Regulation of urokinase receptor function and pericellular proteolysis by the integrin $\alpha_5\beta_1$

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Summary

Interactions between the uPA receptor (uPAR) and various integrins, including $\alpha_5\beta_1$, are known to modulate integrin-dependent cell adhesion, and we have shown that the integrin-associated tetraspanin protein CD82 down-regulates uPAR-dependent plasminogen activation by affecting $\alpha_5\beta_1$ cellular localisation. Here we have investigated whether overexpression of $\alpha_5\beta_1$ directly affects uPAR-dependent pericellular proteolysis. CHO cells overexpressing $\alpha_5\beta_1$ were found to activate plasminogen at a rate up to 18-fold faster than B2CHO cells which are α_5-deficient. This effect was dependent on the activation state of $\alpha_5\beta_1$, as it was maximal in the presence of Mn^{2+}. To determine the role of uPAR-$\alpha_5\beta_1$ interactions in this effect, we determined the adhesion of these cells to immobilised soluble uPAR (suPAR). Neither cell-type was found to adhere to suPAR, but both cell types were found to adhere to an anti-uPAR monoclonal antibody in a uPAR- and integrin-dependent manner. This adhesion was 10-fold greater in the absence of $\alpha_5\beta_1$, possibly implicating the involvement of non-α_5-integrins. Soluble forms of the various components were used to investigate the molecular basis of these effects, but no direct interactions could be demonstrated between $\alpha_5\beta_1$ and either uPAR, uPA or uPA-uPAR complex. This suggests that assembly of these components on the plasma membrane is required to influence uPAR function, increasing uPAR-dependent pericellular proteolysis and decreasing uPAR-dependent cell adhesion. These interactions may be modified by other integrins, suggesting a complex interplay between uPAR and integrins on the cell surface with the potential to regulate invasive cell migration.

Keywords

uPA, uPAR, plasminogen activation, integrins, cell adhesion

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Introduction

The binding of urokinase-type plasminogen activator (uPA) to its specific cell surface receptor uPAR promotes generation of plasmin in the pericellular environment (1–4). In this compartment plasmin is involved in the proteolytic modification of the extracellular matrix (ECM), removing physical barriers to cell migration and further activating latent growth factors and matrix metalloproteases in the ECM (5, 6). In this way plasmin is able to influence cell migration in the pathogenesis of disease states including atherosclerosis and cancer (7). Degradation of ECM components by plasmin impacts on the highly regulated interactions between the ECM and the cytoskeleton, which are mediated through cell surface receptors of the integrin family. In addition to regulating plasmin generation, uPAR is also able to alter how cells interact with the ECM by affecting integrin function (8–12) and by direct interactions with vitronectin (13–15). The integrin $\alpha_5\beta_1$ is of particular interest as putative interactions between this integrin and uPAR have also been linked to uPAR-mediated signalling events (16–19).

We have presented evidence for another level at which the cell surface systems mediating proteolysis and adhesion are coordinated. We found that expression of the tetraspanin protein CD82 in a normal breast epithelial cell line led to 50-fold reduction in uPAR-mediated plasminogen activation despite unaltered expression of uPA or uPAR (20). CD82 was found to cause a redistribution of $\alpha_5\beta_1$ and uPAR, leading to their colocalisation at sites of focal adhesion and physical association as demonstrated by co-immunoprecipitation. Therefore, under these conditions, uPAR was rendered cryptic, i.e. unable to bind uPA, when associated with $\alpha_5\beta_1$. The potential significance of this has led us to investigate the interaction between uPAR and $\alpha_5\beta_1$ and its consequences for uPAR function.

In the present study we show that uPAR function can be modulated by expression of exogenous $\alpha_5\beta_1$ integrin in Chinese hamster ovary (CHO) cells and that this involves cooperation

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with endogenous integrins present on the cell surface. Expression of $\alpha_5\beta_1$ led to a increase in uPAR-dependent plasminogen activation, suggesting that here $\alpha_5\beta_1$ promotes uPA-uPAR interactions. However, uPAR-dependent adhesion of these cells to an immobilised anti-uPAR monoclonal antibody was only observed in the absence of $\alpha_5\beta_1$, suggesting that multiple integrins can differentially affect uPAR function. Furthermore, we find that the effects of $\alpha_5\beta_1$ cannot be recapitulated with purified proteins, demonstrating that these interactions are dependent on assembly of the components on the cell surface.

Materials and methods

Cell lines, antibodies and proteins

CHO cells stably expressing the human α_5-integrin subunit (α5CHO, clone #17) (21) were kindly provided by Prof. Yoshikazu Takada (The Scripps Research Institute, San Diego, CA, USA). $\alpha_5\beta_1$-deficient B2 variant CHO cells (B2CHO) (22) were kindly provided by Prof. Rudy Juliano (University of North Carolina, Chapel Hill, NC, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) foetal calf serum (FCS) and 0.1 mM non-essential amino acids. All cell culture reagents including ECM components were from Invitrogen (Paisley, UK). An isolated 50 kDa fragment of fibronectin containing the integrin binding site and recombinant $\alpha_5\beta_1$ integrin proteins were kindly provided by Prof. Martin Humphries (University of Manchester, UK) (23). These comprised the ectodomain of $\alpha_5\beta_1$ prepared as a fusion with a human γ1 Fc domain to promote heterodimer formation ($\alpha_5\beta_1$-Fc) and $\alpha_5\beta_1$ with the fusion partner removed. The monoclonal antibodies to uPA (clone 5) and uPAR (R4) were kindly provided by Dr. Gunilla Høyer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Secondary antibodies were from Dako (Ely, UK). Soluble recombinant uPAR (suPAR) residues 1–277 expressed in *Drosophila* S2 cells was kindly provided by Dr. Michael Ploug (Finsen Laboratory). Pro-uPA produced by the human kidney cell line TCL-598 and DFP-inactivated uPA were as previously described (1, 24). Lys-plasminogen was from Enzyme Research Laboratories (Swansea, UK). The peptide M25 (9) was prepared by solid phase synthesis. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Sigma (St Louis, MO, USA).

