PAI1 stimulates assembly of the fibronectin matrix in osteosarcoma cells through crosstalk between the αvβ5 and α5β1 integrins

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Summary

The plasminogen activation system regulates matrix remodeling through both proteolytic and non-proteolytic mechanisms. Studies were undertaken to determine the effects of the plasminogen activator inhibitor 1 (PAI1) on the assembly of the fibronectin matrix. The addition of PAI1 to MG-63 cells caused a 1.5- to threefold increase in the rate of fibronectin matrix assembly which was associated with an increase in β integrin activation. PAI1 treatment led to a marked decrease in focal contacts and stress fibers, whereas tensin-containing matrix contacts remained unaffected. The effects of PAI1 on matrix assembly were independent of both urokinase-type plasminogen activator (uPA) and urokinase-type plasminogen activator receptor (uPAR), indicating that the stimulation of matrix assembly by PAI1 does not depend on its anti-proteolytic activity or on the association of uPAR with integrin receptors. Antagonists of the αvβ5 integrin mimicked the effect of PAI1 on cell morphology and fibronectin matrix deposition, indicating that stimulation of matrix assembly by PAI1 required disruption of the interaction between the αvβ5 integrin and vitronectin. Consistent with this conclusion, the Q123K PAI1 mutant which does not bind vitronectin had no effect on matrix assembly. Our data identify PAI1 as a novel regulator of fibronectin matrix assembly, and indicate that this regulation occurs through a previously undescribed crosstalk between the αvβ5 and α5β1 integrins.

Key words: PAI1, Integrin, Fibronectin, Extracellular matrix, Actin

Introduction

Fibronectin is widely expressed by multiple cell types and plays important roles in vertebrate development as well as in cell adhesion, migration, invasion, growth and differentiation. Formation of the fibronectin matrix involves a cell-driven mechanical stretching of fibronectin, which is progressively incorporated into a dense detergent-insoluble fibrillar network through interactions with other cell-associated fibronectin dimers (reviewed in Wierzbicka-Patynowski and Schwarzbauer, 2003). In most cell types, the α5β1 integrin is responsible for the polymerization of fibronectin matrix. The matrix assembly activity of α5β1 integrin can be modulated by changes in the activation state of the integrin that occur in association with changes in integrin conformation (Mould et al., 2002). Changes in the level of activated α5β1 integrins on the cell surface can be induced by modifications in signaling pathways (Brenner et al., 2000) and through the formation of complexes with cytoskeletal components (Giannone et al., 2003). Cell-surface molecules also regulate α5β1-integrin activation. Among them, urokinase-type plasminogen activator receptor (UPAR, also known and hereafter referred to as uPAR), is a GPI-anchored molecule that has been reported to form physical complexes with integrins, including α5β1 integrin and modulate their function (Aguirre-Ghiso et al., 2001; Wei et al., 1996). More recently, we have shown that uPAR can increase fibronectin-matrix deposition and α5β1-integrin activation through a Src-EGFR signaling pathway that is independent of the formation of complexes between uPAR and α5β1 integrin (Monaghan-Benson and McKeown-Longo, 2006).

The plasminogen activator system is involved in matrix remodeling through regulating the activity of urokinase-type plasminogen activator (UROK, also known and hereafter referred to as uPA), which converts plasminogen into plasmin and, thus, mediates pericellular proteolysis (for a review, see Schmitt et al., 1992). Plasminogen activator inhibitor 1 (SERPINE1, also known and hereafter referred to PAI1) is the main in vivo inhibitor of uPA, thus playing a role in extracellular matrix turnover by regulating pericellular plasmin. In addition to plasmin-mediated remodeling, the plasminogen-activator system also modulates cell behavior through non-proteolytic events. Both PAI1 and uPAR bind to the adhesive protein vitronectin. Vitronectin binds PAI1 with high affinity and regulates PAI1 activity by stabilizing PAI1 in its active conformation. The PAI1 binding site on vitronectin lies in the N-terminal SMB domain and functionally overlaps with both the uPAR- and integrin-binding sites. PAI1 is able to competitively inhibit the binding of uPAR and integrin to vitronectin and has been reported to both stimulate and inhibit cell migration (Deng et al., 2001; Okumura et al., 2002; Stefansson and Lawrence, 1996). However, PAI1 can also regulate integrin function through another mechanism which does not require its interaction with vitronectin. PAI1 has been shown to deactivate integrins by binding to the uPA present in uPA-uPAR-integrin complexes on the cell surface resulting in the inactivation of the integrin and the subsequent detachment of cells from the matrix. This activity of PAI requires the binding of PAI to uPA while the latter is bound to uPA (Czekay et al., 2003).

We have shown previously that PAI1 can work synergistically with a uPAR agonist to stimulate fibronectin-matrix assembly in

human osteosarcoma cells (Vial et al., 2006). The current study was undertaken to examine the mechanism that underlies the stimulation of fibronectin-matrix assembly by PAI1. Earlier reports have shown that vitronectin inhibits the assembly of the fibronectin matrix (Hocking et al., 1999; Zhang et al., 1999) and that this inhibition depends on the binding of vitronectin integrins to vitronectin (Zheng et al., 2007). Here, we test the hypothesis that PAI1 stimulates matrix assembly by relieving the inhibitory effects of vitronectin. We now report that PAI1 causes an increase in fibronectin-matrix assembly and in the activation of the α5β1 integrin. The effect of PAI1 on matrix assembly was independent of uPAR and uPA, and resulted from inhibiting the interaction between the αvβ5 integrin and vitronectin. These findings indicate that the regulation of matrix assembly by PAI occurs through a novel crosstalk mechanism between the αvβ5-integrin and α5β1-integrin receptors.

Results

PAI1 increases fibronectin-matrix assembly through a mechanism independent of uPA or uPAR

Incubation of monolayers of MG-63 cells with PAI1 resulted in a dose-dependent increase in fibronectin-matrix assembly (Fig. 1). Stimulation of matrix assembly was seen at 5 nM PAI1 and maximal levels of fibronectin deposition were observed at 100 nM PAI1. Increasing the amount of PAI1 beyond 100 nM resulted in no further stimulation (data not shown). To test whether the binding of PAI1 to either vitronectin or uPA was required for the stimulatory effect of PAI1 on matrix formation, MG-63 cells were incubated with mutant forms of PAI1 that are deficient in specific binding activities. The Q123K mutant, which does not bind to vitronectin, had no effect on fibronectin matrix assembly, indicating that the binding of PAI1 to vitronectin is required to increase assembly of fibronectin matrix. This result was in agreement with our earlier experiments using cells adherent to either vitronectin or fibronectin substrates, in which PAI1 (under conditions of uPAR stimulation) caused an increase in matrix assembly only in the presence of vitronectin (Vial et al., 2006). The PAI1R mutant, which does not bind to uPA but does bind to vitronectin, increased fibronectin polymerization to the same extent as wild-type PAI1, suggesting that the stimulatory effects of PAI1 on matrix assembly requires binding to vitronectin, but does not require the association of PAI1 with uPA. Fig. 1B shows that treatment of cells with PAI1 caused a small, but significant increase in the number of activated β1 integrins. There was no change in the total amount of β1 integrin (data not shown). As reported previously (Vial et al., 2006), ligation of uPAR with the peptide agonist P25 also increased integrin activation and this activation was further enhanced by PAI1.

PAI1 binds vitronectin in close proximity to the uPAR-binding site and can competitively inhibit the binding of uPAR to vitronectin (Deng et al., 1996). To evaluate the role of uPAR on the PAI1 stimulation of matrix assembly, uPAR was knocked down in MG-63 cells by using small interfering RNA (siRNA). Fig. 2A indicates that uPAR expression was almost completely inhibited in MG-63 cells transfected with siRNA targeting uPAR, and uPAR knockdown had no effect on the expression of actin or on the expression of the β5 integrin. Treatment of uPAR-knockdown cells with PAI1-stimulated matrix assembly to levels seen in control cells (Fig. 2B). By contrast, stimulation of matrix assembly by the uPAR ligand P25 was severely attenuated in uPAR-knockdown cells (Fig. 2C). Taken together with the results in Fig. 1, these data demonstrate that the PAI1-dependent stimulation of integrin activation and matrix assembly depend on the binding of PAI1 to vitronectin but do not require either uPA or uPAR.

