αv Integrin, p38 Mitogen-activated Protein Kinase, and Urokinase Plasminogen Activator Are Functionally Linked in Invasive Breast Cancer Cells*

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We reported previously that endogenous p38 MAPK activity is elevated in invasive breast cancer cells and that constitutive p38 MAPK activity is important for overproduction of uPA in these cells (Huang, S., New, L., Pan, Z., Han, J., and Nemerow, G. R. (2000) J. Biol. Chem. 275, 12266–12272). However, it is unclear how elevated endogenous p38 MAPK activity is maintained in invasive breast cancer cells. In the present study, we found that blocking αv integrin functionality with a function-blocking monoclonal antibody or down-regulating αv integrin expression with αv-specific antisense oligonucleotides significantly decreased the levels of active p38 MAPK and inhibited cell-associated uPA expression in invasive breast cancer MDA-MB-231 cells. These results suggest a function link between αv integrin, p38 MAPK activity, and uPA expression in invasive tumor cells. We also found that vitronectin/αv integrin ligation specifically induced p38 MAPK activation and uPA up-regulation in invasive MDA-MB-231 cells but not in non-invasive MCF7 cells. Finally, using a panel of melanoma cells, we demonstrated that the cytoplasmic tail of αv integrin subunit is required for αv integrin ligation-induced p38 MAPK activation.

The degradation of extracellular matrix and basement by tumor-associated proteases is an essential process required for cancer cell invasion and metastasis (1, 2). Urokinase plasminogen activator (uPA) is of particular importance because uPA, through interaction with the uPA receptor (uPAR), facilitates the conversion of plasminogen into plasmin and the activation of metalloproteinases (3, 4). These proteases then allow cancer cells to degrade the surrounding matrix proteins and migrate to the distant sites (5). The overexpression of uPA is detected in various malignancies including breast, prostate, and colon cancers (6–8). Recent studies (9–13) have shown further that a high level of uPA in tumors is associated with a rapid disease progression and poor prognosis in breast cancers. In addition, studies performed in experimental models both in vitro and in vivo demonstrate that the levels of uPA are closely associated with the degree of tumor cell invasion (14–18). Blocking uPA expression or disruption of uPA binding to uPAR has been found to inhibit significantly tumor cell invasion and metastasis in various tumor models (19–22). From these facts, it is apparent that uPA plays a key role in tumor progression and metastasis.

Integrins are heterodimers composed of noncovalently associated α and β subunits. There are at least 14 α and 8 β subunits, forming at least 21 different integrins, which are the major receptors for extracellular matrix proteins (23). The αv integrins are a major subfamily with restricted tissue/cell distribution (23). They have classical integrin functions such as mediating cell attachment and spreading (24, 25), facilitating cell migration (26), and ligand-receptor internalization (27, 28). They also play an important role in tumor progression and metastasis by mediating angiogenesis (29) and promoting tumor cell survival (30, 31). In addition, ligation of αv integrins with their ligands has been reported to regulate the expression of several metalloproteinases (MMPs) and uPA/uPAR in various cancer cell types (32, 33). Suppressing αv integrin expression resulted in the reduction in MMP2, MMP9, and uPAR expression in B-lymphocytes and melanoma cells (32, 34–37). Furthermore, both αvβ3 and αvβ6 integrins physically interact with uPA upon uPA ligation, and these interactions are essential for tumor progression (38–40). Therefore, uPA/uPAR and αv integrins may function in concert to promote tumor metastasis.

The mitogen-activated protein kinases (MAPKs) transduce extracellular signals into cellular responses and play important roles in cell proliferation, apoptosis, differentiation, cell migration, and cytoskeleton remodeling (41–43). Mammalian cells express four types of MAPKs, ERKs, p38 MAPKs, c-Jun NH2-terminal kinases, and big MAPKs. Recent studies have shown that MAPKs including ERK, c-Jun NH2-terminal kinases, and p38 can be activated by integrin ligation, and the induced MAPK activities are important for many integrin-mediated cellular responses. For example, ligation of β1 integrin with fibronectin activates ERK1/2, and this β1 integrin-activated ERK activation is essential for cell cycle progression in NIH-3T3 cells (44). Ligation of αvβ3 integrin with collagen induces the activation of p38 MAPK, and the activity of p38 MAPK is essential for collagen-induced collagenase-1 and -3 expression in human fibroblast cells (45, 46), as well as collagen gene transcription in osteosarcoma cells (47) and NmuMG cell migration (48, 49).

