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Domain 1 of the urokinase receptor (uPAR) is required for uPAR-mediated cell binding to vitronectin

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Abstract In the present paper we have analyzed uPAR-mediated cellular binding to vitronectin using the murine erythroid progenitor cell line 32D. We show that expression of uPAR in 32D cells promotes cellular binding to vitronectin, but fails to support cell spreading. The strength of binding is correlated to the expression level of uPAR and is strongly stimulated by the presence of uPAR ligands. Using a truncated variant of uPAR lacking domain 1 and by antibody inhibition experiments, we demonstrate that domain 1 plays a crucial role in uPAR-mediated cellular binding. The failure of the mutant uPAR to promote cellular binding is paralleled by a strong reduction in the affinity for vitronectin in vitro.

Key words: uPAR; Vitronectin; Adhesion

1. Introduction

The presence of a cellular proteolytic variant of uPAR composed of domain 2 and 3 (D2D3) and, thus, lacking the major uPA binding domain 1 (D1), was first identified in U937 cells [1] and has since then been observed in other cell lines and tumor extracts [2,3]. The formation of the D2D3 uPAR variant may involve the proteolytic cleavage by uPA, which is capable of cleaving the receptor in the linker region between domain 1 and 2 both in vitro and at physiological concentrations in vivo [1,4]. The cleavage of uPAR in this region results in profound changes in the biological properties of uPAR: first, uPAR loses the capacity to bind uPA with a concomitant loss of potential to enhance uPA-mediated cell surface plasmin generation [4,5]. Since the cleavage is mostly expected to occur under conditions of high uPA activity, it might function as a negative feedback mechanism regulating cell surface proteolysis [4]. Second, the cleavage in the hinge region unmasks bioactive epitopes of uPAR that have the potential to induce cellular chemotaxis, cytoskeletal reorganisation and activation of kinases from the *src*-family [6–8].

Cellular binding to vitronectin, mediated by uPAR, was first shown in the human histiocytic lymphoma cell line U937. This cell line becomes strongly adhesive to vitronectin when stimulated with a combination of vitamin D3 and transforming growth factor β1 or with phorbol esters [9,10]. This has later been extended to other cell lines and it has been shown that both cellular and soluble uPAR interact directly with certain forms of vitronectin [10–12]. Besides mediating cellular binding to vitronectin uPAR also modulates the function of adhesion receptors from the integrin family [13–16] and data for a direct interaction between uPAR and purified Mac-1 have been presented [13]. The region of uPAR responsible for the interaction with vitronectin was initially assigned to D2D3 [11] but later data obtained using real-time biomolecular interaction analysis have demonstrated that also D1 is required for the high-affinity interaction in vitro [12]. At present, only little data on the effect of cleavage on the potential of uPAR to mediate cellular binding to vitronectin have been presented [3].

In this paper we analyze the potential consequence of uPAR cleavage on the affinity for vitronectin and on the capacity to mediate cellular binding to vitronectin. Using cellular and soluble uPAR mutant receptors we report that D1 plays a crucial role for the capacity of uPAR to mediate cellular binding to vitronectin as well as for the high affinity interaction with vitronectin in vitro. The system employed, 32D erythroid progenitor cells, is suitable for this analysis as these cells display low intrinsic, integrin-mediated binding to the extracellular matrix proteins fibronectin and vitronectin. Thus, the uPAR-mediated binding is not confused with possible integrin-mediated cell binding and is not affected by cell spreading.

2. Materials and methods

2.1. Reagents

The 32D and WEHI-1 cell lines were obtained from Dr. Jacalyn H. Pierce. COS7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). General chemicals used in this study were obtained from Sigma or Boehringer Mannheim and were of the highest available quality. Cell culture reagents (RPMI, glutamine, penicillin, streptomycin, FCS, trypsin) were purchased from Gibco-BRL, Italy. Recombinant murine IL-3 was obtained from Boehringer Mannheim, Italy. Tissue culture plastic ware was from Costar, Italy. Monoclonal mouse antibodies against human uPAR (mAb: R2, R3, R4 and R5) were obtained from Dr. Gunilla Højer-Hansen at the Finsen Laboratory in Denmark. Monoclonal mouse antibody directed against the FLAG™-epitope (mAb: M2) was purchased from Sigma, Italy. Rabbit polyclonal antibodies against the FLAG-epitope were purchased from Santa Cruz Biotechnology, CA, USA. Human pro-uPA, uPA and ATF was a generous gift of Dr. Jack Henkin (Abbott Laboratories, IL, USA). Human vitronectin was obtained from Molecular Innovations, MI, USA. Fibronectin was

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Abbreviations: ATF, amino-terminal fragment of uPA; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GFD, growth factor domain of uPA; GPI, glycosylphosphatidyl inositol; IL-3, interleukin-3; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMA, 12-*O*-tetradecanoylphorbol-13-acetate; TBS, Tris-buffered saline; (pro)uPA, (pro)urokinase-type plasminogen activator; uPAR, uPA receptor

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obtained from Boehringer Mannheim, Italy. Oligonucleotides were purchased from PRIMM (Italy) and GENSET (France) and had the following sequences (3′–5′): FRA18: ATTATACTCGAGGAAGACGTGCAGGGACCCCGCGCA, FRA19: TATATTGATCATTTAGGTCCAGAGGAGAGTGCCTC, D2.5′T: AATGCATTCGAGGCCCCAAGAGGCTGGGA.