Cell surface plasminogen activation

Plasminogen activation by uPA bound to uPAR on the surface of CHO cells was determined as previously described (2). Briefly, cells were grown to confluence in 48-well plates, washed with phosphate-buffered saline (PBS) to remove unbound uPA and incubated at 37°C with Lys-plasminogen (120 nM) and the plasmin specific fluorogenic substrate H-D-Val-Leu-Lys-AMC (250 µM). Plasmin generation by the endogenously bound uPA was measured continuously as change in fluorescence in a SpectraMAX Gemini microplate reader (Molecular Device, Sunnyvale, CA, USA) at excitation and emission wavelengths of 360 and 440 nm, respectively. Plasmin concentration was determined as δF and plasmin generation represented as δF versus time by reference to standard curves of active-site titrated plasmin. In some experiments endogenously bound uPA was removed by brief treatment with 0.1M glycine/pH 3.0 prior to incubation with pro-uPA (2 nM) for 20 minutes (min) at 37°C and further washing.

Cell adhesion assays

The method used for cell adhesion assays was based on that described by (25). Briefly, 96-well plates were coated with either fibronectin (10 nM), suPAR (50 nM), the anti-uPAR monoclonal antibody R4 (50 nM) or isotype-matched control IgG at the indicated concentrations in coating buffer (15mM Na_2CO_3/35mM $NaHCO_3$/pH 9.6), for 15 hours (h) at 4°C. After washing with PBS, wells were blocked with 1% bovine serum albumin (BSA)/PBS for 30 min at 37°C. Wells were washed again with PBS prior to plating the CHO cells at a density of 1 x 10^5 per well in Hepes-Tyrode buffer (10 mM Hepes/150 mM NaCl/12 mM $NaHCO_3$/0.4 mM NaH_4PO_4/2.5 mM KCl/0.1% (w/v) glucose/0.02% (w/v) BSA/2 mM $MgCl_2$) in the presence or absence of 0.1 mM $MnCl_2$. Cells were incubated at 37°C for 1 h, washed with PBS, fixed with 4% (v/v) formaldehyde for 10 min at room temperature and stained with Methylene Blue in 10 mM sodium borate buffer for 30 min at room temperature. Wells were washed extensively with water, the cell-bound dye released in 50% (v/v) ethanol/0.1% (v/v) HCl and absorbance measured at 650 nm in a ThermoMAX microplate reader (Molecular Device).

Preparation of cell lysates, immunoprecipitation, Western blotting and ligand blotting

Cell lysates were prepared by washing sub-confluent monolayers twice with PBS prior to harvesting in PBS containing complete EDTA-free inhibitors (Roche). Cells were lysed at 5x10^7 cell/ml in 50 mM Hepes pH 7.8/150 mM NaCl containing 1% (v/v) Triton X-100 and protease inhibitors. After 30 min on ice, insoluble material was pelleted by centrifugation at 2,000*g* for 5 min at 4°C. The protein content of the soluble fractions was measured with a BCA protein assay kit according to the manufacturer's instructions (Pierce).

Immunoprecipitation was carried out essentially as described previously (20). Briefly, 100 µl aliquots of fractions precleared with Protein G-Sepharose were incubated with 30 pmole of anti-uPAR R4 or isotype matched IgG for 12 h at 4°C. Fifty µl of 50% (v/v) Protein G-Sepharose was added to each sample, and immune complexes allowed to bind for at least 1 h at 4°C. The beads were washed four times with lysis buffer, and adsorbed material eluted in non-reducing Laemmli sample buffer.

For Western and ligand blotting, samples separated by PAGE were transferred to PVDF membranes (Bio-Rad). Protein bands were detected by incubation with the appropriate antibody followed by HRP-conjugated secondary antibodies (0.65 µg/ml). For ligand blotting to detect endogenous CHO uPAR, the antibody incubations were preceded by an incubation with 2 nM pro-uPA.

Solid phase binding assays

Plates with 96 wells were coated with $\alpha_5\beta_1$-Fc, uPA or suPAR at the indicated concentrations in coating buffer, for 15 h at 4°C. Wells were blocked with 5% BSA in PBS containing either 0.1 mM $MnCl_2$ or 0.1mM EDTA, for 1 h at room temperature with agitation. Wells were then washed three times with PBS/0.1%

(v/v) Tween20. The defined protein samples were prepared in 0.05% (v/v) BSA/PBS with $MnCl_2$ or EDTA and plated for incubation for 1 h as before. Following further washing the primary antibodies were applied at 5 µg/ml in 0.05% (v/v) BSA/PBS with $MnCl_2$ or EDTA and incubated as before. This was repeated with the HRP-conjugated secondary antibody, used at 0.65 µg/ml. After a final washing step the plate was developed by the addition of 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate system (Sigma) per well. Colour was allowed to develop for 10 min and the reaction stopped by the addition of 1M H_2SO_4. Absorbance was measured at 450 nm in a ThermoMAX microplate reader (Molecular Devices).

Native PAGE

Native or non-denaturing PAGE was performed according to the method of Liou and Willison (26). Briefly, equimolar combinations of uPA, suPAR, $\alpha_5\beta_1$ and $\alpha_5\beta_1$-Fc were incubated in Tris-buffered saline (TBS) at 4°C for 10 min prior to separation on a pH 8.8, 6% polyacrylamide gel at 4°C.

Silver staining

Native gels were stained with 0.25% (w/v) Coomassie Brilliant Blue G250/45% (v/v) methanol/10% (v/v) glacial acetic acid for 5 min and destained (45% (v/v) methanol/10% (v/v) glacial acetic acid) for 12 h. Gels were washed in water for 15 min prior to incubation for 5 min with a small amount of $K_3Fe(CN)_6/NaS_2O_3$ in a 5:8 ratio dissolved in water. Gels were washed in water, incubated with 12 mM $AgNO_3$ for approximately 20 min, and further washed in water for 5 min. To develop the silver stain, gels were briefly washed with 270 mM Na_2CO_3 and then incubated in 270 mM Na_2CO_3/0.2% (v/v) formaldehyde until bands had developed; the reaction was stopped with 5% (v/v) acetic acid. All procedures were carried out at room temperature.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Significance was assessed using Student's t-test.

