Motility of Fibronectin Receptor-deficient Cells on Fibronectin and Vitronectin: Collaborative Interactions among Integrins

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Abstract. Cells are capable of adhering to and migrating on protein components of the extracellular matrix. These cell-matrix interactions are thought to be mediated largely through a family of cell surface receptors termed integrins. However, the manner in which individual integrins are involved in cell adhesion and motility has not been fully determined. To explore this issue, we previously selected a series of CHO variants that are deficient in expression of the integrin $\alpha5\beta1$, the "classical" fibronectin receptor. Two sets of subclones of these variants were defined which respectively express ~20% or 2% of fibronectin receptor on the cell surface when compared to wild-type cells (Schreiner, C. L., J. S. Bauer, Y. N. Danilov, S. Hussein, M. M. Sczekan, and R. L. Juliano. 1989. *J. Cell Biol.* 109:3157-3167). In the current study, the variant clones were tested for haptotactic motility on substrata coated with fibronectin or vitronectin. Data from assays using fibronectin show that cellular motility of the 20% variants was substantially decreased (30-75% of wild type), while the motility of the 2% variants was nearly abolished (2-20% of wild type). Surprisingly, a similar pattern was seen for haptotactic motility of both 2% and 20% variants when vitronectin was used (≈20- 30% of wild type). The reduced haptotactic motility of the fibronectin receptor-deficient variant clones on vitronectin was shown not to be due to reduced vitronectin receptor ($\alpha v\beta3$) expression nor to a failure of these variants to adhere to vitronectin substrata. Transfection of the deficient variants with a cDNA for the human $\alpha5$ subunit resulted in normal levels of fibronectin receptor expression (as a human $\alpha5$/hamster $\beta1$ chimera) and restored the motility of the CHO variants on fibronectin and vitronectin. This indicates that expression of the $\alpha5$ subunit is required for normal haptotactic motility on vitronectin substrata and suggests that the fibronectin receptor ($\alpha5\beta1$) plays a cooperative role with vitronectin receptors in cell motility.

INTEGRINS are transmembrane glycoprotein receptors which participate in a wide variety of cellular functions including cell-cell interactions, and the adhesion of cells to extracellular matrix (ECM) proteins such as fibronectin (Fn),[1] laminin, collagen, and vitronectin (Vn) (Akiyama et al., 1990; Hemler, 1990; Hynes, 1987; Juliano, 1987; Ruoslahti and Pierschbacher, 1987). Integrins exist on the cell surface as heterodimers of noncovalently associated α and β subunits. Some integrins interact with their respective ligands through RGD sequences contained within certain ECM proteins, while other integrins recognize non-RGD sites in proteins (Gehlsen et al., 1988; Hall et al., 1990; Sonnenberg et al., 1990). Several integrins which share the $\beta1$ subunit are known to be receptors for Fn; this would include $\alpha5\beta1$, the "classical" Fn receptor (FnR), as well as $\alpha3\beta1$, $\alpha4\beta1$, and the recently described receptor $\alpha v\beta1$ (Brown and Juliano, 1985, 1987; Carter et al., 1990; Dedhar and Gray, 1990; Elices et al., 1991; Guan and Hynes, 1990; Hemler, 1990; Pytela et al., 1985; Straus et al., 1989; Takada et al., 1988; Vogel et al., 1990). Several integrins are known to bind Vn, including the classical Vn receptor ($\alpha v\beta3$) (Suzuki et al., 1986) and the platelet integrin glycoprotein IIb/IIIa (Charo et al., 1986; Phillips et al., 1988; Ponz et al., 1987). Associations of the αv subunit with other novel β chains to form putative Vn receptors have been described recently, but the properties of these receptors are not well defined (Cheresh et al., 1989; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki et al., 1990). The ligand binding patterns of some integrins can be quite promiscuous; for example, the platelet integrin IIb/IIIa binds to fibrinogen, Fn, von Willebrand factor, and Vn, while the $\alpha v\beta3$ VnR reportedly binds von Willebrand factor, thrombospondin, fibrinogen, and Fn (Hemler, 1990; Phillips et al., 1988; Charo et al., 1990; Pytela et al., 1986). By contrast, the $\alpha5\beta1$ integrin has not been reported to bind any ligand other than Fn.

Although integrin receptors have been implicated in several aspects of cell behavior including adhesion and motility, the extent to which individual members of the integrin fam-

1. *Abbreviations used in this paper*: ECM, extracellular matrix; Fn, FnR, fibronectin, fibronectin receptor; HpmF, high powered microscope field; Vn, VnR, vitronectin, vitronectin receptor.

ily are involved is less clear. This study was designed to investigate the role of the classical FnR ($\alpha5\beta1$) in the adhesion and motility of cells on ECM proteins. Most previous studies of the function of the FnR have relied on the use of RGD-containing peptides or protein fragments, or on antibodies that bind Fn or $\alpha5\beta1$, to perturb the interaction of the FnR with its ligand (Brown and Juliano, 1985; Gehlsen et al., 1988; Hayman et al., 1985*a*). As an alternative approach, we have selected and cloned a series of stable FnR-deficient variants from the CHO cell line (Schreiner et al., 1989). Using ELISA and flow cytometric techniques with anti-FnR monoclonal antibodies, two sets of clones were selected which, on average, express either 20% (from here on 20% FnR variants) or 2% (from here on 2% FnR variants) of wild-type FnR levels.

It is clear from many studies that cell adhesion and motility on a substratum coated with a particular ECM protein involves the integrin(s) which recognizes that protein. We believe, however, that this scenario is incomplete. This report provides evidence that the $\alpha5\beta1$, which specifically binds Fn, is involved in the haptotactic motility of cells on substrata other than Fn. Thus, the presence of the $\alpha5\beta1$ FnR is required for motility even when other integrins mediate the adhesion of cells to the substratum.

Methods and Materials

Cell Culture

CHO cells were grown and maintained as previously described (Danilov and Juliano, 1989; Schreiner et al., 1989). Cells were either grown as monolayers in alpha-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Hazelton Research Products, Inc., Denver, PA) and 1% antibiotic–antimycotic mixture (Gibco Laboratories) or in suspension culture where the FBS was reduced to 5%. Cells grown in monolayer were routinely passaged with trypsin-EDTA (Gibco Laboratories) and cell number was determined with celloscope (Electro Zone, Particle Data, Inc., Elmhurst, IL). The CHO FnR-deficient variants used in this series of experiments were previously selected from nonmutagenized CHO populations (Schreiner et al., 1989). Independent selections yielded two subsets of FnR-deficient variants expressing either 20% (clones 1-23, 14-16, and 21-4) or 2% (clones B2, D1, 9, 23, and 25) of FnR when compared to wild-type (control) cells. For these studies we used clones 1-23 and 21-4 from the 20% FnR subset, and clones B2 and 25 from the 2% FnR subset.