2.2. Purification of recombinant soluble uPAR molecules

Cloning and expression of recombinant soluble uPAR mutant proteins was performed as described elsewhere [7]. The recombinant proteins were purified from COS7 conditioned medium by passage over an anti-FLAG™ affinity column (M2 Affinity gel, Sigma). After washing of the column with phosphate buffered saline (PBS), the recombinant proteins were eluted using 0.1 M glycine pH 3.0. The fractions were immediately neutralized using 1/10 volume of 1 M Tris–HCL pH 8.0 and analyzed by enzyme-linked immunosorbent assay (ELISA). Fractions containing the recombinant protein were pooled and dialyzed against PBS.

2.3. General cell culture

All cell culture media used were supplemented with glutamine (5 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). WEHI-1 cells were cultured in complete RPMI medium containing 15% FCS. 32D cells were cultured in complete RPMI medium supplemented with 10% FCS and 10% WEHI-1 conditioned medium as a source of IL-3. For the reparation of conditioned WEHI medium, cultures of proliferating WEHI-1 cells were split 1:100 in fresh medium and allowed to grow until the medium turned acidic (5–6 days). Cells were pelleted by centrifugation and the conditioned medium sterile filtered (0.2 μm) and stored at 4°C until use.

2.4. Construction of expression vectors

Vectors expressing the wild type receptor (uPAR) and a deletion mutant lacking the first 92 amino acids corresponding to domain 1 (D2D3) were constructed by first amplifying the entire uPAR cDNA [17] with the primers FRA18 and FRA19 and cloning the product in a *Eco*RV digested and T-tailed pBluescript SK- vector. The expression vector LTR-2/uPAR was generated by transferring a *Xho*I–*Cla*I fragment, containing the entire uPAR-coding region, from the pBluescript sub-clone to the LTR-2 expression vector digested with same enzymes. The LTR-2/D2D3 expression vector was constructed by first replacing the *Nru*I–*Nsi*I fragment (containing the entire D1 coding region) of the pBluescript uPAR sub-clone with a PCR product generated by amplification of the uPAR cDNA with the primers FRA18 and D2.5′T and digested with the same enzymes (thereby restoring the desired signal-peptide/D2D3 junction amino acid −1/93). Finally, the modified uPAR cDNA was transferred to the LTR-2 vector as described for the wild type cDNA.

2.5. Transfection of 32D cells

32D cells were harvested in mid-log phase (0.5×10^6/ml) and washed twice in RPMI medium. Cells were resuspended in RPMI (50×10^6/ml) and 0.2 ml aliquots were added to electroporation cuvettes (0.4 cm, Bio-Rad) already containing 20 μg of the desired expression vector and 2 μg of pRSVneo (1 μg/ml in water). Cells were electroporated using a single 280 V, 960 μF pulse in an electroporation apparatus (Bio-Rad, Gene Pulser II) and transferred to complete medium for recovery. After 48 h cells were transferred to complete medium containing 0.8 mg/ml G418 for selection of stable transfectants. Single clones of transfectants were obtained by limited dilution of G418 resistant pools into 96-well tissue culture plates. Stable pools and clones of transfected 32D cells were maintained under weak selective pressure in medium containing 0.2 mg/ml G418.

2.6. Adhesion assays

The coating of 96-well tissue culture dishes was performed by incubation with 5 μg/ml vitronectin or 10 mg/ml fibronectin in PBS for 2 h at 37°C or overnight at 4°C. The remaining binding sites were saturated by incubation of the wells with 2% bovine serum albumin in PBS for 1 h at 37°C. Immediately before the adhesion assay, cells were washed twice in RPMI containing 0.1% bovine serum albumin (BSA) and resuspended in the same medium at 2×10^6/ml. Cells (0.05 ml) were added to wells already containing 50 μl RPMI with 0.1% BSA and the agonists/antagonists to be tested. The plates were centrifuged at 1000 rpm for 1 min to sediment the cells and incubated in a humidified incubator at 37°C for 1 h. At the end of the incubation the wells were washed with 37°C-warm RPMI as follows: to the medium already present in the wells was added 2×100 μl medium, at normal pipetting speed, using a multi-well pipette, row by row and from two opposite sites of the wells. The medium (containing the suspended non-adherent cells) was then immediately aspirated from one side of the well and 100 μl of fresh medium added. The procedure of washing was repeated, if necessary, until BSA coated wells contained only a few remaining cells. Adherent cells were fixed for 10 min with 37°C-warm PBS containing 3% (w/v) formaldehyde and the cells stained for 5 min with crystal violet solution (0.5% (w/v) in 20% (v/v) methanol). After washing of the plate by immersion in tap-water, the cells were lyzed in 1% SDS in water and the absorption at 540 nm was measured in an ELISA plate reader. To obtain a measure for specific adhesion the readings were subtracted from the value obtained in BSA coated wells, unless otherwise indicated. All experiments were performed at least three times and the shown values are the mean ± S.D. of triplicate or quadruplicate determinations from a representative experiment.