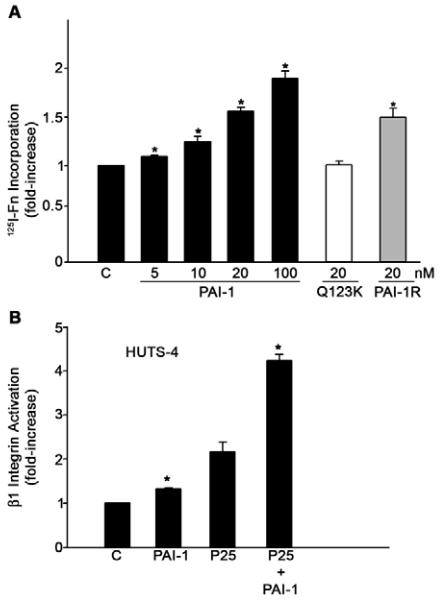

Fig. 1. PAI1 increases fibronectin-matrix assembly and β1-integrin activation in MG-63 cells. (A) MG-63 cells were incubated with [^{125}I]fibronectin for 6 hours in the presence of the indicated concentrations of PAI1, PAI1R or PAI1 (Q123K) in DMEM supplemented with 0.02% BSA and 20 mM HEPES. Cell layers were extracted with 1% DOC, and [^{125}I]fibronectin that was incorporated into the detergent-insoluble matrix was recovered by centrifugation and measured by γ-scintillation. This experiment is representative of three separate experiments. Data are the mean ± s.e. of two experiments performed in duplicate. *P<0.05 (n=4), significantly different than control cells (t-test). (B) MG-63 cells were pretreated with 20 nM PAI1 for 20 minutes and incubated with 50 μM of uPAR agonist P25 or S25 for 1 hour in DMEM. Activation of β1 integrin was assessed by ELISA using the HUTS-4 antibody. Total levels of β1 integrin were measured using the P5D2 antibody against α5β1 integrin. The graph shows the levels of β1-integrin activation after normalization to total β1 levels. Neither P25 nor PAI1 had any effect on the levels of total β1 integrin. Data represent one of three different experiments performed in triplicate. *P<0.05 (n=3), significantly different than control or P25-treated cells (t-test).

Disruption of the αvβ5-integrin–vitronectin interaction increases fibronectin-matrix assembly

Our results indicate that the binding of PAI1 to vitronectin increases fibronectin-matrix deposition by MG-63 cells. The PAI1 binding site on vitronectin is close to the RGD motif of vitronectin and PAI1 binding can sterically inhibit integrin-dependent adhesion to this motif (Lawrence et al., 1994). To determine whether PAI1-mediated effects on matrix assembly resulted from a loss of vitronectin-receptor binding to vitronectin, cells were incubated with blocking antibodies and peptides to disrupt the association of integrin receptors with vitronectin. Treatment of MG-63 cells with the cyclic peptide RGDfV, which disrupts the interaction of αvβ3 and αvβ5 integrins with vitronectin, led to an increase in fibronectin-matrix deposition (Fig. 3A). No effect was observed when the inactive analog RADfV was used. Control adhesion and spreading experiments indicated that the RGDfV peptide completely blocked adhesion of cells to vitronectin but had no effect on adhesion or spreading of cells on fibronectin (data not shown). Incubation of cells with the αvβ3-integrin-blocking antibody LM609 had no effect on fibronectin assembly. However, treatment of cells with the β5-

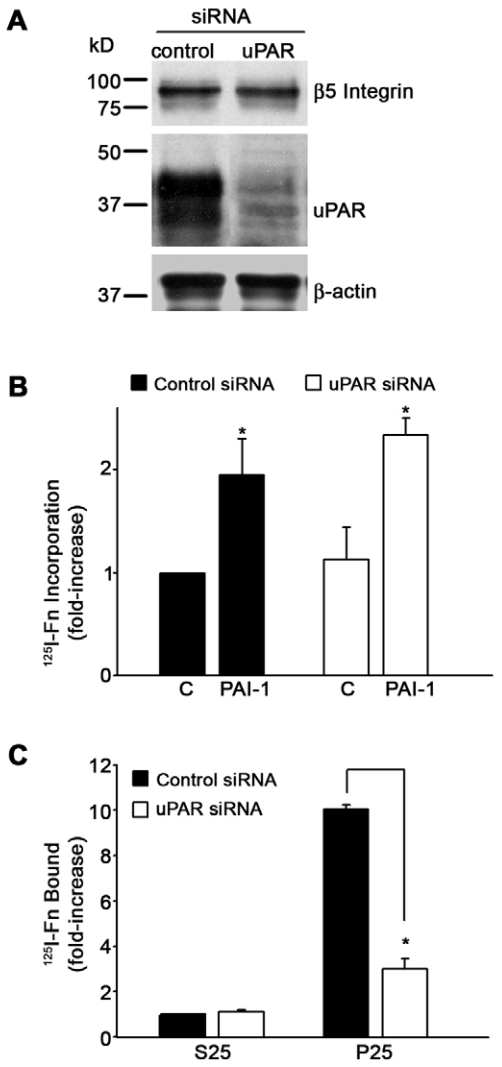

Fig. 2. The PAI1-mediated increase in fibronectin matrix assembly does not require uPAR. (A) Cell lysates were prepared from MG-63 cells transfected with control siRNA or uPAR siRNA. uPAR and β5 integrin levels were analyzed by western blotting. The membrane was stripped and reprobed with anti-β-actin antibody as a loading control. (B) Cells layers from MG-63 cells transfected with control siRNA or uPAR siRNA were incubated with [^{125}I]fibronectin for 6 hours in the absence or presence of PAI1 (20 nM). Cell layers were extracted with 1% DOC, and [^{125}I]fibronectin that had been incorporated into the detergent-insoluble matrix was recovered by centrifugation and measured by γ-scintillation. Data are the mean ± s.e. of two experiments performed in duplicate. *P<0.05 (n=4), significantly different than untreated cells (t-test). (C) Cells layers from MG-63 cells transfected with control siRNA or uPAR siRNA were incubated with [^{125}I]fibronectin for 6 hours in the presence of the uPAR agonist P25 or the control peptide S25. Cell layers were washed and scraped into 1% DOC and cell-layer associated [^{125}I]fibronectin was measured by γ-scintillation. Data are the mean ± s.e. of two experiments performed in duplicate. *P<0.05 (n=4), significantly different than control cells (t-test).

integrin-blocking antibody P1F6 significantly increased fibronectin-matrix assembly (Fig. 3A).

Our previous studies have shown that treatment of MG-63 cells with the uPAR agonist P25 causes a substantial increase in the rate of matrix assembly and addition of PAI1 causes a further increase in matrix assembly. To determine whether the effects of integrin antagonists on matrix assembly occur under conditions

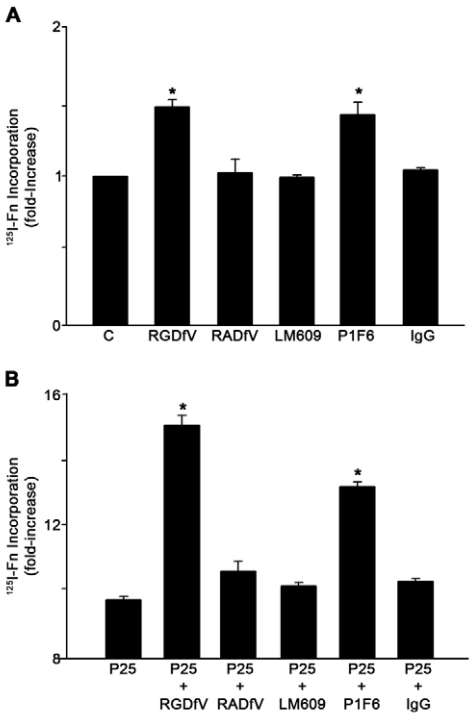

Fig. 3. Inhibition of the interaction between αvβ5-integrin and vitronectin increases fibronectin-matrix assembly. (A) MG-63 cells were pre-incubated for 45 minutes with the cyclic peptide RGDfV (20 μM) or its inactive analog RADfV (20 μM) or for 2 hours with 20 μg/ml of normal mouse IgG, β3-integrin-blocking antibody LM609 or β5-integrin-blocking antibody P1F6; cells were then incubated with [^{125}I]fibronectin (Fn) for 6 hours. The data shown here are representative of three separate experiments. Data are the mean ± s.e. of two different experiments performed in duplicate. *Significantly different than control cells, t-test, P<0.05 (n=4), significantly different than control cells (t-test). (B) Same as A only under conditions of uPAR stimulation with P25. Cell layers were extracted in 1% DOC, and [^{125}I]fibronectin that was incorporated into the detergent-insoluble matrix was recovered by centrifugation and measured by γ-scintillation. This experiment is representative of three separate experiments. Data are the mean ± s.e. of two experiments performed in duplicate. *P<0.05 (n=4), significantly different than P25-treated cells (t-test).

of uPAR stimulation, cells were incubated with P25 in the presence of vitronectin-receptor antagonists RGDfV or P1F6. Incubation of cells with P25 resulted in a ninefold increase in fibronectin matrix assembly. This rate of matrix assembly further increased almost 15-fold above baseline in the presence of peptide antagonists of the vitronectin receptor (Fig. 3B). Similar results were seen when cells were incubated with the αvβ5-integrin-blocking antibody P1F6. These data indicate that the effects of PAI1 on matrix assembly can be mimicked by agents that disrupt αvβ5-integrin binding to vitronectin. Similar effects of PAI1- and integrin-disrupting agents were seen in three other cell lines. As shown in Fig. 4A, treatment of human osteosarcoma cells (HOS or Saos-2) or osteoblasts (hFOB) with either PAI1 or RGDfV caused a 30-50% increase in fibronectin-matrix assembly. PAI1 and RGDfV also increased matrix assembly substantially when added to the cells under conditions of uPAR stimulation (Fig. 4B).

