

Modulation of Cell Migration by Integrin-mediated Cytoskeletal Linkages and Ligand-binding Affinity

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Abstract. Integrin cell surface adhesion receptors play a central role in mediating cell migration. We have developed a model system consisting of CHO cells ectopically expressing the α IIB β 3 integrin to study integrin affinity and cytoskeletal interactions during cell migration. The α IIB β 3 integrins are suited for study of integrin receptors during cell migration because they are well characterized with respect to ligand binding, cytoskeletal interactions, and signal transduction, and mutants with altered receptor function are available. The α IIB β 3 receptor specifically mediates migration of α IIB β 3-transfected CHO cells. The migration of transfected CHO cells was studied on a fibrinogen substrate both by time lapse videomicroscopy and by random and haptotactic transwell assays. Haptotactic and random transwell assays measured distinct aspects of migration, with the random transwell assay correlating most closely with time lapse videomicroscopy. Mutations in the cytoplasmic domains that increase ligand affinity or activation of the α IIB β 3 receptor into a high affinity state by the LIBS6 antibody decreased the migration rate. Likewise, mutations that increase cyto-

skeletal organization without affecting affinity also decreased the migration rate. In contrast, truncation of the β chain, which alters cytoskeletal associations as assayed by absence of focal adhesions, decreased haptotactic migration while increasing random migration. These effects on the migration rate were partially compensated for by altering substrate concentration, demonstrating optimum substrate concentrations that supported maximal migration. For example, cells expressing integrins locked in the high affinity state showed maximal migration at lower substrate concentrations than cells expressing low affinity receptor. Together, these results implicate the strength of adhesion between cell and substrate, as modulated by receptor affinity, organization of adhesive complexes, and substrate concentration, as important regulators of cell migration rate. Further, we demonstrate a dominant effect of high affinity integrin in inhibiting migration regardless of the organization of adhesive complexes. These observations have potential implications for tumor metastasis and its therapy.

CELL migration is central to many normal and pathological processes including embryonic development, wound healing, inflammation, and tumor metastasis. Despite recent progress, we still have a limited understanding of many of the underlying mechanisms that contribute to migration. One of these, a focus of our recent studies, is the regulated and dynamic interaction between the cell and its surrounding substrate (Huttenlocher et al., 1995). Initiation of cell migration, for example, involves protrusion of the leading edge of the cell, which is then stabilized by formation of an adhesive complex. This complex, in turn, generates the traction required for cell movement. Subsequently, adhesions release at the cell

rear, allowing the cell to advance over its substrate (Lee et al., 1994; Stossel, 1993). Optimal migration, therefore, requires coordination between the formation of new adhesions at the cell front and their subsequent release at the cell rear. Intrinsic to this process is an asymmetry, possibly transient, in the strength of the adhesions between the cell front and rear to allow for directional migration (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996). A quantitative model of the relationship between traction forces, adhesive strength, and cell migration points to the importance of adhesion strength as a determinant of migration rates (DiMilla et al., 1991). Thus, the strength of cell-substratum adhesions likely plays a significant role in determining the rate of migration.

Several, somewhat interrelated parameters potentially contribute to the strength of the adhesion between the cell and its surrounding environment. These include the concentration of adhesive ligand or substrate, number of re-

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ceptors, the receptor-ligand affinity, strength of cytoskeletal interactions that link receptors to the cytoskeleton, and spatial organization of the receptors. With one exception, the contributions of these parameters to migration rates have not been investigated systematically. Varying substrate concentration reveals that optimal cell migration occurs at intermediate substrate concentrations (Dimilla et al., 1993; Goodman et al., 1989). This is consistent with the concept that intermediate adhesive strength is important for optimal migration (DiMilla et al., 1991; Duband et al., 1991; Hammarbach et al., 1988; Huttenlocher et al., 1995). A mechanistic interpretation may be that very weak adhesions result in poor traction, whereas very strong adhesions may not release rapidly, resulting in inhibited migration. Together, these observations point to the potential importance of adhesive strength and the need for its systematic investigation.

The present study focuses on the role of integrin receptors during migration. Integrin receptors, which are $\alpha\beta$ heterodimers present on the cell surface, function in migration by acting as a structural link between the extracellular matrix and the actin cytoskeleton (Hynes, 1992). In addition, integrins also initiate signaling processes (Clark and Brugge, 1995; Damsky and Werb, 1992; Juliano and Haskill, 1993), and therefore may contribute to migration by participating in a signal transduction cascade. Studies of the effects of integrin mutations on cell migration are often complicated by the presence of endogenous receptors. To avoid this, we have used a model system consisting of CHO cells ectopically expressing various forms of the integrin α IIb β 3 receptor. This is a very useful system since migration on a fibrinogen substrate is mediated only by the transfected receptor. Therefore, one can study the direct effects of specific integrin mutations on cell migration. This system is also particularly appropriate for the study of the affinities and cytoskeletal interactions of integrin receptors during cell migration since the binding properties of the variant receptors are well characterized.

Mutations in the cytoplasmic domain influence adhesive interactions both by modulating ligand-binding affinity and by mediating cytoskeletal linkages. Modulation of ligand-binding affinity by changes in the integrin cytoplasmic domain, known as inside-out signaling, has been an area of extensive study (Ginsberg et al., 1992; O'Toole et al., 1994). This phenomenon has been studied in the most detail with the α IIb β 3 integrin receptor. For example, mutations in both the α and β chains of the integrin receptor α IIb β 3 can lock the receptor in the high affinity state, as detected by specific binding of the antibody PAC1 (O'Toole et al., 1994). In addition, specific antibodies, e.g., LIBS6, can also lock the receptor in the high affinity state (Frelinger et al., 1991).

The integrin cytoplasmic domain contributes to cell-substratum adhesiveness by acting as a structural link between the extracellular substrate and the actin cytoskeleton. Upon ligand binding, many integrin receptors form highly organized structures, known as focal adhesions, that connect components of the actin cytoskeleton to the extracellular substrate (Burrige et al., 1988). The β -subunit cytoplasmic domain of several integrin receptors localizes integrins to focal adhesions (Hayashi et al., 1990; LaFlamme et al., 1992; Marcantonio et al., 1990; Reszka et al., 1992).

Interestingly, previous studies suggest an inverse correlation between the formation of highly organized focal adhesions and cell migration rate (Dunlevy and Couchman, 1993; Kassner et al., 1995; Pasqualini and Hemler, 1994). Despite this finding, the focal adhesion serves as a model of the general kinds of interactions that form between the integrin receptor and the actin cytoskeleton during migration.

In this study, we explored the effect of altered ligand-binding affinity and cytoskeletal interactions on the cell migration rate as assayed by random and haptotactic transwell assays and by time lapse videomicroscopy. These assays each measure different aspects of migration and provide insight into migratory mechanisms. We found that mutations altering ligand affinity and cytoskeletal linkages had dramatic effects on cell migration. Locking the integrin receptor in a high affinity state by mutation or antibody activation resulted in a significant decrease in cell migration rate. Similarly, increasing cytoskeletal and focal adhesion organization inhibited migration. In contrast, increases in random migration were seen in cells transfected with a mutation in the β cytoplasmic domain that reduces cytoskeletal linkages. These effects depended on substrate concentration, demonstrating the dynamic interplay between cell and environment during cell migration. Overall, our results suggest that the strength of adhesion between cell and substrate, as modulated by multiple factors, is potentially an important contributor to the cell migration rate.