2.7. FACS analysis

32D cells to be analyzed (1×10^6) were pelleted by centrifugation and resuspended in 0.1 ml ice-cold wash buffer (PBS containing 5% FCS) containing 10 mg/ml of the R2 monoclonal antibody and incubated on ice for 30 min. The cells were washed twice with 3 ml ice cold wash buffer and resuspended in 0.1 ml wash buffer containing a FITC conjugated goat anti-mouse antibody (DAKO) diluted 1:50 in wash buffer and incubated for 20 min on ice. After another round of washing, the cells were resuspended in 0.5 ml PBS and analyzed in a Becton Dickinson flow cytometer according to the manufacturers instructions. Relative values for the mean fluorescence were calculated using the Lysis II software package. Non-transfected cells were used as controls for specificity.

2.8. In vitro binding assays

ELISA plates (Nunc Maxisorb) were coated with vitronectin (0.1 ml, 5 μg/ml) in PBS for 2 h at 37°C or overnight at 4°C. The remaining protein binding sites were blocked using 1% blocking reagent (Boehringer Mannheim) in TBS for 1 h at room temperature. All further incubations were performed with gentle agitation at room temperature in 0.1 ml TBS containing 1% blocking reagent (Boehringer Mannheim) and followed by extensive washing with TBS containing 0.1% Tween-20. First, wells were incubated with the FLAG™-tagged soluble uPAR molecules diluted to the indicated concentrations for 1 h at room temperature. In some cases the recombinant suPAR was co-incubated with pro-uPA, ATF and/or antibodies. After washing, wells were probed for the FLAG-epitope by incubation with 0.2 μg/ml rabbit anti-FLAG antibody (Santa Cruz, #sc-807) for 1 h. After washing, wells were probed for bound antibody by incubation with a horseradish conjugated goat anti-rabbit antibody (Amersham) diluted 1:1000. After another round of washing, bound horseradish conjugate was quantified by colorimetric development using the chromogenic substrate ABTS (Boehringer Mannheim). The absorption at 436 nm was recorded in an ELISA plate reader and the signal obtained from wells that were coated with blocking reagent subtracted.

3. Results

3.1. Adhesive properties of 32D cells

To analyze the structural requirements of uPAR as an adhesion receptor we needed a cell system that fulfils two criteria. First, the cell line should be easy to transfect and second the cell line should have low basal binding to vitronectin in order to discriminate between binding caused by the transfected molecules and binding caused by endogenously expressed adhesion receptors.

To evaluate the murine myeloid 32D cell line as a model system for uPAR-mediated adhesion we confronted the adhesive properties of these cells with those of the well-characterized human myeloid U937 cells (Fig. 1A). While U937 cells displayed significant binding to both fibronectin and vitronec-

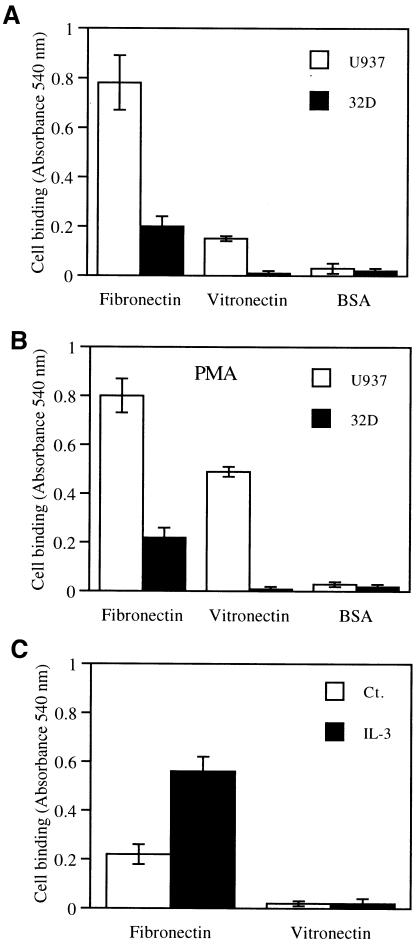

Fig. 1. Adhesive properties of 32D and U937 cells. Non-stimulated (A) or PMA-stimulated (B) 32D cells (closed bars) and U937 cells (open bars) were allowed to adhere to wells coated with fibronectin, vitronectin or BSA under serum free conditions. After 1 h, the wells were washed and bound cells fixed and stained. The numbers of bound cells (in arbitrary units) were calculated by measuring the absorbancy at 540 nm and subtracting the value obtained from wells that received no cells. C: Binding of IL-3-starved 32D cells to vitronectin and fibronectin in the absence (open bars) or presence (closed bars) of 100 U/ml recombinant mouse IL-3. Bound cells were quantified as before and the value obtained in wells coated with BSA subtracted. The data represent the mean ± S.D. of triplicate determinations from a representative experiment.

