The Vitronectin Receptor $\alpha_v\beta_3$ Binds Fibronectin and Acts in Concert with $\alpha_5\beta_1$ in Promoting Cellular Attachment and Spreading on Fibronectin

Israel E Charo,* Lisa Nannizzi,* Jeffrey W. Smith,‡ and David A. Cheresh*‡
*COR Therapeutics, Inc., South San Francisco, California 94080; and ‡Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. The vitronectin receptor ($\alpha_v\beta_3$) is a member of the integrin superfamily of adhesive protein receptors that mediate a wide spectrum of adhesive cellular interactions, including attachment to vitronectin, von Willebrand factor, fibrinogen, and thrombospondin. We have studied the binding of fibronectin to the purified vitronectin receptor, and the role of this receptor in the attachment of cells to fibronectin. A solid-phase microtiter assay was developed to investigate the binding properties of the vitronectin receptor. Purified $\alpha_v\beta_3$ bound fibronectin with high affinity in a saturable, divalent cation-dependent manner. Binding was inhibited by soluble vitronectin, by RGD-containing peptides, and by LM609, a monoclonal antibody against the vitronectin receptor known to inhibit the binding of adhesive proteins to $\alpha_v\beta_3$. Immunoinhibition experiments showed that M21 human melanoma cells, which express the fibronectin receptor, $\alpha_5\beta_1$, as well as $\alpha_v\beta_3$, used both of these integrins to attach and spread on fibronectin. In support of this finding, M21-L cells, a variant cell line that specifically lacks $\alpha_v\beta_3$ but expresses $\alpha_5\beta_1$, attached and spread poorly on fibronectin. In addition, $\alpha_v\beta_3$ from surface-labeled M21 cells was retained, and selectively eluted by RGDS from a fibronectin affinity column. These results indicate that $\alpha_v\beta_3$ acts in concert with $\alpha_5\beta_1$ in promoting fibronectin recognition by these cells. We conclude that fibronectin binds to the $\alpha_v\beta_3$ vitronectin receptor specifically and with high affinity, and that this interaction is biologically relevant in supporting cell adhesion to matrix proteins.

The vitronectin receptor ($\alpha_v\beta_3$) is a member of a superfamly of receptors known as the integrins (Hynes, 1987), which mediate a broad spectrum of cell-cell and cell-substrate interactions. It is now appreciated that such apparently diverse phenomenon as platelet aggregation (Phillips et al., 1980), endothelial cell adhesion to matrix proteins (Charo et al., 1987; Cheresh, 1987), embryogenesis (Duband et al., 1988), and perhaps tumor cell metastasis (Humphries et al., 1988) are mediated by structurally and/or immunologically related integrin receptors expressed on the cell surface.

Integrins are heterodimer complexes of noncovalently associated $\alpha$- and $\beta$-subunits. The integrin superfamily can be divided into subfamilies based on the presence of at least five homologous $\beta$-subunits that can associate with one or more $\alpha$-subunits. The spectrum of ligands bound to each receptor is dictated by the specific $\alpha$- and $\beta$-subunit pairing of the heterodimer complex. For example, $\alpha_v\beta_3$ shows a very specific ligand recognition capability and appears to bind only fibronectin, whereas endothelial cell $\alpha_5\beta_1$ is a more promiscuous receptor and binds both laminin and collagen (Elices and Hemler, 1989; Kirchhofer et al., 1990). In addition, a given cell may express two or more integrins that bind the same adhesive proteins. The ligand binding specificity of the vitronectin receptor has been unclear.

Pytela et al. (1986) found that the vitronectin receptor reconstituted into phospholipid vesicles composed of phosphatidyl choline bound to vitronectin-, but not fibronectin-, fibrinogen-, or thrombospondin-coated surfaces. Subsequent studies found that two different monoclonal antibodies that react with $\alpha_v\beta_3$ blocked the adhesion of human umbilical vein endothelial cells to von Willebrand factor (vWF)° and fibronogen, suggesting that $\alpha_v\beta_3$ interacted with these adhesive proteins as well. Neither of these antibodies blocked the adhesion of endothelial cells, which express both $\alpha_v\beta_3$ and $\alpha_5\beta_1$, to fibronectin (Charo et al., 1987; Cheresh, 1987). Recently, Conforti et al. (1990) found that $\alpha_v\beta_3$ reconstituted into phosphatidyl choline vesicles bound only vitronectin, in agreement with the earlier work of Pytela, and that when a mixture of phosphatidyl serine and phosphatidyl choline was used to form the vesicles $\alpha_v\beta_3$ also bound vWF and fibronectin. It was not determined, however, whether $\alpha_v\beta_3$ in intact cells bound these adhesive proteins, or if this binding was involved in adhesive cellular interactions.

