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Interactions of Upar with Integrin A5β1 Receptor Enhances Fibronectin Deposition and Activates P-Mapk Signaling Pathway

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Mechanism of uPAR cell signaling regulation critically depends on the interactions with other membrane receptors. This paper reveals the role of uPAR- $\alpha_5\beta_1$ integrin interaction in the regulation of MAPK kinase pathway (P-ERK1/2) and fibronectin deposition. Treatment of vitronectin-adherent cells with different concentrations of P25 (10, 50 and 100 μM) for 24 hrs resulted in a dose-dependent increase in ^{125}I-labeled 70-kDa fibronectin (Fn) fragment binding. P25 induced 2.5 fold increase in Fn matrix assembly over control cells on vitronectin. Incubation of A1-F cells seeded on Fn with anti – uPAR antibody, resulted in a suppression of ^{125}I-labeled 70-kDa Fn fragment binding. Prolonged exposure to 100 μM P-25, a concentration leading to maximal effect of fibronectin accumulation, induced sharp increase in phosphorylation of ERK1/2. In contrast, the level of p130 Cas was not elevated revealing that long term elevation of P-ERK1/2 occurs without concomitant activation of p130Cas.

Key words: uPAR, integrin receptors, fibonectin, ERK1/2, vitronectin

Introduction

The extracellular matrix (ECM) is a complex structure of different proteins and proteoglicans that is assembled by cells and provides critical physical support and tissue stability. Cells are attached to the ECM by expressing specific receptors such as integrins. Regulation of cell proliferation, differentiation, as well as different signaling pathways is modulated by cells-ECM interaction. Vitronectin (Vn) is a plasma protein, which is found in association with the extracellular matrix during progression of some tumors [8]. Vn contains an RGD sequence, through which it binds to different integrin receptors such as $\alpha_v\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ and is involved in the cell attachment, spreading and migration [9]. By its localization in the extracellular matrix and its binding to components of plasminogen activation system, Vn can potentially regulate the proteolytic degradation of this matrix. Generation of the serine proteinase plasmin from the extracellular zymogen plasminogen can be catalyzed by either of two other serine proteinases, the urokinase- and tissue-type plasminogen activators (uPA and tPA) [17]. The plasminogen activation system also includes the serpins, plasminogen activator inhibitors types I and II (PAI-1 and PAI-2), and the uPA receptor (uPAR). uPAR is an important regu-

lator of ECM proteolysis, cell-ECM interactions and cell signaling. Coordination of extracellular matrix (ECM) proteolysis and cell signalling by uPAR underlies its important function in cell migration, proliferation and survival and makes it an attractive therapeutic target in cancer and inflammatory diseases. uPAR lacks transmembrane and intracellular domains and so requires transmembrane co-receptors for signaling [17] and can also modulates the adhesive and signaling capacity of the integrins [16]. uPAR interaction with $\alpha_5\beta_1$ integrin can either inhibited β_1-integrin - dependent adhesion to fibronectin (Fn) [19] or increased adhesion to Fn [1]. Incubation of fibroblast monolayers with the P-25 peptide, an uPAR ligand, resulted in a 12–15-fold increase in the accumulation of exogenous fibronectin in the cell layer [13]. However, very little is known about the mechanism by which uPAR may regulate the assembly of the FN matrix and the respective signaling pathways activated. The aim of this study was to investigate the effect of uPAR- $\alpha_5\beta_1$ integrin interaction on fibronectin assembly and intracellular signaling pathways activated. The level of P-ERK was measured, providing direct evidence for the role of integrin–uPAR interaction in the regulation of MAPK signaling pathway.

Materials and Methods

Reagents and Antibodies - Unless otherwise stated, all chemicals were purchased from Sigma. Peptides P-25, sequence AESTYHHLSLGYMYTLN, and S-25, sequence NYHYLESSMTALYTLGH, were synthesized by Cell Essentials (Boston, MA). Anti-uPAR monoclonal antibody was obtained from American Diagnostica. Anti-phospho-ERK1/2 (Th-202/Tyr-204), anti-ERK1/2 antibodies were from Cell Signaling Technology (Beverley, MA). R2 antibody against human uPAR was a gift from Drs. Liliana Ossowski and Julio Aguirre Ghiso (Mt. Sinai School of Medicine, New York). Secondary antibodies goat anti-mouse HRP and goat anti-rat HRP were purchased from Bio-Rad and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Cell Culture - Human foreskin fibroblasts (A1-F) were a gift from Dr. Lynn Allen-Hoffmann (University of Wisconsin, Madison, WI). A1-F cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT). Cells were used at passage 6–12 and unless otherwise noted experiments were performed on monolayer cultures.