tin, 32D cells completely failed to adhere to vitronectin and only displayed moderate binding to fibronectin. Stimulation with cytokines or phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate, PMA) is known to enhance the cellular binding of U937 cells to vitronectin as a result of enhanced expression of uPAR and of its ligand uPA [10]. To analyze the effect of PMA stimulation on the cellular binding of 32D cells we stimulated cultures of 32D and U937 with PMA for 18 h prior to the adhesion assay (Fig. 1B). While the treatment with PMA stimulated the binding of U937 cells to vitronectin approximately two-fold it failed to modulate the binding of the 32D cells to any of the substrates. The 32D cells express a range of integrin receptors including fibronectin and vitronectin receptors [18,19]. The activation state of the integrin receptors and the binding to fibronectin of 32D cells is regulated by IL-3 [19]. To analyze the effect of IL-3 stimulation on binding to fibronectin and vitronectin we performed adhesion assays where the 32D cells had been IL-3-starved for 8 h prior to the experiment (Fig. 1C). Under these conditions the re-addition of IL-3 stimulated the cellular binding of 32D cells to fibronectin approximately two-fold, but failed to modulate the binding to vitronectin.

From these experiments we conclude that the 32D cells represent a suitable model system for the analysis of uPAR-mediated cellular binding to vitronectin as they do not display any notable binding to vitronectin independently of the activation state.

3.2. *uPAR expression promotes 32D cell binding to vitronectin*

To analyze uPAR-mediated cell binding we transfected 32D cells with an expression vector in which we had engineered the full-length human uPAR cDNA [17]. By selection of the transfected cells with G418 we obtained a pool of resistant cells representing a population of clones expressing variable amounts of uPAR on the cell surface as determined by FACS analysis using the anti-uPAR monoclonal antibody R2 (results not shown). During normal passage, the pool of uPAR-transfected cells was notably more adherent to the tissue culture plates than mock-transfected cells (results not shown). To investigate if the increased binding of the uPAR-transfected cells was mediated by vitronectin present in the culture medium we performed adhesion assays to purified vitronectin immobilized in 96-well tissue culture plates under serum free conditions (Fig. 2). Indeed, the pool of uPAR-transfected cells bound stronger to vitronectin than mock-transfected cells over a range of coating concentrations, demonstrating that the expression of uPAR in 32D cells promotes cell binding to vitronectin.

3.3. *The strength of cell binding to vitronectin correlates with the expression level of uPAR*

Even at the highest vitronectin coating concentration less than 10% of the cells in the pool of uPAR transfectants remained bound after washing (not shown) and we speculated that this was caused by the heterogeneous uPAR expression in the pool. To analyze the relationship between the expression level of uPAR and the strength of binding to vitronectin we therefore cloned the pool of transfected cells by end-point dilution. A number of clones representing a range of uPAR expression levels (Fig. 3A) were selected for analysis and their binding to vitronectin determined (Fig. 3B). There was a good

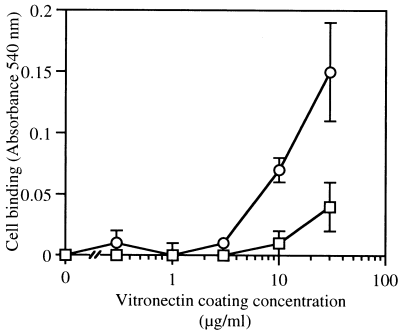

Fig. 2. Expression of uPAR in 32D cells causes increased binding to vitronectin. Pools of G418 resistant cells obtained after transfection with an uPAR expression vector (circles) or empty expression vector (squares) were allowed to adhere to wells coated with increasing concentrations of vitronectin and the bound cells were quantified as before.