Results

We have previously shown that expression of CD82 leads to a 50-fold reduction in uPA-catalysed plasminogen activation, concomitant with a redistribution, colocalisation and physical association between uPAR and $\alpha_5\beta_1$ integrin (20). Here we have directly investigated the effect of $\alpha_5\beta_1$ on uPAR function, using CHO cells expressing different levels of $\alpha_5\beta_1$. α5CHO are transfected with the human α_5-integrin subunit, and have a chimera of human α_5 coupled with endogenous β_1 on their surface (21). B2CHO are essentially α_5 null, containing only 2% of the $\alpha_5\beta_1$ found in parental cells (22).

$\alpha_5\beta_1$ promotes uPAR-mediated plasminogen activation on CHO cells

Plasminogen activation by uPAR-bound uPA on the surface of both α5CHO and B2CHO cells was determined. These experiments were performed both in the presence and absence of Mn^{2+}, which alters the conformational state of certain integrins, including $\alpha_5\beta_1$ (27, 28). Plasminogen activation was barely detectable on B2CHO cells, either in the presence or absence of Mn^{2+} (Fig. 1A). In contrast, robust uPA-dependent plasminogen activation was observed on α5CHO cells. In the absence of Mn^{2+}, plasminogen activation was five-fold higher, increasing to 18-fold higher in the presence of Mn^{2+}, with $\alpha_5\beta_1$ in the activated conformation. The large difference in plasminogen activation between the two cell lines was not a result of differences in the expression of either uPA, as determined by quantification of uPA activity in serum-free conditioned medium from the two cell lines (data not shown), or uPAR, as shown by uPAR ligand blot (Fig. 1A, inset). Plasminogen activation was also determined with the cells saturated with exogenously added uPA (Fig. 1B). Under these conditions the rates of plasminogen activation were higher (approximately six-fold for α5CHO in the presence of Mn^{2+}), and the differences between the varying conditions less marked. However, the overall pattern remained the same, with increased plasminogen activation in the presence $\alpha_5\beta_1$ and with Mn^{2+} having a positive effect. The dependence of the observed plasminogen activation on uPAR was demonstrated by the effect of treating the cells with PI-PLC, which reduced plasmin generation by greater than 70% in both α5CHO and B2CHO cells (data not shown).

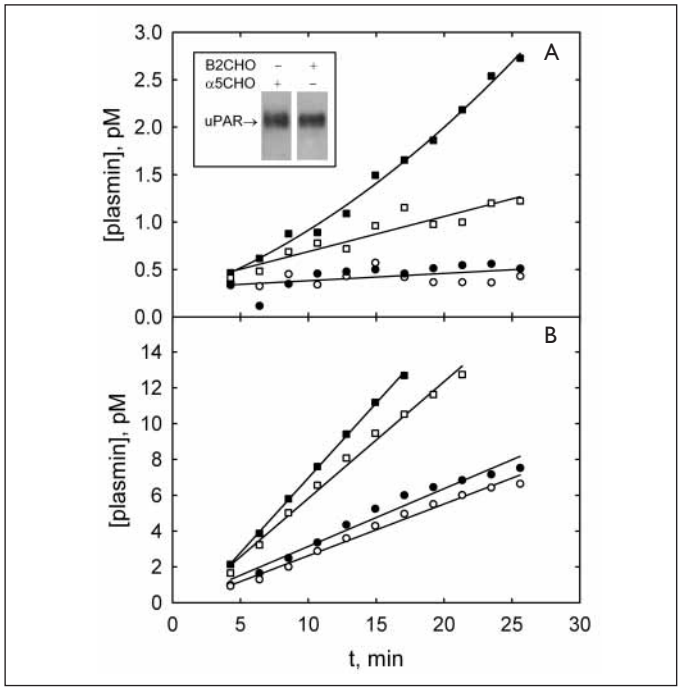

Figure 1: Plasminogen activation on the surface of α5CHO and B2CHO cells. A) The activation of plasminogen by endogenous uPA bound to uPAR on the surface of CHO cells. Plasmin generation was monitored continuously by the hydrolysis of plasmin specific fluorogenic peptide substrate. B) Plasminogen activation on cells saturated with exogenously added pro-uPA. α5CHO (❐), α5CHO + Mn^{2+} (■), B2CHO (○), B2CHO + Mn^{2+} (●). "+ Mn^{2+}" indicates that experiments were performed in the presence of 0.1 mM $MnCl_2$. Under all experimental conditions inclusion of amiloride (100 µM), a selective uPA inhibitor of uPA, reduced plasminogen activation to undetectable levels. The inset shows a uPAR ligand blot of α5CHO and B2CHO cell lysates. The blot was incubated with pro-uPA (2 nM) prior to probing with anti-uPA clone 5 (10 µg/ml) to allow detection of uPAR.