Our previous studies (50) showed that endogenous p38 MAPK activity is elevated in cultured invasive breast cancer cells and that the higher p38 MAPK activity is important for...
breast cancer invasiveness by stabilizing uPA mRNA. However, it is unclear what maintains the elevated endogenous p38 MAPK activity in invasive tumor cells. In this paper, we investigated the role of integrins in the constitutive p38 MAPK activity and uPA expression. In the present study, we demonstrate that blocking αv integrin ligation or down-regulating αv integrin expression decreases endogenous p38 MAPK activity and inhibits uPA expression. Plating cells on vitronectin (Vn)-coated surface activates p38 MAPK and increases uPA expression in invasive MDA-MB-231 cells but not in non-invasive MCF7 cells. These findings suggest that αv integrin ligation specifically activates p38 MAPK and up-regulates uPA expression in invasive cancer cells. In addition, we provided evidence that only the cytoplasmic tail of αv integrin subunit is important for αv integrin-mediated p38 MAPK activation.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture—**Polyclonal antibodies to phospho-p38 and p38 were obtained from Cell Signaling (Beverly, MA). The polyclonal antibody to uPA was obtained from American Diagnostica (Greenwich, CT). Function-blocking monoclonal antibodies (mAbs) to β₁ (P4C10), αvβ₃ (ASC-3), αvβ₅ (P1F6), and αv (AV-1) and the non-function-blocking mAb to αv (LM142) were purchased from Chemicon (Temecula, CA). MDA-MB-231 and MCF7 cell line were obtained from ATCC (Manassas, VA). M21-L4, M21-L12, CS1, CS1/β₁, and CS1/β₅ cell lines were provided by Dr. David Cheres (The Scripps Research Institute). All cells were 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.

**Analyzing the Effect of Function-blocking Integrin Antibodies on p38 MAPK Activity and uPA Expression—**MDA-MB-231 cells were cultured in a 6-well plate for 24 h, and 20 μg/ml function-blocking mAbs (P4C10, ASC-3, P1F6 or AV-1) was added to the cells for 8 h. Cells were lysed in RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO₄, and protease inhibitor mixture). Cell lysates were boiled in nonreducing SDS sample buffer, electrophoresed on 10% polyacrylamide SDS gel, and transferred to a nitrocellulose membrane. The p38 MAPK and activated p38 MAPK were detected with anti-p38 and anti-phospho-p38 MAPK polyclonal antibodies, respectively. The amount of uPA was detected with an anti-uPA polyclonal antibody.

**Analyzing the Effect of αv Integrin Subunit-specific Antisense Oligonucleotide on p38 MAPK Activity and uPA Expression—**We previously developed two efficient αv-specific antisense oligonucleotides (AS1 and AS2) and a non-functional control oligonucleotide (AS3) (37). The sequences for AS1, AS2, and AS3 are GGAGGCGCAAGCCGGGAG (nucleotide –16 to –33 relative to AUG translational start codon), AAAAAACTGCGGAAAG (nucleotide 9–18 relative to AUG translational start codon), and GGAGGCGCAAGCCGGGAG (nucleotide 49 to 32 relative to AUG translational start codon), respectively. To down-regulate αv integrin expression on MDA-MB-231 cells, we added antisense oligonucleotides, AS1, AS2, or AS3 (2 nmol), into overnight MDA-MB-231 cell culture with 10 μl of LipofectAMINE2000 (Life Technologies, Inc.) in a total volume 1 ml for 36 h. Cells were lysed, and the cell lysates were subjected to immunoblotting to detect the levels of activated p38 MAPK and uPA with the respective antibodies.

**Analyzing the Effect of αv Integrin Ligation on the Activities of MAPKs and uPA Expression—**To determine the effect of αv integrin ligation on the activities of MAPKs, cell lines MDA-MB-231 or MCF7 were starved in serum-free medium for 24 h, and 3-cm culture dishes were coated with 1 μg/ml of Vn solution at 4 °C. Cells were detached with 10 mM EDTA, kept in suspension in serum-free medium for 30 min, and then replated onto Vn-coated culture dishes. At varying times, cells were lysed in ice-cold RIPA buffer, and cell lysates were then subjected to immunoblotting to detect the levels of activated p38 MAPK.