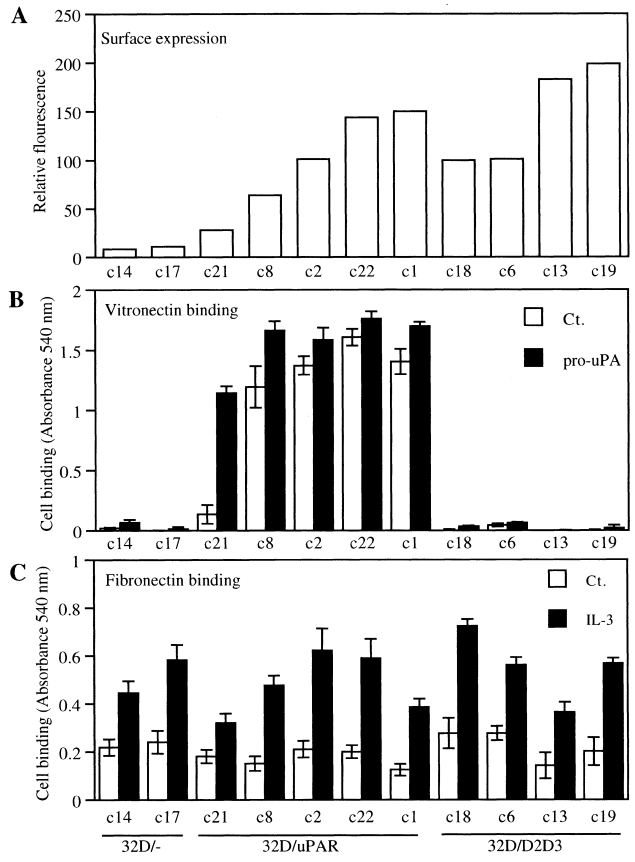

Fig. 3. Binding of 32D uPAR/D2D3 clones to vitronectin. Surface expression (A) of uPAR and D2D3 on cells was evaluated by FACS analysis using the R2 monoclonal antibody that recognizes an epitope on domain 3 of human uPAR. The mean fluorescence (in arbitrary units) is indicated and the clones have been arranged from left to right in order of increasing expression level. Adhesion to vitronectin (B): clones of 32D cells transfected with full-length uPAR (c14, c17, c21, c8, c2, c22, and c1) or with a D2D3 (c18, c6, c13, and c19) were allowed to adhere to vitronectin under serum free conditions in the presence (filled bars) or absence (open bars) of 5 nM pro-uPA. Adhesion to fibronectin (C) was performed as for vitronectin with the exception that it was performed after 8 h of IL-3 starvation and done in the absence (open bars) or presence (filled bars) of 100 U/ml IL-3.

correlation between the expression level of uPAR and the strength of binding to vitronectin as higher expression levels of uPAR were associated with stronger binding to vitronectin. In the highest expressing clones more than 90% of the cells remained bound after washing of the plates (not shown), while the lowest expressing clone (c21) displayed binding comparable to that of the pool of transfected cells. Two clones (c14 and c17) did not express detectable levels of cell surface uPAR as evaluated by FACS analysis and were included in the analysis as negative controls.

3.4. Cell binding is stimulated by uPAR ligands

uPAR-mediated binding of U937 cells to vitronectin is stimulated by uPA [10] and we therefore analyzed the effect of uPA on the binding of uPAR-transfected 32D cells to vitronectin (Fig. 3B). Clones expressing high levels of uPAR bound strongly to vitronectin in the absence of ligand and the addition of pro-uPA to the assay only slightly stimulated binding. However, the binding of clones expressing low and intermediate levels of uPAR was stimulated by pro-uPA and the stimulation was inversely correlated with the expression level of uPAR. A clone expressing low levels of uPAR (c21) was strongly stimulated and clones expressing intermediate to high levels of uPAR (c8, c2, c22 and c1), only weakly stimulated. The presence of pro-uPA during the adhesion assays had no significant effect on the negative control clones expressing undetectable levels of uPAR (c14 and c17), demonstrating that the stimulatory effect of pro-uPA was indeed mediated by uPAR. Stimulation of binding to vitronectin by pro-uPA was dose-dependent with a half-maximal effect at 0.1–0.3 nM pro-uPA (not shown), consistent with the K_d for the interaction between uPAR and pro-uPA. Also two-chain uPA and the amino-terminal fragment (ATF) of uPA stimulated the binding with equal efficiency (results not shown) demonstrating that it was the ligand binding capacity, rather than the catalytic activity that was responsible for the enhancement of binding (not shown).

3.5. Domain 1 of uPAR is required for cell binding to vitronectin

To analyze the effect of uPAR cleavage on uPAR-mediated cell binding to vitronectin we constructed a mutant uPAR cDNA encoding a deletion variant of uPAR where the region encoding domain 1 (amino acids 1–92) was deleted creating a receptor composed of domain 2 and 3 (D2D3). The structure and the domain composition of this recombinant receptor is equivalent to a cleaved form of uPAR observed on various cell lines and tumors [1–3]. We generated clones of 32D cells with the mutant receptor as described for full-length uPAR and identified several clones expressing the truncated receptor at levels comparable to that of full-length uPAR (Fig. 3A). Even though the D2D3 receptor was efficiently expressed it completely failed to promote 32D cell binding to vitronectin (Fig. 3B).

To further analyze the role of domain D1 in uPAR-mediated cell binding to vitronectin, we tested monoclonal antibodies against uPAR for their ability to inhibit the binding of uPAR-transfected 32D cells (Fig. 4A). We used the 32D/uPAR clone 1 cells, as these adhere strongly to vitronectin in the absence of pro-uPA and performed the adhesion assay in the absence of pro-uPA to avoid interference between antibody and pro-uPA binding to uPAR. The possible interference by endogenous 32D pro-uPA can be disregarded because these cells appear not to produce pro-uPA (not shown). In addition, murine pro-uPA would have about a 1000-fold lower affinity for human uPAR [20,21]. Two monoclonal antibodies recognizing epitopes within domain 3 of uPAR (mAb R2 and R4) and a control antibody (M2) did not affect the cell binding to vitronectin. However, both antibodies recognizing epitopes within D1 of uPAR (mAb R3 and R5) strongly inhibited the binding, demonstrating the importance of this domain in uPAR-mediated cell binding to vitronectin.