Materials and Methods

Antibodies and Reagents

The activating anti- α IIb β 3 antibody, LIBS6, and noninhibitory anti- α IIb β 3 antibody, D57, were used as described previously (O'Toole et al., 1994). The inhibitory anti-hamster α 5 antibody, PB1 (Brown and Juliano, 1985, 1988), was supplied by Dr. Rudolph Juliano (University of North Carolina, Chapel Hill); the α IIb β 3 complex-specific antibody, 2G12 (Plow et al., 1985; Woods et al., 1984), was obtained from Dr. Virgil Woods (University of California-San Diego, La Jolla, CA); and the anti-Fn antibody N294 (McDonald et al., 1987) was provided by Dr. John McDonald (Mayo Clinic Scottsdale, Scottsdale, AZ). Fibrinogen was purified as previously described (O'Toole et al., 1994). Anti-vinculin antibodies and cytochalasin D were obtained from Sigma Chemical Co. (St. Louis, MO), and rhodamine phalloidin was obtained from Cappel Organon Teknika Co. (Durham, NC). The α IIb β 3 peptide-specific peptidomimetic inhibitor, RO43-5054 (Alig et al., 1992), was obtained from Dr. B. Steiner (Hoffman-La Roche, Basel, Switzerland).

Cell Culture and Transfection

CHO cells (CHO-K1) cells were obtained from American Type Culture Collection (Rockville, MD). The cells were grown in DME containing 10% FBS, 2 mM glutamine (Sigma Chemical Co.), 1% nonessential amino acids (Sigma Chemical Co.), 100 U/ml penicillin, and 100 μ g/ml streptomycin. The expression vectors in pCDM8 encoding α IIb, α IIb Δ 996, α IIb Δ L, β 3, β Δ 724, and β Δ 717 have been described previously (Hughes et al., 1995; Loftus et al., 1990; O'Toole et al., 1989, 1990, 1994). cDNA constructs were confirmed by DNA sequencing and purified by CsCl centrifugation. Cells were transfected using Lipofectamine (Bethesda Research Laboratories, Gaithersburg, MD) into CHO-K1 cells as described in the manufacturer's protocols. The transfected cells were maintained in DME containing 0.5 mg/ml of G418. Clonal stable cell lines were produced as described (Loftus et al., 1990). Migration studies were performed on more than one clonal line to rule out clonal variation as a cause of the observed alterations in migration. Stable expression of the β Δ 717 mutant was obtained in only one clone, and the results presented are based on this single clone.

Flow Cytometry

Cell surface expression of integrins was analyzed by flow cytometry using the D57 antibody (20 $\mu\text{g}/\text{ml}$) as described (Loftus et al., 1990; O'Toole et al., 1989, 1990, 1994). The cells were washed with PBS and harvested from the plates using 0.02% EDTA in calcium-magnesium-free HEPES-Hank's Buffer (CMF-HH).¹ They were incubated on ice for 30 min with primary antibody, and then washed with blocking buffer (2% BSA in CMF-HH) twice. The cells were then incubated with the secondary antibody, FITC-conjugated sheep anti-mouse IgG (Cappel Organon Teknika Co.). The cells were washed with blocking buffer and resuspended in CMF-HH for flow cytometry. Flow cytometry was performed on a cell sorter (EPICS TM; Coulter Electronics, Inc., Miami Lakes, FL) equipped with Cyclops software for data analysis (Cytomat; Fort Collins, CO). The $\beta\Delta 717$ -, $\beta\Delta 724$ -, and $\alpha\Delta 996$ -expressing CHO cells were sorted for surface expression profiles comparable to the $\alpha\text{IIb}\beta 3$ transfected cells. Migration studies were performed on the sorted populations to confirm that the observed differences in migration rates were not secondary to altered cell surface expression.

Cell Adhesion Assays

Cell adhesion assays were performed using 96-well plates coated with a fibrinogen substrate at 10 $\mu\text{g}/\text{ml}$ for 1 h at 37°C and washed with PBS before blocking with 2% BSA for 1 h. Cells were plated at a density of 2×10^4 cells per well. They were allowed to attach for 30 min and were then submitted to a shake-off procedure of three 300-rpm bursts for 10 s each. They were washed with PBS, and the procedure was then repeated. The cell number adhering was determined by a hexosaminidase colorimetric assay as described (Landegren, 1984).

Cell Migration Assays

Both modified Boyden chamber transwell assays and time lapse videomicroscopy were performed. Cell migration assays were performed in serum-free hybridoma media CCM1 (Hyclone Laboratories, Inc., Logan, UT). The cells were washed with serum-free DME, and then resuspended in the CCM1. The transwell assays were performed using transwell (Co-Star Corp., Cambridge, MA) plates. The membranes were coated with fibrinogen at concentrations ranging from 0.1–100 $\mu\text{g}/\text{ml}$ in PBS for 1 h at 37°C. The assay was used both as a random assay with both the upper and lower surfaces of the membrane coated with substrate and a haptotactic assay with only the lower surface of the membrane coated with substrate. The membranes were then blocked with 2% BSA in PBS for 1 h at 37°C. The membranes were washed with PBS and the cells were then plated in the upper well at a concentration of 1×10^5 in 100 μl of CCM1. The assays were run for 3 h unless otherwise indicated. Similar migration results were obtained at 6 and 10 h (data not shown). The cells in the upper well were removed using a cotton swab, and the migrated cells on the lower surface of the membrane were fixed with methanol for 5 min and stained with methylene blue for 30 min (Fisher leukostat staining kit; Sigma Chemical Co.). The cells on the lower surface were counted using a $\times 10$ grid at high power magnification ($\times 20$). Multiple fields (four to eight) were counted and averaged for each condition studied. Each experiment was performed a minimum of three times.

Time lapse videomicroscopy was performed as previously described (Schmidt et al., 1993). The cells were videotaped for 4–12 h while in CCM1 on non-tissue-culture plates coated with fibrinogen for 1 h and blocked with 2% BSA for 1 h at 37°C. The cells were plated at a low density of 1×10^4 cells per 35-cm plate. The cells were tracked by playing back videos through an image processing system (Biological Detection Systems, Inc., Pittsburgh, PA). We tracked the position of centroids of individual cells at 30-min intervals, within 1–4 h after plating the cells. For each cell tracked, pixel coordinates were determined and correlated with actual distance migrated as $\mu\text{m}/\text{h}$. 25 to 50 cells were tracked for each condition in a minimum of three to five separate experiments.

Immunofluorescence

Coverslips were coated with fibrinogen after acid washing and ethanol treatment. The glass coverslips were coated with fibrinogen at 10 $\mu\text{g}/\text{ml}$, blocked with 2% BSA, and washed with PBS twice before plating the cells. After 3 h, the cells (plated at 1×10^4 cells per well with 24-well plate)

were fixed in PBS containing 3% formaldehyde (Ted Pella, Inc., Irvine, CA) for 15 min. The coverslips were then washed with PBS, quenched in PBS containing 0.1 M glycine for 15 min, treated with 1% Triton X-100 in PBS, and blocked in PBS containing 5% goat serum. After incubating with the primary antibody, anti-vinculin antibody, the cells were washed with PBS three times, and then incubated with the fluorescein-conjugated secondary antibody, FITC sheep anti-mouse at 1:200 with rhodamine-phalloidin-conjugated goat anti-mouse diluted 1:500 in blocking buffer (Cappel Organon Teknika Co.). The coverslips were mounted and observed using a fluorescent microscope at $\times 63$ (Axioplan; Carl Zeiss, Inc., Thornwood, NY). Pictures were taken using TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Results

Ectopically Expressed $\alpha\text{IIb}\beta 3$ Integrin Mediates Migration of CHO Cells on Fibrinogen