Purification and Derivatization of Proteins - Human plasma fibronectin was purified by ion exchange chromatography on DEAE-cellulose (Amersham Biosciences) as described previously [13] and further purified by affinity chromatography with gelatin-agarose and heparin-agarose. The 70-kDa amino-terminal fragment of fibronectin was generated by limited digestion of intact fibronectin with cathepsin D followed by gelatin affinity chromatography as described previously. Vitronectin was purified from human serum by heparin-Sepharose (Amersham Biosciences) affinity chromatography according to the methods of Yatohgo *et al.* [23]. Purified 70-kDa fibronectin (100 µg), was iodinated with 1 mCi of Na ^{125}I (PerkinElmer Life Sciences). Iodinated proteins were mixed with bovine albumin, 1 mg/ml, dialyzed against TRIS-buffered saline, and frozen at -80 °C until used.

Matrix Incorporation Assays - ^{125}I-Fibronectin assembly into a detergent- insoluble matrix was determined as described previously [11]. Cultures were incubated with ^{125}I-fibronectin (1 µg/ml; 1 x 10^6 cpm/ml) in DMEM at 37 °C in the presence of either P-25 or S-25. Incubation times and peptide doses and/or inhibitors are as designated in the figure legends. After incubation, cells were rinsed three times in PBS, and cell layers were scraped directly into 1 N NaOH to determine the total cell layer-associated fibronectin.

Fibronectin Fragment Binding Assays - Cell layers were preincubated with peptides for 1 h prior to the addition of ^{125}I-labeled 70-kDa fragment (100 ng/ml) or ^{125}I-labeled 120-kDa (12.5–100 ng/ml) fragment in serum-free medium. Following incubation with fragments, cell layers were washed three times with PBS, solubilized in 1 N NaOH, and cell-associated radioactivity was determined by γ-scintillation. Nonspecific binding of the 70- and 120-kDa fragments was determined in the presence of excess (50 or 100 μg/ml) unlabeled protein and was subtracted from total binding. To minimize endogenous fibronectin levels during experimental procedures, cell layers were washed three times with serum-free DMEM and pretreated for 3.5 h with cycloheximide (20 μg/ml) in DMEM containing ITS_{2+} (Sigma) as described previously [10]. To coat substrates, fibronectin or vitronectin was diluted to 10 μg/ml in PBS and coated onto tissue plates (Corning/Costar) for 3 h at 37 °C.

Western Blot Analysis—Cells were collected in 0.5 ml of lysis buffer containing 125 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromphenol blue, and 2% β-mercaptoethanol. Proteins from total cell lysates were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad). Equal loading was ensured by Ponceau S staining. Membranes were blocked in phospho- buffered saline, 0.05% Triton X-100 containing 5% skim milk powder and were then probed overnight with specific primary antibodies (1:2000 p-ERK, 1:1000 p130Cas or 1:1000 ERK1/2). Antibodies were detected with the corresponding horseradish peroxidase-linked secondary antibodies. Blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce) detection reagents. Membranes were stripped with stripping buffer (2% SDS, 100 mM Tris-HCl, 0.1% β-mercaptoethanol) for 45 min at 60 °C and re-probed with the corresponding antibodies to total ERK1/2 for loading control. The membranes were then exposed to x-ray films for various time intervals.

Results

To estimate whether uPAR might effectively regulate FN matrix assembly in skin fibroblasts, cells were incubated with P-25, a peptide, known to bind to uPAR and modulate activity of β1 integrins [1]. The addition of 50 μM P-25 to monolayers of skin fibroblasts resulted in dramatic increase in the accumulation of exogenous fibronectin into fibroblast monolayers as compared with control experiments done in the presence of a scrambled peptide, S-25 **(Fig. 1)**. Assembly of fibronectin into a matrix depends on the binding of the amino-terminal region of FN to matrix assembly sites on the cell surface [12].