3.6. Domain 1 and uPAR ligand are required for high affinity binding of soluble uPAR to vitronectin in vitro

The above data suggest that the defect of D2D3 receptor to mediate cellular binding to vitronectin was caused by a reduced or abolished ability to interact with vitronectin. To address this point directly we analyzed if the D2D3 receptor also has a reduced affinity for vitronectin in vitro. To do this we performed in vitro binding assays to immobilized vitronec-

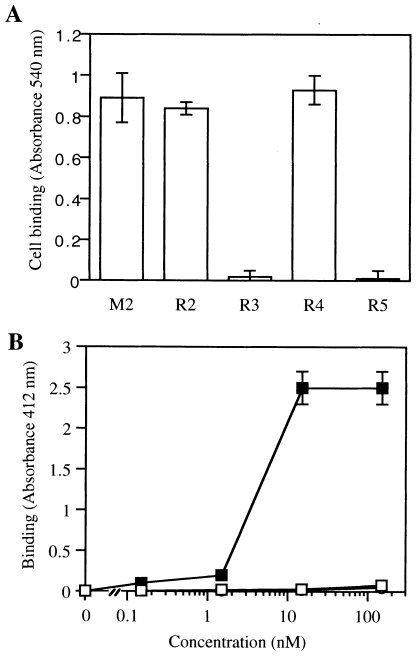

Fig. 4. Domain 1 of uPAR is involved in uPAR binding to vitronectin both in vivo and in vitro. A: 32D/uPAR clone 1 cells expressing high levels of uPAR were allowed to adhere to vitronectin in the presence of monoclonal antibodies against either uPAR domain 3 (mAb R2 and R4), uPAR domain 1 (mAb R3 and R5) or a negative control antibody (mAb M2). After washing, bound cells were quantified as described in the legend to Fig. 1. B: Binding of soluble uPAR to vitronectin. ELISA plates were coated with vitronectin (5 μg/ml) and the remaining binding sites blocked with BSA. Wells were incubated with increasing concentrations of FLAG tagged soluble uPAR (squares) or D2D3 mutant (circles). The binding was performed in the presence (filled) or absence (open) of 50 nM pro-uPA. After washing, the amount of bound protein was quantified by sequential incubations with an anti-FLAG antibody; a horseradish peroxidase conjugated secondary antibody and a colorimetric peroxidase substrate. The absorbancy at 412 nm was measured in an ELISA reader and taken as a measure of bound proteins. The data represent the medium value of duplicate determinations (±S.D.) from a representative experiment. The curves for the D2D3 protein cannot be seen very well as they are superimposed with the curve for soluble uPAR in the absence of pro-uPA.

tin using a soluble form of full-length uPAR and of the mutant D2D3 receptor. The construction, expression and purification of these soluble receptors have been described in detail elsewhere [7]. These soluble uPAR variants have the same domain structure as those used for transfection of 32D cells but the GPI-anchoring sequence has been replaced with a short peptide sequence (the FLAG epitope) recognized by specific antibodies. We incubated vitronectin-coated wells with increasing concentrations of the recombinant receptors in the presence or absence of pro-uPA and quantified the bound proteins by virtue of the FLAG-tag (Fig. 4B). Even though we could detect binding of the full-length receptor down below 1 nM in the presence of pro-uPA we failed to observe any notable binding in the absence of pro-uPA, suggesting that uPAR occupancy is indeed a major determinant in the affinity of uPAR for vitronectin. The soluble D2D3 receptor mutant failed to bind to vitronectin over the entire concentration range and did so both in the presence or absence of pro-uPA.

3.7. uPAR-mediated binding to vitronectin is independent of integrin activation state

In uPAR-transfected HEK293 cells uPAR-mediated vitronectin adhesion depends upon integrin activation as it is inhibited at low temperatures and by the introduction of dominant negative integrins [13]. To analyze the requirement for integrin function in the 32D-cell system, we performed adhesion assays in which we regulated the integrin activation state using IL-3 and temperature (Fig. 5A). The binding to vitronectin was not significantly affected by IL-3 stimulation and was not reduced at 4°C, suggesting that uPAR-mediated cell