To determine the effect of αv integrin ligation on uPA expression, MDA-MB-231 or MCF7 cells were plated on Vn-coated dishes and cultured for 1 or 2 days. Cells were lysed, and cell lysates were subjected to immunoblotting to detect the levels of uPA.

**Construction and Transfection of Cytoplasmic Tail-deleted αv, β₅, and β₅ Integrin Subunit Expression Vectors—**To prepare cells expressing the cytoplasmic tail-deleted αv integrin subunit, the expression vector pcDNA/αvΔ995 was transfected into M21L12 cells with LipofectAMINE according to manufacturer's protocol. The pcDNA/αvΔ995 contains the αv integrin subunit with carboxy-terminal deletion up to GFFKR sequence (provided by Zhuohua Zhang, Burnham Institute).

Cytoskeletal Tail-generated αv, β₅, and β₅ integrins were designated as CS1/β₁, CS1/β₅, and CS1/β₅, respectively. The amount of uPA was detected with an anti-uPA polyclonal antibody.

**Results**

αv Integrin Function/Expression Is Essential for Endogenous p38 MAPK Activity in Invasive Breast Cancer Cells—Extracellular matrix proteins including Vn, osteopontin, fibronectin, and laminin have been reported to up-regulate uPA expression in various cell types (51–56). Integrin ligation has also been shown to activate p38 MAPK in mast cells and fibroblasts (45, 46, 57). We thus hypothesized that integrin ligation may contribute to the elevated endogenous p38 MAPK activity and up-regulated uPA expression in invasive breast cancer cells. To test this hypothesis, we treated the invasive MDA-MB-231 cells with function-blocking mAbs to β₁, αv, αvβ₅, and αvβ₄ integrins. Immunoblotting with an anti-phospho-p38 antibody showed that the levels of activated p38 MAPK were moderately inhibited by function-blocking mAb to β₁ integrin subunit (P1F6), and not affected by function-blocking mAbs to αvβ₅ integrin (ASC-3) (Fig. 1A). In contrast, function-blocking mAb to αv integrin (AV-1) and αvβ₅ integrin (P1F6) significantly inhibited p38 MAPK phosphorylation (Fig. 1A). Because MDA-MB-231 cells expressed significant levels of each of these integrins (data not shown), these results suggest that the functionality of αv integrins is important for the sustained endogenous...
p38 MAPK activity in MDA-MB-231 cells.

To examine further the functional correlation between \( \alpha_v \) integrins and p38 MAPK activity, we also treated MDA-MB-231 cells with two previously developed \( \alpha_v \)-specific antisense oligonucleotides (AS1 and AS2) (37) to down-regulate \( \alpha_v \) integrin expression. As determined by flow cytometry, AS1 and AS2 significantly inhibited the levels of endogenous \( \alpha_v \) integrin expression (data not shown). Treatment of cells with AS1 or AS2 at 2 \( \mu \)M reduced over 80% of cell surface \( \alpha_v \) integrin expression (data not shown). Treatment of cells with AS1 or AS2 significantly inhibited the levels of endogenous p38 MAPK activity (Fig. 1B); in contrast, MDA-MB-231 cells treated with the non-functional AS3 displayed a similar level of p38 MAPK phosphorylation as the untreated cells (Fig. 1B). These results suggest that \( \alpha_v \) integrins are essential for elevated endogenous p38 MAPK activity in invasive breast cancer cells.

\( \alpha_v \) Integrin Function/Expression Is Essential for uPA Expression in Invasive Breast Cancer Cells—We next examined the role of \( \alpha_v \) integrin on cell-associated uPA expression. Various amounts of \( \alpha_v \) integrin function-blocking mAb (AV-1) were added to cultures of attached MDA-MB-231 cells for 8 h. After treatment, the cells were lysed, and the levels of uPA were analyzed by immunoblotting with an anti-uPA antibody. The amount of uPA was reduced by mAb AV-1 in a dose-dependent manner. At least 80% of cell-associated uPA expression was blocked with 20 \( \mu \)g of AV-1 (Fig. 2A). In contrast, a non-function-blocking mAb to \( \alpha_v \) integrins (LM142) showed no inhibitory effect on the levels of cell-associated uPA (Fig. 2A).