The urokinase receptor uPAR formed stable complexes with $\alpha_5\beta_1$ integrin, modulating its function and promoted cell adhesion to vitronectin via a ligand binding site on uPAR. We therefore did experiments to evaluate whether uPAR could affect assembly of the fibronectin matrix. To examine the effect of different modulators of Vn – integrin interactions and regulation of Fn matrix assembly site expression we have used P 25 for disruption of uPAR – β_1 integrin interaction (**Fig.2.**). Previous studies have identified P 25 as a specific inhibitor of uPAR – integrin interaction [19]. Peptide 25 binds to uPAR, but do not interfere directly with the binding of uPAR to Vn or uPA. Matrix assembly site expression on fibroblasts cells was measured in a 1-h binding assay using ^{125}I-labeled 70-kDa Fn fragment. Cycloheximide-pretreated fibroblasts were allowed to adhere and spread on tissue culture wells coated with either Vn or Fn. Treatment of vitronectin-adherent cells with different concentrations of P25 (10, 50 and 100 μM for 24 hrs resulted in a dose-dependent increase in ^{125}I-labeled 70-kDa Fn fragment binding.

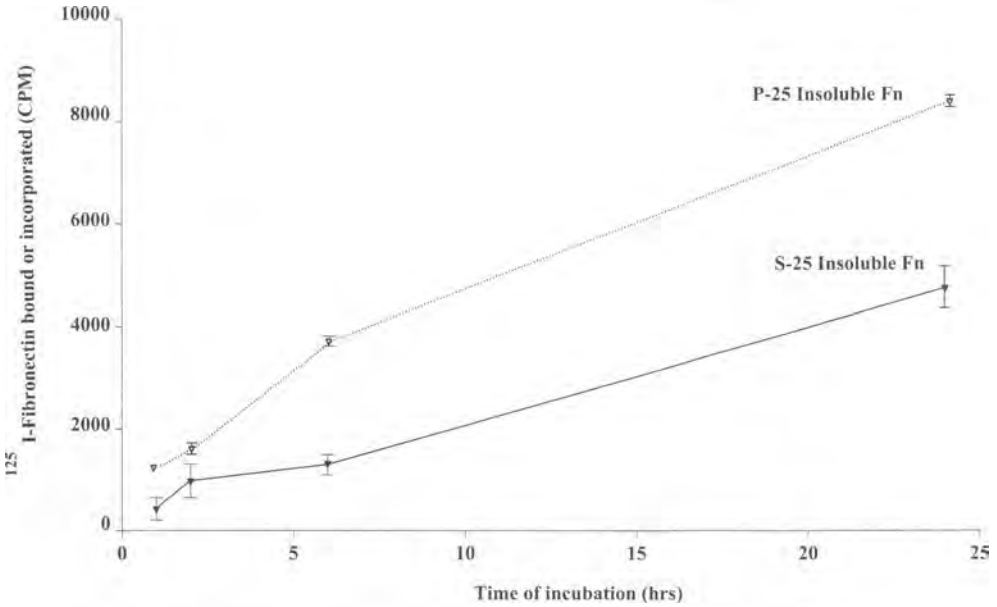

Fig. 1. Effect of P-25 peptide on fibronectin matrix assembly. Confluent fibroblast monolayers were incubated in DMEM containing 125I-fibronectin in the presence of 50 μM P-25 or S-25. At the indicated time points, the medium was removed from the cells, the cells were rinsed in PBS, and the cell layer was solubilized in 1 N NaOH. 125I-FN associated with the cell layer was determined by scintillation.

Fn matrix assembly was induced 2.5 fold over control cells on Vn substrate with 100 μM P25. The scrambled version (100 μM S25), when added to adherent cells on Vn, wasn't capable of inducing elevation in Fn matrix assembly. These studies indicate that disruption of uPAR-integrin interaction by P25 can reverse the matrix assembly inhibition induced by Vn.