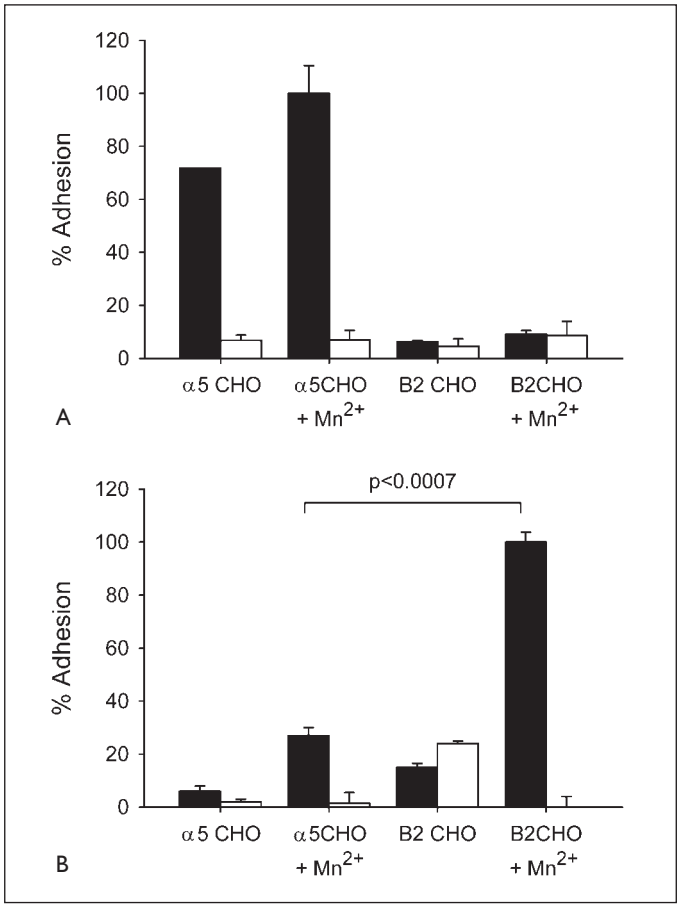

Figure 2: Adhesion of α5CHO and B2CHO to fibronectin, suPAR and R4 anti-uPAR monoclonal antibody. CHO cells were allowed to adhere for 1 hour at 37°C to wells coated with either fibronectin (10 nM), suPAR (50 nM) or the antibody R4 (50 nM). "+ Mn^{2+}" indicates that experiments were performed in the presence of 0.1 mM $MnCl_2$. Cells were fixed and stained for analysis. A) Adhesion of α5CHO and B2CHO to fibronectin (closed bars) and suPAR (open bars). Data are presented as "% Adhesion" where 100% is defined as the adhesion of α5CHO to fibronectin in the presence of $MnCl_2$. B) Adhesion of α5CHO and B2CHO to the anti-uPAR R4(closed bars) and an isotype matched control IgG (open bars). Data are presented as "% Adhesion" where 100% was defined as the adhesion of B2CHO to R4 in the presence of $MnCl_2$. The adhesion of B2CHO to R4 was typically 60–80% of the adhesion of α5CHO to fibronectin.

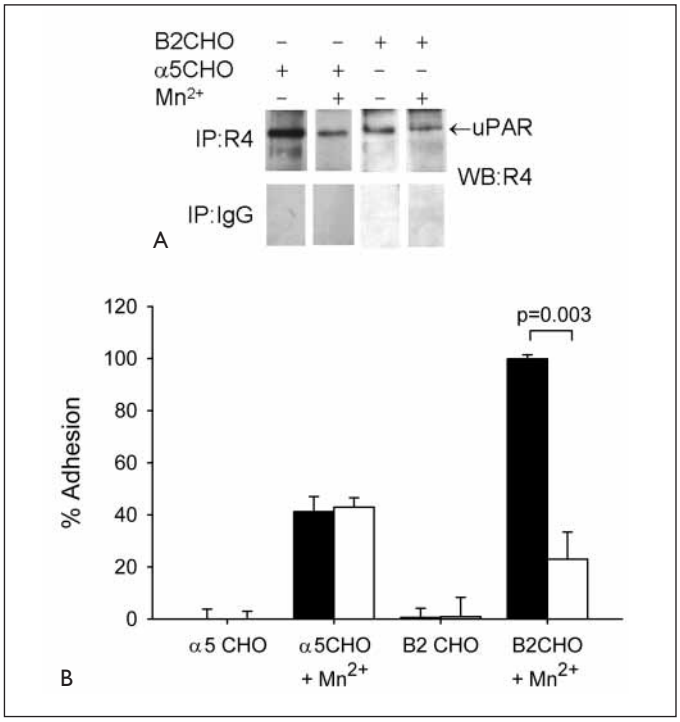

Figure 3: Endogenous uPAR on B2CHO cells mediates adhesion to the anti-uPAR antibody R4. A) Immunoprecipitation of CHO uPAR by the anti-uPAR monoclonal antibody R4. α5CHO and B2CHO cells were extracted in 1% Triton X-100, immunoprecipitated with anti-uPAR R4 or isotype matched control and uPAR detected by Western blot. Where Mn^{2+} is indicated 0.1 mM $MnCl_2$ was added to the culture media for 24 hours prior to extraction. B) Effect of PI-PLC on cell adhesion. α5CHO and B2CHO cells were treated with PI-PLC prior to assay of adhesion to R4-coated wells. Cells treated with PI-PLC (open bars) are shown in comparison to untreated cells (closed bars). "+ Mn^{2+}" indicates that experiments were performed in the presence of 0.1 mM $MnCl_2$. Data are presented as "% Adhesion" where 100% is defined as the adhesion of B2CHO to R4 in the presence of 0.1 mM $MnCl_2$.

These observations clearly demonstrate that cell surface integrins influence uPAR function. uPAR has no requirement for $\alpha_5\beta_1$ to enable it to bind uPA, as shown by many experiments in purified and cellular systems, yet its presence here appears to promote uPAR-mediated plasminogen activation. Therefore these observations can are most readily interpreted as a reduced ability of the cells to support uPAR-mediated plasminogen activation in the absence of $\alpha_5\beta_1$.

Soluble uPAR is not a ligand for $\alpha_5\beta_1$ on the cell surface

It has previously been reported that cell-surface integrins can engage with soluble uPAR as a ligand (29). Therefore, we immobilised suPAR on microtitre plates to investigate differences in its interaction with cellular integrins by measuring cell adhesion. However, neither cell type was found to adhere to immobilised suPAR (Fig. 2A). This was not due to steric or other effects caused by direct immobilisation of suPAR, as adhesion was not observed when suPAR was bound to immobilised anti-uPAR antibodies. Addition of exogenous uPA did not promote adhesion (data not shown). Adhesion of cells to the $\alpha_5\beta_1$ ligand fibronectin was used as a control, and as expected α5CHO adhered more avidly than B2CHO (11-fold), and this was enhanced by the presence of Mn^{2+} (Fig. 2A) and abolished by the presence of EDTA (data not shown). Therefore, in these experiments, the interaction between $\alpha_5\beta_1$ and uPAR that has previously been reported to occur on the cell surface cannot be recapitulated under conditions mimicking *trans*-interactions.