Disruption of αvβ5-integrin–vitronectin interaction leads to an increase in β1-integrin activation

As shown in Fig. 1B, the addition of PAI1 to MG-63 cells caused a significant increase in activated β1 integrin on the cell surface.

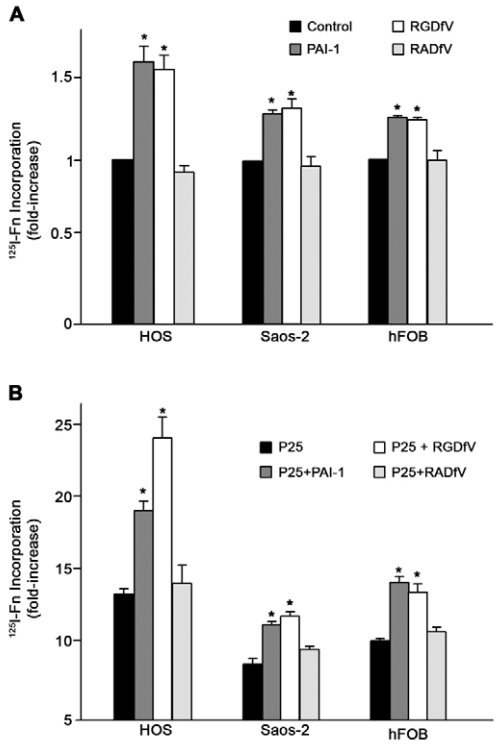

Fig. 4. PAI1 stimulates fibronectin-matrix assembly in osteosarcoma and osteoblast cells. (A) Cells (HOS, Saos-2, hFOB) were pre-incubated for 20 minutes with PAI1 (20 nM), or for 45 minutes with the cyclic peptide RGDfV (20 μM) or its inactive analog RADfV (20 μM); cells were then incubated with [^{125}I]fibronectin (Fn) for 6 hours. Cell layers were extracted in 1% DOC, and [^{125}I]fibronectin that had been incorporated into the detergent-insoluble matrix was recovered by centrifugation and measured by γ-scintillation. This experiment is representative of three separate experiments. Data are the mean ± s.e. of two different experiments performed in duplicate. *P<0.05 (n=4), significantly different than control cells (t-test). (B) Same as A only under conditions of uPAR stimulation with 50 μM P25. This experiment is representative of three separate experiments. Data are the mean ± s.e. of two experiments performed in duplicate. *P<0.05 (n=4), significantly different than P25-treated cells (t-test).

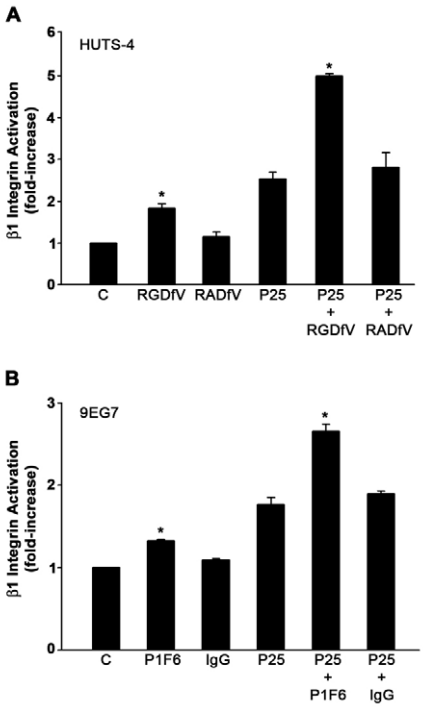

Fig. 5. Activation of β1 integrin by β5-integrin-blocking agents. (A) MG-63 cells were pretreated for 45 minutes with 20 μM of the cyclic peptides RGDfV or RADfV and incubated with 50 μM of P25 or S25 in DMEM. Activation of β1 integrin was assessed by ELISA using the HUTS-4 antibody. (B) MG-63 cells were preincubated for 2 hours with 20 μg/ml of normal mouse IgG, or β5 integrin blocking antibody P1F6 before treatment with P25 or S25. Integrin activation was assessed using monoclonal antibody 9EG7. All graphs show the levels of β1-integrin activation after normalization to total β1 levels as described in Fig. 1. Total β1-integrin levels did not change. Data represent one of three different experiments performed in triplicate. *P<0.05 (n=3), significantly different than control or P25-treated cells (t-test).

To determine whether antagonists of αvβ5 integrin can modulate the activation of α5β1 integrin, ELISA assays were performed. Cells were treated with either the cyclic peptide RGDfV or with an αvβ5-integrin-blocking antibody (PIF6) and the amount of activated β1 integrin was assessed using antibodies that recognize the active conformer (Fig. 5). The RGDfV peptide and the PIF6 antibody (Fig. 5A and B, respectively) increased β1 activation under basal conditions, and under conditions of uPAR stimulation with P25. β1-integrin activation was increased to levels similar to that seen with PAI1 (compare Fig. 5 with Fig. 1B). There was no change in total levels of β1 integrin (data not shown). The control peptide RADfV and mouse IgG had no effect on β1-integrin activation. These data suggest that the disruption of the αvβ5-integrin–vitronectin interaction leads to an increase in both β1-integrin activation and fibronectin-matrix assembly.

PAI1 disrupts αvβ5-integrin-containing adhesions and actin stress fibers

To evaluate whether the addition of exogenous PAI1 to cells disrupts the binding of αvβ5 integrin to vitronectin, αvβ5-integrin-receptor localization was visualized by indirect immunofluorescence. As shown in Fig. 6, control cells express numerous clusters of αvβ5 integrin, which are colocalized with paxillin in focal contacts (Fig. 6a,e,i). Treatment of cells with PAI1 lead to a marked decrease in the number of focal contacts with fewer clusters of β5 integrins and decreased paxillin staining (Fig. 6b,f,j). Similar results were seen when cells were treated with the PAI1 mutant PAI1R, which binds to vitronectin but not to uPA (Fig. 6c,g,k). Treatment of cells with the PAI1 mutant (Q123K), which does not bind vitronectin, failed to disrupt the focal contacts (Fig. 6d,h,l). These data have been quantified in Table 1 and show that >90% of the cells exhibit marked decreases in focal-contact numbers. The PAI1-mediated disruption of focal contacts was also associated with a change in the organization of actin filaments. As shown in Fig. 7, cells treated with PAI1 exhibited a loss of stress fibers associated with the appearance of actin aggregates at the periphery of the cell (compare Fig. 7, panels a and b). The PAI1R mutant also disrupted actin-filament structure (Fig. 7c) indicating that the effect of PAI1 on stress fibers did not require uPA binding. The PAI1 Q123K mutant had no effect on actin organization (Fig. 7d) consistent with a requirement for vitronectin binding in the effect of PAI1 on cytoskeletal organization. The PAI1-induced loss of stress fibers was accompanied by fewer clusters of β5-integrin receptors (Fig. 7e-h). These data indicate that the addition of PAI1 to MG-63 cells results in the loss of both focal contacts and actin stress fibers, and that this activity requires the binding of PAI1 to vitronectin.