To address the question about the role of uPAR in the regulation of Fn matrix assembly site, the monoclonal antibody (#3936, American Diagnostica) which recognizes the human urokinase receptor (uPAR) was tested for its ability to inhibit 70 kDa binding. This antibody binds with high affinity to both unoccupied and occupied uPAR on the cell surface and blocks the binding of uPA. A1-F cells coated on Fn were incubated with various concentrations of this mAb. As shown on **Fig. 3**, incubation of the cells for 24 hrs with 10 and 30 μg monoclonal Ab resulted in a suppression of ^{125}I-labeled 70-kDa Fn fragment binding. These results demonstrate that uPAR is involved in regulation of matrix assembly site expression

Enhancement of fibronectin deposition induced by P-25 raises the question, which signaling pathway could be activated as a result of uPAR-integrin complex disruption. We examined the effect of prolonged exposure to 100 μM P-25, a concentration leading to maximal effect of fibronectin accumulation. Results shown in **Fig. 4** reveal a sharp increase in phosphorylation of ERK1/2 by 15 min of treatment. Longer exposure for 30 min increased the level of phosphorilation and continuation until 45 min leads to small decrease. In contrast, the level of p130 Cas was not elevated at any time point between

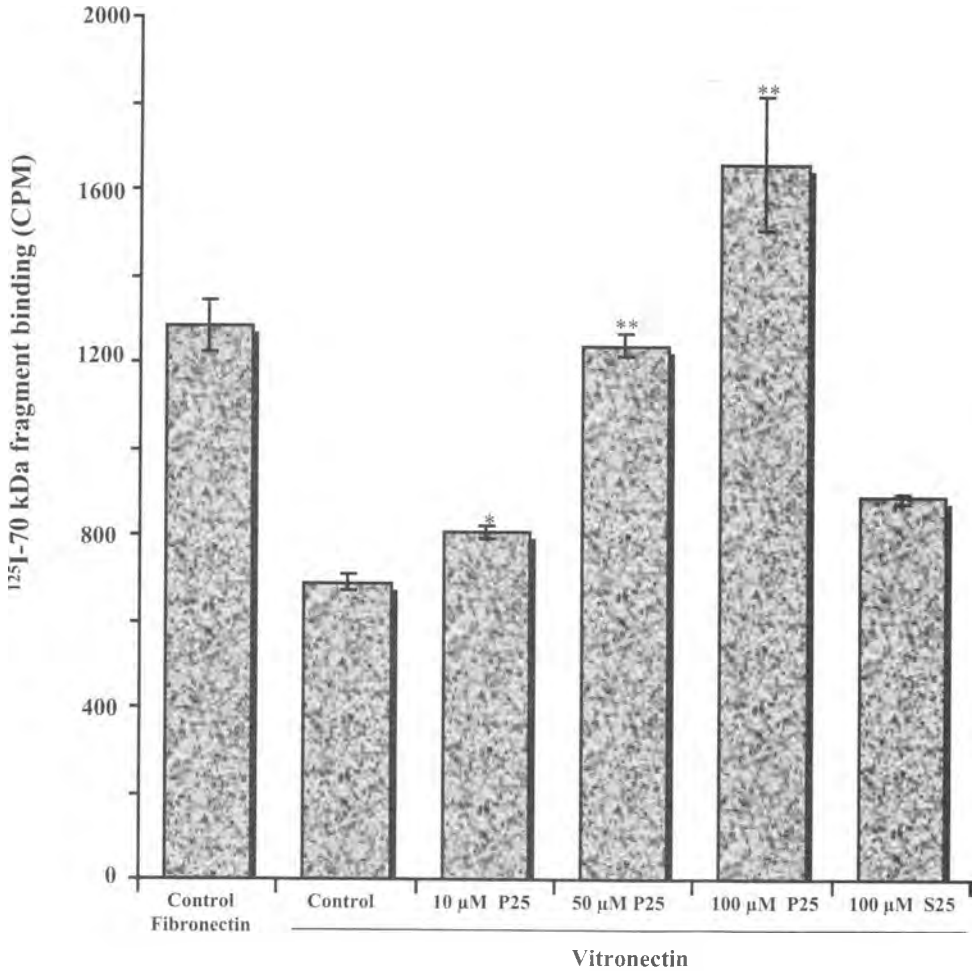

Fig. 2. Effect of uPAR - α1 integrin interaction disrupting peptide P25 on 125I-labeled 70- kDa fragment binding to A1-F cells. Cycloheximide-pretreated A1-F fibroblasts, were seeded at confluence on vitronectin-coated tissue cultured wells. The cells were treated for 24 hours with 10, 50 and 100 µM peptide 25, (P25) or 100 µM scrambled version, (S25). Data are presented as 125I-labeled 70- kDa fragment binding radioactivity per well S. E. $P<0.01$ compared with control.