Genetic analysis of integrin receptor function during cell migration is complicated by the presence of endogenous integrin receptors on the cell surface. The $\alpha\text{IIb}\beta 3$ -transfected CHO cells represent a useful system for the study of interactions between integrins and the substrate during cell migration because untransfected CHO cells do not express an endogenous receptor for fibrinogen. We found that untransfected CHO cells were unable to attach to or spread on a fibrinogen substrate for the first 4–6 h after plating. They maintained a rounded morphology and failed to migrate on fibrinogen during this time period. In contrast, CHO cells expressing the $\alpha\text{IIb}\beta 3$ integrin attached and showed a spread morphology on fibrinogen. The migration of these $\alpha\text{IIb}\beta 3$ -expressing CHO cells on a fibrinogen substrate was mediated by the $\alpha\text{IIb}\beta 3$ receptor. A specific fibrinogen peptide inhibitor, RO43-5054, and a function-blocking antibody specific to the $\alpha\text{IIb}\beta 3$ receptor, 2G12, inhibited migration (Fig. 1). An $\alpha\text{IIb}\beta 3$ antibody that does not block function (D57) and an $\alpha 5\beta 1$ integrin function-blocking antibody (PB1) both failed to block migration on fibrinogen. In contrast, PB1 blocked migration of CHO cells on fibronectin (data not shown). In addition, CHO B2 cells (which lack endogenous $\alpha 5\beta 1$ integrin) ectopically expressing $\alpha\text{IIb}\beta 3$ showed migration comparable to the $\alpha\text{IIb}\beta 3$ -expressing CHO cells, suggesting that the $\alpha\text{IIb}\beta 3$ -mediated migration was not significantly altered by the presence of endogenous $\alpha 5\beta 1$ integrin. Although differences in fibronectin assembly have been demonstrated for $\alpha\text{IIb}\beta 3$ and its mutants after extended times in culture (Wu et al., 1995), we found no detectable organized fibronectin in CHO cells expressing $\alpha\text{IIb}\beta 3$ and its mutants under our short-term migration conditions (data not shown). These studies demonstrated that the $\alpha\text{IIb}\beta 3$ integrin specifically mediated the migration of $\alpha\text{IIb}\beta 3$ -expressing CHO cells on a fibrinogen substrate.

Migration Assays: Haptotaxis vs Random Migration

Two different assays were used to study migration, including the modified Boyden chamber assay and time lapse videomicroscopy. The modified Boyden chamber, or transwell assay, is generally used in a haptotactic configuration. Migration is directed toward a fixed substratum by coating only one (the lower) surface of the membrane with substrate. It can also be used as a random assay with both the upper and lower surfaces of the membrane coated with

1. Abbreviation used in this paper: CMF-HH, calcium-magnesium-free HEPES-Hank's buffer.

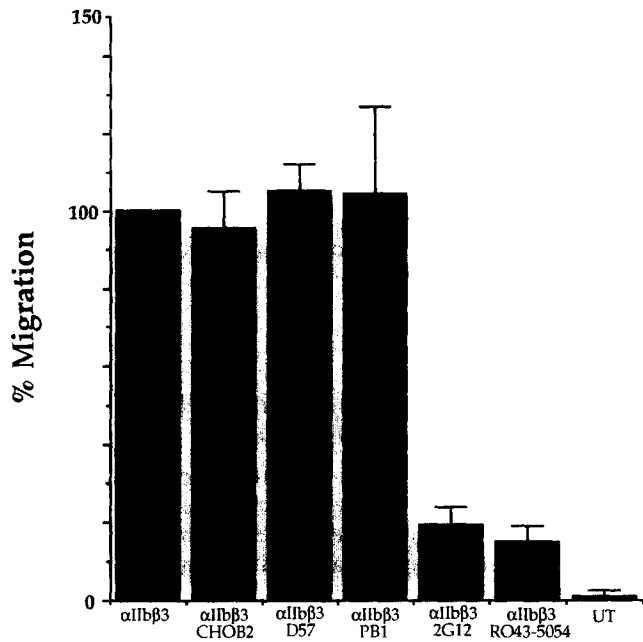


Figure 1. The α IIb β 3 integrin mediated migration of CHO cells expressing α IIb β 3 on fibrinogen. Migration of CHO cells expressing wild-type α IIb β 3 integrin was studied by random transwell assay, with both the upper and lower surface of the membrane coated with fibrinogen at 10 μ g/ml. Cells were plated in serum-free hybridoma media CCM1, and cell migration was quantitated by counting the cell number on the lower surface of the membrane after 3 h, as described in Materials and Methods. Migration was expressed as the percentage of migration of the α IIb β 3-expressing CHO cells, and each data point represented the average of a minimum of three separate experiments with error bars showing SD. Cells were pretreated for 20 min before plating with D57, a non-function-perturbing anti- α IIb β 3 antibody, PB1, an α 5 β 1 function-perturbing antibody, 2G12, a function-perturbing anti- α IIb β 3 antibody, or RO43-5054, an α IIb β 3 function-perturbing peptide, as indicated. Migration of CHO cells expressing α IIb β 3 was specifically inhibited by 2G12 and RO43-5054, but was not altered by α IIb β 3 expression in CHOB2 cells or by inhibition with PB1, demonstrating that migration on fibrinogen was independent of the α 5 β 1 integrin receptor. Untransfected cells (UT) did not migrate on fibrinogen.

substrate. There are distinct differences between these two assays. Random migration measures the ability of substrate to support cell movement without a gradient in substrate concentration. In contrast, the haptotactic assay is a directional assay and measures the ability of a cell to sense and to respond to gradients in substrate concentration. We observed differences between the assays using CHO cells expressing the α IIb β 3 integrin. Both assays showed a dependence of the migration rate on substrate concentration. Random migration showed an optimum at intermediate substrate concentrations, with decreased migration rates at both higher and lower substrate concentrations. In contrast, haptotaxis required higher substrate concentration for maximum migration than random assay. In addition, the increase in migration rate seen at higher substrate concentrations persisted rather than displaying a clear optimum (Fig. 2). Migration observed by time lapse videomicroscopy correlated most closely with random migration in transwell assays. The migration of cells visualized by time

lapse videomicroscopy was random and nondirectional, as expected in the absence of a gradient in substrate concentration. Similar differences between the assays were also observed with CHO cells expressing mutant forms of the α IIb β 3 receptors. A possible interpretation of these differences is that the haptotactic assay may be more dependent on the ability of cells to form strong adhesive contacts at the cell front, whereas the random assay may be more limited by the ability of cells to release adhesions at the cell rear.

Migration Rate Depends on Substrate Concentration and Cell Surface Integrin Expression Levels

The α IIb β 3-expressing CHO cells showed optimum random migration at intermediate fibrinogen concentrations. Fig. 2 shows that CHO cells ectopically expressing α IIb β 3 migrated maximally at a fibrinogen substrate concentration of 5 μ g/ml. There was a large decrease in the migration rate at both higher (>10 μ g/ml) and lower (<1 μ g/ml) substrate concentrations. The migration speed was clearly sensitive to incremental changes in substrate concentration. Studies by time lapse videomicroscopy similarly demonstrated maximum migration at an intermediate substrate concentration of \sim 5 μ g/ml (Table I). The migration speed of α IIb β 3-transfected cells tracked by time lapse videomicroscopy showed significant variability, ranging between 11 and 90 μ m/h (average 31 μ m/h) for the motile population on 5 μ g/ml of fibrinogen. Observations by time lapse videomicroscopy revealed that the stationary cells are spread, while the motile population showed a more rounded phenotype. Higher substrate concentrations were associated with a higher population of spread cells and correlated with a decrease in the motile population (Table I).

The cell migration rate was also influenced by variations in the level of α IIb β 3 integrin cell surface expression. The role of cell surface expression levels on migration in the α IIb β 3-transfected cells was measured by sorting the cells into two groups, the low expressors (lowest third of positive cells) and high expressors (highest third of positive

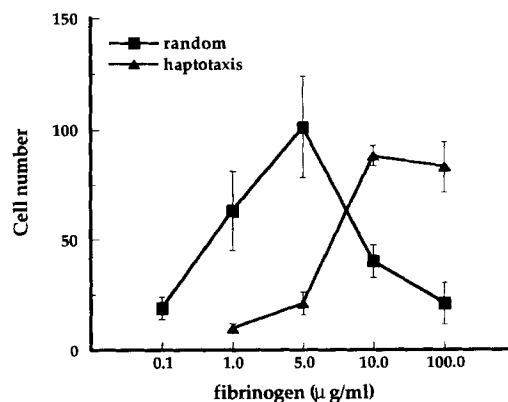


Figure 2. Migration of α IIb β 3-expressing CHO cells was substrate concentration dependent. Migration of α IIb β 3-expressing CHO cells was studied by both random and haptotactic transwell assays with fibrinogen concentrations ranging from 0.1–100 μ g/ml. Each data point represented the mean \pm SD from a minimum of three separate experiments. In the random assay, migration was biphasic. In the haptotactic assay, migration required a higher substrate concentration to initiate migration and was not biphasic.