In this paper we present evidence that purified $\alpha_v\beta_3$ is capable of recognizing fibronectin in a specific and RGD-dependent manner, and that it and the fibronectin receptor, $\alpha_5\beta_1$, act in concert in mediating the attachment and spreading of cells on fibronectin.
spreading of human melanoma cells on a fibronectin substrate. These data provide evidence for a novel biological role for the vitronectin receptor, αβ₃, in mediating the adhesion of cells to fibronectin in the extracellular matrix.

Materials and Methods

Purification of Proteins

The vitronectin receptor (αβ₃) was purified from human placenta as described (Smith and Cheresh, 1988). Briefly, one or two fresh human placentas were homogenized in a food processor in the presence of 20 mM Tris-HCl, 150 mM NaCl, 2.0 mM CaCl₂, 100 mM octyl-glucopyranoside, 0.05% NaN₃, pH 7.4. The homogenates were centrifuged at 100 g to remove the tissue, and again at 10,000 g. The supernatants were then passed over an LM609 affinity column to selectively retain the αβ₃ complex, and eluted with 10 mM acetate, 150 mM NaCl, 0.1% NP-40, 2 mM CaCl₂, pH 2.3. The eluted material was further purified by chromatography on a wheat germ affinity column (Sigma Chemical Co., St. Louis, MO). We obtained ~1 mg of highly purified αβ₃ from each placenta. This material was homogeneous on overloaded SDS-polyacrylamide gels, and did not contain detectable levels of platelet GPIIb-IIIa when assayed by ELISA.

Human vitronectin was purified from plasma as described (Yatogba et al., 1988). Briefly, human plasma was clotted in a glass beaker in the presence of protease inhibitors, and applied to a heparin-Sepharose column in the presence of 8 M urea. Purified vitronectin was eluted with 10 mM Na-phosphate buffer (pH 7.2) containing EDTA (5 mM), urea (8 M), and NaCl (0.5 M). Human plasma fibronectin was purified by gelatin-agarose affinity chromatography as described (Engvall et al., 1978). Further purification was performed by gel-filtration on a Sephacryl-S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4. Peak fractions were analyzed on reduced SDS-gels, and were free of detectable fibrinogen. Human fibrinogen and vWF were purified as described (Cheresh et al., 1989).

Antibodies

The αβ₃ monoclonal LM609 was produced as described (Cheresh and Spiro, 1987). The fibronectin receptor antibody BIIG2 (Werb et al., 1989) was provided by Dr. Caroline Damsky (University of California, San Francisco, CA) and the GPIIb-IIIa monoclonal 10E5 (Coller et al., 1983) was provided by Dr. Barry Coller (State University of New York at Stony Brook, NY).

Biotinylation of Adhesive Proteins

For biotinylation, proteins were dialyzed into 0.1 M NaHCO₃, 0.1 M NaCl, pH 8.2 and centrifuged in a table top ultracentrifuge (model TL-100; Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 30 min at 4°C to remove any particulate matter, and the protein concentration was adjusted to a concentration of 1 mg/ml. Sulfo-NHS-Biotin (No. 21217; Pierce Chemical Co., Rockford, IL) was added as a solid (12 mg of biotin ester per mg of biotin sensitive protein), and gently mixed on an end-over-end Nutorator (Clay Adams, Parsippany, NJ) for 30 min at room temperature. Unreacted biotin ester was removed by exhaustive dialysis against 50 mM Tris-HCl, 100 mM NaCl, 0.05% NaN₃, pH 7.4, at 4°C, and the biotinylated protein was stored at 4°C until used.