Transfection of FnR-deficient Variants

A 2% FnR-variant clone, B2, was chosen for reversion of the FnR-deficient phenotype. Cells were reverted by transfecting a plasmid (pECE-$\alpha5$) which is an expression vector for the human $\alpha5$ subunit (Giancotti and Ruoslahti, 1990). Cells were cotransfected with a neomycin-resistant plasmid, pSVNeo, for selection purposes. B2 cells were transfected by electroporation as described in the *Bio-Rad Methods in Electroporation* manual (Bio-Rad Laboratories, Richmond, CA). Briefly, cells were grown in monolayer, harvested with trypsin-EDTA, and washed twice in ice cold electroporation media. The cells were then resuspended at a density of 1×10^7 cells/ml. Cells were then added to electroporation 0.4-cm Gene Pulser cuvettes (Bio-Rad Laboratories) and 20 μg of pECE-$\alpha5$ and 2 μg of pSVNeo were added to the curvettes and incubated with the cells on ice for 10 min. before electroporation. Both plasmids had been linearized within a PVU-I restriction site before addition to the cells to facilitate insertion of the plasmids into host chromosomal DNA. Cells were pulsed using voltages between 200 and 800 V with a constant capacitance of 25 μF. Cells were selected with G418 (1,000 μg/ml) and isolated using cloning rings. After cloning, transfectants were maintained in normally supplemented alpha-MEM containing 400 μg/ml G418. Cells were screened for expression of the chimeric human $\alpha5$/hamster $\beta1$ FnR by ELISA and by flow cytometry using the B1E5 anti-human $\alpha5$ antibody (generous gift of C. Damsky, University of California at San Francisco) (Werb et al., 1989). Five subsets of clones were obtained from the transfection (B2B1–B2B5). Based on ELISA data, clone B2B4 showed the highest level of $\alpha5\beta1$ expression.

Integrin Analysis

The expression of the $\alpha5\beta1$ FnR, VnR, and the integrin $\beta1$ subunit was evaluated by indirect immunofluorescence using flow cytometric techniques. PB1 (a mouse monoclonal anti-FnR antibody specific for the intact CHO $\alpha5\beta1$ complex), 7E2 (a mouse monoclonal antibody specific for the CHO $\beta1$ integrin subunit), B1E5 (a rat monoclonal anti-human $\alpha5$ antibody), and rabbit polyclonal anti-VnR ($\alpha v\beta3$) antibody (Suzuki et al., 1986) were used as primary antibodies, and FITC-conjugated IgG (anti-mouse or anti-rabbit) was used as a secondary reagent. The PB1 and 7E2 monoclonals have been fully described elsewhere (Brown and Juliano, 1985, 1988). Background staining was assessed by omitting the primary antibody. The FnR-deficient cells were screened by an ELISA technique as described (Schreiner et al., 1989) to confirm the phenotype. The FnR-deficient variants were periodically screened thereafter by ELISA using PB1, to ensure stability of the variant phenotype.

Extracellular Matrix Proteins

Fn was prepared from human plasma as described (Schwartz and Juliano, 1984). Vn was also prepared from human plasma in a modification (Danilov and Juliano, 1989) of a previously published procedure (Ruoslahti et al., 1987).

Adhesion Assay

Tissue culture plates (48-well, Costar Corp., Cambridge, MA) were coated for 2 h with 250 μl of PBS containing Fn or Vn at varying concentrations. The plates were then washed with PBS, blocked with 3% BSA in PBS, and finally washed four times with alpha-MEM containing 1% BSA and 20 mM Hepes, pH 7.3 (adhesion buffer). Cells growing in suspension were metabolically labeled overnight with ^{35}S-Trans label (2–5 μCi/ml, 1,000 Ci/mM; ICN Biomedical, Inc., Costa Mesa, CA). Cells were washed three times and resuspended at 1–5 $\times$ 10^5 cells/ml in adhesion buffer. Cells were added to the plates and allowed to attach to the substrata for 1 h at 37°C. At the end of this time, unattached cells were removed by washing the plates with adhesion buffer. Attached cells were lysed with 2% SDS and the lysates were transferred to scintillation vials for counting. All assays were performed in triplicate and adhesion was expressed as a percentage of positive control.

Motility Assays

The haptotactic motility experiments were performed using Transwell motility chambers (Costar Corp.) in a slight modification of a previously described assay (Albini et al., 1987; Schreiner et al., 1989). The Transwell motility chambers contain an upper and lower well separated by a tissue culture-treated polycarbonate membrane (polyvinyl-pyrrolidone free) with an 8-μm-diam pore size. Motility experiments were performed by first precoating the undersurface of the polycarbonate membrane with various concentrations of Fn or Vn in PBS. Cells growing in suspension were washed three times and resuspended in alpha-MEM containing 1% BSA; the same medium was also placed in the lower chamber. Cells (2×10^5) were added to the upper chamber and incubated at 37°C for varying times. At the end of the incubation period, the cells on the upper surface of the membrane were removed using a cotton-tipped applicator, and visually inspected under a microscope to ensure complete removal. The membranes were fixed with neutral buffered formalin (2%) and stained with hematoxylin. The membranes were mounted on glass slides and the cells from at least 10 random microscopic fields were counted. All experiments were run in triplicate and motility was expressed in terms of cells per high powered microscopic field (HPMF). This assay measures haptotaxis, i.e., directional motility in the presence of a gradient of bound protein. Extensive cell movement through the membrane was observed only when the adhesive protein was present exclusively on the side of the filter facing the lower chamber; when the adhesive protein was present on both sides of the filter, movement through the filter was sharply reduced (Bauer, J., unpublished observations). Kinetic motility experiments were preformed as described above except the Transwell motility inserts were coated with fixed concentrations of Fn (30 μg/ml) or Vn (5 μg/ml). Motility experiments performed in the presence of PB1 anti-FnR antibody were also carried out essentially as described above except the washed cells were incubated with excess PB1

in alpha-MEM containing 1% BSA at 37°C for 30 min. The cells were then added to the upper chamber of the motility inserts and incubated for 8 h at 37°C.

In some cases, additional motility studies were done by observing the ability of cells to move randomly on a uniform substratum of Fn or Vn using a modification of an assay which has been fully described elsewhere (Akiyama et al., 1989). In this assay, cells are allowed to migrate from an agarose plug onto a substratum initially uniformly coated with an ECM protein. The size of the cell outgrowth is then measured as a function of time; in our case this was done using a microscope eyepiece with a measuring grid. With CHO cells this assay requires 3-7 d, and several cell doublings occur. The medium used was alpha-MEM supplemented with insulin, transferrin, and selenium, but without serum. The substrata were coated with 10 μg/ml of Vn or Fn.

*Table I. Integrin Expression Compared to Wild Type**

Clone	Primary Antibody		
	PB1	anti-VnR	7E2
1-23	22.0	96.0	30.0
21-4	20.0	120.0	23.0
B2	1.0	104.0	18.0
25	1.0	138.0	35.0

* Results are expressed as a percentage of the level of CHO wild-type cells calculated from the mean channel fluorescence values obtained by immunofluorescent staining followed by flow cytometric analysis.

Results

Variant Cell Selection and Integrin Receptor Analysis

The FnR-deficient variants were selected from a normally growing population of CHO cells (Schreiner et al., 1989). Flow cytometric analysis of several independent clones is shown in Fig. 1. Variants 1-23 and 21-4 exhibit a significant decrease in FnR expression when compared to control (wild type), while expression of the FnR in variants B2 and 25 is nearly abolished. These results are more apparent when the data from the flow cytometer are quantitated and compared; i.e., 1-23 and 21-4 (termed 20% FnR variants) show ~20% of control FnR expression, while B2 and 25 (termed 2% FnR variants) show 1-2% (Table I, first column). The 20% and 2% phenotypes of the variants were also confirmed by ELISA (data not shown). Earlier work with the FnR variants indicates that the defect in FnR expression is at the level of mRNA of the α5 subunit (Schreiner et al., 1989). The β1 subunit is also present on the cell surface in reduced amounts (Table I, third column); the reduction in β1 expression is due to the decrease in intact α5β1 FnR expression in the variant cells.