![Fig. 2. The functionality of \( \alpha_v \) integrins is required for uPA expression in MDA-MB-231 cells.](image)

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These data suggest that functional \( \alpha_v \) integrins are required for uPA expression in MDA-MB-231 cells. In a parallel study, we also treated MDA-MB-231 cells with the \( \alpha_v \)-specific antisense oligonucleotide, AS2, or control oligonucleotide, AS3, and followed the effect on uPA expression by immunoblotting. The amount of cell-associated uPA was significantly decreased in AS2-treated cells, whereas AS3 showed no inhibitory effect on uPA levels (Fig. 2B). Taken together, these studies suggest a functional link between \( \alpha_v \) integrins, p38 MAPK activity, and uPA expression in invasive breast cancer cells.

\( \alpha_v \) Integrin Ligation Activates p38 MAPK and Induces uPA Expression in Invasive Breast Cancer Cells—We next examined whether \( \alpha_v \) integrin ligation was capable of activating p38 MAPK in both invasive MDA-MB-231 and non-invasive MCF7 breast cancer cells. Cells were first starved overnight and then suspended in serum-free medium for 30 min to reduce endogenous p38 MAPK activity. Subsequently, we plated cells on Vn-coated surfaces for varying times (10 min to 2 h). Immunoblotting with an antibody specific to phospho-p38 MAPK showed that \( \alpha_v \) integrin ligation with Vn induced dramatic p38 MAPK phosphorylation as early as 10 min and reached maximal p38 MAPK activity at 20 min in MDA-MB-231 cells (Fig. 3A). Interestingly, \( \alpha_v \) integrin ligation did not activate p38 MAPK in non-invasive MCF7 cells (Fig. 3B). These results suggest that \( \alpha_v \) integrin ligation may specifically induce p38 MAPK activation in invasive breast cancer cells.

To examine the effect of \( \alpha_v \) integrin ligation on uPA expression, MDA-MB-231 or MCF7 cells were plated on Vn-coated plates for 1–2 days. Cells were then lysed, and the amount of uPA protein was quantitated by immunoblotting. MDA-MB-231 cells cultured on a Vn-coated surface exhibited a much higher amount of cell-associated uPA protein than those cultured on an uncoated surface (Fig. 4). In contrast, no difference in the levels of uPA expression could be detected between MCF-7 cells cultured on Vn-coated and uncoated surface (Fig. 4). These results suggest that Vn may specifically induce uPA expression in invasive breast cancer cells.

The \( \alpha_v \) Integrin Subunit Cytoplasmic Tail Is Essential for \( \alpha_v \) Integrin Ligation-induced p38 MAPK and ERK Activation—We also investigated the importance of \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins in Vn-induced p38 MAPK activation using human melanoma M21 and CS1 cell systems. In the M21 cell system, the M21-L12 line does not express the \( \alpha_v \) integrin subunit, thus no \( \alpha_v \) integrin is present at the cell surface; the M21-L4 line was established by the stable transfection of the \( \alpha_v \) integrin subunit in M21-L12 cells, and this line expresses both \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins (58). In the CS1 cell system, the CS1 line expresses neither \( \beta_3 \) nor \( \beta_5 \) integrin subunit; the CS1/\( \beta_3 \) and CS1/\( \beta_5 \) lines were created by...
the stable transfection of β3 subunit or β5 subunit in CS1 cells, thus these lines express either αvβ3 or αvβ5 integrin, respectively (59, 60). Allowing M21-L4, CS1/αv, or CS1/β3 cells to attach on Vn-coated surface for 30 min resulted in a significant increase in p38 MAPK phosphorylation (Fig. 5, A and B). However, plating M21-L12 or CS1 cell on Vn did not lead to p38 increase in p38 MAPK activation (Fig. 5, A and B). These results suggest that both αvβ3 and αvβ5 integrins are capable of mediating p38 MAPK activation.

To determine which integrin subunit is important for p38 MAPK activation, we examined the effect of Vn on p38 MAPK activation in M21-L12 cells transfected with αv integrin subunit lacking its intracellular domain (αvΔ995). Although both this line and M21-L4 line adhered equally well to Vn (data not shown), the Vn-induced p38 MAPK activation was completely abolished in M21/αvΔ995 cells (Fig. 5A). These results suggest that the cytoplasmic tail of αv subunit is required for αv integrin ligation-induced p38 MAPK activation. Subsequently, we also examined Vn-induced p38 MAPK activation in CS1 cells transfected with intracellular domain-lacking β3 (β3Δ716) or β5 subunit (β5Δ720) plasmids, and we found that Vn-induced p38 MAPK activation was not disabled in these two lines (Fig. 5B). These results strongly suggest that the intracellular domain (the cytoplasmic tail) of the αv subunit, rather than the intracellular domain of the β3 or β5 subunit, is essential for αv integrin-mediated p38 MAPK activation.