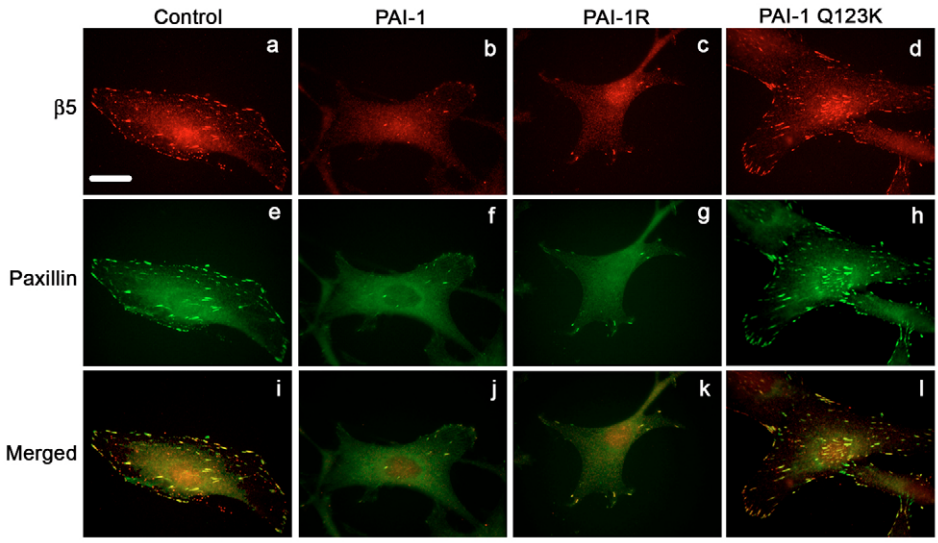

Fig. 6. PAI1 disrupts focal adhesions. Monolayers of MG-63 cells were plated overnight onto coverslips in complete medium and then incubated for 3 hours with 20 nM of PAI1 or PAI1 mutants (PAI-1R and PAI-1 Q123K). Cells were subsequently fixed, permeabilized and stained for 1 hour with the clone 15F11 antibody against β5 integrin (panels a-d). After 1-hour staining using Alexa-Fluor-594-derivatized anti-mouse secondary antibody, cells were washed and labeled for an additional hour with a paxillin-FITC antibody (panels e-h). Panels i-l show merged images that indicate colocalization of β5 integrin with paxillin. Scale bar, 20 μm.

uPAR also binds to vitronectin and is present within the αvβ5-integrin–vitronectin contacts (Fig. 8Aa-c) (see also Salasznyk et al., 2007). To evaluate whether uPAR was required for the PAI1-induced disruption of αvβ5-integrin–vitronectin contacts, PAI1 was added to cells in which uPAR had been knocked down. Treatment of cells with siRNA targeting uPAR (see also Fig. 2A) results in a complete loss of uPAR staining in focal contacts (Fig. 8Ba,d) with no loss of paxillin staining (Fig. 8Bb,e), indicating that uPAR knockdown does not affect the localization of paxillin to focal adhesions. uPAR knockdown also had no effect on β5-integrin clustering (Fig. 8Cb) indicating that it does not affect the binding of the αvβ5 integrin to vitronectin. Incubation of uPAR knockdown cells with PAI1 shows nearly complete loss of clustered β5 integrin (Fig. 8Cd). These data suggest that the disruption of αvβ5 integrin binding to vitronectin by PAI1 is independent of uPAR.

PAI1 does not affect matrix adhesion

Although treatment of MG-63 cells with PAI1 disrupted the association between αvβ5 integrin and vitronectin, there was no cell detachment. As shown in Fig. 9A (panel a), MG-63 cells assembled a fibronectin matrix and this matrix was still present on PAI1-treated cells under conditions where β5 clustering was disrupted (Fig. 9Ac,d). Furthermore, Fig. 9B shows the colocalization of fibronectin with activated β1 integrins (Fig. 9Ba-c). This colocalization was still present after PAI1 treatment (Fig. 9Bd-f), suggesting that PAI1 treatment does not disrupt fibronectin-based matrix adhesions. To evaluate this possibility further, cells were stained for tensin as a marker for matrix adhesions. As shown in Fig. 10A, MG-63 cells exhibited prominent staining for tensin which co-distributed extensively with matrix fibronectin, indicating the presence of matrix adhesions on these cells. These adhesions were present after treatment of cells with PAI1. Fig. 10B shows that control cells have both paxillin and tensin containing adhesions. The merged figure shows little colocalization of these adhesions indicating that they represent distinct structures. Treatment of cells with PAI1 caused a nearly complete loss of paxillin staining, whereas considerable tensin staining remained (Fig. 10B). These data indicate that the addition of PAI to cells disrupts αvβ5-integrin-containing and paxillin-containing focal contacts, whereas α5β1-integrin-containing and tensin-containing matrix adhesions are maintained.

β5-integrin knockdown attenuates PAI1-mediated increases in matrix assembly

To confirm the role of the αvβ5 integrin in the PAI1 stimulation of matrix assembly, the β5 integrin subunit was knocked down in MG-63 cells using siRNA targeting β5 integrin. Fig. 11A indicates that knockdown of the β5-integrin subunit decreased expression of β5-integrin protein. Scanning of western blots from cell lysates showed that the extent of knockdown was 50% (data not shown). β5-integrin knockdown resulted in a 50% increase in basal (control) levels of matrix assembly (Fig. 11B). The addition of PAI1 to cells transfected with control siRNA resulted in a twofold increase in fibronectin-matrix assembly. However, matrix assembly following PAI1 addition was increased by 25% in cells transfected with β5-integrin siRNA compared with control-transfected cells (Fig. 11C). Taken together our data suggest that PAI1 increases fibronectin matrix deposition by inhibiting the interaction between αvβ5

Table 1. Quantification of focal contacts in MG-63 cells treated wild-type PAI1 or PAI1 mutants

	Percentage of cells with		
Treatment	1-10 focal contacts	10-20 focal contacts	>20 focal contacts
None	0	0	100
PAI-1	98	2	0
PAI-1R	94	6	0
Q123K	0	0	100

To examine the number of focal contacts, MG-63 cells were plated onto coverslips, kept overnight in complete medium, and incubated for 3 hours with 20 nM PAI-1 or either of the PAI-1 mutants (PAI-1R and Q123K). Cells were fixed, permeabilized and stained for paxillin. Focal contacts were visualized using an Olympus BMX-60 microscope (100×). For each treatment, 200 cells were analyzed.

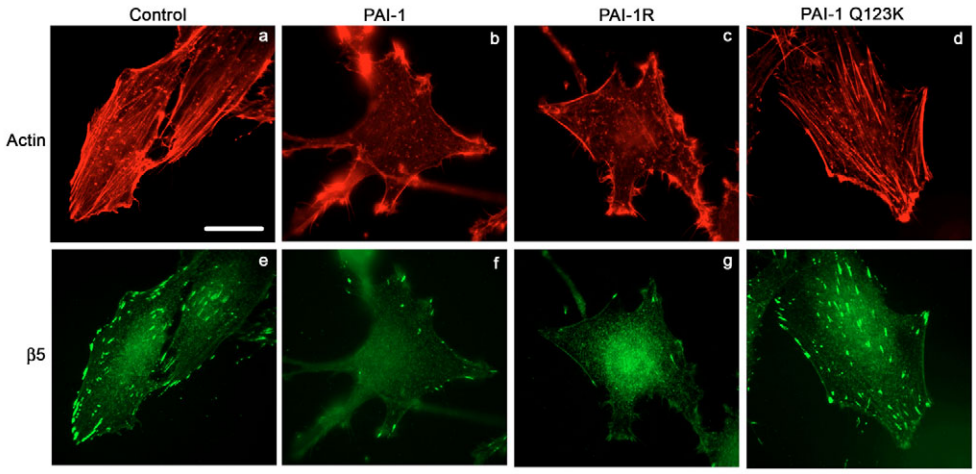

Fig. 7. PAI1 disrupts actin stress fibers. MG-63 cells were plated overnight onto coverslips in complete medium and then incubated for 3 hours with 20 nM of PAI1 or PAI1 mutants (PAI-1R and PAI-1 Q123K). Cells were subsequently fixed, permeabilized and stained for 1 hour with the clone 15F11 antibody against β5 integrin. Cells were then washed and labeled for an additional hour using Alexa-Fluor-488-derivatized anti-mouse secondary antibody and Alexa-Fluor-594–phalloidin. Actin and β5 integrin were visualized by indirect immunofluorescence. Scale bar, 20 μm.

integrin and vitronectin and provide evidence for a previously unrecognized crosstalk between the αvβ5 and α5β1 integrins.

