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13. ABSTRACT (Maximum 200 Words)

Ovarian cancer is the leading gynecological cancer that results in death. Due to the lack of symptom in the early stage of disease, most ovarian cancers are diagnosed in the late stage. Current therapy such as chemotherapy is not very effective for curing ovarian cancer. This proposal aims to develop an adenovirus-based gene therapy for suppression ovarian malignancies. In our studies, we have found that the interaction between vitronectin and αvβ3 integrin is essential for ovarian cancer cell survival and invasion. We thus developed potent small interfering RNA targeting vitronectin and β3 integrin subunit. We inserted these specific siRNAs to adenovirus vector and test whether Ad-delivered siRNA could inhibit ovarian malignancies. We demonstrate that Ad-delivered siRNA can significantly reduce ovarian cancer cell survival and invasiveness as determined by in vitro assays. Using SCID mouse model, we further show that Ad-delivered siRNA also inhibit in vivo tumor development and prolong animal survival. Our study provide the basis to develop an alternative therapeutic modality for ovarian cancer.

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Introduction

This proposal is based on our previous finding that the expression of vitronectin and αvβ3 integrin and their interaction promotes ovarian cancer cell survival in suspension condition. In the studies performed in the first year of the funding period, we demonstrate that the interaction of vitronectin and αvβ3 integrin induces NF-κB activation and the induced NF-κB activity is essential for vitronectin/αvβ3 integrin-mediated ovarian cancer cell survival. In the second year of the funding period, we successfully developed specific and potent short interfering RNA (siRNA) against vitronectin and β3 integrin subunit. In the third year of the funding period, our goal was to use recombinant adenovirus to deliver vitronectin and β3 integrin subunit-specific siRNA to ovarian cancer cells and further evaluate the efficacies of the Ad-delivered siRNAs to suppress ovarian tumorigenecity in both *in vitro* and *in vivo* experimental models.

Body

In the previous year, we have developed mammalian siRNA vectors for both vitronectin and β3 integrin subunit (pSUPER/VN and pSUPER/β3). To prepare recombinant Ad vector containing siRNA, we cleaved vitronectin and β3 siRNA expression cassettes from these pSUPER vectors using BamH I/Kpn I. The released fragments were cloned in adenovirus shuttle vector, pShuttle (QBIOGEN), and subsequently cotransformed with pAd.Easy-1 (QBIOGEN) into recombination-competent *E.Coli* strain BJ5183. After analyzing the formed colonies, the plasmids with correct recombination were transfected into 293 cells to obtain siRNA-containing Ad vectors. The abilities of these siRNA-Ad vectors to downregulate vitronectin and β3 integrin expression were determined in ovarian cancer OVCAR5 cells and over 90% of inhibition in vitronectin and β3 integrin expression was detected with cells infected with Ad/VN-siRNA and Ad/β3-siRNA (Fig.1). These results demonstrate that siRNA can be successfully delivered to ovarian cancer cells to block targeted gene expression.

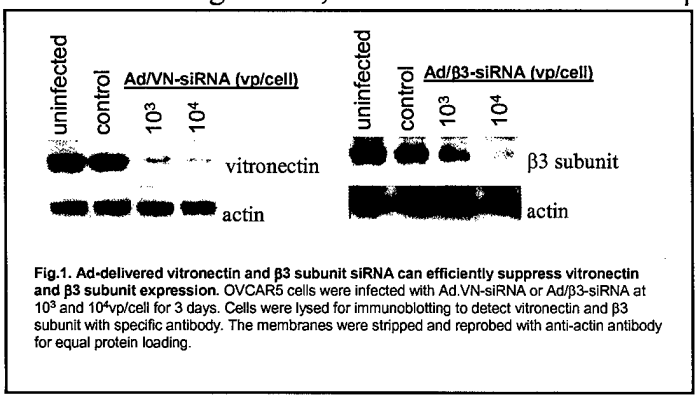

Fig.1. Ad-delivered vitronectin and β3 subunit siRNA can efficiently suppress vitronectin and β3 subunit expression. OVCAR5 cells were infected with Ad.VN-siRNA or Ad/β3-siRNA at 10^3 and 10^4vp/cell for 3 days. Cells were lysed for immunoblotting to detect vitronectin and β3 subunit with specific antibody. The membranes were stripped and reprobed with anti-actin antibody for equal protein loading.

We next examined the ability of Ad-delivered siRNAs to induce cell death in suspension. OVCAR5 cells were infected by either Ad/VN-siRNA or Ad/β3-siRNA or together for 48 hrs, and subsequently cultured in non-adhesion condition (in polyHEMA-treated surface). At varying times (1-4 days), a portion of cell suspension was counted under microscope with trypan blue. The expression of vitronectin and β3 integrin subunit siRNA resulted in 40 and 65% of reduction in cell number comparing to the control cells. The combined expression of both vitronectin and β3 integrin subunit siRNAs led to even greater reduction in cell number (78%) (Fig.2A). These results suggest that vitronectin and β3 integrin subunit siRNAs are capable of suppressing ovarian cancer cell growth in

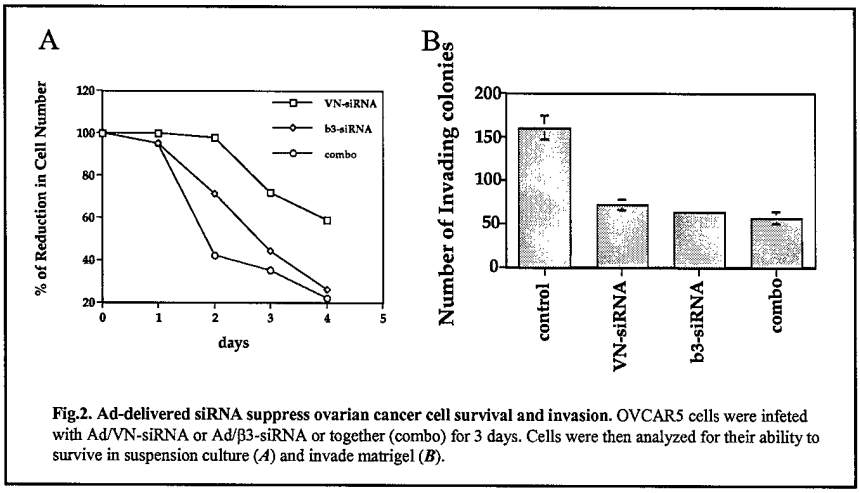

Fig.2. Ad-delivered siRNA suppress ovarian cancer cell survival and invasion. OVCAR5 cells were infeted with Ad/VN-siRNA or Ad/β3-siRNA or together (combo) for 3 days. Cells were then analyzed for their ability to survive in suspension culture (*A*) and invade matrigel (*B*).

suspension condition. In a parallel experiment, we also determine the effect of vitronectin and β3 integrin subunit siRNAs on OVCAR5 cell invasiveness using matrigel invasion chamber (Becton-Dickson). Vitronectin and β3 integrin subunit siRNAs blocked 55% and 61% of OVCAR5 cell invasion respectively, and these two siRNA together conferred 65% inhibition in the ability of OVCAR5 cells to invade matrigel (Fig.2B). These results suggest that vitronectin and β3 integrin subunit siRNAs are also capable of inhibiting ovarian cancer cell invasion.