15 and 45 min of P-25 treatment. Densitometry indicated that there was about a 15-fold increase in P-ERK1/2 with prolonged incubation for 30 min. The results suggest that long term elevation of P-ERK1/2 occurs without concomitant activation of p130Cas.

Discussion

Many findings, strongly suggest an important and causal role for uPA-catalyzed plasmin generation in cancer cell invasion through the extracellular matrix [3]. uPA system is also involved in cancer cell-directed tissue remodeling. Moreover, the system also supports cell migration and invasion by plasmin-independent mechanisms, including multiple interactions between uPA, uPAR, PAI-1, extracellular matrix proteins, integ-

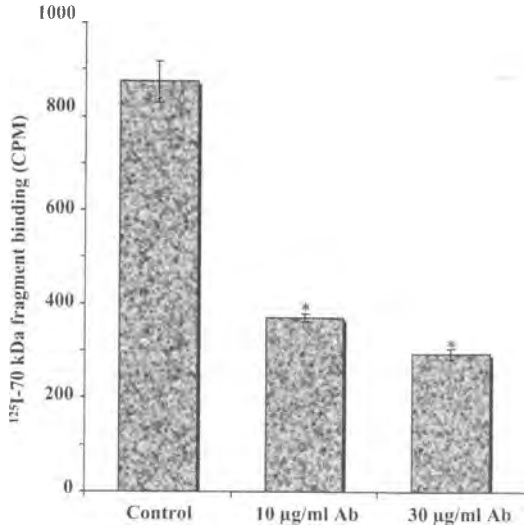

Fig. 3. Effect of anti-uPAR mAb on 125I-70 kDa fragment binding to A1-Fs cells. Serum-starved, cyclohexamide- treated fibroblasts were resuspended in serum free medium and seeded onto fibronectin-coated tissue culture wells. Cells were incubated overnight in the presence of 10 µM and 30 µM anti-uPAR mAb or control IgG. Data are presented as 125I-labeled 70-kDa fragment binding radioactivity per well, S. E. $P<0.01$ compared with controls.

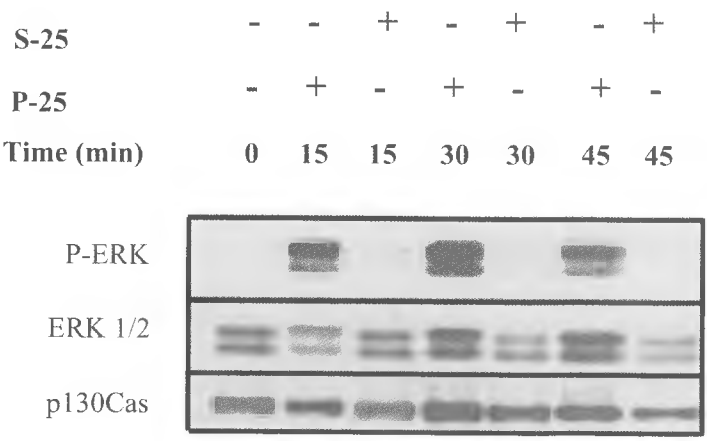

Fig. 4. Effect of P-25 and S-25 treatment on ERK1/2 and p130Cas phosphorylation. A1-F's fibroblast cells were incubated with 200 M nicotine for the indicated time points. The cell lysates were subjected to Western blot analysis with antibodies to P-ERK1/2 or p130Cas. Nitrocellulose filters were stripped and incubated with ERK1/2 antibody for loading control. Representative Western blots are shown.

rins, endocytosis receptors, and growth factors. These interactions seem to allow temporal and spatial reorganizations of the system during cell migration and a selective degradation of extracellular matrix proteins during invasion. uPAR can function as an adhesion receptor for Vn and can also form complexes with several integrins including β_1, β_2, β_3 [22] and β_5 integrins [24]. uPAR contain two different binding sites, with the Vn binding site being distinct from the urokinase-binding site and is located in heparin-binding domain [20].