$\alpha_5\beta_1$ influences uPAR-mediated cell adhesion

Although neither cell line could adhere to suPAR bound to immobilised antibodies, it was observed in control experiments that in the absence of suPAR both cell lines adhered specifically to the anti-uPAR monoclonal antibody R4, but not to isotype-matched control IgG (Fig. 2B) or other anti-uPAR antibodies (data not shown). The adhesion of both cell types was increased

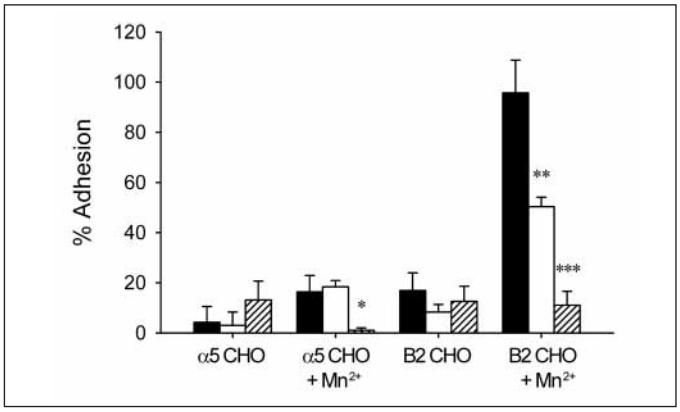

Figure 4: The uPAR-binding peptide M25 blocks adhesion of B2CHO cells to the anti-uPAR antibody R4. α5CHO and B2CHO were treated with 10 μM M25 (open bars), 100 μM M25 (shaded bars) or carrier control (solid bars) for 20 minutes at 37°C prior to being allowed to adhere to R4-coated wells. After 1 hour at 37°C cells were fixed and stained for analysis. Data are presented as "% Adhesion" where 100% was defined as the adhesion of B2CHO to R4 in the presence of 0.1mM $MnCl_2$. *, $p < 0.02$; **, $p < 0.006$; ***, $p = 0.0003$. In control experiments M25 was shown to have no direct effect on the interaction between suPAR and the R4 antibody.

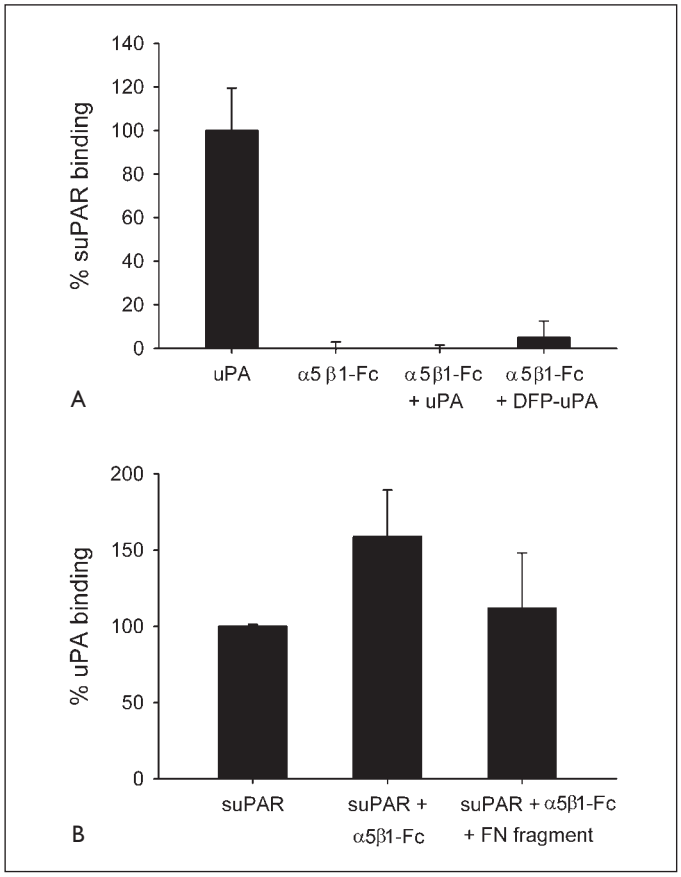

Figure 5: Solid phase assays of the interactions between uPAR, uPA, $\alpha_5\beta_1$ and fibronectin. Solid phase binding assays were performed to measure: A) suPAR binding to immobilised uPA and $\alpha_5\beta_1$-Fc. Binding of suPAR (100 nM) to wells coated with either uPA (100 nM) or $\alpha_5\beta_1$-Fc (5 nM) was detected with R4 as the primary antibody R4. In samples indicated, equimolar uPA or DFP-uPA was added to the suPAR incubation mixture. In all cases the binding of suPAR to $\alpha_5\beta_1$-Fc was significantly different from the binding of suPAR to uPA ($p \leq 0.001$). Controls with fibronectin indicated that the integrin was active and able to bind its ligand (not shown). B) uPA binding to immobilised suPAR +/- $\alpha_5\beta_1$-Fc/50kDa fibronectin fragment. Binding of uPA (50 nM) to wells coated with suPAR (50 nM) was detected using the primary anti-uPA clone 5. In samples indicated, equimolar $\alpha_5\beta_1$-Fc or $\alpha_5\beta_1$-Fc + fibronectin fragment was added to the uPA incubation mixture. There were no statistically significant differences between the data. The experiments shown were performed in the presence of 0.1 mM $MnCl_2$. Data are expressed as "% Binding" with 100% defined as the binding of uPA and suPAR.

in the presence of Mn^{2+}, suggesting the involvement of activated integrins. However, the adhesion of the B2CHO cells was at least three-fold greater than the adhesion of the cells expressing $\alpha_5\beta_1$.

To demonstrate that endogenous hamster uPAR on the surface of CHO cells could recognise the anti-human uPAR antibody R4, immunoprecipitation experiments were performed (Fig. 3A). uPAR could be immunoprecipitated from both α5CHO and B2CHO cells, both in the presence and absence of Mn^{2+}. uPAR could also be immunoprecipitated by other anti-human uPAR monoclonal antibodies (data not shown). The uPAR-dependence of the observed adhesion was also confirmed by treating the cells with PI-PLC to remove GPI-anchored uPAR prior to the adhesion experiments. PI-PLC reduced the adhesion of B2CHO cells in the presence of Mn^{2+} by more than four-fold, while the adhesion of α5CHO cells was unaffected (Fig. 3B). These observations confirm that uPAR was responsible for the observed integrin-dependent differential adhesion to the antibody.

To confirm that the observed adhesion of CHO cells to the antibody R4 was integrin dependent, cells were treated with M25, a uPAR-binding peptide which has been shown to disrupt uPAR-integrin interactions in other cell types (9). M25 reduced the adhesion of B2CHO cells by up to 85% in a dose-dependent manner (Fig. 4), but had a much lesser effect on the adhesion of α5CHO and only at high concentrations of peptide. The minor adhesion of both cell types in the absence of Mn^{2+} was unaffected. Therefore, in B2CHO cells uPAR-dependent adhesion in the absence of $\alpha_5\beta_1$ is both promoted by Mn^{2+} and inhibited the M25 peptide. This strongly implicates the involvement of activated integrins other than $\alpha_5\beta_1$ in facilitating the observed uPAR-dependent adhesion to the anti-uPAR antibody. The lack of effect of M25 on the adhesion of α5CHO cells is in good agreement with our previous observations with HB2 breast epithelial cells, in which M25 did not interfere with the CD82-induced interaction between $\alpha_5\beta_1$ and uPAR (20).