We also investigated the ability of vitronectin and β3 integrin subunit siRNA vectors to inhibit ovarian cancer cell growth using SCID mouse model. OVCAR5 cells (10^7 cells/mouse) were intraperitoneally injected to animal and tumors were observed four weeks after OVCAR5 cell injection. We divided animals into four experiment groups (12 animals in each group): animals receiving control virus alone, animals receiving Ad/VN-siRNA, animals receiving Ad/β3-siRNA, and animals receiving the combination of Ad/VN-siRNA and Ad/β3-siRNA. At four weeks after animals receiving Ad vectors, six animals from each group were sacrificed, the tumors were excised and weighed. Animals receiving Ad/VN-siRNA, Ad/β3-siRNA and combination of both Ad vectors displayed the reduction in tumor mass of 51%, 67%, and 69% respectively comparing to animals receiving the control virus (Fig.3A). These results suggest vitronectin and β3 integrin subunit siRNA are capable of suppressing ovarian cancer development.

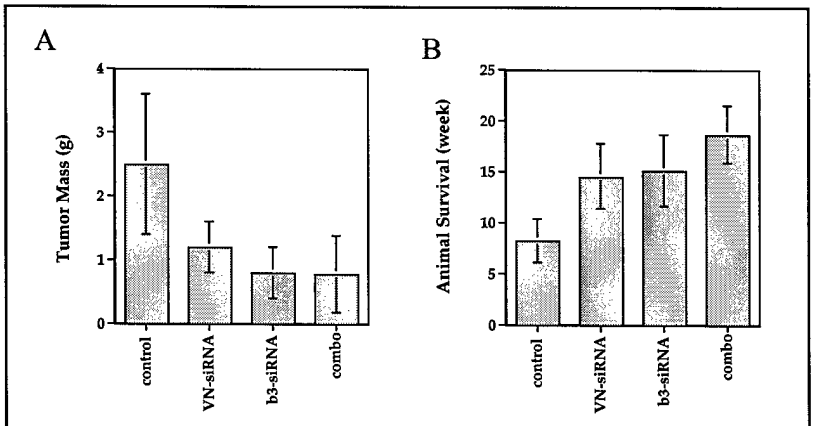

Fig.3. Ad-delivered siRNA suppress ovarian malignancies. OVCAR5 cells were injected to SCID mice and tumors were allowed to form for 4 weeks. Control Ad or Ad vectors containing vitronectin or β3 integrin subunit siRNA were then injected into peritonium. Animals were either sacrificed at 4 weeks to measure the tumor weight (*A*) or allowed to live until they die (*B*).

To determine the effect of siRNA Ad vectors on the survival of tumor-bearing SCID mice, we monitored the remaining six animals in each group daily until their death. Animals receiving control virus lived average of another 8.3

weeks. In contrast, animals receiving Ad/VN-siRNA and Ad/β3-siRNA lived average of 14.6 and 15.2 weeks respectively (Fig.3B). In six animals receiving the combination of both Ad vectors, two animals are still alive after 21 weeks. The four deceased mice lived average of 18.7 weeks (Fig.3B). These results suggest that downregulating vitronectin and αvβ3 integrin expression using Ad-delivered siRNA can significantly prolong ovarian cancer-bearing animal survival.

Key Research Accomplishment

- We have constructed Ad vector containing vitronectin and β3 integrin subunit siRNAs. Infecting ovarian cancer cells with these siRNA Ad vectors resulted in significant reduction in vitronectin and β3 integrin subunit expression. Moreover, ovarian cancer cells treated with these Ad vectors showed reduced ability to survive in suspension culture and to invade matrigel. Using SCID mouse model, we found that these Ad vector can inhibit tumor development and prolong animal survival.

Reportable Outcomes

One published manuscript was partially supported by this grant:

Han, Q., Leng, J., Bian, D., Mahanivong, C., Carpenter, K.A., Pan, Z.K., Han, J., and **Huang, S.** (2002). Rac1-MKK3-p38-MAPKAPK2 signaling pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. *J.Biol.Chem.*, 277:48379-48385.

Two other manuscripts currently in preparation are also partially supported by this grant. Personnel receiving pay from this research effort were: Shuang Huang, Ph.D., Jian Chen, Ph.D., Robert Cheng and Qiwei Han

Conclusions

We have generated Ad vectors containing vitronectin and β3 integrin subunit siRNAs. In our experiments, we found that these vectors can suppress ovarian cancer cell tumorigenicity. With further validation on the efficacies of these vectors to suppress ovarian malignancies, these vectors may provide an alternative therapeutic modality for ovarian cancer treatment.

References

N/A

Appendices

Han, Q., Leng, J., Bian, D., Mahanivong, C., Carpenter, K.A., Pan, Z.K., Han, J., and **Huang, S.** (2002). Rac1-MKK3-p38-MAPKAPK2 signaling pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. *J.Biol.Chem.*, 277:48379-48385.

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Rac1-MKK3-p38-MAPKAPK2 Pathway Promotes Urokinase Plasminogen Activator mRNA Stability in Invasive Breast Cancer Cells*

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We reported previously that down-regulating or functionally blocking αv integrins inhibits endogenous p38 mitogen-activated protein kinase (MAPK) activity and urokinase plasminogen activator (uPA) expression in invasive MDA-MB-231 breast cancer cells whereas engaging αv integrins with vitronectin activates p38 MAPK and up-regulates uPA expression (Chen, J., Baskerville, C., Han, Q., Pan, Z., and Huang, S. (2001) *J. Biol. Chem.* 276, 47901–47905). Currently, it is not clear what upstream and downstream signaling molecules of p38 MAPK mediate αv integrin-mediated uPA up-regulation. In the present study, we found that αv integrin ligation activated small GTPase Rac1 preferentially, and dominant negative Rac1 inhibited αv integrin-mediated p38 MAPK activation. Using constitutively active MAPK kinases, we found that both constitutively active MKK3 and MKK6 mutants were able to activate p38 MAPK and up-regulate uPA expression, but only dominant negative MKK3 blocked αv integrin-mediated p38 MAPK activation and uPA up-regulation. These results suggest that MKK3, rather than MKK6, mediates αv integrin-induced p38 MAPK activation. Among the potential downstream effectors of p38 MAPK, we found that only MAPK-activated protein kinase 2 affects αv integrin-mediated uPA up-regulation significantly. Finally, using β-globin reporter gene constructs containing uPA mRNA 3′-untranslated region (UTR) and adenosine/uridine-rich elements-deleted 3′-UTR, we demonstrated that p38 MAPK/MAPK-activated protein kinase 2 signaling pathway regulated uPA mRNA stability through a mechanism involving the adenosine/uridine-rich elements sequence in 3′-UTR of uPA mRNA.