Table 1. Average Migration Speed for CHO Cells Expressing α IIB β 3 and Its Mutants

Mutant	Fibrinogen concentration	Percentage motile	Directional migration rate
	$\mu\text{g/ml}$	%	$\mu\text{m/L}$
α IIB β 3	5	74 (25/34)	31
	10	11 (3/27)	13
α IIB β Δ 724	5	74 (32/43)	36
	10	38 (16/42)	22
α IIB Δ 996 β 3	5	9 (3/34)	11
α L Δ β 3	5	7 (2/30)	11
α IIB β Δ 717	5	21 (6/29)	20

Directional migration speed was determined using time lapse videomicroscopy with cell tracking. Cells were tracked at 30-min intervals as described in Materials and Methods. The percentage motile is defined as % with migration speed $\geq 10 \mu\text{m/h}$. Average speed is the average directional speed of the motile population.

cells). The overall range of expression was ~ 10 -fold. Although the two populations displayed significant differences in random or haptotactic migration rates, they were less than twofold. Cells with high levels of integrin surface expression migrated faster, while the lower expressors exhibited decreased migration rates (data not shown). This modest difference in the migration rate was smaller than the differences seen between wild type and the various mutants described here. Therefore, within the range of receptor densities used in this study, differences in receptor density did not account for the differences in cell migration observed with the various mutants. In addition, where indicated, integrin expression levels were controlled by sorting the cells into populations with similar expression levels.

Receptor Affinity Modulates the Migration Rate

Changes in the ligand-binding affinity of the integrin receptor may regulate cell migration. For example, certain cell types, such as immune cells, have transient activation of receptor associated with increased migration. In addition, it is also possible that a cycle of high affinity followed by low affinity at the cell rear is required for rapid migration. We studied the migratory capacity of cells with the integrin receptor fixed in the high affinity state. The wild-type α IIB β 3 integrin receptor transfected into CHO cells is present in an inactive or low affinity state. The α IIB β 3 receptor may be fixed in the high affinity state by antibody activation with LIBS6 or by specific mutations in the integrin cytoplasmic domain.

Activation of the α IIB β 3 receptor by LIBS6 inhibited random migration by ~ 4 -fold on a fibrinogen substrate (Fig. 3 A). Haptotactic migration was also inhibited, but to a lesser extent than random migration (Fig. 3 B). Similarly, a mutation in the integrin receptor cytoplasmic domain that results in constitutive activation of the receptor resulted in decreased random and haptotactic migration in transfected CHO cells, as assayed by both transwell assays and time lapse videomicroscopy (Fig. 3, A and B). The affinity-activating mutant, α L Δ , has a deletion of the membrane proximal GFFKR sequence of the α chain, which results in the constitutive high affinity state of the integrin receptor. Surface expression of the α L Δ , α L full, and α IIB β 3 is shown in Fig. 3 C. The decrease in migration of α L Δ -transfected CHO cells was maintained over the 10-h

time period studied (data not shown). Observations by time lapse videomicroscopy demonstrated that the α L Δ -expressing CHO cells extend lamellipodia but do not progress along the substrate with the rear of the cell remaining fixed on the substratum (data not shown). A potential mechanism suggested by these observations is that these cells have inhibited release of adhesions at the cell rear. These studies show that freezing the integrin receptor in the high affinity state, by antibody activation or by a mutation that results in constitutive activation, inhibits both random and haptotactic cell migration.

Alterations in Cellular Morphology and Focal Adhesion Organization Correlate with the Migration Rate

Previous studies suggest that increased migration rates correlate with decreased cell spreading and focal adhesion organization (Dunlevy and Couchman, 1993; Kassner et al., 1995; Pasqualini and Hemler, 1994). We probed this relationship in the CHO system using integrin cytoplasmic domain mutations. CHO cells expressing mutations in the α IIB β 3 integrin receptor when plated on fibrinogen each display characteristic cell spreading and organization of their focal adhesions and actin stress fibers. Cells expressing the mutant α IIB Δ 996 β 3, which contains an α -subunit cytoplasmic domain truncation (Table II), showed more prominent focal adhesions and larger, more centrally distributed actin stress fibers when compared with CHO cells expressing the wild-type α IIB β 3 integrin (Fig. 4). These cells also showed decreased migration by random and haptotactic assays (Fig. 5). Likewise, cells expressing the α L Δ β 3 mutant were also more spread and had more brightly staining focal adhesions when compared with the α IIB β 3-transfected cells (Fig. 4). These morphological features are not likely due to the increased affinity of the receptor, since CHO cells expressing α IIB β 3, when activated into the high affinity state by LIBS6, did not show a significant change in either the organization of the actin cytoskeleton or focal adhesions (data not shown). Secondly, α IIB Δ 996 β 3 is known to be in the low affinity state (Ylänne et al., 1993). The cells expressing α L Δ also showed decreased migration rates that differed in character from those seen for the LIBS6-treated cells and correlated positively with the increased organization of the actin filaments and focal adhesions (see below). Together, these results further support the inverse relationship between the cell migration rate and spreading, organization of the actin cytoskeleton, and focal adhesions.

CHO cells expressing the β Δ 724 integrin, which has a truncation in the β -subunit cytoplasmic domain, did not have organized focal adhesions or stress fibers (data not shown). Yet, previous studies have shown that these cells have no significant reduction in binding to fibrinogen (Ylänne et al., 1993). Cells expressing α IIB β 724 showed increased random migration rates (Fig. 6). Although the results were similar with four different clones, the random migration rates of the α IIB β Δ 724-expressing CHO cells ranged from 75–200% of the α IIB β 3-expressing cells. These results were likely due to differences in surface expression seen with the different clones, since the clones with decreased expression showed slower migration rates. The results presented are based on a single clone that had