αvβ3 was also examined in the wild-type and variant CHO cells. Vn is another ECM component and is important in promoting the attachment and spreading of cells on the ECM (Hayman et al., 1985*b*). Previous experiments in our laboratory (Bauer, J. S., and R. L. Juliano, unpublished data) have shown that CHO cells adhere to and migrate on Vn. Thus the VnR was chosen to study the effects of decreased FnR expression on the functionality of other integrins. VnR was examined by flow cytometric analysis using an anti-VnR polyclonal antibody (Fig. 2). Both the 20% and 2% FnR variants show levels of VnR expression similar to wild-type cells. This again is clearly evident when the data from the flow cytometer are quantitated and differentially compared. (Table I, second column).

Adhesion to Substrata

Wild-type CHO cells adhered maximally to Fn substrata at a coating concentration of 2.5 μg/ml or greater (Fig. 3 *A*). The 20% FnR variants showed levels of adhesion to Fn similar to wild-type cells suggesting that 100% of the FnR is not necessary to promote maximal adhesion of CHO cells to Fn. Adhesion of the 2% FnR variants to Fn, even at coating concentrations of 10 μg/ml, is nearly abolished. An apparent "spare receptor" phenomenon (Brown and Juliano, 1987) exists for the adhesion of CHO cells to Fn measured under the conditions described here; however, the amount of receptor becomes limiting in the adhesion process as the expression of FnR drops to very low levels. If the shear stress applied in the adhesion assay were higher, a greater fraction of the total FnR might be required to maintain adhesion. Similar adhesion experiments were also performed with Vn as the

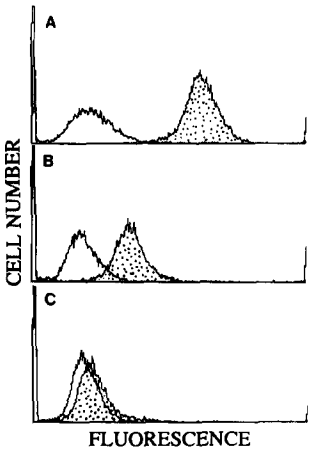

Figure 1. FACS histograms of FnR expression on FnR variants. Equal numbers of wild-type and FnR variants were washed with cold PBS, then treated with anti-FnR mAb (PB1) followed by staining with fluorescein-conjugated sheep anti-mouse IgG (*stippled histograms*). Cells not treated with PB1 and stained with fluorescein-conjugated sheep anti-mouse IgG were used as negative controls (*clear histograms*). A leftward shift of the stippled histogram relative to wild-type cells indicates a decrease in receptor expression. Each peak represents 10^4 cells. Histograms *A*, wild type; histograms *B*, 20% FnR variant (1-23); and histograms *C*, 2% FnR variant (B2). The abscissa represents the logarithm of the fluorescence intensity, while the ordinate represents the number of cells at each level of fluorescence.

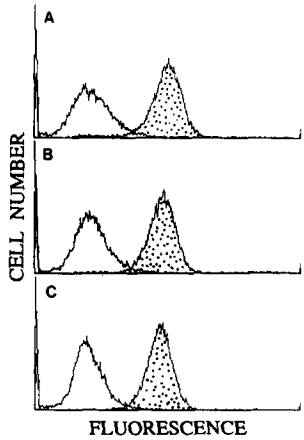

Figure 2. FACS histograms of VnR expression on FnR variants. Equal numbers of wild-type and FnR variants were washed with cold PBS, then treated with anti-VnR polyclonal antibodies followed by staining with fluorescein-conjugated goat anti-rabbit IgG (*stippled histograms*). Cells not treated with the anti-VnR antibodies before staining with the fluorescein-conjugated goat anti-rabbit IgG were used as negative controls (*clear histograms*). Each peak represents 10^4 cells. Histograms *A*, wild-type; histograms *B*, 20% FnR variant (1-23); and histograms *C*, 2% FnR variant (B2). The abscissa represents the logarithm of the fluorescence intensity, while the ordinate represents the number of cells at each level of fluorescence.

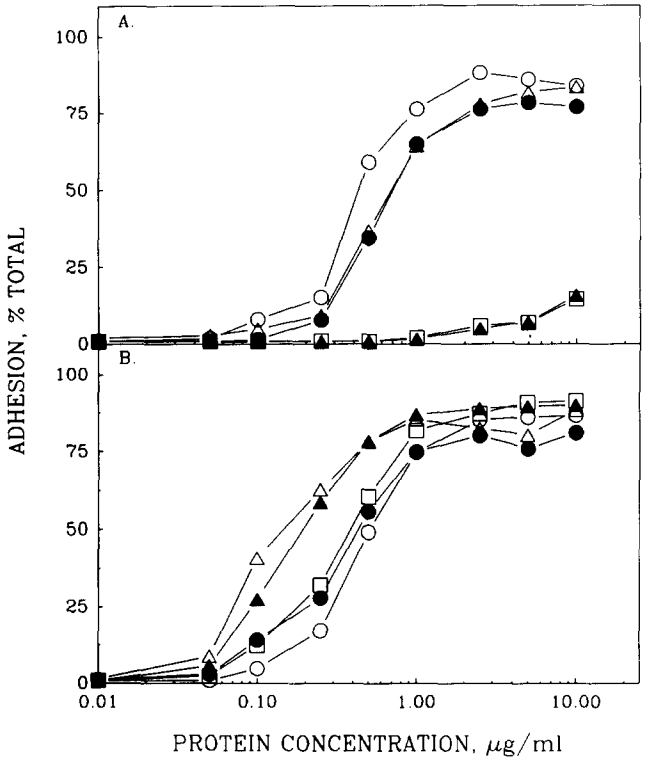

Figure 3. Efficiency of adhesion of CHO FnR variants to Fn- and Vn-coated substrata. Tissue culture plates were coated with various concentrations of (*A*) Fn or (*B*) Vn for 2 h at 37°C in PBS, then blocked with BSA as described in Materials and Methods. Cells that were metabolically labeled overnight with ^{35}S-Trans label were washed and resuspended at 5 × 10^5 cells/ml in adhesion buffer. Cells were allowed to adhere for 60 min. The adherent cells were recovered and analyzed for radioactivity as described in Materials and Methods. (○—○) wild-type; (●—●) 1-23; (△—△) 21-4; (▲—▲) B2; (□—□) 25.