Urokinase plasminogen activator (uPA)[1] is overexpressed in various malignancies including breast, ovarian, and colon cancer (1–6). Both *in vitro* and *in vivo* studies have demonstrated clearly that the levels of uPA are associated closely with the degree of tumor cell invasion and that uPA plays a key role in tumor progression and metastasis (7–15). When uPA binds to its cell surface receptor, uPA rapidly converts plasminogen into plasmin, which then degrades a variety of extracellular matrix proteins and also initiates the activation of a metalloproteinase cascade (16, 17). In addition, the interaction of uPA with the uPA receptor also mediates a variety of cell responses including adhesion, migration, proliferation, and transcription of specific genes (18–26), and these processes have potential impact in tumor invasion and metastasis.

The p38 MAPKs include four isoforms (α, β, γ, and δ) (27, 28). Despite the structural similarity among the members of p38 MAPK family, differences in activation profile and substrate specificity have been observed (28, 29). The p38 MAPKs can be activated by a wide spectrum of stimuli, including cellular stress, proinflammatory cytokines, and growth factors (29, 30). The activation of p38 MAPKs is regulated by upstream MAPKK (MKK3, MKK6, and probably MKK4) via phosphorylation of a TGY phosphorylation site (28, 31). The p38 MAPK effects are carried out by downstream substrates including protein kinases and transcription factors (28, 30). Recently, p38 MAPK signaling pathway has also been demonstrated to play an important role in regulating mRNA stability (32–34).

The concentration of an mRNA is a function of its rates of synthesis and degradation (35). The regulation of mRNA stability is therefore an important means of modulating gene expression. Generally, mRNA stability is controlled by *cis*-acting sequences within 5′- or 3′-untranslated regions (UTRs) or, in some cases, within the coding region (35, 36). The best characterized regulatory elements are the adenosine/uridine-rich elements (ARE) within 3′-UTR of cytokine, growth factor, and proto-oncogene mRNAs, and these elements often contain several copies of the motif AUUUA (36). Attaching ARE within a uridine-rich context results in the instability of otherwise stable reporter mRNA such as β-globin mRNA (37, 38). Furthermore, mRNA stability is regulated by *trans*-acting RNA binding factors that interact with AREs (36), and these include AUF1 (39, 40), HuR (41, 42), and tristetraprolin (32, 43).

Our previous studies showed that engaging αv integrins with vitronectin (Vn) activates p38 MAPK and up-regulates uPA expression in invasive MDA-MB-231 cells and that the expression/function of αv integrins is essential for constitutive p38 MAPK activity and uPA expression in invasive cancer cells

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[1] The abbreviations used are: uPA, urokinase plasminogen activator; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; UTR, untranslated region; ARE, adenosine/uridine-rich element; Vn, vitronectin; mAb, monoclonal antibody; Ad, adenovirus; PBD, Rac1/Cdc42 binding domain; RBD, Rho binding domain; MAPKAPK2, MAPK-activated protein kinase 2; MKK, MAPK kinase; GST, glutathione *S*-transferase; MEK, MAPK/extracellular signal-regulated kinase kinase; MSK, mitogen and stress-activated kinase; MNK, MAPK-interacting kinase; pfu, plaque-forming unit; IL, interleukin; PRAK, p38 MAPK-activated kinase.

(44). We and others (45, 46) have also shown that p38 MAPK regulates uPA expression by promoting uPA mRNA stability. However, the upstream and downstream signaling molecules of p38 MAPK involved in Vn/αv integrin ligation-mediated uPA up-regulation remain to be elucidated. In the present study, we demonstrate that Vn/αv integrin ligation activates Rac1 preferentially and that the Rac1 activity is important for Vn-induced p38 MAPK activation. We also show that although both constitutively active MKK3 and MKK6 enhance uPA expression, only dominant negative MKK3 blocks Vn/αv integrin ligation-induced p38 MAPK activation and uPA up-regulation. Among the potential p38 MAPK downstream kinases, only dominant negative MAPKAPK2 inhibits uPA expression and destabilizes uPA mRNA. In the parallel experiments, we find that constitutively active MAPKAPK2 restores uPA expression and prolongs uPA mRNA stability in p38 MAPK-inhibited MDA-MB-231 cells. These findings suggest that a signaling pathway involving Rac1-MKK3-p38-MAPKAPK2 mediates Vn/αv integrin-mediated uPA up-regulation. In addition, we provide evidence that the ARE-containing 3′-UTR of uPA mRNA is essential for p38 MAPK/MAPKAPK2-regulated uPA mRNA stability.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Polyclonal antibodies to phospho-p38, p38, phospho-MKK3/6, and phospho-MKK4 were purchased from Cell Signaling (Beverly, MA). The polyclonal antibody to uPA was obtained from American Diagnostica (Greenwich, CT). Rac1 monoclonal antibody (mAb), anti-active MAPKAPK2, and MAPKAPK2 polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Cdc42, RhoA mAbs, and MKK3, MKK4, MKK6, myc tag polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p38 MAPK inhibitor, SB203580, and control 201474 were obtained from Calbiochem (San Diego, CA). MDA-MB-231 cell line was obtained from ATCC (Manassas, VA) and was maintained in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2.

Recombinant Adenovirus Construction—To construct recombinant adenovirus (Ad) encoding dominant negative Ras and Rho GTPase, cDNAs for Myc-tagged H-Ras (N17), RhoA (N19), Rac1 (N17), and Cdc42 (N17) cDNAs were cloned into the adenovirus shuttle vector pAd.CI (containing cytomegalovirus promoter). Ad vector containing Myc-tagged wild-type and constitutively active Rac1 (V12) were constructed similarly. To construct Ad vector encoding dominant negative PRAK, MAPKAPK2, MAPKAPK3, MNK1, MSK1 and MSK2, cDNAs of these kinases containing an alanine mutation in lysine residue in the kinase domain were subcloned into pAd.RSV vector. Ad vectors were prepared by cotransfecting these vectors with pJM17 into 293 cells as described previously (47). Construction of the control Ad vector (Ad.RSV) and Ad vectors containing constitutively active or dominant negative MAPKKs have been described elsewhere (47).

Analyzing the Effect of αv Integrin Ligation on the Activity of Rho GTPase—Activated Rho GTPase can be detected by analyzing GTP-bound Rho GTPase. Rac1/Cdc42 and RhoA activities were determined by the recently developed PBD and RBD assays, respectively (48, 49). In Rac1/Cdc42 assays, the PBD from the effector protein p21-activated kinase is used as a probe to specifically isolate the active form of Rac1 or Cdc42 (48). In RhoA assays, the RBD from Rhotekin is used as probe to interact specifically with active RhoA (49). To determine the effect of αv integrin ligation on the activities of Rac1, Cdc42, and RhoA, MDA-MB-231 cells were starved in serum-free medium for 24 h, and 3-cm culture dishes were coated with 2 μg/ml of Vn solution at 4 °C. Cells were detached with Verssene (Invitrogen), kept in suspension in serum-free medium for 1 h and then plated onto Vn-coated dishes. At varying times, cells were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 5% glycerol, 5 mM NaF, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, and proteinase inhibitor mixture). Cell lysates were incubated with GST-PBD or GST-RBD (20 μg) beads at 4 °C for 1 h, and the beads were subsequently washed five times with lysis buffer. Bound Rac1, Cdc42, or RhoA were detected by immunoblotting using specific mAbs, respectively.