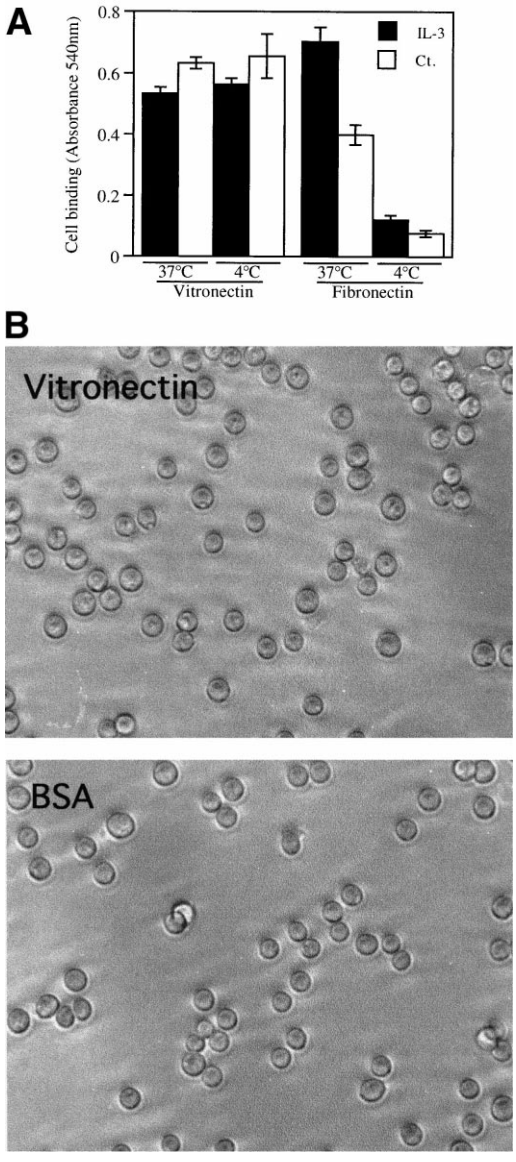

Fig. 5. uPAR-dependent vitronectin binding of 32D cells is independent of integrin activation and does not lead to cell spreading. A: IL-3-starved 32D/uPAR clone 1 cells were allowed to adhere to vitronectin or fibronectin in the presence (filled bars) or absence (open bars) of IL-3 (100 U/ml) at 37 or 4°C as indicated. After washing, bound cells were quantified as described in Fig. 1. B: uPAR-transfected 32D cells (clone 1) were allowed to adhere to plastic dishes coated with BSA (BSA) or vitronectin (VTN) for 1 h at 37°C after which the cells were photographed. To reveal the morphology of cells plated on BSA the dishes were not washed prior to photography.

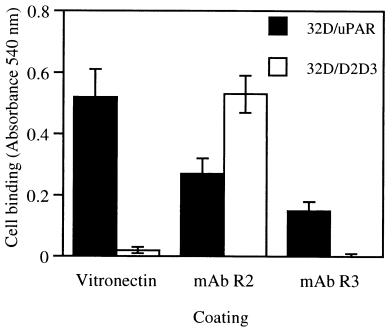

Fig. 6. Cell binding to anti-uPAR antibodies. 32D cells transfected with uPAR (filled bars) or the mutant D2D3 receptor (open bars) were allowed to adhere to wells coated with vitronectin or antibodies against uPAR (5 μg/ml). After washing of the plates bound cells were quantified as before.

binding to vitronectin does not require integrin activation. The integrin-mediated binding of 32D cells to fibronectin was enhanced by IL-3 stimulation but this stimulation was almost completely inhibited at 4°C demonstrating the requirement for metabolic energy in this type of cell binding. Inhibition of integrin function by EDTA and peptides containing the RGD motif also failed to inhibit the binding of uPAR-expressing cells to vitronectin while they completely blocked the binding to fibronectin (not shown). In support of the lack of integrin involvement in the uPAR-mediated cell binding to vitronectin we also failed to observe cell spreading of the uPAR-transfected 32D cells (Fig. 5B).

In HEK293 cells the expression of uPAR inhibits integrin-mediated adhesion to fibronectin, suggesting that uPAR also regulates cell adhesion by modulation of integrin function [13]. To investigate if this is also the case in the transfected 32D cells we assayed the binding of the different 32D clones to fibronectin in the absence or presence of IL-3 (Fig. 3C). 32D clones expressing both uPAR and D2D3 adhered to fibronectin and binding was further stimulated by IL-3 demonstrating that the surface expression of uPAR does not strongly impair IL-3-dependent integrin function and activation. However, a rather high degree of clonal variation in adhesion to fibronectin was observed, preventing the determination of possible minor effects.

Taken together the data suggest that uPAR-mediated binding to vitronectin of transfected 32D cells occurs independently of integrin function and activation and hence may simply reflect an affinity between the cells and the tissue culture plastic mediated by the interaction between uPAR (on the cell surface) and vitronectin (on the plastic). To investigate this possibility we performed adhesion assays to plates that we had coated with monoclonal antibodies against uPAR (Fig. 6). In fact, uPAR-transfected 32D cells adhered strongly to wells coated with antibodies against both domain 1 (mAb R3) and domain 3 (mAb R2). The 32D cells transfected with the mutant D2D3 receptor, which failed to bind to vitronectin (Fig. 3B), bound to the uPAR antibody directed against an epitope within domain 3 (mAb R2). The results thus demonstrated that in 32D cells any high affinity interaction between a cell surface molecule (namely uPAR) and an immobilized protein (vitronectin or anti-uPAR antibodies) can mediate cellular binding, indicating that uPAR acts as a surface binding site for vitronectin without intrinsic cell adhesion functions.