Soluble uPAR and uPA do not interact with the isolated ectodomain of $\alpha_5\beta_1$

The observations here, and in our previous study (20), demonstrate that $\alpha_5\beta_1$ integrin can modulate the interaction of uPAR with its ligand uPA in a manner dependent on the activation state of the integrin. However, divergent effects were observed in the two different experimental systems used. Here, α_5-transfected CHO cells display increased uPAR-dependent plasminogen activation, whereas previously, in HB2 cells, transfection with CD82 led to a strong association of uPAR with $\alpha_5\beta_1$ and a concomitant reduction in uPA binding and plasminogen activation.

In an attempt to determine the molecular basis of these effects, the interaction between suPAR and recombinant isolated

ectodomain of human $\alpha_5\beta_1$ was investigated. $\alpha_5\beta_1$-Fc fusion protein was immobilised on microtitre plates and the binding of suPAR determined using the uPAR antibody R4. This ELISA-type detection format avoids potential problems of direct labelling of suPAR modifying key residues of potential functional importance. In control experiments, suPAR bound efficiently to immobilised uPA (Fig. 5A) and fibronectin bound efficiently to $\alpha_5\beta_1$-Fc when either protein was immobilised (data not shown). However, binding of suPAR to immobilised $\alpha_5\beta_1$-Fc could not be detected (Fig. 5A), nor could the binding of $\alpha_5\beta_1$-Fc to immobilised suPAR (data not shown). uPAR-ligand complexes, formed by preincubation of suPAR with either uPA or DFP-inactivated uPA, also failed to bind to immobilised $\alpha_5\beta_1$-Fc (Fig. 5A). These experiments were performed in the presence of Mn^{2+} to ensure that $\alpha_5\beta_1$-Fc was in an active conformation, but the observations were unchanged in the presence of EDTA, although as expected EDTA prevented the binding of fibronectin to $\alpha_5\beta_1$-Fc and did not affect the binding of uPA to suPAR (data not shown).

As we could not detect an interaction between suPAR and $\alpha_5\beta_1$ directly, we investigated whether $\alpha_5\beta_1$ could interfere with the interaction between uPA and suPAR. However, the binding of uPA to immobilised suPAR was unaffected by the presence of either $\alpha_5\beta_1$-Fc or $\alpha_5\beta_1$-Fc in complex with the 50 kDa fibronectin fragment (Fig. 5B). Similarly the binding of suPAR to immobilised uPA was unaffected by the presence of $\alpha_5\beta_1$-Fc (data not shown).

To address whether the inability to detect an interaction between purified uPAR and $\alpha_5\beta_1$ resulted from artefacts due to protein immobilisation, proteins were combined in solution and complex formation analysed by native PAGE. This non-denaturing technique separates proteins on the basis of their charge as well as their hydrodynamic size, and can be used to detect protein-protein interactions with "band shifts" being observed on complex formation (30). This is demonstrated by the observation that the complex between uPA and suPAR is clearly distinct from the bands due to either protein alone (Fig. 6A). Similarly, when $\alpha_5\beta_1$ or $\alpha_5\beta_1$-Fc were combined with the 50 kDa fibronectin fragment, an integrin-fibronectin complex was detected (Fig. 6B). In the case of $\alpha_5\beta_1$ the complex was indicated by the appearance of a new band, whereas the complex between $\alpha_5\beta_1$-Fc and the fibronectin fragment was indicated by the disappearance of the fibronectin fragment band, and a sharpening of the $\alpha_5\beta_1$ band. Using this technique there was no evidence for complex formation between uPAR and $\alpha_5\beta_1$ as no new bands were detected and the mobility and appearance of the integrin and suPAR bands remained unchanged (Fig. 6C).

The control experiments (Fig. 6A and B) demonstrate that this technique can detect interactions of both high affinity (uPA-uPAR, K_d 0.1–0.3 nM) and low affinity ($\alpha_5\beta_1$-fibronectin, K_d 0.1–1 μM), but fail to detect a suPAR-$\alpha_5\beta_1$ interaction at reactant concentrations of up to 2 μM. However, to ensure that lack of detection of an $\alpha_5\beta_1$-uPAR interaction was not due to complex dissociation during electrophoresis, the effect of $\alpha_5\beta_1$ on uPA-suPAR complex formation was determined. The mobility of suPAR was detected by Western blotting and was unchanged by $\alpha_5\beta_1$-Fc, either in the presence or absence of uPA (Fig. 6D). Similarly suPAR did not alter the interaction between $\alpha_5\beta_1$-Fc and the 50 kDa fibronectin fragment (data not shown).

The lack of an interaction between suPAR and $\alpha_5\beta_1$ in solution was further supported by the observation that soluble $\alpha_5\beta_1$ had no effect on plasminogen activation initiated by pro-uPA either in the presence or absence of suPAR (data not shown).

Discussion

In the present study we show that $\alpha_5\beta_1$ integrin has a profound influence on uPAR function, affecting its roles in both pericellular proteolysis and cell adhesion. We have shown previously that the ability of uPAR to support plasminogen activation at the cell sur-