Analyzing the Effect of αv Integrin Ligation on the Activities of MAPKKs—To determine the effect of αv integrin ligation on the activities of MAPKKs, MDA-MB-231 cells were starved for 24 h and kept in suspension for 1 h in serum-free medium. Cells were then plated on Vn-coated plates and at varying times lysed in ice-cold radioimmune precipitation assay buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and protease inhibitor mixture). Cell lysates were boiled in non-reducing sample buffer and subjected to immunoblotting to detect total and active MKK3/6 or MKK4 with respective antibodies.

Analysis of p38 MAPK Activity and uPA Expression—To determine the effect of dominant negative Ras, Rho GTPase, and MAPKKs on αv integrin ligation-induced p38 MAPK activity, MDA-MB-231 cells were infected individually with dominant negative H-Ras, RhoA, Rac1, Cdc42, MEK1, MKK3, MKK4, or MKK6 Ad vectors (100 pfu/cell) for 24 h. After another 24 h of serum starvation, cells were plated on Vn-coated surface for 30 min and subsequently lysed for immunoblotting to detect active p38 MAPK with phospho-p38 polyclonal antibody.

To determine the effect of dominant negative p38 MAPK downstream kinases on Vn-up-regulated uPA expression, MDA-MB-231 cells were infected with dominant negative PRAK, MAPKAPK2, MAPKAPK3, MNK1, and MSK1 or MSK2 Ad vector (100 pfu/cell) for 24 h and then plated on Vn-coated dishes and cultured for 24 h. Cells were lysed, and cell lysates were subjected to immunoblotting to detect the levels of uPA. To determine whether constitutive active MAPKAPK2 was able to rescue SB203580-inhibited uPA expression, MDA-MB-231 cells were infected with Ad vector containing constitutive active PRAK (100 pfu/cell) or MAPKAPK2 (10 and 100 pfu/cell) for 24 h. Cells were then treated with SB203580 (5 μM) for 24 h prior to cell lysis and immunoblotting to detect uPA.

Analyzing the Effect of Constitutively Active Rac1 on MKK3 and MKK6 Activities—MDA-MB-231 cells were infected with constitutively active or wild-type Rac1 Ad vector (100 pfu/cell) for 48 h and subsequently lysed in radioimmune precipitation assay buffer. Cell lysates were precleared with Gamma-bind beads (Amersham Biosciences) and then immunoprecipitated with either anti-MKK3 or MKK6 polyclonal antibody. The immunoprecipitates were boiled in sample buffer and subjected to immunoblotting with anti-phospho-MKK3/6 polyclonal antibody to detect the levels of active MKK3 or MKK6, respectively.

Analyzing the Effect of Constitutively Active MKK3 on MAPKAPK2 Activity—To determine the effect of constitutively active MKK3 on MAPKAPK2 activity, MDA-MB-231 cells were infected with constitutively active MKK3 Ad vector (100 pfu/cell) for 48 h and subsequently lysed for immunoblotting to detect active MAPKAPK2 with anti-phospho-MAPKAPK2 antibody.

To determine whether p38 MAPK is involved in MKK3-induced MAPKAPK2 activation, MDA-MB-231 cells were first infected with constitutively active MKK3 or MKK4 Ad vector (100 pfu/cell) for 24 h and followed by addition of SB203580 or SB202474 (5 μM) for another 24 h. Cells were lysed, and the immunoblotting was performed to detect active MAPKAPK2.

uPA mRNA Reporter Plasmids and mRNA Stability Analysis—Plasmid pBBB was provided by Dr. Ann-Bin Shyu (University of Texas School of Medicine, Houston, TX). The entire and truncated forms of uPA 3′-UTR were synthesized by reverse transcriptase PCR using total RNA isolated from MDA-MB-231 cells. The PCR products were digested with *Bam*HI or *Bam*HI/*Bgl*II and subcloned into the unique *Bgl*II site of pBBB. A common 5′-primer, CTCTGAGG**A**TCCCCAGGGAGGAAACGGGCA, corresponding to nucleotides 1367–1396 of human uPA mRNA sequence was used in the amplification of both PCR products. This 5′-primer contains a mutation at position 1375 (G → A) to generate a *Bam*HI site. The 3′-primers used for PCR are as follows. 1) For construct pBBB/3′-uPA (containing the entire 928-bp uPA 3′-UTR), a primer, ATCA**GA**T**C**TATTTAAAATTACAAAAATATAAATAAAAATAG, corresponding to nucleotide 2282–2242 of human uPA mRNA sequence was synthesized, which contains mutations at positions 2278, 2277, and 2275 (C → G, T → A, and T → C) to generate a *Bam*HI site. 2) For construct pBBB/3′-uPAΔARE (pBBB containing ARE-deleted 3′-UTR of uPA mRNA), a primer, TAAGGA**TTC**GTCCTCACCCCACCCAGTGAG, corresponding to nucleotides 2192–2222 was synthesized. This primer includes mutations at positions 2215 and 2213 (G → T and G → C) to generate a *Bgl*II site.

To determine the effect of p38 MAPK activity on uPA mRNA stability, MDA-MB-231 cells were cotransfected with pBBB and pBBB/3′-uPA for 24 h using LipofectAMINE 2000 (Invitrogen) in medium containing 10% fetal calf serum. SB203580 (10 μM) or control compound SB202474 (10 μM) was then added to cells for 1 h. Cells were switched to serum-free medium containing SB203580 or SB202474, and poly(A)$^+$ RNA was isolated at various time points (1–4 h). The levels of β-globin transcripts were determined by Northern blotting. To determine

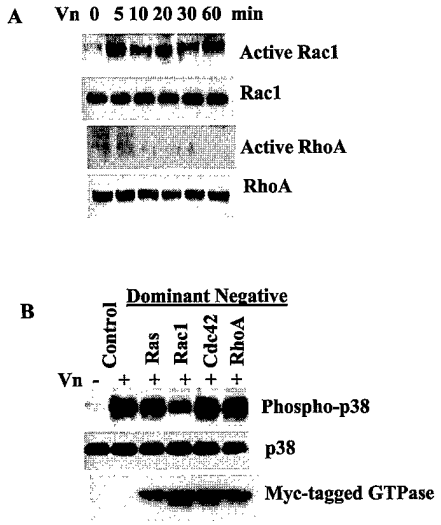

FIG. 1. **Rac1 is involved in Vn-induced p38 MAPK activation.** *A*, MDA-MB-231 cells were starved overnight and plated on Vn-coated surfaces for various times. Cells were lysed, and the cell lysates were incubated with GST-PBD or GST-RBD beads at 4 °C. Rac1 and RhoA activities were determined by the amount of PBD-bound Rac1 and RBD-bound RhoA, respectively. The levels of total Rac1 and RhoA were determined by immunoblotting using cell lysates with the respective mAbs as described under "Experimental Procedures." *B*, MDA-MB-231 cells were infected with dominant negative H-Ras, Rac1, Cdc42, or RhoA-containing Ad vector for 48 h. Cells were plated on Vn-coated surface for 30 min and subsequently lysed for immunoblotting to detect phosphorylated p38 MAPK. The membrane was stripped and first reprobed for p38 MAPK to ensure equal protein loading and then reprobed for Myc-tagged dominant negative H-Ras, Rac1, Cdc42, and RhoA expression.