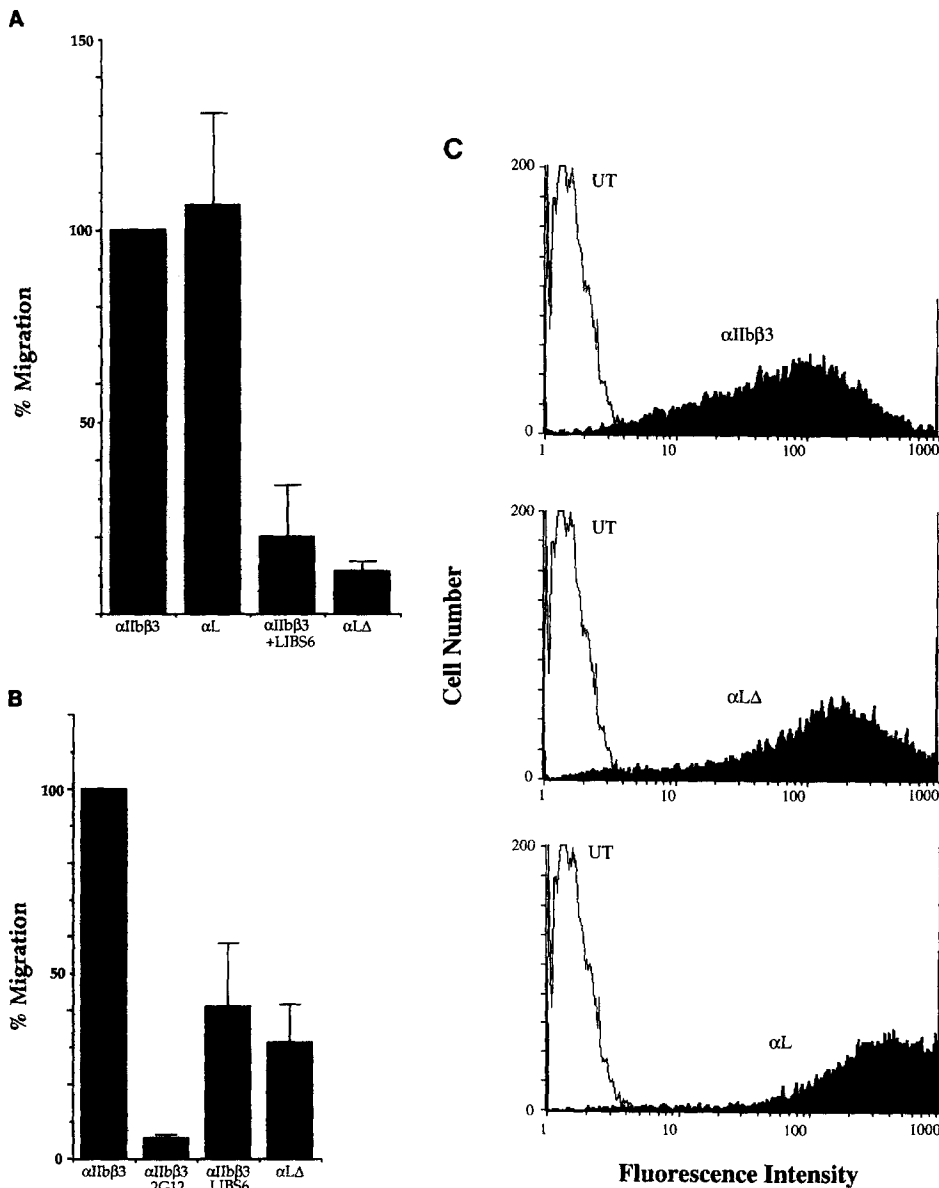


Figure 3. High affinity integrin inhibited migration. Migration was studied by both random and haptotactic transwell assays on fibrinogen (10 μ g/ml) as described in Materials and Methods. The results were expressed as a percentage of α Ib β 3-mediated migration. The data points represented the mean of three separate experiments with error bars showing SD. The integrin receptor was activated by pretreatment of α Ib β 3-expressing CHO cells with the antibody LIBS6 (100 μ g/ml) for 20 min. A cytoplasmic deletion mutant, α L Δ , also locked the receptor in the high affinity state. Freezing the integrin receptor in the high affinity state inhibited both random (A) and haptotactic (B) migration. Pretreatment of the α Ib β 3-expressing CHO cells with the α Ib β 3 function-perturbing antibody, 2G12, also inhibited the α Ib β 3-mediated haptotactic migration. The levels of surface expression of α Ib β 3 integrin and mutants are shown (C). The cells were stained with the anti- α Ib β 3 antibody, D57, and analyzed by flow cytometry as described in Materials and Methods.

consistently high surface expression levels that were comparable to the α Ib β 3-expressing cells. The migration of β Δ 724-expressing cells was inhibited by the function-blocking mAb, 2G12, the inhibitory peptide, RO43-5054, and LIBS6, demonstrating the participation of this recep-

tor in the observed migration (Fig. 6 A). Observations by time lapse videomicroscopy showed that the β Δ 724-expressing cells extended small lamellipodia and migrated with very little cell spreading. In contrast, α Ib β 3-expressing CHO cells treated with cytochalasin D showed a disrupted

Table II. Sequences of the Cytoplasmic Tails of the α Ib, β 3, and the Mutants

α Ib	989
α Ib Δ 996	KVGFFKRNRP PLEEDDEEGE
α L	KVGFFKR*
α L Δ	KVGFFKRNLK EKMEAGRVPNGIPAEDSEQLASGQAEGDP
	GCLKPLHEKDSSEGGGKD
	<u>KGFFKRN</u> LKEKMEAGRVPNGIPAEDSEQLASGQAEGDP <u>GCLKPLHEK</u> D
	SESGGGKD
β 3	716
β Δ 724	KLLITIHDRKEFAKFEERARAKWDTANNPLYKEATSTFTNITYRGT
β Δ 717	KLLITIH*
	K*

Underline represents deletion.

* Stop codon.