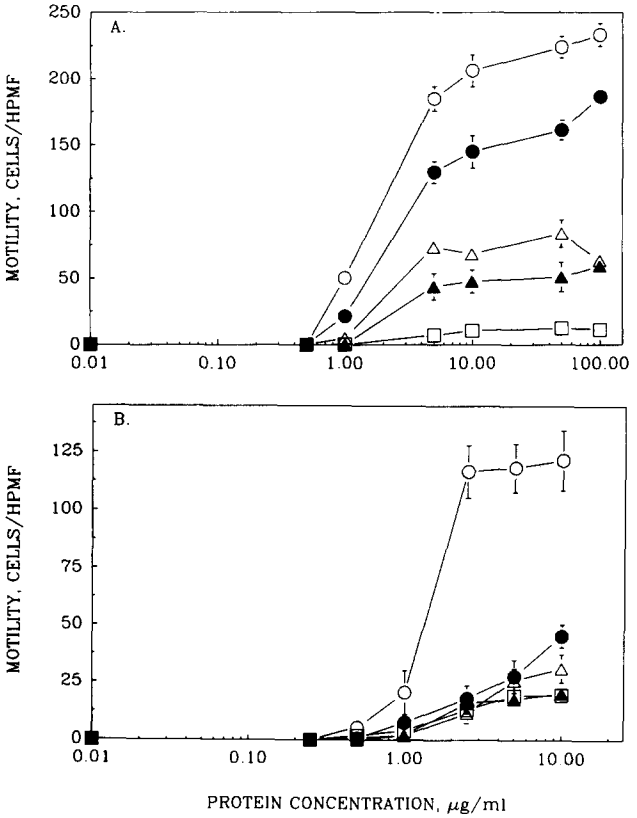

Figure 4. Motilities of CHO FnR variants on Fn and Vn. The undersides of Transwell motility chamber inserts were coated with various concentrations of (*A*) Fn or (*B*) Vn in PBS. Cells were washed and resuspended in alpha-MEM containing 1% BSA. 2 × 10^5 cells were added to the upper chamber and the motility chambers were incubated at 37°C for 8 h. Motility was quantitated by counting the number of cells that migrated to the undersides of the membranes. The results are averages of at least 10 random high powered microscopic fields. ((○—○) wild-type; (●—●) 1-23; (△—△) 21-4; (▲—▲) B2; (□—□) 25.

coating protein. Clones 1-23 and 25 showed control levels of adhesion to Vn, while clones 21-4 and B2 exhibited slightly greater adhesion to Vn than wild type (Fig. 3 *B*). These data indicate that the FnR defect does not affect the adhesion of CHO cells to Vn.

Motility of CHO Cells on Fn and Vn

It has been shown that the α5β1 FnR is involved in the motility of cells on Fn substrata and the motility response is proportional to the Fn concentration (Furcht et al., 1984; Lacovara et al., 1984; McCarthy et al., 1985; Straus et al., 1989). Several types of motility exist including haptotaxis (directed migration on ligands fixed to a substratum), chemotaxis (directed migration towards a soluble ligand), and random, nondirectional movement. Attempts have been made to study haptotaxis and chemotaxis independently, but an uncertainty still exists as to whether there are any fundamental differences between the two processes (Straus et al., 1989).

Here we have examined the role of the FnR in the haptotactic motility of CHO cells on Fn and Vn. Wild-type CHO cells migrated on Fn (Fig. 4 *A*) at coating concentrations >1.0 μg/ml and showed maximal motility (235 cells/HPMF) at 100 μg/ml or greater. Clone 1-23 showed a decreased maximal motility on Fn, exhibiting ~75% of wild-type level. The other 20% FnR variant, 21-4, showed ~30% of maximal wild-type motility. The motility of 2% FnR variants on Fn was even less; clone B2 showed ~20% of maximal wild-type motility, while the motility response of clone 25 was nearly abolished.

Wild-type CHO cells showed maximal haptotactic motility (125 cell/HPMF) on Vn at coating concentrations of 2.5 μg/ml or greater (Fig. 4 *B*). Migration of wild-type cells on Vn was evident at protein coating concentrations >0.5 μg/ml. Motility data from the FnR variants on Vn were discordant with expected results. Both the 20% and 2% FnR variants showed similar but markedly reduced motility on Vn when compared to wild-type cells. Even at Vn coating concentrations of 10 μg/ml, the motility of the FnR variants never exceeded 30% of wild-type. Thus, although FnR deficient cells adhered normally to Vn, their haptotactic motility on Vn was significantly impaired.

Kinetics of Motility

The motility results presented thus far have been from experiments which were allowed to continue for 8 h. To explore the possibility that the FnR variants require additional time to achieve maximal migration, experiments were extended

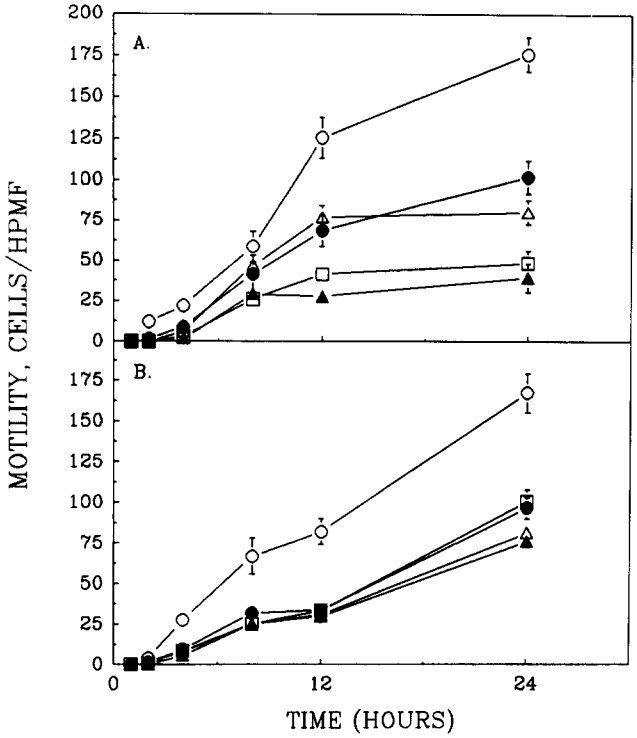

Figure 5. Kinetics of CHO FnR variant motility on Fn and Vn. Transwell motility inserts were coated with either 30 μg/ml Fn (*top*) or 5 μg/ml Vn (*bottom*). 1 × 10^5 cells were added to the upper chamber and incubated for various times at 37°C. Motility was quantitated as before. (○—○) wild-type; (●—●) 1-23; (△—△) 21-4; (▲—▲) B2; (□—□) 25.

to 24 h. For these experiments 1.0 × 10^5 cells were added per well instead of 2.0 × 10^5 cells, and higher concentrations of proteins (30 μg/ml and 5 μg/ml, for Fn and Vn, respectively) were used to coat the polycarbonate membranes. Wild-type CHO cells showed greater motility on Fn than the FnR-deficient variants (Fig. 5 *A*) at all time points tested, except for the initial 2-h period. This initial lag period is probably due to the adherence and spreading of cells on the polycarbonate membrane followed by recognition of the protein gradient. Further, even at 24 h neither the 20% FnR variants nor the 2% FnR variants achieved wild-type levels of motility (exhibiting 46 and 21% of wild-type cell motility, respectively). When the kinetics of motility of the FnR variants on Vn were tested (Fig. 5 *B*), a pattern similar to the results presented in Fig. 4 were obtained; i.e., neither the 20% nor the 2% FnR variants were able to achieve control levels of motility despite the fact that normal levels of VnR are present on the surface of the variants. From these results, we conclude that the decrease in haptotactic motility of the FnR variants on Fn or Vn was not due to an increased initial lag period.