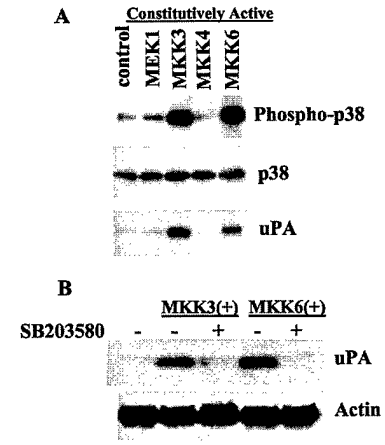

FIG. 2. **Constitutively active MKK3 and MKK6 can up-regulate uPA expression.** *A*, MDA-MB-231 cells were infected with constitutively active MEK1-, MKK3-, MKK4-, or MKK6-containing Ad vector in serum-free condition for 48 h. Cells were lysed, and immunoblotting was performed to detect phosphorylated p38 MAPK and uPA. *B*, MDA-MB-231 cells were infected with constitutively active MKK3- or MKK-6 containing Ad vector for 24 h. SB203580 (5 μM) was then added to cells for 24 h and subsequently lysed for immunoblotting to detect cell-associated uPA expression. The membrane was stripped and reprobed for actin to ensure equal protein loading.

whether ARE in the 3′-UTR of uPA mRNA affects p38 MAPK-regulated uPA mRNA stability, pBBB/3′-uPAΔARE vector was transfected into MDA-MB-231 cells in complete medium for 24 h. Cells were then treated with SB203580 (10 μM) for 1 h and then switched to serum-free medium containing SB203580. poly(A)$^+$ RNA was isolated, and Northern blotting was performed to detect β-globin transcripts.

To determine the effect of constitutively active MAPKAPK2 and PRAK on uPA mRNA stability in p38 MAPK-inhibited condition, MDA-MB-231 cells were infected with control Ad or Ad vector containing constitutive active MAPKAPK2 or PRAK2 for 24 h and followed by transfection with pBBB/3′-uPA for another 24 h. SB203580 was added to cells for 1 h, and cells were then switched to serum-free medium containing SB203580 for 1–4 h followed by poly(A)$^+$ RNA extraction.

RESULTS

Rac1 Is Involved in αv Integrin-induced p38 MAPK Activation—Our previous studies showed that Vn/αv integrin ligation induced p38 MAPK activation (44). Several recent studies have implicated that Rho GTPases including Rac, Cdc42, and RhoA are involved in integrin-mediated cellular responses (50–54) and that Rac1 and Cdc42 both activate p38 MAPK (55, 56). To determine whether Rho GTPases were involved in αv integrin-mediated p38 MAPK activation, we first examined the effect of VN/αv integrin ligation on Rac1, Cdc42, and RhoA activities. MDA-MB-231 cells were starved overnight and then suspended in serum-free medium for 1 h. Cells were plated on Vn-coated surfaces for varying times (2 to 60 min) and subsequently lysed for PBD or RBD assay to determine Rac1/Cdc42 and RhoA GTPase activity, respectively. Rac1 was activated by Vn/αv integrin ligation as early as 2 min and remained active in the entire 60 min (Fig. 1*A*). The activity of Cdc42 was not detected even though Cdc42 protein expression was readily detectable in these cells (data not shown). Interestingly, the activity of Rho A decreased upon Vn/αv integrin ligation (Fig. 1*A*). These results suggest that Vn/αv integrin ligation may induce Rac1 activation preferentially.

We next examined the importance of Rac1 activity in Vn/αv integrin ligation-induced p38 MAPK activation. Dominant negative mutants of H-Ras or Rho GTPases (Rac1, Cdc42, and RhoA) were expressed in MDA-MB-231 cells using recombinant adenovirus for 48 h, and cells were then plated on Vn-coated surfaces for 30 min. Immunoblotting with an antibody specific for phospho-p38 MAPK showed that the expression of dominant negative Rac1 (N17), but not dominant negative Cdc42, RhoA, or H-Ras, inhibited Vn/αv integrin-induced p38 MAPK phosphorylation (Fig. 1*B*) significantly. These results suggest that Vn/αv integrin ligation may signal through Rac1 to activate p38 MAPK in invasive breast cancer cells.

MKK3 Mediates Vn/αv Integrin Ligation-induced p38 MAPK Activation and uPA Up-regulation—MKK3, MKK6, and MKK4 have been reported capable of activating p38 MAPK (28, 31). We thus determined whether these MAPKKs were able to increase uPA expression in invasive breast cancer cells. Constitutively active mutants of MEK1, MKK3, MKK4, and MKK6 were expressed in MDA-MB-231 cells using recombinant adenovirus for 48 h. Total cellular protein was isolated from the cells and analyzed for the levels of uPA expression. Immunoblotting analyses showed that both constitutively active MKK3 and MKK6 activated p38 MAPK and also up-regulated uPA expression significantly (Fig. 2*A*). In contrast, constitutive active MEK1 or MKK4 exhibited no stimulatory effect in p38 MAPK activity or uPA expression. To determine whether p38 MAPK is indeed required for constitutively active MKK3/6-induced uPA expression, SB203580 (a specific p38 MAPK inhibitor) was added to constitutively active MKK3 or MKK6-expressing MDA-MB-231 cells. SB203580 at 5 μM concentration abrogated MKK3/6-induced uPA expression almost completely (Fig. 2*B*). These results suggest that both MKK3 and MKK6 can up-regulate uPA expression through p38 MAPK.