**DISCUSSION**

Our previous studies (50) have demonstrated that the endogenous p38 MAPK activity is elevated in invasive breast cancer cells and that constitutive p38 MAPK activity is essential for uPA/uPAR expression and matrix invasion by breast cancer cells. However, it is not clear how elevated p38 MAPK activity is maintained in invasive breast cancer cells. Early studies (51–56) have shown that extracellular matrix proteins including vitronectin, osteopontin, fibronectin, and laminin can up-regulate uPA expression in various cell types including melanoma, macrophage, and breast cancer cells. Several recent studies (45, 57) have also demonstrated that engaging integrins with their ligands can activate p38 MAPK in mast and fibroblast cells. These findings prompted us to investigate whether integrin ligation contributes to the elevated p38 MAPK activity in invasive breast cancer cells. We found that treatment of highly invasive MDA-MB-231 cells with function-blocking mAb to αv integrins greatly reduced the level of p38 MAPK activity and uPA expression (Figs. 1A and 2A). Similarly, down-regulating αv integrin expression with antisense oligonucleotides also significantly inhibited p38 MAPK activity and uPA expression in MDA-MB-231 cells (Figs. 1B and 2B). These findings suggest a function link between αv integrin, p38 MAPK, and uPA expression in invasive breast cancer cells.

Whereas αv integrins are expressed in both invasive and noninvasive breast cancer cells, we detected elevated p38 MAPK activity and uPA overproduction in most of invasive breast cancer cells but not in non-invasive cells (50). We thus compared the extent of αv integrin ligation-induced p38 MAPK activation and uPA up-regulation in both invasive and non-invasive cells. We have shown that plating invasive MDA-MB-231 cells on Vn-coated surface induces dramatic p38 MAPK phosphorylation (Fig. 3A) and up-regulates uPA expression (Fig. 4A); in contrast, engaging αv integrins with immobilized Vn neither activated p38 MAPK (Fig. 3B) nor induced uPA expression in non-invasive MCF7 cells (Fig. 4B). A recent study reported that αv integrins can be present in two functional states in breast cancer cells, and only the activated state of αv integrins is expressed in metastatic cells (61). Functional studies further showed that only the activated state of αv integrins were able to promote breast cancer cell invasion and metastasis (61, 62). Therefore, we consider the possibility that only the activated state of αv integrins is capable of mediating p38 MAPK activation and uPA up-regulation.

Both integrin subunits are capable of mediating signaling events. In the present study, we have shown that only the cytoplasmic tail of αv integrin subunit is essential for Vn-induced p38 MAPK activation. A recent study (47) has shown that engaging the αvβ3 integrin with a three-dimensional col-

**Fig. 4.** Adherence to Vn specifically induces uPA expression in invasive breast cancer cells. Invasive MDA-MB-231 and non-invasive MCF-7 cells were plated on Vn-coated or noncoated surfaces for 1 or 2 days. Cells were then lysed, and immunoblotting was performed to detect cell-associated uPA.

**Fig. 5.** The cytoplasmic tail of the αv integrin subunit is essential for αv integrin ligation-induced p38 MAPK activation. Cells were starved overnight and kept in suspension for 30 min. Cells were then plated on Vn-coated surface for 30 min and then lysed, and active p38 MAPK was detected by immunoblotting. A, M21-L12, M21-L4, and M21/αvΔ995. B, CS1, CS1/β3, CS1/β3Δ716, CS1/β5, and CS1/β5Δ720.
lagen gel activates p38 MAPK. In another study (49), the cytoplasmic tail of αv integrin subunit was found to be specific for collagen I-stimulated p38 MAPK activation in NMuMG cells. Furthermore, the amino acid residues essential for p38 MAPK activation are distinct from those required for ERK activation (48). We currently are investigating the role of the αv integrin cytoplasmic tail in uPA up-regulation.

Substantial evidence has been generated which indicates that uPA plays an important role in tumor invasion and metastasis (3, 4). Experimental models have also shown that inhibiting uPA function can significantly block tumor progression in animals (19–21). However, broad inhibitors of uPA (serpin) have not been considered as therapeutic agents because of their general effect on the fibrinolytic system (4). In the present study, we demonstrate that the amino acid residues essential for p38 MAPK activation in NmuMG cells.

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