Binding Assay

The binding of adhesive proteins to immobilized αβ₃ was performed using a modification of the method recently described for platelet GPIIb-IIIa (Steiner, B., and P. Hadavry. 1988. Circ. Suppl. 78[Pt. 2]:2478[Abstr.]; Charo et al., 1990). The vitronectin receptor (1 mg/ml) was diluted 1:100 with a Triton X-100-free buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, 0.02% NaN₃, and then immediately added to 96-well microtiter plates (Linbro E.I.A. H Plus Microtitration plate; Flow Laboratories, Inc., McLean, VA) at 0.1 ml (0.5 μg) per well, and incubated overnight at 4°C. Microtiter plates were stored at 4°C for up to 2 mo before being used. At the time of the experiment, the wells were washed once with 50 mM Tris, 100 mM NaCl, 2 mM CaCl₂, 0.02% NaN₃, 1 mg/ml BSA, pH 7.4 (binding buffer) and then incubated in 0.1 ml of a solution of binding buffer with 35 mg/ml BSA for 2 h at 30°C to block nonspecific binding, followed by an additional wash with binding buffer (with 1 mg/ml BSA).

Biotinylated adhesive proteins (0.1 ml/well) were added and incubated for 3 h at 30°C in binding buffer (with 1 mg/ml BSA). After the incubation the wells were aspirated completely and washed once with 250 μl of binding buffer. Bound ligand was quantitated by the addition of 0.1 ml of an antibio-

Binding of αβ₃ to Immobilized Fibronectin

Human fibronectin was digested with chymotrypsin (enzyme/substrate ratio of 1:20) to produce the 120-kD cell-binding domain fragment, as described (Pytel et al., 1985a), which was coupled to cyanoemodine activated Sepharose (Pharmacia Fine Chemicals). M21 cells were surface labeled using lactoperoxidase-catalyzed iodination (Charo et al., 1986), and lysed with 200 mM n-octyl α-D-glucopyranoside (n-octyl α-glucoside) in 25 mM TBS. The cell lysate was slowly passed (at room temperature) over the 120-kD affinity column equilibrated in TBS (pH 7.4) with 50 mM octylglucoside and 2 mM MgCl₂. It was then washed with the same buffer, and eluted with RGES (1 mg/ml) and then RGDS (1 mg/ml), as described by Pytel et al. (1985a). Each fraction (0.5 ml) was immunoprecipitated with LM609 (20 μg/ml) and run on reduced 7.5% SDS-polyacrylamide gels, which were then examined by autoradiography.

Cell Adhesion

M21 and M21-L cells (Cheresh and Spiro, 1987) were grown in RPMI supplemented with 20% (vol/vol) FCS and antibiotics. The cells were partially depleted of methionine by incubation for 2-4 h in RPMI plus PBS-dialyzed serum, and then labeled by incubation with 250 μCi of [³⁵S]methionine overnight. The cells were then washed three times with serum-free media, harvested, and added to vitronectin-coated microtiter wells. After 30-60 min at 37°C (in the presence or absence of antibodies) the cells were washed three times and solubilized (0.1 M NaOH, 60 min, room temperature). Bound cells were quantitated by counting beta emissions. In the control wells ~50% of the added cells attached to the adhesive protein. Adhesion of cells to BSA-coated wells was <5% of the adhesion to vitronectin. All measurements were performed in triplicate (or quadruplicate), and the standard deviations of these quadruplicates were ~10% of the mean or less.

Results

Ligand Binding to the Purified Vitronec

To investigate the ligand binding properties of αβ₃, a solid-phase microtiter assay was developed. In this assay purified αβ₃ was immobilized on the bottoms of microtiter wells, and biotinylated adhesive protein ligands were added and incubated with the receptor. As seen in Fig. 1, there was high affinity, saturable binding of vitronectin, fibronectin, vWF, and fibrinogen to the purified vitronectin receptor. In contrast, there was little or no binding of laminin or BSA to αβ₃.

The binding of biotinylated adhesive proteins to αβ₃ was specific. Fibronectin and vitronectin binding to αβ₃ was blocked by a monoclonal antibody (LM609) known to inhibit the binding of cells to vitronectin (Cheresh and Spiro, 1987), but not by monoclonals to the fibronectin receptor (BIIG2) or to the platelet GPIIb-IIIa complex (10E5) (Fig.)
Figure 1. Binding of adhesive proteins to the purified vitronectin receptor (α,β3). Biotinylated adhesive proteins were added at the indicated concentrations to microtiter wells coated with purified α,β3, and incubated for 3 h at 30°C. After unbound ligand was removed by washing, the bound adhesive protein was determined by ELISA. Data points shown are the mean of quadruplicate determinations.