We next examined the effect of Vn/αv integrin ligation on MKK3/6 activity. Serum-starved MDA-MB-231 cells were plated on Vn-coated surface for varying times (5 min to 1 h), and immunoblotting was then performed to detect the active MKK3/6 and MKK4 (phosphorylated MAPKKs) with the relative antibodies. Vn/αv integrin ligation induced rapid increase in MKK3/6 phosphorylation; in contrast, the levels of MKK4

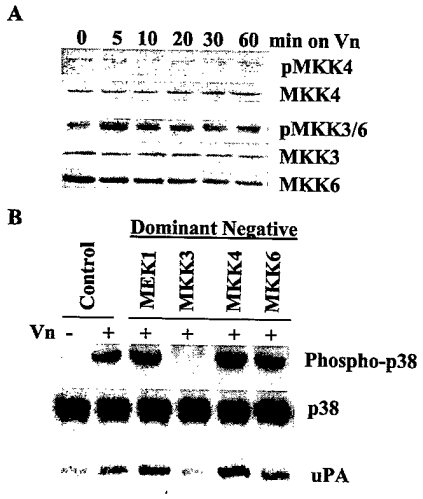

FIG. 3. **Vn-induced p38 MAPK activation and uPA up-regulation are signaled through MKK3.** *A*, MDA-MB-231 cells were plated on Vn-coated surface for various times. Cells were then lysed, and immunoblotting was performed to detect phosphorylated MKK4 or MKK3/6 (*pMKK4* or *pMKK3/6*). *B*, MDA-MB-231 cells were infected with dominant negative MEK1-, MKK3-, MKK4-, or MKK6-containing Ad vector for 48 h. Cells were then plated on Vn-coated surface for 30 min and followed by immunoblotting to detect phosphorylated p38 MAPK and cell-associated uPA.

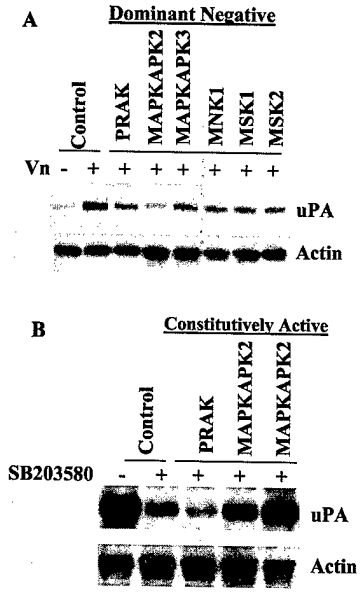

FIG. 4. **MAPKAPK2 is the main p38 MAPK downstream effector for uPA up-regulation.** *A*, MDA-MB-231 cells were infected with dominant negative PRAK-, MAPKAPK2-, MAPKAP3-, MNK1-, MSK1-, or MSK2-containing Ad vector for 48 h. Cells were lysed, and immunoblotting was performed to detect cell-associated uPA. *B*, MDA-MB-231 cells were infected with constitutively active PRAK (1,000 pfu/cell) or MAPKAPK2 (200 and 1,000 pfu/cell) for 24 h. SB203580 (5 μM) was added to cells for 24 h, and cells were then lysed for immunoblotting to detect cell-associated uPA. Membranes were stripped and reprobed for actin to ensure equal protein loading.

phosphorylation were not altered significantly (Fig. 3*A*). In the subsequent experiment, we expressed dominant negative mutants of MEK1, MKK3, MKK4, or MKK6 in MDA-MB-231 cells and determined their effect on αv integrin-mediated p38 MAPK activation and uPA up-regulation. Only the dominant negative MKK3 reduced αv integrin-mediated p38 MAPK activation and uPA up-regulation (Fig. 3*B*). These results suggest that MKK3, rather than MKK6, mediates Vn/αv integrin ligation-induced p38 MAPK activation and uPA expression in MDA-MB-231 cells although both MKK3 and MKK6 can up-regulate uPA expression.

MAPKAPK2 Is Involved in p38 MAPK-regulated uPA Expression—Up to six kinases (PRAK, MAPKAPK2, MAPKAPK3, MNK1, MSK1, and MSK2) are activated by p38 MAPK (28, 31) and can potentially serve as the p38 MAPK downstream effectors. To determine which of these molecules is involved in p38 MAPK-regulated uPA expression, dominant negative mutants of PRAK, MAPKAPK2, MAPKAPK3, MNK1, MSK1, or MSK2 were expressed in MDA-MB-231 cells using recombinant adenovirus. Cellular protein was extracted from these cells 48 h post-Ad infection and analyzed for the levels of uPA expression. Immunoblotting showed that a dominant negative MAPKAPK2 inhibited ~80% of Vn-up-regulated uPA expression in MDA-MB-231 cells (Fig. 4*A*). In contrast, only a slight reduction in the levels of uPA protein can be observed in cells expressing dominant negative PRAK, MAPKAPK3, MNK1, MSK1, or MSK2 in comparison with control (Fig. 4*A*). In a parallel study, we examined whether constitutively active MAPKAPK2 could rescue uPA expression in SB203580-treated (p38 MAPK-inhibited) MDA-MB-231 cells. Constitutively active MAPKAPK2 was expressed in cells using recombinant adenovirus for 24 h and followed by addition of SB203580 (5 μM) to the cells for another 48 h. Immunoblotting using anti-uPA antibody showed that SB203580 inhibited uPA expression significantly (Fig. 4*B*). However, the expression of constitutively active MAPKAPK2 (at 10^3pfu), but not constitutively active PRAK, restored at least 80% of SB203580-decreased uPA level (Fig. 4*B*). These results suggest that MAPKAPK2 is the main downstream effector of p38 MAPK for regulating uPA expression.

Rac1-MKK3-p38 MAPK-MAPKAPK Pathway Exists in Invasive Breast Cancer Cells—Because Vn-mediated uPA up-regulation is blocked by dominant negative Rac1, MKK3, and MAPKAPK2 (see Figs. 1, 3, and 4), and SB203580-inhibited uPA expression is restored largely by constitutively active MAPKAPK2 (Fig. 4), it suggests strongly that a signaling pathway involving Rac1-MKK3-p38 MAPK-MAPKAPK2 is responsible for Vn-mediated uPA up-regulation in MDA-MB-231 cells. To confirm the presence of this signaling pathway, we first investigated the effect of active Rac1 on MKK3 and MKK6 activities. Wide-type or constitutively active Rac1 was expressed in MDA-MB-231 cells with the aid of adenovirus, and cells were then lysed and subjected immunoprecipitation with anti-MKK3 and -MKK6 antibodies. Immunoblotting on these immunoprecipitates with active MKK3/6 antibody showed that wide-type Rac1 did not alter the level of active MKK3 or MKK6 (Fig. 5*A*). In contrast, the levels of active MKK3, rather than MKK6, were elevated significantly by the constitutively active Rac1 expression (Fig. 5*A*). These results suggest that active Rac1 activates MKK3 specifically in MDA-MB-231 cells and are consistent with the results that only dominant negative MKK3 blocks Vn-mediated p38 MAPK activation and uPA up-regulation (Fig. 3).