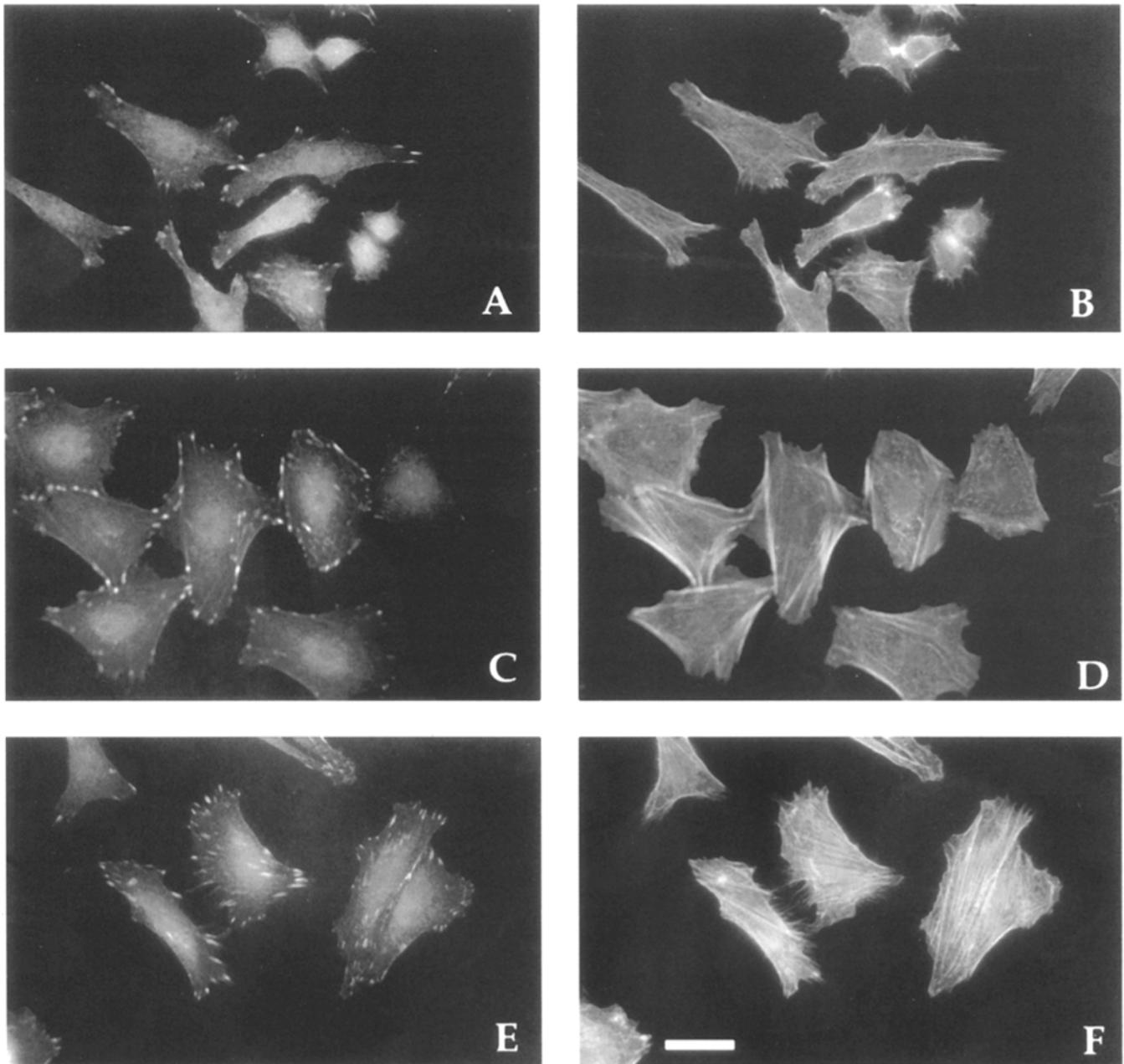


Figure 4. Cellular morphology of CHO cells expressing $\alpha\text{IIb}\beta 3$, $\alpha\text{IIb}\Delta 996\beta 3$, and $\alpha\text{L}\Delta\beta 3$. CHO cells transfected with $\alpha\text{IIb}\beta 3$, $\alpha\text{IIb}\Delta 996\beta 3$, and $\alpha\text{L}\Delta\beta 3$ were cultured for 4 h on fibrinogen-coated (10 $\mu\text{g}/\text{ml}$) coverslips in serum-free media (CCM1) and immunostained with vinculin (A, C, and E) and phalloidin (B, D, and F). CHO cells expressing $\alpha\text{IIb}\beta 3$ (A and B) showed focal contacts (A) and a peripheral organization of actin stress fibers (B). CHO cells expressing $\alpha\text{L}\Delta\beta 3$ showed increased cell spreading, bright adhesions (C), and a peripheral distribution of the actin stress fibers (D). CHO cells expressing $\alpha\text{IIb}\Delta 996\beta 3$ showed numerous bright focal adhesions (E) and a more central distribution of the actin stress fibers (F). Bar, 20 μm .

cytoskeleton and significant cell rounding; however, there was no lamellipodia extension, and cell migration was inhibited as assayed by both time lapse videomicroscopy and transwell assays (data not shown).

In contrast with random migration, CHO cells expressing $\beta\Delta 724$ showed decreased haptotaxis compared with the $\alpha\text{IIb}\beta 3$ -expressing CHO cells (Fig. 6 B). Furthermore, the $\beta\Delta 724$ -expressing cells exhibited more random than haptotactic migration. In this way, this mutant was unique among all of the other mutants studied. Presumably, the different haptotactic response reflected an inability to form suffi-

ciently strong adhesions at the cell front to mediate migration through the narrow pores in the transwells (see Discussion).

Similar studies were also performed on the migration of CHO cells transfected with another β cytoplasmic mutation, $\beta\Delta 717$, which has a more extensive deletion of the β -subunit cytoplasmic domain. These cells not only have no organized focal adhesions or stress fibers, but also the receptor is locked in the high affinity state (Hughes et al., 1995) (data not shown). The $\beta\Delta 717$ -transfected cells showed a significant decrease in both random and haptotactic cell migration in transwell assays or time lapse videomicroscopy.

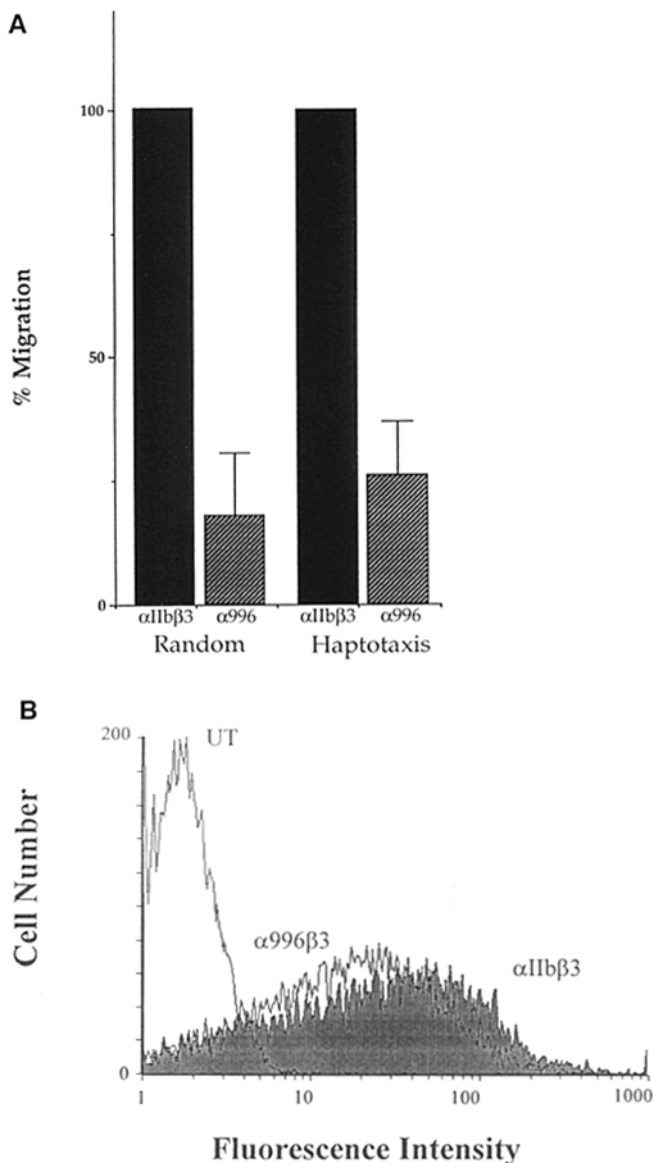


Figure 5. Migration was inhibited in CHO cells expressing the αIIb cytoplasmic domain truncation $\alpha\text{IIb}\Delta\text{996}\beta\text{3}$. Random and haptotactic migration was studied as described in Materials and Methods on 10 $\mu\text{g/ml}$ of fibrinogen. The results represented the mean of three separate experiments and are expressed as a percentage of $\alpha\text{IIb}\beta\text{3}$ migration, with error bars showing SD. Migration of CHO cells expressing $\alpha\text{IIb}\Delta\text{996}\beta\text{3}$ was inhibited in both random and haptotactic transwell assays (A). The levels of surface expression of $\alpha\text{IIb}\Delta\text{996}\beta\text{3}$ and $\alpha\text{IIb}\beta\text{3}$ are shown (B). Cells were sorted by FACS[®], as described in Materials and Methods, to obtain similar surface expression levels.

In addition, activating the $\alpha\text{IIb}\beta\text{724}$ receptor with LIBS6 also inhibited the random migration. Therefore, locking the integrin receptor in the high affinity state appears to inhibit cell migration even in cells lacking focal adhesions.

Cells Expressing Different Integrin Mutants Display Characteristic Substrate Concentration Optima for Migration

Alterations in integrin–ligand affinity and cytoskeletal in-

teractions clearly have major effects on the CHO cell migration rate. A potential mechanism for these effects is that both ligand affinity and cytoskeletal organization contribute to the overall strength of adhesion of these cells to their substratum. In this view, varying other parameters, like substrate density, that also affect adhesion strength would reverse the effects of altered integrin affinity or cytoskeletal associations. We pursued this possibility by assaying the migration of cells expressing wild-type and mutant integrins on substrates of varying ligand densities. In general, our results demonstrate that the effects of the integrin modifications on migration are, at least in part, due to a shift in the concentration optimum for migration. This supports the notion of adhesive strength as a regulator of migration and demonstrates an interrelation of the parameters that regulate migration.

The migration of cells with the integrin receptor locked in the high affinity state showed a substrate concentration optimum for migration that differed from that of the wild-type transfectant. CHO cells ectopically expressing $\alpha\text{IIb}\beta\text{3}$, locked in the high affinity state by either the αLA mutation or the LIBS6 antibody, migrated maximally at a fibrinogen-coating concentration of 1 $\mu\text{g/ml}$ (Fig. 7 A). This is lower than the optimum fibrinogen-coating concentration of 5 $\mu\text{g/ml}$ seen for wild-type transfectants. However, while the substrate concentration optimum for the αLA -expressing cells was shifted, the migration rate at that optimum was about fourfold less than that of the wild type at its optimum. This contrasted the effect of the LIBS6 antibody, which also locks the receptor in the high affinity state. When LIBS6 is added to wild-type cells, their optimum migration rate diminished only modestly. As reported above, the αLA mutant has increased cytoskeletal and focal adhesion organization that is not seen in LIBS6-treated cells; this difference provides a likely explanation for why migration of the αLA mutant was inhibited even at its concentration optimum.