As expected for a member of the integrin superfamily of receptors, chelation of divalent cations with EDTA also completely blocked binding. In addition, binding was blocked by the tetrapeptide RGDS, but not by RGES (not shown). The dodecapeptide from the gamma chain of fibrinogen (amino acids 400-411) did not block fibronectin or vitronectin binding to α,β3, although it is known to block the binding of adhesive proteins to GPIIb-IIIa on activated platelets (Kloczewiak et al., 1984). The binding of vWF and fibrinogen to α,β3 was examined with the same antibodies and peptides, and the results were exactly as found for fibronectin and vitronectin (data not shown). Thus, immobilized α,β3, binds adhesive proteins with the properties expected of the cellular vitronectin receptor.

The binding of vitronectin and fibronectin to α,β3 was mutually exclusive. Fibronectin blocked the binding of biotinylated vitronectin (15 nM) to α,β3, and half-maximal inhibition was achieved at a fibronectin concentration of 25 nM, which was comparable to the concentration of vitronectin required to inhibit this binding (Fig. 3). Similarly, vitronectin very effectively blocked the binding of fibronectin to α,β3 (data not shown). These results indicate that fibronectin and vitronectin compete when binding to α,β3, and suggest that vitronectin binds with somewhat higher affinity.

Additional evidence for a specific interaction between α,β3 and fibronectin was obtained by passing the lysate of surface-labeled M21 melanoma cells, which express both the vitronectin receptor (α,β3) and the fibronectin receptor (α,β1) (Cheresh et al., 1989) over a fibronectin affinity column, as described by Pytela et al. (1985b). The vitronectin receptor was retained on this column, and was eluted by RGDS, but not by RGES (Fig. 4).

The Vitronectin Receptor Is Involved in the Adhesion of Melanoma Cells to Fibronectin

To determine whether the binding of fibronectin to the vitronectin receptor was functionally significant in the context of a cell membrane we examined the adhesion of two related melanoma cell lines to fibronectin-coated surfaces. M21 cells attached and spread well on fibronectin-coated slides (Fig. 5). M21 cell adhesion to fibronectin was not significantly inhibited by antibodies against the fibronectin receptor (BI1G2) or the vitronectin receptor (LM609), but was blocked by the combination of the two antibodies. This suggested that α,β3 and α,β1 were both involved in M21 cell adhesion to fibronectin. To further investigate the role of α,β3 in cellular attachment to fibronectin experiments were performed with M21-L cells, which is a mutant cell line that specifically lacks α,β3, but expresses normal levels of the
Table I. Inhibition of Cell Adhesion to Fibronectin

| Antibody or Peptide | M21 Cells | M21-L Cells |
|---------------------|-----------|-------------|
| Control             | 405,997   | 101,338     |
| BIIG2               | 324,932   | 37,323      |
| LM609               | 325,026   | 132,608     |
| BIIG2 + LM609       | 106,612   | 51,492      |
| GRGDSPK             | 62,704    | 33,205      |
| RGES                | 365,283   | 109,925     |

Adhesion of metabolically labeled M21 and M21-L melanoma cells to fibronectin was measured in the presence or absence of antibodies to the αvβ3 fibronectin receptor (BIIG2; 20 μg/ml), the αvβ3 vitronectin receptor (LM609; 20 μg/ml), and peptides GRGDSPK and RGES (300 μM). Very similar results were obtained when each antibody was used at 10 μg/ml. Data is from one of three similar experiments.