To determine the effect of active MKK3 on MAPKAPK2 activity, constitutively active MKK3 or MKK4 was introduced into MDA-MB-231 cells using recombinant Ad vector for 2 days. Immunoblotting with phospho-MAPKAPK2-specific antibody showed that the levels of MAPKAPK2 phosphorylation in cells expressing constitutively active MKK4 was very similar to the control (Fig. 5*B*). In contrast, the expression of constitutively active MKK3 enhanced the levels of MAPKAPK2 phosphorylation significantly (Fig. 5*B*). To determine whether p38 MAPK participates in MKK3-induced MAPKAPK2 activation, MDA-MB-231 cells were infected with constitutively active MKK3 Ad vector for 24 h, and SB203580 or control compound 202474 (5 μM) was then added to cells for another 24 h. Cells

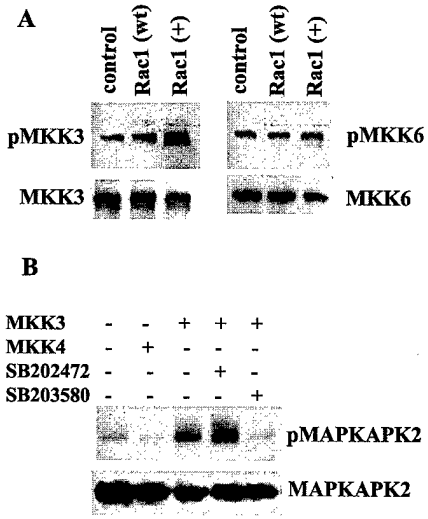

FIG. 5. **Rac1-MKK3-p38 MAPK-MAPKAPK2 pathway is present in MDA-MB-231 cells.** *A*, MDA-MB-231 cells were infected with control, wild-type, or constitutively active Rac1 Ad vector for 48 h. Cells were lysed, and cell lysates were immunoprecipitated with MKK3 or MKK6 polyclonal antibody. The immunoblotting was then performed with these immunoprecipitates to detect active MKK3 or MKK6 (phospho-MKK3 or phospho-MKK6; *pMKK3* or *pMKK6*). Membranes were stripped and reprobed with MKK3 or MKK6 antibody to ensure equal loading. *B*, MDA-MB-231 cells were infected with constitutively active MKK3 or MKK4 for 24 h and then switched to medium in the absence or presence of SB203580 or SB202474 for another 24 h. Cells were lysed, and immunoblotting was performed to detect active MAPKAPK2 (phospho-MAPKAPK2; *pMAPKAPK2*) using phospho-MAPKAPK2-specific antibody. The membrane was stripped and reprobed with MAPKAPK2 polyclonal antibody to detect cellular MAPKAPK2 protein.

were lysed, and immunoblotting was performed to determine the levels of MAPKAPK2 phosphorylation. The treatment of SB203580, but not control SB202474, abrogated the constitutively active MKK3-induced MAPKAPK2 activation almost completely (Fig. 5*B*). These results suggest that p38 MAPK mediates MKK3-induced MAPKAPK2 activation.

p38 MAPK-regulated uPA mRNA Stability Requires the ARE in 3′-UTR of uPA mRNA—Highly conserved ARE have been found in 3′-UTR of uPA mRNAs and reported to be involved in p38 MAPK-regulated uPA mRNA stability (46, 57). To determine the importance of uPA 3′-UTR and its ARE in p38 MAPK-regulated uPA mRNA stability, we subcloned uPA 3′-UTR and ARE-deleted 3′-UTR in plasmid pBBB. The pBBB vector contains the rabbit β-globin cDNA under the control of the c-*fos* promoter. Because c-*fos* promoter is a serum-dependent promoter, the presence or absence of serum can be used to control promoter activity, and thus the stability of β-globin mRNA can be monitored without the addition of actinomycin D. Also, by inserting the sequence of interest in pBBB, the effect of this sequence on mRNA stability can be determined by monitoring otherwise stable β-globin mRNA (37, 38). The pBBB vector containing uPA 3′-UTR (pBBB/3′-uPA) was transfected into MDA-MB-231 cells, and the transfected cells were then treated with SB203580 or control compound SB202474 (5 μM). poly(A)$^+$ RNA was isolated at various times (1–4 h), and Northern blotting was conducted to detect the levels of β-globin transcripts. Both β-globin and β-globin/uPA 3′-UTR RNA transcripts were equally stable in cells treated with control SB202474 (Fig. 6). In contrast, SB203580 destabilized β-globin/uPA 3′-UTR RNA transcript significantly whereas β-globin mRNA stability was not affected (Fig. 6). Interestingly, the pBBB vector containing ARE-deleted 3′-UTR (removal of the last 68 bp) (pBBB/3′-uPAΔARE) could no longer destabilize β-globin RNA transcript in SB203580-treated MDA-MB-231 cells (Fig. 6). These results suggested that p38 MAPK pathway

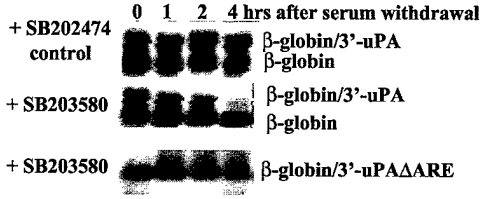

FIG. 6. **p38 MAPK-regulated uPA mRNA stability depends on the ARE in 3′-UTR.** MDA-MB-231 cells were either cotransfected with phospho-BBB and phospho-BBB/3′-uPA or transfected with phospho-BBB/3′-uPAΔARE. Cells were switched to serum-free medium, and poly(A)$^+$ RNA was isolated at varying times. Northern blotting was performed to detect β-globin transcripts.

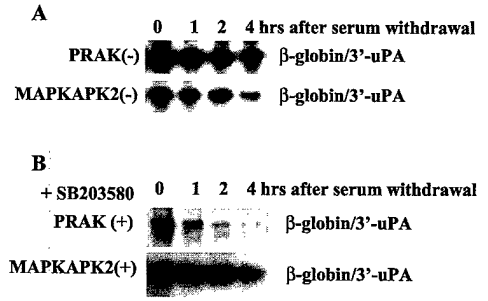

FIG. 7. **MAPKAPK2 regulates uPA mRNA stability through an ARE-targeted mechanism.** *A*, MDA-MB-231 cells were infected with dominant negative PRAK or MAPKAPK2-containing Ad vector for 48 h. Cells were switched to serum-free medium, and poly(A)$^+$ RNA was isolated at varying times. Northern blotting was performed to detect β-globin transcripts. *B*, MDA-MB-231 cells were infected with constitutively active PRAK or MAPKAPK2 for 48 h, followed by 1 h of SB203580 (5 μM) treatment. Cells were then switched to serum-free medium containing SB203580, and poly(A)$^+$ RNA was isolated at varying times. Northern blotting was performed to detect β-globin transcripts.

regulates uPA mRNA stability through an ARE-targeted mechanism.