Discussion

Our earlier studies have demonstrated that the assembly of the fibronectin matrix is under complex regulation by the plasminogen-activator system. PAI1 and uPAR have been shown to synergistically stimulate fibronectin polymerization (Monaghan et al., 2004; Monaghan-Benson and McKeown-Longo, 2006; Vial et al., 2006). In the current study, we show that PAI1 stimulates matrix assembly by activating a previously undescribed crosstalk between the αvβ5 and α5β1 integrins. PAI1 stimulation of matrix

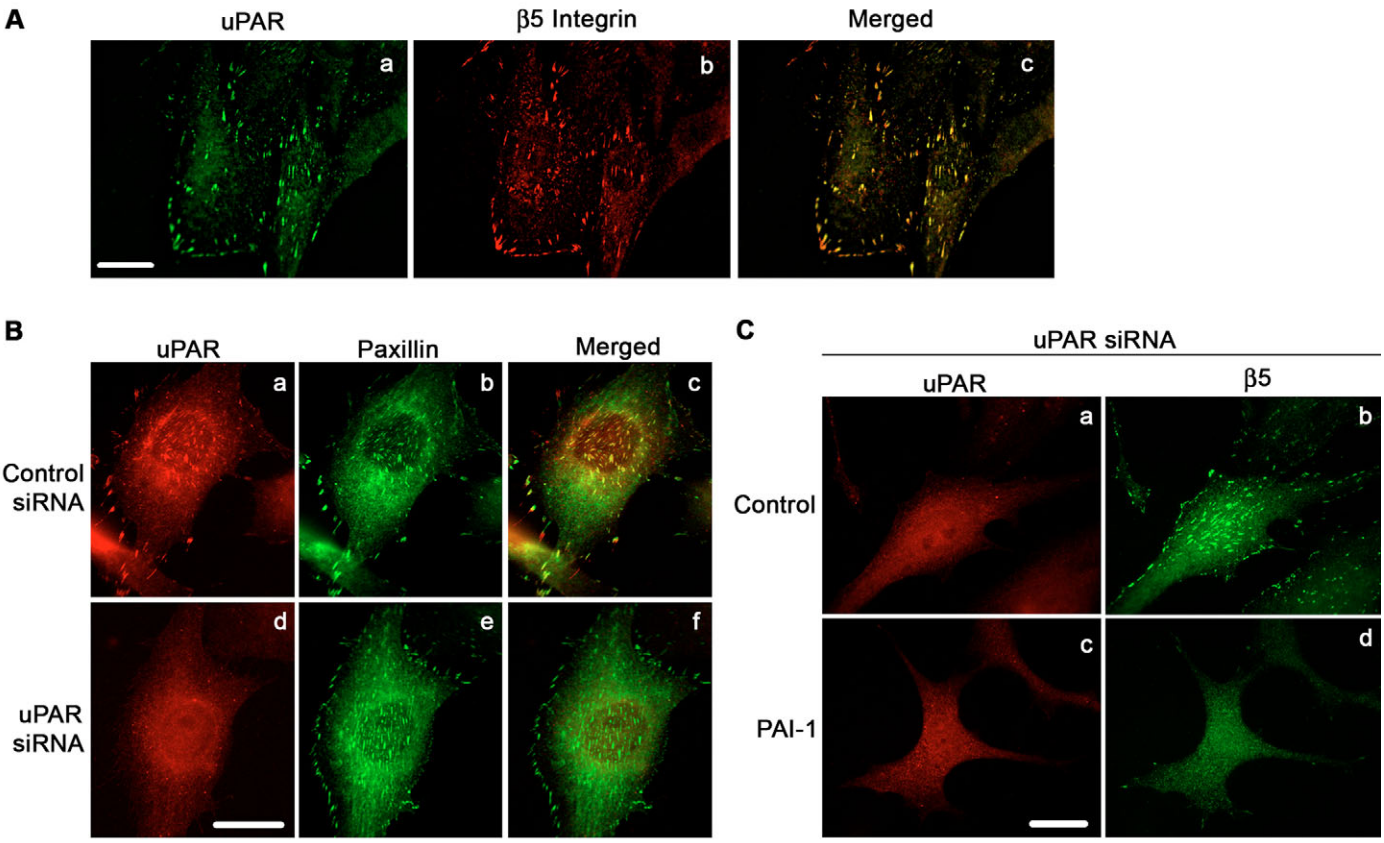

Fig. 8. PAI1-mediated disruption of focal adhesions does not require uPAR. (A) MG-63 cells were plated overnight in complete medium. Cells were subsequently fixed, permeabilized and stained with uPAR polyclonal antibody (panel a) and 15F11 monoclonal antibody against β5 integrin (panel b). uPAR and β5 integrin were visualized by indirect immunofluorescence. Panel c shows the merged image. Scale bar, 20 μm. (B) Cells stained for uPAR and paxillin. Panels a and b show uPAR and paxillin staining in cells transfected with control siRNA. Panels d and e show uPAR and paxillin staining in uPAR knockdown cells. Panels c and f are the merged images. Yellow indicates colocalization of uPAR and β5 integrin. Scale bar, 20 μm. (C) Cells transfected with uPAR siRNA were plated overnight in complete medium and then incubated for 3 hours in DMEM in the presence or absence of PAI1. Scale bar, 20 μm. Cells were subsequently fixed, permeabilized and stained. uPAR and β5 integrin were visualized by indirect immunofluorescence in untreated cells (panels a and b) and PAI1 treated cells (panels c and d).

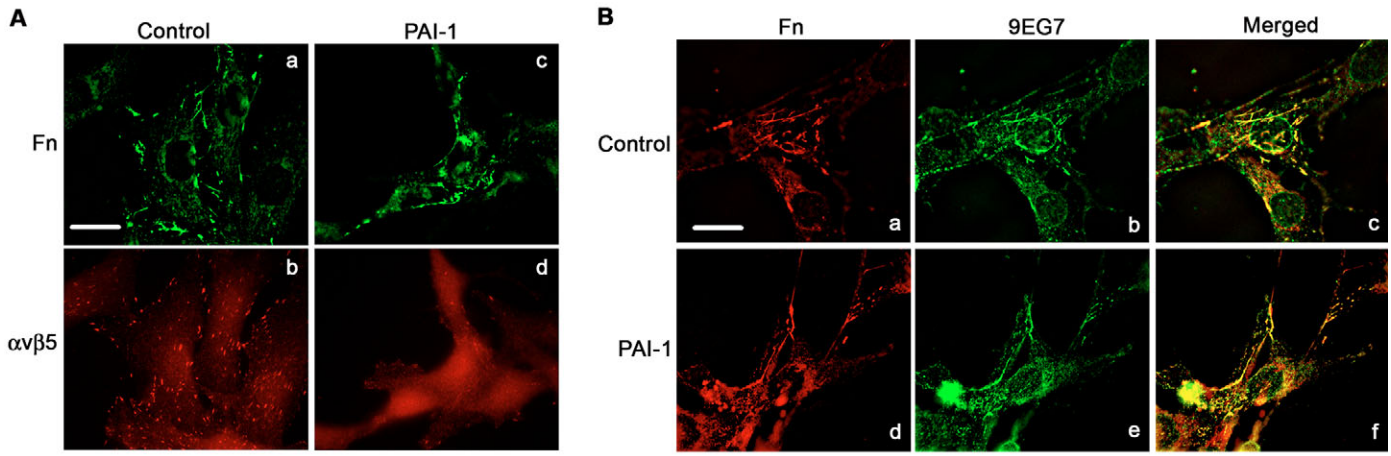

Fig. 9. PAI1 does not disrupt the association of α5β1 integrin with fibronectin matrix. Cells were incubated for 3 hours in DMEM in the presence or absence of PAI1, subsequently fixed, permeabilized and immunostained. (A) Fibronectin matrix and β5 integrin were visualized by indirect immunofluorescence in untreated cells (panels a and b) and PAI1 treated cells (panels c and d). Scale bar, 20 μm. (B) Fibronectin and activated β1 integrin (detected with 9EG7 antibody) were visualized by indirect immunofluorescence in untreated cells (panels a and b) and PAI1 treated cells (panels d and e). Panels c and f shows merged pictures. Yellow staining indicates areas of colocalization of β1 integrin with fibronectin matrix. Scale bar, 20 μm.

assembly occurred over the range of 5-100 nM. Earlier studies have shown that the binding of PAI1 to vitronectin occurs at nM concentrations (Seiffert and Loskutoff, 1991), and that PAI1 is present in the subcellular matrix at nM levels (Higgins et al., 1989). These data indicate that the effects of PAI1 on matrix assembly are seen at physiologically relevant concentrations. The effects of PAI1 on matrix assembly are dependent on vitronectin, but do not require uPAR. The stimulation of matrix assembly by PAI1 is relatively modest (1.5- to 2.5-fold) but is similar to previously reported effects of PAI1 on the stimulation of cell migration (Degryse et al., 2005; Isogai et al., 2001; Takahashi et al., 2005).