CHO cells expressing mutations that disrupt cytoskeletal associations, $\beta\Delta\text{724}$ and $\beta\Delta\text{717}$, were similarly examined. They also showed an optimum substrate concentration that shifts with varying substrate concentration (Fig. 7 B). The $\beta\Delta\text{724}$ -transfected cells migrated maximally at a slightly higher substrate-coating concentration, 10 $\mu\text{g/ml}$, than did the wild-type transfectants. The migration rates of the mutant and wild-type cells at their substrate concentration optima were similar. In contrast, the $\beta\Delta\text{717}$ -transfected cells, which have a disrupted cytoskeleton and are also locked in the high affinity state, migrated maximally at a lower substrate concentration, 1 $\mu\text{g/ml}$. However, their maximal migration rate was nearly threefold less than that of the control. These observations again demonstrate an interrelation among the different parameters that modulate adhesion; they also point to a dominant effect of locking receptors in the high affinity state.

The adhesion of the mutant and wild-type cells was measured to determine whether relative adhesivity correlated with the migration rates. CHO cells expressing different forms of the $\alpha\text{IIb}\beta\text{3}$ integrin receptor resulted in altered adhesion on fibrinogen (Fig. 8). The $\alpha\text{IIb}\beta\text{3}$ -expressing cells showed intermediate levels of adhesion, while cells expressing the high affinity mutants had increased adhesion and, as described above, a correlated decrease in cell

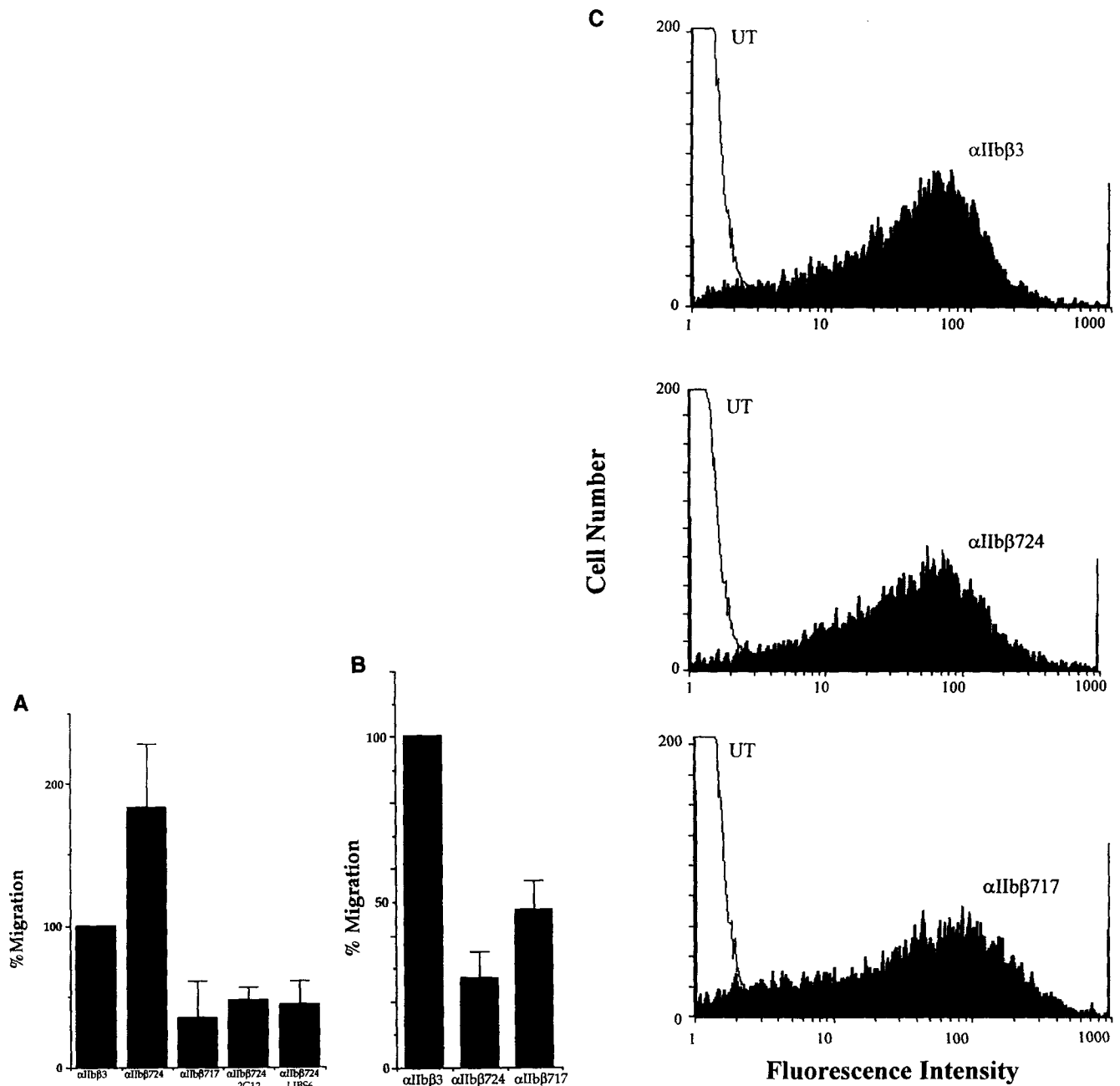


Figure 6. Truncation of the β 3 cytoplasmic domain inhibited haptotactic but not random migration. Migration studies were performed on 10 μ g/ml of fibrinogen as described in Materials and Methods. Migration was expressed as a percentage of α I**Ib** β 3-mediated migration. Each data point represented an average of a minimum of three separate experiments with error bars showing SD. CHO cells expressing a truncation mutant of β 3, α I**Ib** β Δ 724, showed normal to increased random migration (A) and decreased haptotactic migration (B). The random migration of CHO cells expressing β Δ 724 was specifically inhibited by pretreatment with the α I**Ib** β 3 function-perturbing antibody, 2G12 (20 μ g/ml), or by the activating antibody, LIBS6. Further truncation of the β cytoplasmic domain (β Δ 717), which locks the receptor in the high affinity state, also inhibited random migration. Haptotactic migration was inhibited in CHO cells expressing both the α I**Ib** β Δ 724 and α I**Ib** β Δ 717 integrin (B). The levels of surface expression of CHO cells expressing α I**Ib** β 3 and mutants are shown (C).

migration rate. In contrast, cells expressing the β Δ 724 mutants, with disrupted cytoskeletal linkages, showed slightly decreased adhesion. Although it is clearly not the only factor, migration rates are altered by changes in cell-substratum adhesiveness with migration generally maximized at intermediate levels of adhesiveness.

Discussion

In this study, we addressed the effects of altered integrin affinity and cytoskeletal linkages on random and haptotactic migration. We demonstrated that locking the integrin receptor in a high affinity state by an activating antibody

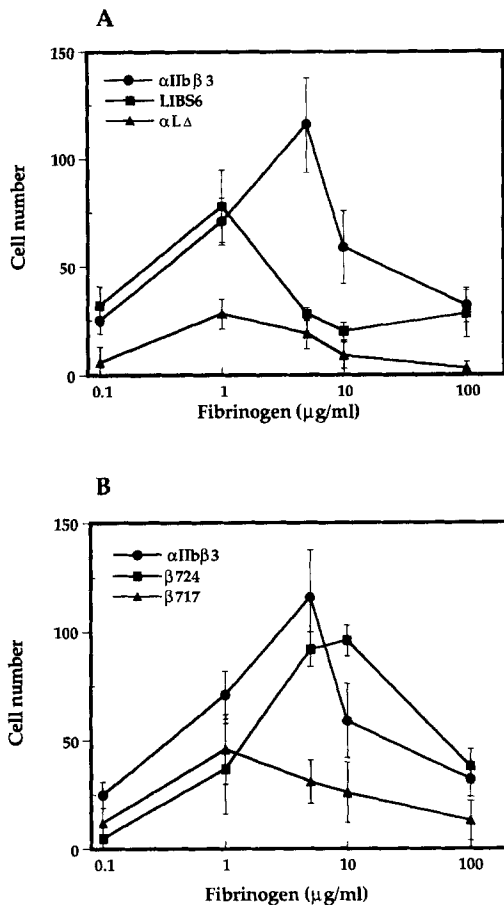


Figure 7. Integrin perturbations altered the optimum substrate concentration for maximal migration. Migration was assayed by random transwell assay on fibrinogen ranging from 0.1–100 μg/ml in CCM1, as described in Materials and Methods. The results represented the average from three separate experiments with error bars showing SD. There was a shift in the substrate concentration–migration curve to the left with high affinity integrin (A). CHO cells expressing αIIbβΔ724, with disrupted focal contacts, migrated maximally at higher substrate concentrations, with a shift in the curve to the right (B).

