of actin stress fibers, which does not occur until 4 hours after addition of TGFβ1 (Bhowmick et al., 2001a).

Inhibitors of p38MAPK and dominant-negative MKK3AL impaired TGFβ-induced changes in cell morphology and reorganization of the actin cytoskeleton. Expression of the dominant-negative mutant of p38α also blocked TGFβ-mediated EMT. Together, these results suggest that the p38MAPK pathway contributes to TGFβ-induced alterations in the actin cytoskeleton and the cell shape during EMT. Consistent with this hypothesis, p38MAPK has been shown to mediate regulation of the actin cytoskeleton in smooth muscle myocytes in response to TGFβ (Hedges et al., 1999), and in H_2O_2-induced rapid reorganization of the actin cytoskeleton in endothelial and mesenchymal cells (Huot et al., 1998). A recent study reported involvement of p38MAPK in TGFβ-mediated EMT (Bhowmick et al., 2001b). In this report, adenoviral transduction of dominant-negative p38β inhibited TGFβ-mediated EMT at the step of disruption of junctional complexes but did not alter actin reorganization. We found that p38MAPK inhibitors and dominant-negative MKK3AL affected actin stress fiber formation (Fig. 6). TGFβ and Alk5T204D activated both MKK3 and MKK6 in NMuMG cells (Figs 3, 4). This suggests that TGFβ may activate multiple p38MAPK isoforms in NMuMG cells as MKK3 preferentially activates p38α and p38γ, while MKK6 activates p38MAPKs α, β and γ (Enslen et al., 1998). Recent studies showed that p38α and p38β may have different functions (Wang et al., 1998) and different subcellular localization (Lee et al., 2000). p38MAPK inhibitors block activity of both p38α and p38β (Enslen et al., 1998) and MKK3AL impaired phosophorylation of p38MAPK in NMuMG cells as measured with an antibody that recognizes both α and β isoforms. Therefore, multiple p38MAPKs may be involved in TGFβ-induced EMT and mediate different aspects of EMT, potentially explaining the discrepancies with previous studies (Bhowmick et al., 2001b).

EMT is a complex process involving restructuring of the cell cytoskeleton, cell membrane and cell-cell junctions. Previous studies implicated several molecules in different aspects of EMT. Smad transcription factors have been shown to synergize with Alk5 in induction of EMT but no specific function has been associated with these factors (Piek et al., 1999a). PI3K/Akt may contribute to dissolution of tight junctions and to TGFβ transcriptional responses (Bakin et al., 2000). RhoA/Rock signaling has been implicated in the actin stress fiber formation (Bhowmick et al., 2001a). What aspect of EMT can be mediated by p38MAPK? p38MAPK can regulate the actin organization via HSP27 (Hedges et al., 1999; Huot et al., 1998). Therefore, p38MAPK may function in TGFβ-induced reorganization of the actin cytoskeleton in parallel or upstream of the RhoA/Rock pathway since dn-RhoA and Y27632, a Rock kinase inhibitor, did not affect activation of p38MAPK by TGFβ (data not shown). In addition, p38MAPK may contribute to the expression of TGFβ target genes that are casually involved in EMT because p38MAPK has been implicated in TGFβ-transcriptional responses by activating ATF2 and Sp1 (Park et al., 2000; Raingeaud et al., 1996; Sano et al., 1999).

Finally, we investigated the role of p38MAPK in TGFβ-induced migration of mouse and human mammary epithelial cells. The p38MAPK inhibitors blocked TGFβ-stimulated migration of NMuMG, MDA-MB-231 and 4T1 cells. These results are consistent with the proposed role of p38MAPK in TGFβ-mediated chemotaxis of human neutrophils (Hannigan et al., 1998) and smooth muscle cells (Hedges et al., 1999). Interestingly, Smad3-deficient keratinocytes and monocytes are impaired in the chemotactic response to TGFβ (Ashcroft et al., 1999), whereas p38MAPK inhibitors did not affect Smad2 phosphorylation (Fig. 2). These data suggest that the p38MAPK pathway may act in parallel or in cooperation with a Smad-dependent pathway in chemotactic responses to TGFβ.

The data presented suggest that p38MAPK signaling plays a critical role in TGFβ-induced EMT and cell migration. This pathway may be considered as a potential target of therapeutic interventions in neoplastic and inflammatory disorders associated with TGFβ-mediated EMT.

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