Effects of Antibodies on Motility

A concern in these studies was possible contamination of our Vn preparation by Fn, or cross-recognition of Vn by the FnR (Basara et al., 1985) in the absence of Fn on the substratum. To explore these possibilities, PB1, a monoclonal antibody which binds to and inhibits the intact FnR, was used in motility experiments to block the involvement of the FnR. Motil-

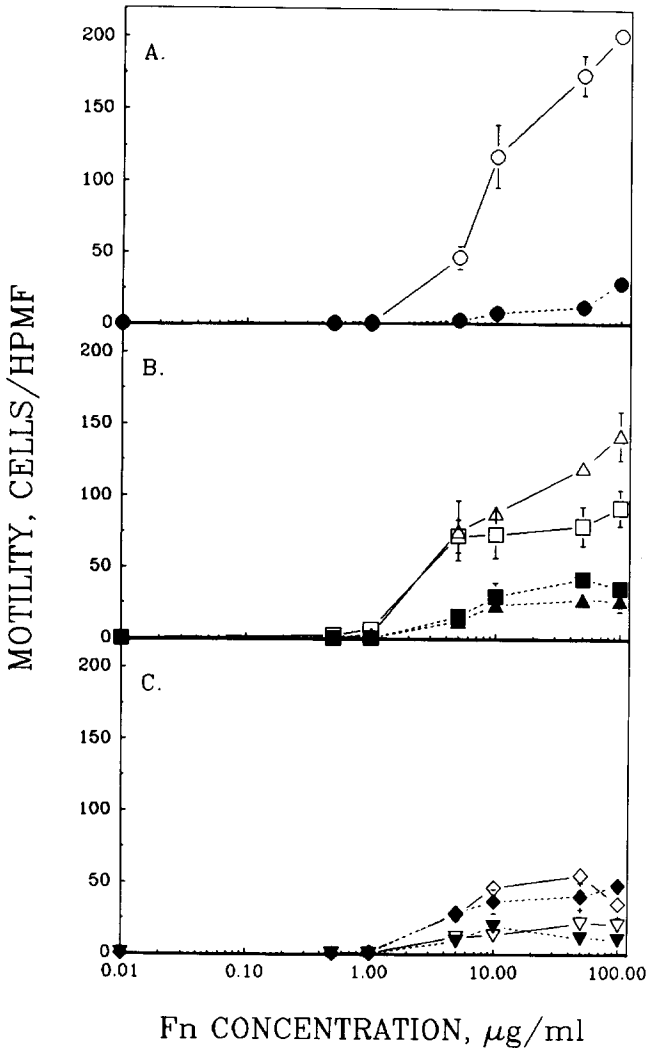

Figure 6. The effect of anti-FnR antibody on CHO FnR variant motility on Fn. Transwell motility inserts were coated with various concentrations of Fn. Cells were washed and resuspended in alpha-MEM containing 1% BSA. Excess PB1 antibody was added to 2 × 10^5 cells and incubated for 30 min at 37°C. The cells were then added to the upper chamber and incubated for 8 h at 37°C. Motility was quantitated as before. (*A*) (○—○), wild-type (−PB1); (●- - -●), wild-type (+PB1). (*B*) (△—△), 1-23 (−PB1); (▲- - -▲), 1-23 (+PB1); (□—□), 21-4 (−PB1); (■- - -■), 21-4 (+PB1). (*C*) (◇—◇), B2 (−PB1); (◆- - -◆), B2 (+PB1); (▽—▽), 25 (−PB1); (▼- - -▼), 25 (+PB1).

ity of wild-type cells on Fn in the presence of PB1 was nearly abolished (Fig. 6 *A*). Motility of the 20% and 2% FnR variants was also markedly reduced in the presence of the PB1 antibody (Fig. 6 *B* and *C*). Thus, reduction in cell motility on Fn appeared to be directly related to the reduction in the amount of functional FnR present caused by the inhibitory action of the PB1 antibody. The basis of the small degree of residual mobility in the presence of PB1 is unknown; possibly cell surface heparan sulfate proteoglycans, or alternatively, expression of the $\alpha 3\beta 1$ integrin may permit a low level of interaction with Fn (Giancotti and Ruoslahti, 1990), especially when $\alpha 5\beta 1$ is not available (Elices et al., 1991).

Data from motility experiments performed on Vn (Fig. 7) in the presence of PB1 show that inhibition of the binding ca-

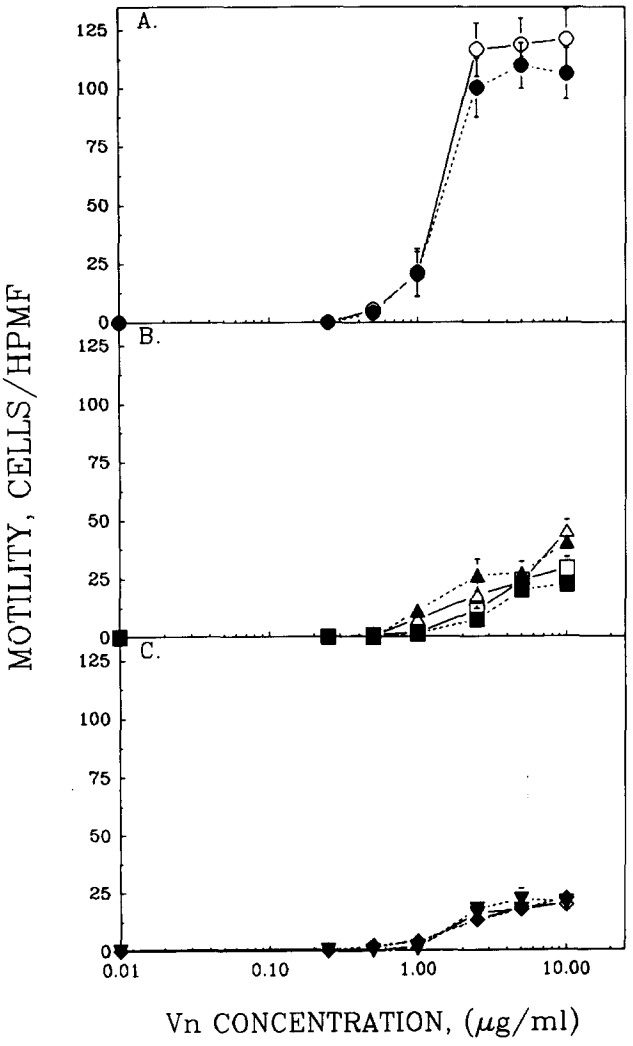

Figure 7. The effect of anti-FnR antibody on CHO FnR variant motility on Vn. Transwell motility inserts were coated with various concentrations of Vn. Cells were washed and resuspended in alpha-MEM containing 1% BSA. Excess PB1 antibody was added to 2 × 10^5 cells and incubated for 30 min at 37°C. The cells were then added to the upper chamber and incubated for 8 h at 37°C. Motility was quantitated as before. (*A*) (○—○), wild-type (−PB1); (●- - -●), wild-type (+PB1). (*B*) (△—△), 1-23 (−PB1); (▲- - -▲), 1-23 (+PB1); (□—□), 21-4 (−PB1); (■- - -■), 21-4 (+PB1). (*C*) (◇—◇), B2 (−PB1); (◆- - -◆), B2 (+PB1); (▽—▽), 25 (−PB1); (▼- - -▼), 25 (+PB1).

pacity of the FnR does not affect the motilities of either wild-type cells or the FnR variants on this substratum. The motility of the wild-type cells remains about fivefold greater than both the 20% and 2% FnR variants. From these data it can be concluded that blocking the binding ability of the FnR does not affect the motility of cells on Vn.

Other experiments were performed to examine the possibility of colocalization of the FnR with the VnR in CHO cells during the formation of adhesion plaques on Vn. These experiments were performed using indirect immunofluorescence using PB1 or the polyclonal anti-VnR antibody. The results from these experiments clearly demonstrate that the FnR does not colocalize with the VnR in CHO cell focal contacts (data not shown), in agreement with results in other cell types (Fath et al., 1989).