Despite the controversy surrounding whether uPAR and integrins interact directly, many studies show that uPAR signaling requires integrin co-receptor [17]. It was also shown that some non-integrin co-receptors of uPAR cooperate with integrins in signaling or influence uPAR-integrin interactions [5]. Purified uPAR and $\alpha_5\beta_1$ integrin *in vitro* can form complexes [21], although other studies could not replicate these data [4].

Experimental evidence has indicated that vitronectin in the matrix may inhibit the polymerization of fibronectin [10]. The polymerization of Fn matrix is a cell-dependent process that is regulated by the expression of specific matrix assembly sites on the surface of substrate-attached cells [14]. In one of the initial steps of matrix assembly, cell surfaces bind the amino-terminal region of Fn in a reversible and saturable manner [12]. In many transformed cell types, fibronectin matrix assembly is down-regulated. Tumor cells are generally less adhesive than normal cells and deposit less extracellular matrix [15]. Therefore, the levels of fibronectin in the matrix regulate basic events associated with early tumor progression. The major receptor responsible for Fn matrix assembly is the $\alpha_5\beta_1$ integrin. Transfection of α_5 integrin into CHO cells leads to a large increase in fibronectin deposition in their extracellular matrix [7]. Transfected cells migrated less than control cells and showed reduced saturation density and reduced ability to grow in soft agar. The results indicate that extracellular matrix recognition by the$\alpha_5\beta_1$ integrin plays a role in the control of cell proliferation and suggest that a reduction of this fibronectin receptor may be responsible for the acquisition of anchorage independence by transformed cells. Although it has been well documented that decreased Fn matrix assembly occurs in tumor-derived cell lines, little is known about the extracellular signals that regulate matrix assembly activity in normal fibroblasts. Earlier studies have demonstrated that cells adherent to Vn exhibit decreased levels of cell surface matrix assembly sites compared with Fn-adherent cells [25].

The role of integrins as well as the relationship between different intracellular signaling pathways and the regulation of matrix assembly is not clear. It has been shown that V-ras down regulates matrix assembly by affecting integrin affinity for Fn. Other intracellular signaling pathways that have been known to affect matrix assembly are p38 MAP kinase and signaling from Ras through ERK [6]. Signalling events downstream of uPAR- β_1 integrin interactions promote Tyr phosphorilation of FAK, leading to activation of Ras-ERK pathway [2].

The adaptor protein p130Cas (also know as BCAR1), has been shown to be involved in different cellular processes including cell adhesion, migration and transformation. Cas interacts with focal adhesion plaques and is phosphorylated by the tyrosine kinases FAK and Src. It was shown that uPAR promotes formation of the p130Cas-Crk complex to activate Rac [18]. A number of effector molecules have been shown to interact with Cas and play a role in its function, including c-crk and v-crk, two adaptor proteins involved in inracellular signaling. Cas function is dependent on tyrosine phosphorylation of its substrate domain, suggesting that tyrosine phosphorylation of Cas in part regulates its control of adhesion and migration.

Our observations suggest that a variety of treatments, including haparin-binding domain of vitronectin, anti-uPAR mAb and P25, peptide disrupting uPAR - β_1-integrin interaction can change 70- kDa fragment binding to A1-F cells. Vitronectin's heparin-

binding domain down-regulates matrix assembly site expression, thus indicating a role for the heparin-binding domain in the regulation of fibronectin polymerization. Treatment of vitronectin-adherent cells with different concentrations of P25 (10, 50 and 100 μM for 24 hrs resulted in a dose-dependent increase in ^{125}I-labeled 70-kDa Fn fragment binding. 100 μM P25 induced 2.5 fold increase Fn matrix assembly over control cells on Vn.

Our data also support the suggestion that uPAR-integrin receptor interaction can affect fibronectin matrix assembly site and activates MAPK signaling pathway. This is a novel finding that warrants further study to characterize how explicitly this interaction regulates downstream signaling molecules and modulates fibronectin component of ECM assembly.

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