MAPKAPK2 Affects uPA mRNA Stability through uPA 3′-UTR—We also investigated the effect of dominant negative MAPKAPK2 and PRAK on β-globin/uPA 3′-UTR transcript stability. MDA-MB-231 cells were transfected with pBBB containing uPA 3′-UTR and followed by a 2-day infection with control Ad vector or Ad vector encoding dominant negative MAPKAPK2 or PRAK. Although the stability of β-globin/uPA 3′-UTR transcript was not affected significantly by the expression of dominant negative PRAK, it was reduced greatly by the expression of dominant negative MAPKAPK2 (Fig. 7*A*). In further experiments, we examined the ability of constitutively active MAPKAPK2 to restore SB203580-induced uPA mRNA stability. Constitutively active MAPKAPK2 was introduced into β-globin/uPA 3′-UTR RNA transcript-expressing MDA-MB-231 cells prior to SB203580 treatment for various times. Subsequently, poly(A)$^+$ RNA was isolated, and Northern analysis was performed to detect β-globin transcripts. Although SB203580 reduced β-globin/uPA 3′-UTR RNA transcript stability greatly, the expression of constitutively active MAPKAPK2 prolonged β-globin/uPA 3′-UTR RNA transcript stability significantly in SB203580-treated MDA-MB-231 cells (Fig. 7*B*). In a parallel control experiment, we also determined the effect of constitutively active PRAK on β-globin/uPA 3′-UTR transcript stability in SB203580-treated cells and found that it was unable to prolong β-globin/uPA 3′-UTR transcript stability (Fig. 7*B*). These results suggest that MAPKAPK2 is the main p38 MAPK downstream effector for promoting uPA mRNA stability in invasive breast cancer cells.

DISCUSSION

Our previous studies have demonstrated that the elevated endogenous p38 MAPK is essential for uPA up-regulation in

invasive breast cancer (58). We have further shown that the elevated p38 MAPK activity requires αv integrin expression/function, and αv integrin ligation activates p38 MAPK and up-regulates uPA expression (44). In this report, we identified the upstream and downstream signaling molecules of p38 MAPK that participate in p38 MAPK-regulated uPA mRNA stability. Small GTPase Rac1 and Cdc42 are capable of activating p38 MAPK (55, 56) and are important for many integrin-mediated cellular responses (50–54). We thus investigated whether Rho GTPases including Rac1, Cdc42, and RhoA were involved in αv integrin-mediated p38 MAPK activation. We found that Vn/αv integrin ligation activated Rac1 preferentially (Fig. 1*A*). Further studies also showed that dominant negative Rac1, rather than dominant negative Cdc42 and RhoA, inhibited αv integrin ligation-induced p38 MAPK activation significantly (Fig. 1*B*). These findings suggest strongly that αv integrin signals through Rac1 for p38 MAPK activation. Several recent studies reported that α2β1 integrin ligation can activate p38 MAPK in various cell types (59–61). Furthermore, α2β1-induced p38 MAPK activation requires Cdc42 in human osteosarcoma cells (59) and Rac1 in murine NmuMG cells (60). Therefore, we consider the possibility that Rac1 or Cdc42 as a common signaling molecule for mediating integrin-induced p38 MAPK activation.

The specific upstream activator kinases for p38 MAPK are MKK3 and MKK6. MKK4, a known activator kinase for JNK, has also been implicated in p38 MAPK activation *in vitro* (27, 28). Although MKK3 and MKK6 are 80% homologous to each other and, in many cases, mediate same signals for p38 MAPK activation, they have been reported to exhibit differential involvement in other cellular events. For example, MKK6, rather than MKK3, is required for FasL and c-Abl-induced cell death in Jurkat T and NIH3T3 cells, respectively (47, 62). Also, MKK6 is important for IL-12-induced p38 MAPK activation and STAT4 activation in T and NK cells (63). However, MKK3, rather than MKK6, mediates tumor necrosis factor α and lipopolysaccharide-induced p38 MAPK activation and cytokine expression in both fibroblast and macrophages (64, 65). We showed that dominant negative MKK3, but not dominant negative MKK6, was capable of inhibiting αv integrin ligation-induced p38 MAPK activation and uPA up-regulation significantly (Fig. 3), suggesting that integrin-induced p38 MAPK activation may be mediated strictly by MKK3. Our study provides additional evidence for distinct involvement of MKK3 and MKK6 for p38 MAPK-mediated cellular responses.

p38 MAPK signaling pathway is involved in a wide spectrum of cellular functions, and the diversified role of p38 MAPK may be explained by the existence of various p38 MAPK downstream signaling molecules. Multiple kinases and transcription factors have been identified as p38 MAPK substrates and are potentially downstream effectors of p38 MAPK (28, 29). In fact, p38 MAPK/MSK-1 pathway is found to mediate vascular endothelial cell growth factor-induced cAMP-response element-binding protein phosphorylation and activation in human endothelial cells (66), p38 MAPK/MNK1 pathway is essential for stress-induced eukaryotic initiation factor-4E activation (67, 68), and phosphorylation of 5-lipoxygenase appears to require p38 MAPK/MAPKAPK2 and MAPKAP3 pathways (69). Our studies showed that dominant negative MAPKAPK2 inhibited αv integrin ligation-induced uPA up-regulation specifically (Fig. 4*A*), and constitutively active MAPKAPK2 rescued uPA expression in p38 MAPK-inhibited cells (Fig. 4*B*). These results suggest strongly that MAPKAPK2 is the main p38 MAPK downstream effector in regulating uPA expression in invasive breast cancer cells. These studies also further support the notion that the diversified role of p38 MAPK is determined by the variety of downstream signaling molecules.

In the past several years, growing evidence has revealed clearly that the ARE in 3′-UTR of mRNA can affect mRNA stability significantly (70, 71). The minimum consensus sequence for ARE is UUAUUUAUU or UUAUUU(A/U)(A/U) (36), and this sequence is present in the 3′-UTR of uPA mRNA (57). Early studies have shown that p38 MAPK pathway regulates uPA mRNA stability in an ARE-targeted mechanism (46), and this finding was further confirmed by our studies (Fig. 6). We demonstrated that the stability of uPA mRNA 3′-UTR-containing β-globin transcript was decreased specifically by dominant negative MAPKAPK2 in MDA-MB-231 cells by (Fig. 7*A*), and prolonged by, constitutively active MAPKAPK2 in p38 MAPK-inhibited MDA-MB-231 cells (Fig. 7*B*). These results suggest that MAPKAPK2 is the downstream effector of p38 MAPK for regulating uPA mRNA stability. Several recent studies have also demonstrated the importance of MAPKAPK2 in regulating mRNA stability. Blocking MAPKAPK2 activity was reported to inhibit cytokine/stress-induced IL-8 and IL-8 expression by destabilizing their mRNA (72). In MAPKAPK2-deficient cells, the half-life of IL-2 mRNA was reduced more than 10-fold, and re-expressing MAPKAPK2 in these cells restored IL-2 mRNA stability (73). Furthermore, tristetraprolin, a protein regulating mRNA stability, was found to be a direct substrate of MAPKAPK2 (32). Taken together, it is very likely that MAPKAPK2 may be a common p38 MAPK downstream signaling molecule for regulating ARE-mediated mRNA stability.

In conclusion, we have defined the signaling pathway linking αv integrin ligation, p38 MAPK activation to uPA up-regulation in invasive breast MDA-MB-231 cells. Because uPA plays a significant role in tumor invasion and progression, the therapeutic approaches may be developed by intercepting the signaling pathway responsible for uPA overexpression.

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