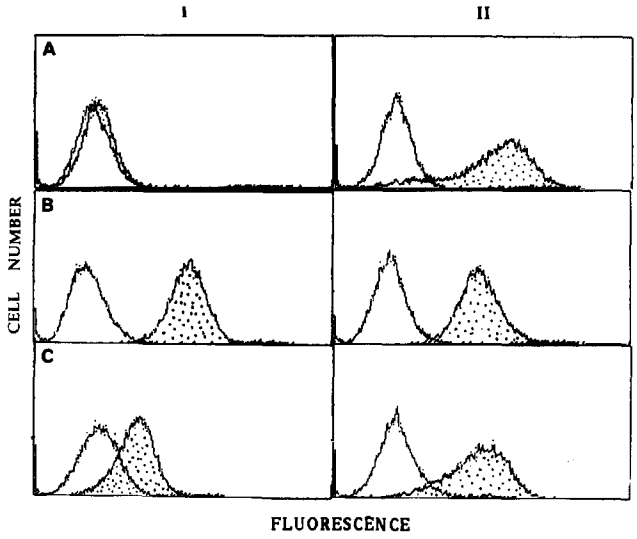

Figure 8. (*A*) FACS histograms of α5 expression on the 2% FnR variant, B2, and on the α5 transfectant (B2B4). Equal numbers of each cell type were washed with cold PBS, then treated with anti-α5 mAb B1E5, followed by staining with fluorescein-conjugated goat anti-rat IgG (*stippled histograms*). Cells not treated with B1E5 and stained with fluorescein-conjugated rabbit anti-mouse IgG were used as negative controls (*clear histograms*). (*B*) FACS histograms of VnR expression on B2 and B2B4 cells. Equal numbers of each cell type were washed with cold PBS, then treated with anti-VnR polyclonal antibodies followed by staining with fluorescein-conjugated goat anti-rabbit IgG (*stippled histograms*). Cells not treated with the anti-VnR antibodies before staining with the fluorescein-conjugated rabbit anti-mouse IgG were used as negative controls (*clear histograms*). (*C*) FACS histograms of β1 expression on B2, and B2B4 cells. Equal numbers of each cell type were washed with cold PBS, then treated with anti-β1 mAb, 7E2, followed by staining with fluorescein-conjugated sheep anti-mouse IgG (*stippled histograms*). Cells not treated with 7E2 and stained with fluorescein-conjugated sheep anti-mouse IgG were used as negative controls (*clear histograms*). Each peak represents 10^4 cells. Histograms I, 2% FnR variant, B2; histograms II, α5 transfectant, B2B4. The abscissa represents the logarithm of the fluorescence intensity, while the ordinate represents the number of cells at each level of fluorescence.

Transfection of FnR-deficient Variants with α5 cDNA

The data thus far suggest a cooperativity between the VnR and FnR in the mobility of CHO cells on Vn. As stated above, previous studies have shown the nature of the defect in the FnR-deficient variants to be reduced expression of the α5 subunit of FnR (Schreiner et al., 1989). To correct this defect and to determine whether this was the only defect causing the decrease in motility on Vn, a 2% FnR variant, B2, was cotransfected with a plasmid containing a cDNA for the human α5 subunit of the FnR, and a plasmid for neomycin resistance. Five transfected clones were isolated and subsequently screened by ELISA for α5 and β1 cell surface expression (data not shown). The clone showing the greatest level of α5β1 expression, B2B4, was chosen for further study. Based on the ELISA data, α5β1 expression in the B2B4 clone was slightly greater than in wild-type levels. From the flow cytometry results using the B1E5 anti-α5 antibody, it is evident that the B2B4 clone expresses much more α5 than the original B2 clone (Fig. 8 *A*). Further, the levels of β1 expression in the B2B4 clone were also increased (Fig.

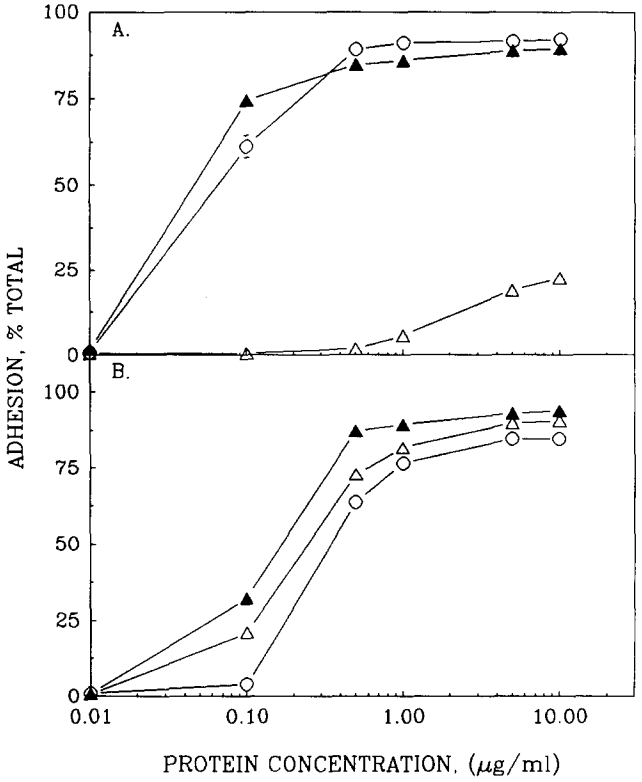

Figure 9. Efficiency of adhesion of α5 transfectants to Fn- and Vn-coated substrata. Tissue culture plates were coated with various concentrations of (*A*) Fn or (*B*) Vn for 2 h at 37°C in PBS, then blocked with BSA as described in Materials and Methods. Cells that were metabolically labeled overnight with ^{35}S-Trans label were washed and resuspended at 5×10^5 cells/ml in adhesion buffer. Cells were allowed to adhere for 60 min. The adherent cells were recovered and analyzed for radioactivity as described in Materials and Methods. The upper graph shows adhesion of cells to Fn-coated substrata. The lower graph shows adhesion of cells to Vn. (○—○) wild-type; (△—△) B2; (▲—▲) B2B4.

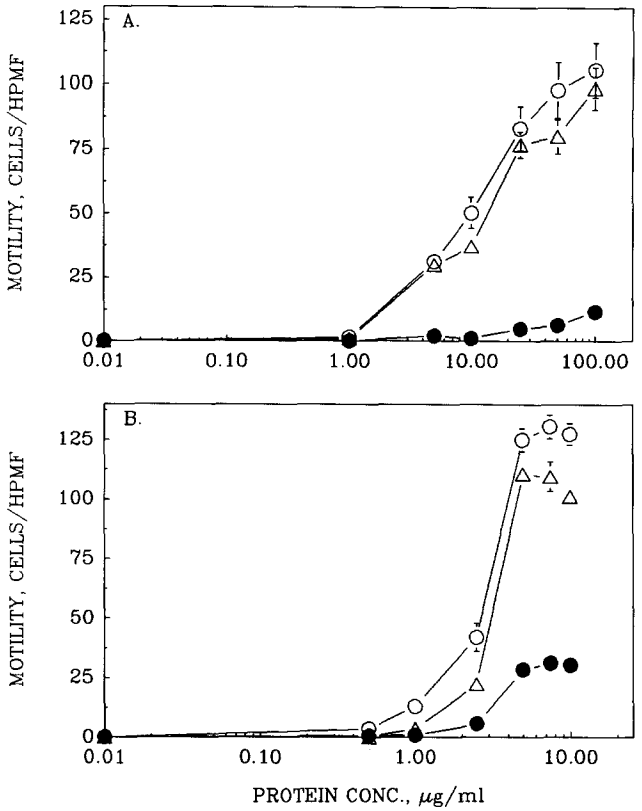

Figure 10. Motilities of α5 transfectants on Fn and Vn. The undersides of Transwell motility chamber inserts were coated with various concentrations of either (*A*) Fn or (*B*) Vn in PBS. Cells were washed and resuspended in α-MEM containing 1% BSA. 2×10^5 cells were added to the upper chamber and the motility chambers were incubated at 37°C for 8 h. Motility was quantitated by counting the number of cells that have migrated to the underside of the membrane. The results are expressed as averages of at least 10 random high powered microscopic fields. (○—○) Wild-type; (●—●) B2; (△—△) B2B4.