The disruption of focal contacts by PAI1 can be mimicked by antagonists of the αvβ5 integrin, suggesting that PAI1 is displacing the integrin from vitronectin. The PAI1-binding site on vitronectin functionally overlaps with the integrin-binding site; however, it is unlikely that PAI is actively displacing previously bound integrin. More conceivable, PAI1 might bind to vitronectin when a binding site becomes available following vitronectin-receptor trafficking out of the focal contact. Studies indicate that active remodeling of contacts occurs and that the expected half-life of a focal contact can be as short as a few minutes (Edlund et al., 2001; Franco et al., 2006). Vitronectin has been shown to negatively regulate matrix assembly (Hocking et al., 1999; Zhang et al., 1999) through a mechanism depending on vitronectin binding to integrins (Zheng et al., 2007). This suggests that PAI1 is relieving an inhibitory activity of αvβ5 integrin. The loss of

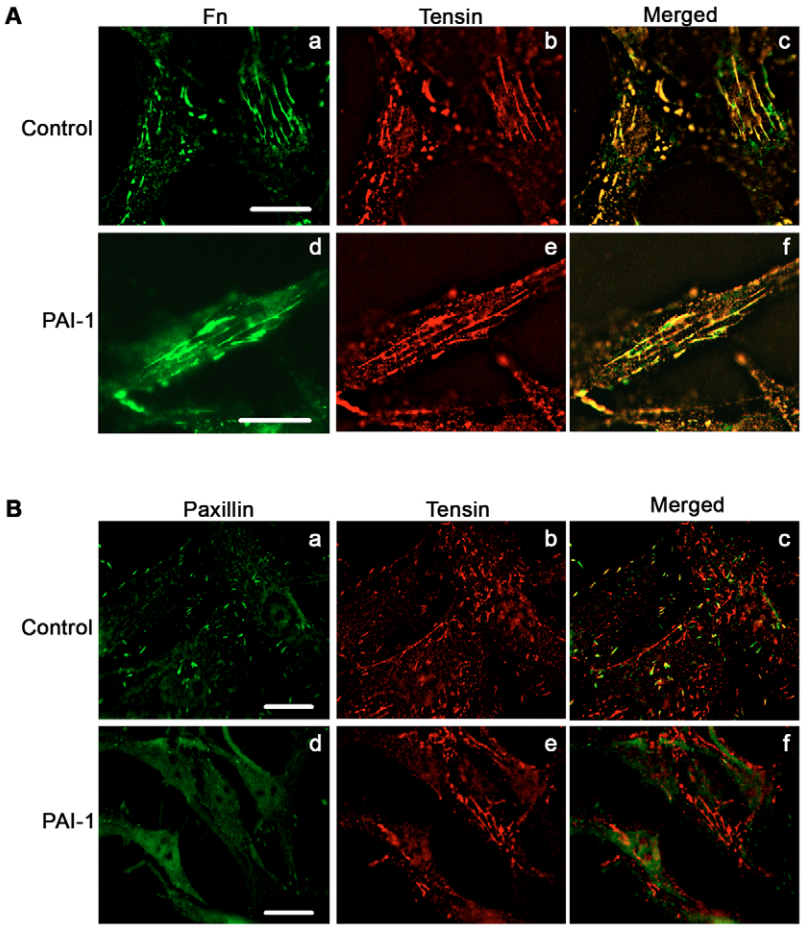

Fig. 10. PAI1 does not disrupt matrix adhesions. Cells were incubated overnight on coverslips in complete medium and then incubated for 3 hours in DMEM in the presence or absence of PAI1 (20 nM). Cells were subsequently fixed, and permeabilized. (A) Fibronectin and tensin were visualized by indirect immunofluorescence in untreated cells (panels a and b) and PAI1 treated cells (panels d and e). Panels c and e show the merged pictures. Yellow staining represents area of colocalization of tensin and fibronectin. Scale bar, 20 μm. (B) Paxillin and tensin were visualized by direct and indirect immunofluorescence in untreated cells (panels a and b) and PAI1 treated cells (panels d and e). Panels c and f shows merged pictures. Scale bar, 20 μm.

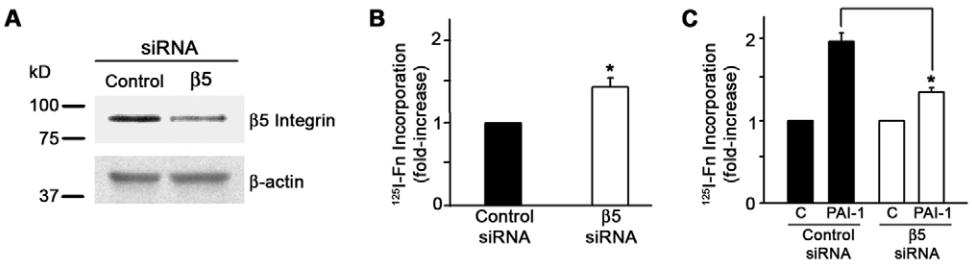

Fig. 11. β5 knockdown inhibits PAI1 stimulation of matrix assembly. (A) Lysates from MG-63 cells transfected with either control siRNA or β5 siRNA were analyzed for β5 integrin and actin using western blotting. (B,C) MG-63 cells treated with either control or β5 siRNA were incubated with [^{125}I]fibronectin for 6 hours in the presence of 20 nM PAI. Cells were extracted with 1% DOC, and [^{125}I]fibronectin that had been incorporated into detergent-insoluble matrix was recovered by centrifugation and measured by γ-scintillation. Experiments are representative of separate experiments performed in triplicate. Data are the mean ± s.e. of one experiment performed in triplicate. $P<0.05$ ($n=3$), *t*-test. All values were normalized to protein content. No significant difference in protein content was observed between control siRNA and β5 siRNA-treated cells.

vitronectin-receptor binding results in a loss of actin stress fibers and a decrease in paxillin-containing focal adhesions. PAI1 treatment had no effect on matrix adhesions because tensin and α5β1 remained colocalized with the fibronectin matrix. These data indicate that the effects of PAI1 on cell adhesion are selective, causing a loss of focal adhesions but having no effect on matrix adhesions. The data also indicate that β1-integrin activation occurs in the absence of stress fibers, and that focal contacts, but not matrix adhesions, support stress fiber organization in these cells. These findings are consistent with earlier reports in HT-1080 cells, which showed that actin stress fibers are not required for fibronectin-matrix assembly (Brenner et al., 2000). Our data suggest that the disengagement of αvβ5 integrin from vitronectin stimulates the assembly of fibronectin matrix by increasing the number of activated α5β1 integrins on the cell surface. The mechanism by which the disruption of the αvβ5-integrin–vitronectin interaction by PAI1 regulates α5β1-integrin function is unclear. The disengagement of αvβ5 integrins results in organizational changes in actin, which might impact the association of the α5β1 integrin with cytoskeleton-associated proteins that are involved in integrin activation (reviewed in Arnaout et al., 2007).

Our earlier studies have shown that uPAR agonists can stimulate matrix assembly through inside-out signaling pathways, leading to integrin activation (Monaghan-Benson and McKeown-Longo, 2006). PAI1 can further stimulate matrix assembly under conditions of uPAR ligation resulting in synergistic increases in matrix assembly rates (Vial et al., 2006). In this study, we show that treatment of cells with PAI1 stimulates matrix assembly and integrin activation when uPAR is knocked down. Our results suggest that PAI and uPAR regulate matrix assembly through independent pathways, which converge at the level of integrin activation. Previous studies have shown that the addition of PAI1 to HT-1080 cells causes the inactivation of both β3 and β1 integrins (Czekay et al., 2003). In those studies, integrin inactivation by PAI1 required the binding of PAI1 to uPA while the latter was bound to uPA. However, in our studies PAI1 could disrupt the association of αvβ5 integrin with vitronectin, but we found no requirement for uPA or uPAR in the PAI1-mediated loss of αvβ5-integrin-containing adhesions. We also found that PAI1 strengthened rather than disrupted the association of β1 with the fibronectin matrix. The basis for this discrepancy is not clear but might have to do with the amounts of PAI1 used in each of the studies. PAI1-mediated integrin inactivation required 40 times higher levels of PAI1 (800 nM) than the amounts used in our studies, in which disruption of focal contacts and stimulation of matrix assembly were seen at 20 nM PAI1.