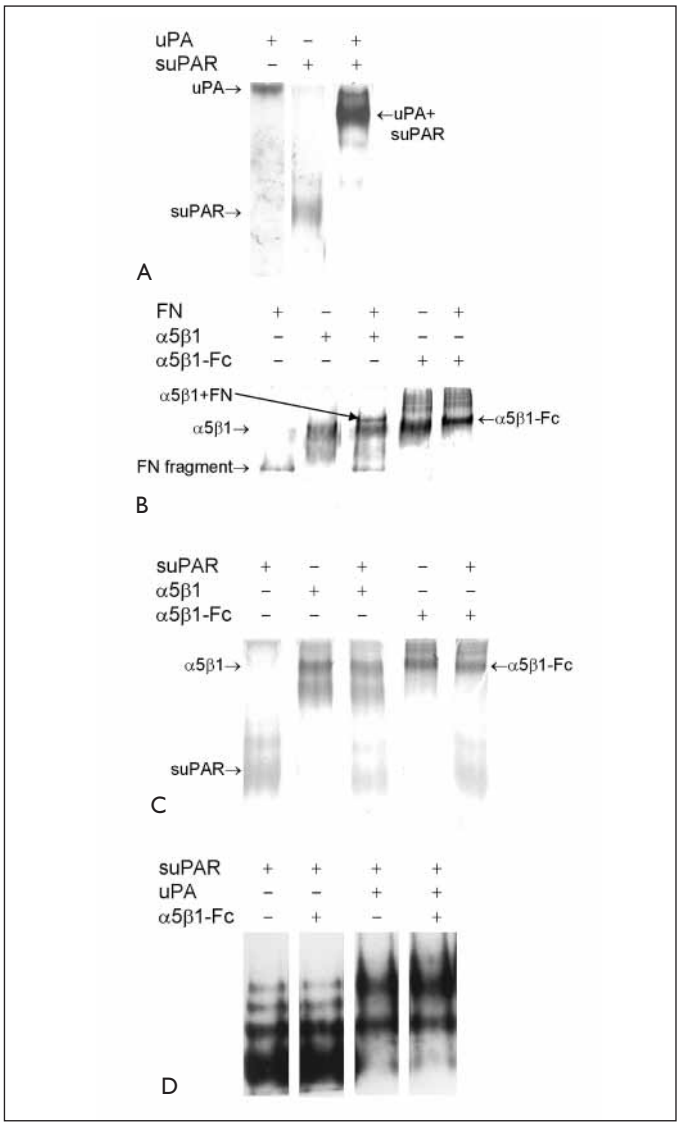

Figure 6: Native PAGE analysis of the interactions between uPAR, uPA, $\alpha_5\beta_1$ and fibronectin. Equimolar (2 μM) combinations of uPA, suPAR, $\alpha_5\beta_1$, $\alpha_5\beta_1$-Fc and fibronectin fragment, as indicated, were incubated for 10 minutes at 4°C prior to analysis by native PAGE at pH 8.8. Panels A-C show silver stained gels. D) Western blot performed with the anti-uPAR R4. Bands corresponding to $\alpha_5\beta_1$, $\alpha_5\beta_1$-Fc, $\alpha_5\beta_1$ + Fn fragment, suPAR, uPA, uPA+suPAR are indicated (A-C). The multiple suPAR bands seen in the Western blot (D) result from the differently glycosylated forms in the preparation.

face can be markedly affected by other membrane proteins involved in the regulation of cell adhesion. We found that the tetraspan protein CD82 reduced the ability of uPAR to bind uPA, leading to a 50-fold reduction in plasminogen activation (20). However, we also found that the effect of CD82 was indirect, and that it promoted a stable interaction between uPAR and $\alpha_5\beta_1$, thereby implicating this integrin in the modulation of uPAR function. Here we have directly investigated the effect of $\alpha_5\beta_1$, both by over-expression in CHO cells and using recombinant soluble integrin. Although we find no direct evidence for a molecular interaction between uPAR and $\alpha_5\beta_1$, the presence of $\alpha_5\beta_1$ on the cell surface affects uPAR function, both increasing uPAR-mediated plasminogen activation and decreasing uPAR-mediated cell adhesion.

Plasminogen activation on $\alpha_5\beta_1$-expressing cells was increased by up to 18-fold in comparison to B2CHO cells, despite both cell lines expressing equivalent levels of uPAR. This situation is clearly different to our previous observation of greatly reduced plasminogen activation as a consequence of CD82-mediated uPAR-$\alpha_5\beta_1$ interactions (20), and consistent with this we found no evidence for uPAR-$\alpha_5\beta_1$ interactions in α5CHO cells. Other studies have reported that the integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$ can increase plasminogen activation. The leukocyte-specific integrin $\alpha_M\beta_2$ has been observed to bind both uPA and plasminogen directly, leading to an increase in plasminogen activation (31, 32). $\alpha_V\beta_3$ was also observed to bind uPA directly and to mediate plasminogen activation on the surface of uPAR-depleted CHO cells (33). Here we found no evidence for direct binding of uPA to $\alpha_5\beta_1$, and purified $\alpha_5\beta_1$ had no effect on uPA-catalysed plasminogen activation, and therefore similar uPAR-independent mechanisms are unlikely to be involved in the observed cellular effects of $\alpha_5\beta_1$.

We also found no evidence for *trans*-interactions between cell surface $\alpha_5\beta_1$ and immobilised suPAR. However, the presence of $\alpha_5\beta_1$ was observed to alter the adhesion of these cells specifically to the anti-uPAR monoclonal antibody R4. Although not of direct biological relevance, this adhesion gives some insights into the role of integrins in modulating uPAR function. The adhesion to the antibody was demonstrated to be uPAR-dependent, as it was abolished by PI-PLC treatment of the cells, and the antibody was shown to recognise hamster uPAR in immunoprecipitation experiments. This adhesion was also integrin-dependent as it was greatly promoted in the presence of Mn^{2+}. This cooperation between uPAR and integrins suggests that adhesion to the antibody is mediated by interactions between these proteins. This conclusion is further supported by the observation that the uPAR-binding peptide M25, which has previously been shown to disrupt the interaction between uPAR and multiple integrins (9), effectively abolished this cell adhesion. Therefore, the observation that adhesion of the cells expressing $\alpha_5\beta_1$ was reduced in comparison to the α_5-deficient cells suggests that this integrin does not interact with uPAR in a manner supporting this adhesion. Furthermore, $\alpha_5\beta_1$ appears to disrupt interactions between uPAR and integrins endogenously present on these cells that do support uPAR-mediated adhesion.