8 *B*). Lastly, VnR levels in the B2B4 clone were found to be unchanged by the transfection procedure (Fig. 8 *C*).

Adhesion of the α5 Transfectants to Fn and Vn

Although the transfected α5 subunit was recognized by the BIE5 antibody, it remained to be seen whether the chimeric α5β1 (human α5/hamster β1) could form a functional FnR within CHO cells. Adhesion experiments were performed to study the functionality of the hybrid α5β1. Results from these experiments show that the B2B4 cells adhered to Fn as well as wild-type (Fig. 9 *A*); as before, B2 cells failed to adhere appreciably to Fn. Expression of the chimeric α5β1 had only minor effects on the adhesion of cells to Vn (Fig. 9 *B*).

Motility of α5 Transfectants on Fn and Vn

Haptotactic motility of the B2B4 cells on Fn was nearly identical to wild-type cells, while B2 cells showed little motility on Fn (Fig. 10 *A*). The results presented in Fig. 10 *B* show that by bolstering α5β1 expression in α5β1 FnR-deficient cells, haptotactic motility on Vn is restored. Motility of the B2 clone on Vn, which had been attenuated presumably because of a lack of FnR expression, was restored after the α5 transfection. This clearly suggests an involvement of the α5β1 FnR in haptotactic cell motility on Vn. To ensure that the decrease in motility on Vn was not due to an inability of the B2 to spread after attachment, observations were made of cellular spreading on 10 μg/ml Fn and Vn. The photographs show that both wild-type and B2B4 cells spread well on Fn while B2 cells remain rounded (Fig. 11). All three cell types, however, spread equally well on Vn.

Random Motility of FnR-deficient Cells and α5 Transfectants on Fn and Vn

A somewhat different picture emerged when we examined nondirectional cell motility rather than haptotaxis. In these studies we examined cell outgrowth from an agar plug onto a uniform protein-coated substratum. As seen in Table II, the FnR-deficient clone B2 was very poorly motile on a Fn substratum, while in clone B2B4 (the α5 transfectant) motility was restored to wild-type levels. On Vn substrata, all of the clones were rather poorly motile, but the differences between wild-type cells and clones B2 or B2B4 were not significant. This suggests that the presence or absence of the α5β1 FnR may affect directional motility more than random motility.

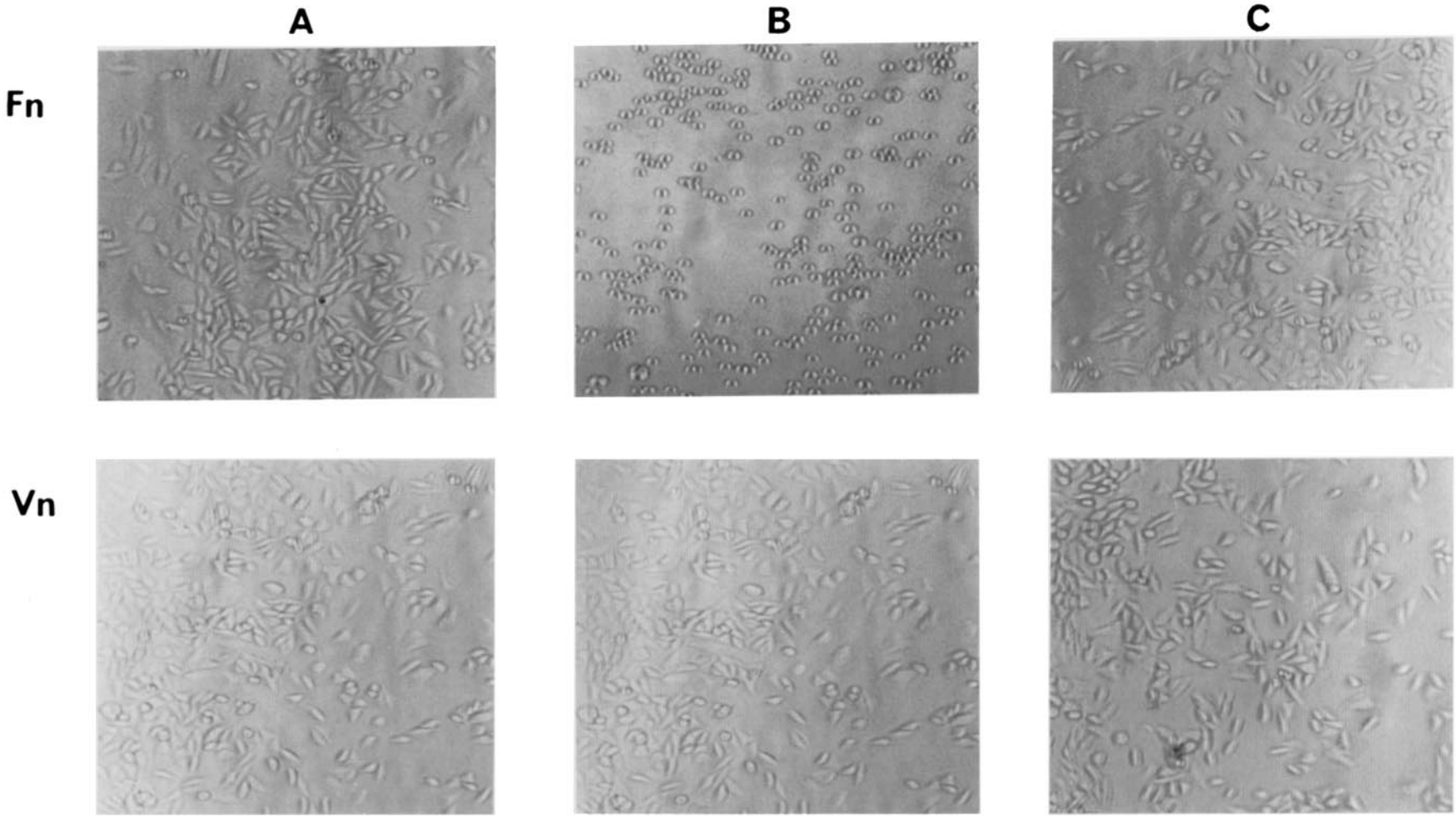

Figure 11. Spreading of cells on Fn and Vn. Cells were allowed to attach to tissue culture plastic coated with either 10 μg/ml Fn (*upper*) or Vn (*lower*) for 90 min. Photographs were taken using a 40× objective. (*A*) wild-type; (*B*) B2; (*C*) B2B4.