A recent study has shown that cells adhering to vitronectin in the presence of PAI1 are only partially inhibited from attachment (Stefansson et al., 2007). Those cells that can overcome the inhibitory effects of PAI1 on adhesion are partially spread and do not assemble either focal contacts or stress fibers. Adhesion of these cells to vitronectin in the presence of PAI1 required polymerized microtubules and actin. Our results are consistent with these findings, and extend to show that the addition of PAI1 to adherent monolayers of osteosarcoma cells causes a fairly rapid (within <3 hours) disruption of previously formed focal contacts and stress fibers. Taken together, these results suggest that cells can modulate the effects of PAI1 on cell adhesion or matrix assembly by altering the association of vitronectin integrin receptors with the cytoskeleton.

Increased PAI1 levels are seen in many cancers and are positively correlated with disease progression (Chambers et al., 1998; Foekens et al., 2000). The balance between local PAI1 concentrations and integrin activation may have important implications for processes, such as angiogenesis and/or tumor cell metastasis, where leaky blood vessels can contribute to increased amounts of vitronectin in the matrix. Previous studies using haptotaxis assays have shown that preincubation of vitronectin with PAI1 blocks cell binding to vitronectin and promotes migration of cells toward fibronectin (Isogai et al., 2001). Our studies show that the incubation of adherent cells with PAI1 stimulates the activation of the α5β1 integrin and increases the rate of fibronectin polymerization. The effects of PAI1 on matrix assembly and cell motility are both dependent on vitronectin and suggest that the two PAI1-dependent phenomena function in concert to support directed cell migration by coupling the recycling of vitronectin receptors to the polymerization of the fibronectin matrix.

Materials and Methods

Reagents and antibodies

Unless otherwise stated, all chemicals were purchased from Sigma (St Louis, MO). The uPAR ligand, peptide P25, sequence AESTYHHLSLGYMYTLN, and the scrambled peptide, S25, sequence NYHYLESSMTALYTLGH, were synthesized by Cell Essentials (Boston, MA). The uPAR small interfering RNA (siRNA) (GGTGAAGAAGGGCGTCCAA), β5 integrin siRNA (GAACAACGGTGGA-GATTTT) and non-targeting control siRNA pool were purchased from Dharmacon. The RGDfV peptide, selective for vitronectin integrins, and the inactive analog RADfV were purchased from Sigma. The selectivity of this peptide was verified in control experiments, which showed that RGDfV had no effect on α5β1-integrin function. The anti-β1-integrin antibodies, clones P5D2 and HUTS-4 were purchased from

Chemicon (Temecula, CA). The 9EG7 antibody, anti-tensin and FITC-labeled anti-paxillin antibodies were obtained from Pharmingen (San Diego, CA). The anti-β5-integrin antibody clones P1F6 and 15F11 (used for the immunofluorescence experiments) were from Chemicon (Temecula, CA). The rabbit anti-β5-integrin antibody (used for western blotting) was from Abcam (Cambridge, UK). Control mouse IgG and antibody against β-actin were obtained from Sigma. Secondary goat anti-mouse and goat anti-rabbit HRP antibodies were purchased from Bio-Rad (Hercules, CA). Alexa-Fluor-594-labeled goat anti-rat antibody was obtained from Molecular Probes (Eugene, OR). Recombinant active PAI1 and the non-vitronectin-binding PAI1 mutant Q123K, were from Molecular Innovations (Southfield, MI). The dual mutant PAI1R (T333R and A335R) that does not bind uPA was kindly provided by D. Lawrence (Internal Medicine, University of Michigan Medical School, Ann Arbor, MI). The anti-uPAR polyclonal antibody was a kind gift from Andrew Mazar (Attenuon, San Diego, CA).

Cell culture

MG-63 and HOS human osteosarcoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), containing antibiotics (penicillin-streptomycin) and 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT). Saos-2 human osteosarcoma cells were from ATCC and were cultured in DMEM containing 15% FBS. Human fetal osteoblasts (hFOB) cells were obtained from ATCC and were cultured in DMEM/F12 medium without Phenol Red but supplememted with 10% FBS, as described previously (Harris et al., 1995).

RNA interference experiments

For the knockdown of β5 integrin, cells were electroporated using SIPORT electroporation buffer from Ambion. In each electroporation cuvette (Genesee Scientific, San Diego, CA), 1.5 μg of siRNA was added to 9×10^4 cells, and electroporation was performed at 450 V, four pulses 100 μseconds, separation between pulses 100 mseconds. Cells were then cultured in six-well plates for 4 days in complete medium. For the uPAR RNA interference (RNAi) experiments, cells were transfected as above and then retransfected on day 2 using Dharmafect 2 as recommended by the manufacturer (Dharmacon). Twenty-four hours later, uPAR-knockdown cells were replated and experiments were performed the following day.

Purification of fibronectin and vitronectin

Human plasma fibronectin was purified from a fibronectin- and fibrinogen-rich by-product of factor VIII production by ion-exchange chromatography on DEAE-cellulose (Amersham Biosciences), and iodinated as described previously (Zheng and McKeown-Longo, 2002). Vitronectin was purified from fibronectin- and fibrinogen-depleted human plasma by heparin-Sepharose-affinity chromatography according to the method of Yatohgo et al. (Yatohgo et al., 1988). Iodinated fibronectin was mixed with bovine albumin, 1 mg/ml, dialyzed against phosphate-buffered saline, and frozen at –80°C until used. Fibronectin (2 mg/ml) was derivatized with Alexa Fluor 488 according to the manufacturer's protocol (Molecular Probes).

Matrix incorporation assay

Cells were seeded onto six-well plates (2.2×10^5 cells per well) in complete medium. The following day, cultures were incubated for 6 hours with [^{125}I]fibronectin (2 μg/ml; 1×10^6 cpm/ml) in DMEM supplemented with 0.02% BSA and 20 mM HEPES in the presence or the absence of PAI1, PAI1 mutants, or various peptides or antibodies, as described in the figure legends. For isolation of detergent insoluble matrix, cells were rinsed in PBS, extracted in 1% deoxycholate (DOC) (in 20 mM Tris buffer pH 8.8) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM iodoacetic acid). Detergent-insoluble matrix was obtained by centrifugation at 39,000 ***g*** for 40 minutes, and associated radioactivity present in the pellet measured using γ-scintillation. To determine the amount of total cell-layer-associated fibronectin, cells were rinsed three times in PBS, scraped directly into 1 ml of 1% deoxycholate and the total cell layer associated [^{125}I]fibronectin was determined by γ-scintillation.

Fluorescence microscopy

MG-63 cells were cultured overnight (30×10^3 cells per coverslip) in DMEM containing 10% FBS serum. Cell were washed and incubated for 3 hours in DMEM supplemented with 0.02% BSA and 20 mM HEPES in the absence or presence of 20 nM PAI1 (or PAI1 mutants), RGDfV or its inactive analog RADfV (20 μM). Cells were rinsed, fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 10 minutes and blocked for 1 hour with 3% BSA in PBS. Coverslips were incubated in PBS supplemented with 1% BSA using the primary antibody as described in the figure legends. Cells were washed in PBS for 5 minutes and incubated with Alexa-Fluor-488- or Alexa-Fluor-594-labeled secondary antibody. After staining, coverslips were mounted with Prolong Antifade according to manufacturer's instructions (Molecular Probes). Slides were examined using an Olympus BMX-60 microscope equipped with a cooled CCD sensi-camera (Cooke, Auburn Hills, MI). Images were acquired using Slidebook software (Intelligent Imaging Innovation, Denver, CO) and processed with Photoshop program (Adobe).

Cell lysis and immunoblot analysis

Cell layers were washed with PBS before solubilization in cell-lysis buffer containing 20 mM Tris-HCl pH 7.4, 1% Triton X-100, 0.5% NP-40, 0.15 M NaCl, 1 mM Na_3VO_4, and one mini-tablet of complete protease inhibitor per 10 ml (Roche, Indianapolis, IN). After incubation on ice for 30 minutes, cell lysates were centrifuged at 20,800 ***g*** for 15 minutes at 4°C and the insoluble pellets discarded. The protein concentration of the lysate was determined using a BCA protein assay reagent (Pierce, Rockford, IL). Aliquots of cell lysates containing equal amounts of protein were subjected to SDS-PAGE on a 8% polyacrylamide gel under non-reducing conditions, followed by transfer to nitrocellulose membrane. Proteins were detected by western blot with a chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Integrin-activation assay

Activation of β1 integrin was assessed by ELISA using either the HUTS-4 or the 9EG7 antibody as described previously (Monaghan et al., 2004). These antibodies recognize the activated conformation of the β1 integrin. Total levels of β1 integrin were measured using the P5D2 antibody that recognizes all forms of β1 integrin. Cells were incubated with antibodies (100 ng/ml) for 1 hour at 37°C, rinsed with PBS and fixed with 3% paraformaldehyde. Following blocking with 3% BSA, cells were incubated for 1 hour with HRP-conjugated goat anti-mouse antibody. Freshly prepared substrate (0.1 M citrate buffer pH 5.0, 0.5 mg/ml *o*-phenylenediamine, 1 μl/ml 30% hydrogen peroxide) was added to each well, and the color was allowed to develop. The reaction was stopped with the addition of 2 N sulfuric acid, and the OD_{490} was measured. OD measurements were corrected for light scattering by subtracting the values for OD_{650}.