When comparing the observations here to those we have made previously in cells overexpressing CD82 (20), it is apparent that the effects ascribed to $\alpha_5\beta_1$ are divergent, although in both cases $\alpha_5\beta_1$ is intimately linked to the regulation of uPAR-mediated plasminogen activation. We previously showed that CD82 promoted stable interactions between uPAR and $\alpha_5\beta_1$, which could be detected by co-immunoprecipitation, leading to a reduction in uPAR-mediated plasminogen activation. In the present study, despite overexpression of $\alpha_5\beta_1$, there is no evidence for a similar stable association, and plasminogen activation is not reduced by $\alpha_5\beta_1$. Therefore, in CHO cells there is no evidence that $\alpha_5\beta_1$ interacts with uPAR to directly regulate plasminogen activation. The reduced plasminogen activation observed in the absence of$\alpha_5\beta_1$ may possibly involve interactions between uPAR and integrins other than $\alpha_5\beta_1$ present on the surface of these cells, i.e. those that support uPAR-mediated cell adhesion.

Evidence for interactions between uPAR and $\alpha_5\beta_1$ has previously come from a variety of observations. Adhesion of $\alpha_5\beta_1$-expressing cells to immobilised suPAR in an activation-dependent manner has been demonstrated, and interpreted as *trans*-interactions between the proteins (although this adhesion was considered to be weaker than that observed on over-expression of several other β_1 and β_3 integrins) (29). Adhesion of the same cells to uPA was found to be dependent on both uPAR and $\alpha_5\beta_1$, suggesting lateral or *cis* uPAR-$\alpha_5\beta_1$ interactions (18). The binding of radio-iodinated suPAR to purified $\alpha_5\beta_1$ has also been demonstrated to require the presence of uPA (18), although binding of biotinylated suPAR to immobilised purified $\alpha_5\beta_1$ in the absence of uPA has also been demonstrated (10). In the present study we have found no adhesion of either α5CHO or B2CHO cells to suPAR, either directly or indirectly immobilised via an antibody, and we have been unable to detect any interaction between the purified proteins, either in the presence or absence of ligands for both receptors, using a range of techniques in which suPAR is neither labelled nor chemically modified. It should be noted that biotinylation of suPAR is known to decrease its specific binding capacity and to increase non-specific binding (G. Høyer-Hansen, personal communication). However, our inability to detect an interaction between uPAR and $\alpha_5\beta_1$ in purified systems does not exclude the occurrence of direct interactions on the cell surface where high local concentrations of the proteins could promote *cis*-interactions, for example in lipid rafts (34). uPAR and $\alpha_5\beta_1$ have been shown to co-imunoprecipitate in certain tumour cells (16), and we have previously observed this in the presence of CD82 (12, 20); although not here in α5CHO cells. Indirect evidence for uPAR-$\alpha_5\beta_1$ interactions comes from observations on the effect of uPAR on various aspects of $\alpha_5\beta_1$ function, including signalling, migration and fibronectin matrix assembly (11, 12, 16–19, 35). In many of these examples, uPA binding to uPAR is thought to promote the interaction with $\alpha_5\beta_1$. However, our previous observations on the effect of CD82 are not consistent with this mechanism, as uPAR in the resulting stable complex with $\alpha_5\beta_1$ was unable to bind uPA (20). An important aspect of the two model systems that we have used is that we are observing the function of endogenously expressed uPAR, and not the consequences of uPAR over-expression as often used in other studies.

Although we have interpreted our observations as implicating non-α_5 integrins in increasing uPAR-mediated cell adhesion, and possibly in interacting with uPAR and reducing uPAR-mediated plasminogen activation, we have not identified these inte-

grins. How these putative interactions might be modified by $\alpha_5\beta_1$ is unclear, but, from our previous observations with CD82 (20) and the known role of tetraspanins in modulating the localisation and functional activity of integrins (36, 37), a role for these proteins in the observed effects is possible.

A major conclusion that can be drawn from the data presented here, and which is supported by our previous observations (20), is that the presence of uPAR on the cell surface, even in the presence of excess uPA, does not inevitably lead to uPA binding and plasminogen activation. We have previously referred to uPAR that is unavailable for uPA binding as "cryptic", while others have termed it "latent" (38), and a rational basis for this can be proposed based on the crystal structure of uPAR. The structure of uPAR has been solved both in complex with a peptide antagonist of uPA binding (39) and with the amino-terminal fragment of uPA (40). These structures differ in the relative orientation of the three homologous domains of uPAR, all of which are necessary for uPA binding (41, 42), suggesting a significant degree of conformational flexibility in uPAR that is controlled by ligand binding (38). Therefore, it could be envisaged that interactions between uPAR and integrins, including $\alpha_5\beta_1$, could alter the conformation of uPAR to influence uPA binding. Direct evidence that conformational changes in uPAR can abolish uPA binding comes from the observation that certain anti-uPAR monoclonal antibodies can non-competitively dissociate preformed uPA-uPAR complexes (43). On the cell surface such conformational changes in uPAR could result from either direct interactions with integrins or integrin-mediated clustering of uPAR.

In summary we have shown that the integrin $\alpha_5\beta_1$ plays a key role in the regulation of uPAR function at the cell surface, affecting its role both as a receptor mediating plasminogen activation and as a putative adhesion receptor, and extending our previous observations (20). Together with the known role of uPAR-integrin interactions in modulating integrin function, these observations demonstrate that bi-directional communication exists between these two protein systems involved in pericellular proteolysis and cell adhesion. This communication has the potential to be dynamic, as no irreversible or covalent changes to the proteins are involved, and may have important role in mediating invasive cell migration. Understanding how the apparently promiscuous interactions between uPAR and integrins at the cell surface are regulated will require further investigation.

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What is known about this topic?

- uPAR has a key role in mediating plasminogen activation on the cell surface.
- Interactions between uPAR and adhesion receptors of the integrin family, including $\alpha_5\beta_1$, interact with uPAR on the cell surface to alter integrin function.
- uPAR-mediated plasminogen activation is down-regulated by CD82, an integrin-associated tetraspanin protein, by redistributing $\alpha_5\beta_1$ to colocalise and associate with uPAR on the cell surface.

What does this paper add?

- We show that expression of $\alpha_5\beta_1$ in Chinese hamster ovary cells leads both to an increase in uPAR-mediated plasminogen activation and a decrease in uPAR- and integrin-dependent adhesion to an anti-uPAR monoclonal antibody.
- Despite these effects, direct interactions between uPAR and $\alpha_5\beta_1$ could not be found, either on the cell surface or using purified proteins in solution.
- $\alpha_5\beta_1$ and other integrins cooperate to regulate uPAR-mediated proteolysis, and also uPAR-mediated cell adhesion, potentially influencing invasive cell migration.

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