Table II. Random Motility of FnR Variants and Alpha 5 Transfectants

Cell type	Radius of Outgrowth on Fibronectin		
	Day 0	Day 3	Day 7
CHO WT	0	6.5 (2.2)	15.5 (1.3)
B2	0	0	0
B2B4	0	5.2 (1.1)	18.3 (1.3)

Cell type	Radius of Outgrowth on Vitronectin	
	Day 0	Day 7
CHO WT	0	5.3 (2.6)
B2	0	4.4 (2.0)
B2B4	0	7.1 (1.4)

Random motility was evaluated by measuring cell outgrowth from an agar plug attached to a protein-coated substratum, as described in Materials and Methods. The results represent the means and standard errors (in parenthesis) of triplicate determinations. Results are expressed as the difference between the radius of the outgrowth and the radius of the plug (in microns). The 3-d outgrowth on Vn was too small to measure accurately.

Discussion

Cells have been shown to adhere to and migrate on a variety of ECM proteins. These behaviors are mediated in part by integrins which act as receptors for ECM proteins. The classic FnR, $\alpha5\beta1$, is thought to exclusively bind Fn and to mediate adhesion and motility of cells on substrata coated with this protein (Akiyama et al., 1990; Brown and Juliano, 1985; Hemler, 1990; Pytela et al., 1985; Straus et al., 1989). This manuscript confirms previous observations, i.e., that the $\alpha5\beta1$ FnR is important in the adhesion and motility of cells on Fn, but goes further to show that expression of FnR may also be required for the directional motility of cells on other substrata, particularly Vn. In CHO clones which express normal levels of VnR, judged both by flow cytometry and by adhesion to Vn, the lack of $\alpha5$ expression leads to impaired haptotactic motility on Vn. Transfection of FnR-deficient cells with a human $\alpha5$ subunit leads to a restoration of haptotactic motility on Vn. Thus, expression of the $\alpha5$ subunit at normal levels seems to be required for normal motility on Vn, even though the $\alpha5\beta1$ integrin does not bind Vn.

An earlier study found that the expression of the $\alpha5\beta1$ integrin in CHO cells reduced motility (Giancotti and Ruoslahti, 1990). However, only cells which expressed very high levels of the integrin were affected in this manner. In a similar vein, Akiyama et al. (1989) reported that an anti-$\alpha5$ monoclonal enhanced motility in human fibroblasts. It may be that while some level of expression of $\alpha5\beta1$ is required for motility, very high levels become inhibitory; thus in some cases partial blockade of $\alpha5\beta1$ may lead to enhanced motility. Further, results with motility assays are often very dependent on the nature of the assay used (Varani et al., 1978). The assays used by Giancotti and Ruoslahti (1990) and by Akiyama et al. (1989) were cell outgrowth assays reflecting migration on a uniform substratum; in our case we have primarily investigated directional motility. Our results suggest that directional motility may be more strongly affected by the level of $\alpha5\beta1$ expression than is random cell movement.

At this time we are unable to offer a definitive biochemical explanation for the effects of FnR expression on directed motility on Vn, but several reasonable hypotheses can be delineated. One possibility stems from the fact that integrins, while responsible for recognizing and binding ECM proteins extracellularly, also are involved in the reorganization of cytoskeletal elements. When cells adhere to a substratum coated with ECM proteins, focal adhesion plaques as well as more transient adhesive contacts are initiated by the clustering of integrin receptors. Within the cell, cytoskeletal proteins (e.g., actin, α-actinin, vinculin, and talin) associate with the cytoplasmic region of these integrin clusters (Burridge et al., 1988; Nuckolls et al., 1990; Otey et al., 1990). These cytoskeletal interactions play a role in cell adhesion and may play an even more critical role in directional motility of cells. It seems possible that in the absence of the $\alpha5\beta1$ FnR, the cytoskeleton may fail to organize in a manner suitable for haptotactic motility, even though initial recognition of substratum proteins is adequately provided by other integrins such as the $\alpha v\beta3$ VnR. Thus cell adhesion is maintained but directional cell motility is impaired.

Another possibility is that in the wild-type CHO cell, the α and β subunits are produced in balanced amounts, but that in the absence of $\alpha5$ production in the variants, excess $\beta1$ subunit exists. This excess $\beta1$ may then complex with other α subunits, particularly the αv subunit of the VnR. The resultant $\alpha v\beta1$ complex apparently does not recognize Vn (Dedhar and Gray, 1990; Vogel et al., 1990), and its formation, instead of $\alpha v\beta3$, might thus limit motility on Vn. Previous results (Schreiner et al., 1989) suggest that cell motility is more sensitive to decreased integrin expression than is cell adhesion. Thus reduction in the amount of $\alpha v\beta3$ could markedly reduce motility on Vn without affecting adhesion on this substrate. However, it seems unlikely that the level of $\beta1$ subunit in the variants would necessarily impair $\alpha v\beta3$ formation, since wild-type CHO cells also seem to express an excess of $\beta1$ (Giancotti and Ruoslahti, 1990). Further, surface labeling and immunoprecipitation experiments with anti-$\alpha v\beta3$ antibody followed by SDS-PAGE under conditions where $\beta3$ and $\beta1$ are clearly resolved, did not show any reduction in the ratio of $\beta3$ to αv in the precipitates from B2 or B2B4 cells as compared to wild-type cells, nor any evidence for the presence of $\alpha v\beta1$ complex (Bauer, J., and J. Varner, unpublished data). While we cannot rule out small changes in the association of αv with other beta subunits, there do not seem to be dramatic differences.

Yet another possibility relates to the growing evidence that the $\alpha5\beta1$ FnR has the ability to transduce signals to the cell interior which can regulate gene expression and tumorigenicity (Giancotti and Ruoslahti, 1990; Kornberg et al., 1991; Schreiner et al., 1991; Werb et al., 1989). While the nature of these signals and their relation to cell adhesion and motility are as yet poorly defined, it seems conceivable that they might also regulate the directed motility of cells on a substratum.

Possibilities that have been clearly eliminated include Fn contamination of the Vn substratum or $\alpha5\beta1$ FnR-mediated recognition of Vn. As shown in Figs. 6 and 7, treatment of wild-type CHO cells with the PB1 monoclonal antibody, which blocks the function of the $\alpha5\beta1$ FNR, essentially completely inhibits haptotactic motility on Fn substrata, but has no effect on haptotactic motility on Vn substrata. Thus, it is unlikely that part of the motility of wild-type cells on Vn could be attributed to contamination by Fn. Further, the fact that the PB1 antibody blocks adhesion and motility on

Fn but not Vn, militates against the possibility that $\alpha5\beta1$ recognizes Fn. The possibility of direct physical association of the FnR and VnR also seems unlikely. Colocalization of FnR and VnR was explored using indirect immunofluorescence with antibodies to each receptor. Results clearly showed that focal contacts are formed independently by the receptors recognizing each protein, in agreement with previous results (Fath et al., 1989).

In summary, our results indicate that the expression of the $\alpha5\beta1$ FnR is required for directed cell motility on Vn. Cells which are deficient in FnR expression adhere well to Vn substrata but are markedly impaired in their ability to be motile on Vn gradients. Expression of a functional human $\alpha5$/hamster $\beta1$ FnR on the surface of the FnR-deficient variants by transfection reverts the defect in directional motility. Although there is no evidence for close juxtaposition of FnR and VnR in cells adherent on Vn, the presence of the FnR may be necessary to organize the cytoskeleton for motile activity, or to transmit critical signals for motility. This function does not seem to require that the FnR bind its ligand, since blocking the binding function of FnR with a monoclonal antibody did not impair motility on Vn.

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