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**Title and Subtitle:**
Vitronectin and Integrin αvβ3 in Ovarian Carcinoma

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**Abstract:**
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 BamHI/KpnI. The released fragments were cloned in adenovirus shuttle vector, pShuttle (QBIOPGEN), and subsequently cotransformed with pAd.Easy-1 (QBIOPGEN) 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.

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...
integron 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 (10⁷ 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.

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:


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**

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., Basikerville, 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 or 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 effects 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) 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 TOY 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 AUFI (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.
(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/av integrin ligation-mediated uPA up-regulation remain to be elucidated. In the present study, we demonstrate that Vn/av integrin ligation activates Rac1 preferentially and that the Rac1 activity is important for Vn/av-induced p38 MAPK activation. We also show that both constitutively active MKK3 and MKK6 enhance uPA expression, only dominant negative MKK3 blocks Vn/av 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/av 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-MK4 were purchased from Cell Signaling (Beverly, MA). The polyclonal antibody to uPA was obtained from American Diagnostics (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 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% CO2.

Recombinant Adenosine Construction—To construct recombinant adenosovirus (Ad) encoding dominant negative Rac and Rho GTPase, cDNAs for Myc-tagged H-Ras (N17), RhoA (N19), Rac1 (N17), and Cdc42 (N17) cDNAs were cloned into the adenosovirus shuttle vector pAd.CI (containing cytomegalovirus promoter). Ad vector containing Myc-tagged Rac1 and constitutively active Rac1 (V12) were constructed similarly. To construct Ad vector encoding dominant negative PRAK, MAPKAPK2, MAPKAPK3, MK1, MK1, and MK2, 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 an 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 RBD and RBD assays, respectively (48, 49). In Rac1/Cdc42 assays, the GDP 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 Rhoetkin 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 5 μg/ml of Vn-integrin (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and protease inhibitor mixture). Cells lysates were boiled in non-reducing sample buffer and subjected to immunoblotting to detect total and active MKK3/6 or MKK4 with respective antibodies.

Analyzing the Effect of Constitutively Active Rac1 on MKK3 and MKK6 Activities—MDA-MB-231 cells were 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. After another 24 h of serum starvation, cells were plated on Vn-coated dishes 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/av-integrin-mediated uPA expression, MDA-MB-231 cells were infected with dominant negative PRAK, MAPKAPK2, MAPKAPK3, MK1, and MK1 or MK2 Ad vector (100 pfu/cell) for 24 h and then plated on Vn-coated dishes and cultured for 24 h. Cells were lysed, and lysates were subjected to immunoblotting to detect the levels of uPA. To determine whether constitutively active anti-vPAK MKAPK2 was able to rescue SB203580-inhibited uPA expression, MDA-MB-231 cells were infected with Ad vector containing constitutive active RhoA (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 MKK3 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 24 h and then treated with SB203580 or SB202474 (5 μM) for another 24 h. Lysates were then 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 Activities 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 RBD and RBD assays, respectively (48, 49). In Rac1/Cdc42 assays, the GDP 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 Rhoetkin 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 5 μg/ml of Vn-integrin (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and protease inhibitor mixture). Cells lysates were boiled in non-reducing sample buffer and subjected to immunoblotting to detect total and active MKK3/6 or MKK4 with respective antibodies.
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 reprobed for p38 MAPK to ensure equal protein loading and then reprobed for Myc-tagged dominant negative H-Ras, Rac1, Cdc42, and RhoA expression.

whether ARE in the 3'-UTR of uPA mRNA affects p38 MAPK-regulated uPA mRNA stability, pBBB3'-apAAARE 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 pBBB3'-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. 1A). 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. 1A). These results suggest that Vn/αv integrin ligation may induce Rac1 activation preferentially.

**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 next 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. 2A). In contrast, constitutively 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 MKK3/6-expressing MDA-MB-231 cells. SB203580 at 5 μM concentration abrogated MKK3/6-induced uPA expression almost completely (Fig. 2B). 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...
phosphorylation were not altered significantly (Fig. 3A). 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. 3B). These results suggest that MKK3, rather than MKK6, mediates αv/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. 4A). 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. 4A). 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. 4B). However, the expression of constitutively active MAPKAPK2 (at 10^4 pfu), but not constitutively active PRAK, restored at least 80% of SB203580-decreased uPA level (Fig. 4B). These results suggest that MAPKAPK2 is the main downstream effector of p38 MAPK for regulating uPA expression.
Regulation of uPA mRNA Stability by p38 Pathway

ARE-deleted 3'-UTR in plasmid pBBB. The pBBB vector contains 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 regulating uPA mRNA stability, we subcloned uPA 3'-UTR and found in 3'-UTR of uPA mRNAs and reported to be involved in MKK3/6 and MKK4. 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. 5B). 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 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 MAPKAPK2 (Fig. 7A). 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. 7B). 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. 7B). 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/av integrin ligation activated Rac1 preferentially (Fig. 1A). 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. 1B). 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 (29, 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/MPKAPK2 and MAPKAPK3 pathways (69). Our studies showed that dominant negative MPKAP2 inhibited αv integrin ligation-induced uPA up-regulation specifically (Fig. 4A), and constitutively active MAPKAPK2 rescued uPA expression in p38 MAPK-inhibited cells (Fig. 4B). 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 MAPKAP2 in MDA-MB-231 cells by (Fig. 7A), and prolonged by, constitutively active MAPKAPK2 in p38 MAPK-inhibited MDA-MB-231 cells (Fig. 7B). 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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