Discussion

In this study we present biochemical and functional evidence that the vitronectin receptor αvβ3 mediates cellular interactions with fibronectin. Using a sensitive and reproducible solid-phase assay we have found specific and high affinity binding of vitronectin, vWF, fibrinogen, and fibronectin to the purified vitronectin receptor. We have further found that αvβ3 mediated the adherence and spreading of a human melanoma cell line to fibronectin and bound specifically to a fibronectin affinity column. We conclude from these studies that αvβ3 is a multifunctional receptor that binds at least four RGD-containing adhesive proteins, including fibronectin.
Microtiter plates coated with purified receptors provide a simple and rapid method for studying the binding properties of integrins. We have recently used a similar solid-phase assay to identify a peptide derived from GPIIIa that inhibits the binding of fibronectin to the platelet GPIb-IIIa complex (Charo et al., 1990). In using this assay we have found that vitronectin, vWF, fibrinogen, and fibronectin bind to αβ₃ in a similar manner. The binding of these adhesive proteins to purified αβ₃ was divalent cation dependent, and was inhibited by RGD peptides and a monoclonal antibody known to inhibit vitronectin binding to αβ₃ on whole cells. In contrast, ligand binding to αβ₃ was not blocked by the dodecapeptide from the gamma chain of fibronectin (at concentrations up to 500 μM), which is consistent with recent reports that the dodecapeptide interacts with GPIIb (αm), and not GPIIIa (β₃) (D’Souza et al., 1990). In contrast, there was little binding of laminin and no binding of BSA to αβ₃. These results confirm our earlier work (Charo et al., 1987; Cheresh, 1987) that αβ₃ mediated endothelial cell adherence to fibronectin and vWF, and provide direct evidence that αβ₃ binds fibronectin and other RGD-containing adhesive proteins specifically and with high affinity.

To determine if the binding of fibronectin to αβ₃ had biological implications we examined the adherence of two related melanoma cell lines to fibronectin. Although M21 and M21-L cells express high levels of the fibronectin receptor (αβ₃), it was clear that the M21 cells attached and spread much more readily on fibronectin than did the M21-L cells. This suggested that αβ₃, which is present on the M21 cells, but not on the M21-L cells, mediated cellular interactions with fibronectin. Antibody inhibition studies using LM609 confirmed this hypothesis. We conclude from this data that the vitronectin receptor binds fibronectin, and that this binding is functionally significant in as much as it mediates cellular attachment and spreading on fibronectin. Thus, αβ₃ acts in concert with αβ₃, in promoting M21 cell adhesion and spreading on fibronectin.

The vitronectin receptor appears to be a receptor designed to bind the RGD sequence. We have shown that fibronectin, fibrinogen, and vWF, in addition to vitronectin, bind to αβ₃. Lawler et al. (1988) found that αβ₃ binds thrombospondin. Indeed, it is noteworthy that αβ₃ was originally purified on an RGD affinity column (Pytel et al., 1985b), which suggests that this receptor binds RGD in multiple adhesive proteins with high affinity. In contrast, the fibronectin receptor (αβ₃) interacts exclusively with fibronectin (Pytel et al., 1986), and thus appears to recognize the RGD sequence only as presented by fibronectin. In a solid-phase assay similar to the one presented in this paper we have found that purified αβ₃ binds fibronectin, but does not bind fibrinogen, vitronectin, or vWF (Charo, I. F., and L. Nannizzi, unpublished observations).

Earlier studies by Pytel et al. (1985a) found that αβ₁ was the predominant cellular receptor in MG-63 osteosarcoma cells that bound to the 120-kD fragment of fibronectin immobilized on Sepharose. This study did not report the binding of αβ₁ to fibronectin, under conditions very similar to those used in this study, nor did it specifically exclude this interaction. We used immunoprecipitation with LM609 to specifically follow the binding and elution of αβ₁ from the fibronectin column, since the electrophoretic mobility of αβ₁ and αβ₃ on SDS gels is very similar. In addition, the high level of expression of αβ₁ on M21 cells has facilitated the detection of this interaction.

The relaxed ligand binding specificity of the vitronectin receptor may facilitate the interaction of αβ₃, bearing cells, such as malignant melanoma cells, with multiple proteins in the extracellular matrix. It is reasonable to speculate, therefore, that αβ₃ may be important in metastases of tumors. In addition, Savill et al. (1990) have recently reported that opsonization of senescent polymorphonuclear leukocytes by macrophages occurs via the macrophage αβ₃ receptor. Interestingly, opsonization was inhibited by soluble fibronectin as well as by soluble vitronectin. The interaction between fibronectin and αβ₃ may thus be of considerable physiological significance.

The existence of multiple integrins on a given cell capable of binding collagen (Santoro, 1986; Collier et al., 1989), fibronectin (Wagner et al., 1989), and vitronectin (Lam et al., 1989) has been described. However, little data exists as to the possible biological relevance of this phenomenon. In this report we demonstrate that receptors from two subfamilies of the integrin superfamily, the vitronectin receptor αβ₃ and the fibronectin receptor αβ₁, can act in concert to mediate cell adhesion and spreading on fibronectin.

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