4. Discussion

Firm cellular attachment to the extracellular matrix is a complex process, which involves numerous molecular interactions between extracellular proteins, cell surface receptors and the intracellular cytoskeleton. The process is initiated by the cellular binding to the extracellular matrix, mediated by the interaction between cellular receptors and extracellular matrix proteins and is followed by a complex process of signaling, receptor activation and cytoskeleton reorganization, eventually leading to firm attachment and cell spreading.

Expression of uPAR in 32D cells promotes cellular binding to vitronectin but not cell spreading. This is in contrast to the epithelial HEK293 cell line in which expression of uPAR leads to firm cell adhesion and spreading on vitronectin [11]. The apparent discrepancy between these observations may be caused by differences between the two cell lines. HEK293 grow firmly adherent and therefore clearly possess the necessary cell program and machinery to promote firm cell adhesion and spreading. The 32D cells are highly undifferentiated, grow in suspension and are only expected to undergo firm adhesion and cell spreading upon differentiation. It is therefore likely that these cells do not possess the required cell program and/or machinery to support cell spreading. Expression of uPAR in 32D cells evidently promotes the initial cellular binding to vitronectin, but fails to initiate the process of cell spreading (Fig. 5B). The strength of binding of uPAR-transfected 32D cells to vitronectin and anti-uPAR antibodies was comparable, suggesting that the only requirement for binding is a sufficiently high number of interactions between the cell and the substrate. When starved 32D cells are treated with IL-3, they bind more strongly to fibronectin (Figs. 1C and 3C and [19]). However, both basal- and IL-3-stimulated binding to fibronectin is not strongly affected by the expression of uPAR (Fig. 3C) suggesting that uPAR does not modulate integrin function in the 32D cells.

The structural requirement for a high affinity interaction between vitronectin and uPAR has been addressed in a couple of studies [11,12]. Wei et al. have suggested that a region within the D2D3 part of uPAR is responsible for the interaction. This conclusion is based on experiments with a D2D3 fragment purified from chymotrypsin-cleaved suPAR and inhibition by the R4 antibody (recognizing an epitope within domain 3). Højer-Hansen et al. have, based on real-time biomolecular analysis, concluded that intact uPAR is required for the high affinity interaction between uPAR and vitronectin. Our data are in accordance with the latter, as we do not observe any significant binding of D2D3 to immobilized vitronectin. A likely explanation for the discrepancy between the observations could be the presence of low levels of contaminating full-length soluble uPAR in the D2D3 preparations used by Wei et al. In our experimental set-up this potential problem has been eliminated using recombinant uPAR fragments expressed in cells that do not produce any full-length suPAR.

We observe a strict requirement for the pro-uPA to obtain significant binding of suPAR to immobilized vitronectin in vitro. This observation contradicts data published by others who observe no difference [4] or a moderate to high stimulation [11] by uPAR ligands. The reason for this discrepancy may lie in the different assays employed and in the different soluble uPAR molecules or vitronectin preparations used in

the experiments. The binding of suPAR to vitronectin is not a simple first order binding reaction and probably involves conformational changes of both uPAR and vitronectin [4]. Indeed, Wei et al. suggest that uPAR is in equilibrium between a high and a low affinity conformation, with respect to vitronectin binding, which is pushed towards the high affinity state upon ligand binding. A number of modifications such as radio labeling, biotinylation or the engineered addition of epitopes used for purification and detection can potentially affect this equilibrium.

In contrast to our data in vitro, we did not observe a strict requirement for ligand binding in the process of cellular binding to vitronectin in vivo, except only partially in clones expressing low uPAR levels. This apparent difference may have a number of explanations. First, the local concentration of membrane bound uPAR at the interface between the cell and the substratum might be high enough to allow a sufficient number of low affinity interactions between uPAR and vitronectin to obtain the required resistance to the hydrodynamic stress applied in the adhesion assay. Second, the high affinity conformation of uPAR could be stabilized by the GPI-anchor, which is not present on soluble uPAR or by the interaction with other membrane proteins. However, these explanations only apply to cells that express high levels of uPAR. Low-uPAR 32D cells required ligand to bind to vitronectin.

uPAR antagonist are molecules that inhibit the uPA/uPAR interaction through binding to uPAR. These molecules include non-catalytic derivatives of uPA (ATF, GFD and derivatives), antibodies as R3 and R5 [22] and different low molecular weight compounds [23]. uPAR antagonists are therapeutically interesting as they are able to inhibit tumor growth and metastasis in certain model systems (reviewed in [24]). The anti-cancer effect of these molecules has been assigned to the capacity to block the binding of uPA to uPAR with its concomitant reduction in plasminogen activation potential of the cells. However, it is likely that the effect of these compounds might, at least partially, be caused by their pro- or anti- adhesive properties. A great deal of work is done in the pharmaceutical industry to develop non-toxic low molecular weight uPAR antagonists and the 32D/uPAR cell system is perfect to test the pro- or anti-adhesive properties of these compounds.

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