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References

Aguirre-Ghiso, J. A., Liu, D., Mignatti, A., Kovalski, K. and Ossowski, L. (2001). Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol. Biol. Cell* **12**, 863-879.

Arnaout, M. A., Goodman, S. L. and Xiong, J. (2007). Structure and mechanics of integrin-based cell adhesion. *Curr. Opin. Cell Biol.* **19**, 495-507.

Brenner, K. A., Corbett, S. A. and Schwarzbauer, J. (2000). Regulation of fibronectin matrix assembly by activated Ras in transformed cells. *Oncogene* **19**, 3156-3163.

Chambers, S. K., Irvins, C. M. and Carcamgiu, M. L. (1998). Plasminogen activator inhibitor-1 is an independent poor prognostic factor for survival in advanced stage epithelial ovarian cancer patients. *Int. J. Cancer* **79**, 449-454.

Czekay, R.-P., Aertgeerts, K., Curriden, S. A. and Loskutoff, D. (2003). Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J. Cell Biol.* **160**, 781-791.

Degryse, B., Resnati, M., Czekay, R.-P., Loskutoff, D. J. and Blasi, F. (2005). Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. *J. Biol. Chem.* **280**, 24792-24803.

Deng, G., Curriden, S. A., Wang, S., Rosenberg, S. and Loskutoff, D. J. (1996). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J. Cell Biol.* **134**, 1563-1571.

Deng, G., Curriden, S. A., Hu, G., Czekay, R. P. and Loskutoff, D. J. (2001). Plasminogen activator inhibitor-1 regulates cell adhesion by binding to the somatomedin B domain of vitronectin. *J. Cell. Physiol.* **189**, 23-33.

Edlund, M., Lotano, M. A. and Otey, C. A. (2001). Dynamics of a-actinin in focal adhesion and stress fibers visualized with a-actinin-green fluorescent protein. *Cell Motil. Cytoskeleton* **48**, 190-200.

Foekens, J. A., Peters, H. A., Look, M. P., Portengen, H., Schmitt, M., Kramer, M. D., Brunner, N., Janicke, F., Meijer-van Gelder, M. E., Henzen-Logmans, S. C. et al. (2000). The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Res.* **60**, 636-643.

Franco, P., Vocca, I., Carriero, M. V., Alfano, D., Cito, L., Longanesi-Cattani, I., Grieco, P., Ossowski, L. and Stoppelli, M. P. (2006). Activation of urokinase receptor by a novel interaction between the connecting peptide region of urokinase and αvβ5 integrin. *J. Cell Sci.* **119**, 3424-3434.

Giannone, G., Jiang, G., Sutton, D. H., Critchley, D. R. and Sheetz, M. P. (2003). Talin1 is critical for force-dependent reinforcement of initial integrin-cytoskeleton bonds but not tyrosine kinase activation. *J. Cell Biol.* **163**, 409-419.

Harris, S., Enger, R. J., Riggs, B. L. and Spelsberg, T. C. (1995). Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *J. Bone Miner. Res.* **10**, 178-186.

Higgins, P. J., Ryan, M. P. and Chaudhari, P. (1989). Cytochalasin D-mediated hyperinduction of the substrate-associated 52-kilodalton protein p52 in rat kidney fibroblasts. *J. Cell. Physiol.* **139**, 407-417.

Hocking, D. C., Sottile, J., Reho, T., Fassler, R. and McKeown-Longo, P. J. (1999). Inhibition of fibronectin matrix assembly by the heparin-binding domain of vitronectin. *J. Biol. Chem.* **274**, 27257-27264.

Isogai, C., Laug, W. E., Shimada, H., Declerck, P. J., Stins, M. F., Durden, D. L., Erdreich-Epstein, A. and DeClerck, Y. A. (2001). Plasminogen activator inhibitor-1

promotes angiogenesis by stimulating endothelial cell migration toward fibronectin. *Cancer Res.* **61**, 5587-5594.

Lawrence, D. A., Berkenpas, M. B., Palaniappan, S. and Ginsburg, D. (1994). Localization of vitronectin binding domain in plasminogen activator inhibitor-1. *J. Biol. Chem.* **269**, 15223-15228.

Monaghan, E., Gueorguiev, V., Wilkins-Port, C. and McKeown-Longo, P. J. (2004). The receptor for urokinase-type plasminogen activator regulates fibronectin matrix assembly in human skin fibroblasts. *J. Biol. Chem.* **279**, 1400-1407.

Monaghan-Benson, E. and McKeown-Longo, P. J. (2006). uPAR regulates a novel pathway of fibronectin matrix assembly requiring Src dependent transactivation of EGFR. *J. Biol. Chem.* **281**, 9450-9459.

Mould, A. P., Askari, J. A., Barton, S., Kline, A. D., McEwan, P. A., Craig, S. E. and Humphries, M. J. (2002). Integrin activation involves a conformational change in the α1 helix of the β subunit A-domain. *J. Biol. Chem.* **277**, 19800-19805.

Okumura, Y., Kamikubo, Y., Curriden, S. A., Wang, J., Kiwada, T., Futaki, S., Kitagawa, K. and Loskutoff, D. J. (2002). Kinetic analysis of the interaction between vitronectin and the urokinase receptor. *J. Biol. Chem.* **277**, 9395-9404.

Salasznyk, R. M., Zappala, M., Zheng, M., Yu, L., Wilkins-Port, C. and McKeown-Longo, P. J. (2007). The uPA receptor and the somatomedin B region of vitronectin direct the localization of uPA to focal adhesions in microvessel endothelial cells. *Matrix Biol.* **26**, 359-370.

Schmitt, M., Janicke, F., Moniwa, N., Chucholowski, N., Pache, L. and Graeff, H. (1992). Tumor-associated urokinase type plasminogen activator: biological and clinical significance. *Biol. Chem. Hoppe Seyler* **373**, 611-622.

Seiffert, D. and Loskutoff, D. J. (1991). Kinetic analysis of the interaction between type 1 plasminogen activator inhibitor and vitronectin and evidence that the bovine inhibitor binds to a thrombin-derived amino-terminal fragment of bovine vitronectin. *Biochim. Biophys. Acta* **1078**, 23-30.

Stefansson, S. and Lawrence, D. A. (1996). The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin. *Nature* **383**, 441-443.

Stefansson, S., Su, E. J., Ishigami, S., Cale, J. M., Gao, Y., Gorlatova, N. and Lawrence, D. A. (2007). The contributions of integrin affinity and integrin-cytoskeletal engagement in endothelial and smooth muscle cell adhesion to vitronectin. *J. Biol. Chem.* **282**, 15679-15689.

Takahashi, T., Suzuki, K., Ihara, H., Mogami, H., Kazui, T. and Urano, T. (2005). Plasminogen activator inhibitor type 1 promotes fibrosarcoma cell migration by modifying cellular attachment to vitronectin via αvβ5 integrin. *Semin. Thromb. Hemost.* **31**, 356-363.

Vial, D., Monaghan-Benson, E. and McKeown-Longo, P. J. (2006). Coordinate regulation of fibronectin matrix assembly by the plasminogen activator system and vitronectin in human osteosarcoma cells. *Cancer Cell Int.* **6**, 8.

Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V. and Chapman, H. A. (1996). Regulation of integrin function by the urokinase receptor. *Science* **273**, 1551-1555.

Wierzbicka-Patynowski, I. and Schwarzbauer, J. E. (2003). The ins and outs of fibronectin matrix assembly. *J. Cell Sci.* **116**, 3269-3276.

Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M. (1988). Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* **13**, 281-292.

Zhang, Q., Peyruchaud, O., French, K. J., Magnusson, M. K. and Mosher, D. F. (1999). Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a Rho-dependent signal pathway. *Blood* **93**, 2984-2990.

Zheng, M. and McKeown-Longo, P. J. (2002). Regulation of HEF1 expression and phosphorylation by TGF-β1 and cell adhesion. *J. Biol. Chem.* **277**, 39599-39608.

Zheng, M., Ambesi, A., Yu, L. and McKeown-Longo, P. J. (2007). Quantification of fibronectin matrix assembly sites using a novel ELISA assay. *Matrix Biol.* **26**, 330-333.