or mutations in the integrin cytoplasmic domain significantly inhibited both types of cell migration. We further showed that mutations in the cytoplasmic domain of the β chain, which alter cytoskeletal associations without changing ligand-binding affinity, also affected the migration rate. These alterations occurred, in part, through shifts in the substrate concentration optima for maximal migration. We found that transwell assays, when used in both the haptotactic and random configurations, provided complementary information that reflected the role of adhesion strength in migration. These results demonstrate the interplay of different parameters in determining migration rates and support the concept of an optimal adhesive strength as an important regulator of cell migration. Finally, we demonstrated a dominant effect of receptor activation in inhibiting migration regardless of the organization of adhesive complexes or cytoskeletal associations.

Our observations suggest that alterations in the integrin affinity for extracellular ligands play an important role in

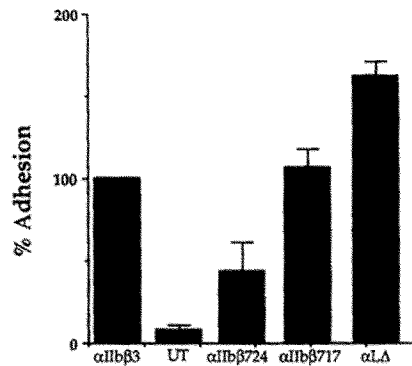


Figure 8. Adhesion assays demonstrated differences in the adhesion of αIIbβ3 and its mutants. Adhesion assays were performed in 96-well plates with cells plated on 10 μg/ml of fibrinogen in CCM1 for 30 min before the shake-off procedure and colorimetric determination described in Materials and Methods. Adhesion was expressed as a percentage of αIIbβ3-mediated adhesion. Each data point represented the mean ± SD from three separate experiments. Nonspecific adhesion of cells to BSA-coated wells was subtracted. CHO cells expressing the high affinity integrin, αLA, showed increased adhesiveness on fibrinogen. CHO cells expressing αIIbβΔ724 showed decreased adhesiveness as compared with the αIIbβ3-expressing CHO cells.

regulating cell migration. We found that a high affinity integrin inhibited migration in the αIIbβ3–CHO system. Observations by time lapse videomicroscopy showed that CHO cells expressing the high affinity mutant had the rear of the cell fixed to the substratum despite lamellipodial projections, suggesting that there may have been inhibited release of adhesions at the cell rear. These findings would be consistent with the notion that the normal migratory cycle may use transient activation of the integrin receptor at the cell front, with subsequent deactivation at the cell rear. Antibody activation of the α4β1 integrin inhibits haptotactic migration of eosinophils, a result similar to ours with the αIIbβ3 integrin expressed in CHO cells (Kuijpers et al., 1993). However, leukocyte migration via the β2 integrins requires receptor activation, although this activation is transient and may in fact be part of a cycle of activation at the cell front with deactivation at the rear (Springer, 1995). In contrast to β2-mediated leukocyte migration, αIIbβ3-mediated migration in CHO cells occurs with the receptor in the low affinity state, with inhibition seen with the high affinity integrin. Clearly, different classes of integrin receptors have different intrinsic affinities for their ligand, and it now seems likely that migration is supported only within a prescribed range of receptor–ligand affinities (Diamond and Springer, 1994). It is intriguing to speculate that spontaneous mutations affecting integrin affinity may occur and potentially increase or decrease the migration rates of certain cell types, such as tumor cells. These observations also suggest potential therapeutic approaches, e.g., gene therapy or novel drug treatments, to control pathological cell migration, such as tumor metastases, by altering integrin receptor affinity. The β3 integrin, in particular, is often expressed on highly motile cells, such as melanoma cells (Albelda et al., 1991), and therefore may be a useful target to control pathological cell migration.

We also found an inverse relationship between focal adhesion organization and cell migration rates. One of the mutations that we studied, α IIB Δ 996 β 3, produced increased organization of focal adhesions and actin microfilament bundles. We found a correlated decrease in migration in CHO cells expressing α IIB Δ 996 β 3, despite having a receptor in the low affinity state. Previous observations also suggest an inverse correlation between the organization of focal adhesions and actin filaments and the cell migration rate. Fibroblast migration decreases as focal adhesion organization increases (Dunlevy and Couchman, 1993). Furthermore, cells that move slowly like fibroblasts have more highly organized adhesive structures than do highly migratory cells, like neural crest cells and neutrophils. Destabilizing focal adhesions may in fact be a mechanism by which certain factors, like growth factors (Dunlevy and Couchman, 1995; Matsumoto et al., 1994) and antiadhesive factors, promote migration (Murphy-Ullrich, 1995; Murphy-Ullrich and Hook, 1989).

Our examination of the relationship between reduced focal adhesion organization and migration rates is complex since we found assay-dependent differences in migration rates. CHO cells expressing the β Δ 724 and β Δ 717 truncations showed greatly reduced focal adhesion organization. When compared with the wild-type α IIB β 3 control, cells expressing the β Δ 717 truncation, which is also locked in a high affinity state, showed inhibited migration. Cells expressing the β Δ 724 truncation have a migration rate that, when compared with the control, was moderately increased by random migration assays but decreased in haptotactic assays. This assay-dependent difference in migration is unique to this mutation. We interpret this as follows: random migration may be limited by the ability of a cell to release adhesions at the cell rear. On the other hand, the geometry of the haptotactic transwell configuration requires that a process emanating from one side of the well migrates through the pore, makes an adhesion, and then facilitates the movement of the cell body through the pore. This would require the cell to form strong adhesive contacts at the cell front and to be less limited by release at the cell rear. Therefore, in the β Δ 724-expressing cells, random migration may increase because of an increased rate of adhesive release at the cell rear. However, in the haptotactic assay, migration may be inhibited either because of an inability to deliver the signals required for haptotaxis or an inability to form adhesions at the cell front that are strong enough to mediate the migration.

The β -subunit truncation mutations are thought to affect focal adhesion organization via impaired linkage of integrins to the actin cytoskeleton. These truncations likely influence not only focal adhesion organization but also the strength of the linkages thought to mediate migration. A recent study shows that the NPXY motif, which is required for the localization of the β 3 integrin to focal contacts, is also necessary for normal haptotactic migration (Filardo et al., 1995). Our results are consistent with their observations since the α IIB β Δ 724-expressing CHO cells showed markedly inhibited haptotactic migration. It is interesting that β Δ 724-expressing cells migrated rapidly by random assays despite a large truncation of the cytoplasmic domain. Since antibodies and peptides that inhibit adhesion through the α IIB β 3 receptor also inhibited migration, the migra-

tion on fibrinogen was in fact mediated by the α IIB β Δ 724 receptor. It is important to also note that the CHO cells have no endogenous β 3 integrin that associates with the transfected α IIB integrin. Therefore, it is likely that either other regions in the β -subunit cytoplasmic domain, the α -subunit cytoplasmic domain, or another receptor that associates with the α IIB β 3 receptor mediates the cytoskeletal linkages. With regard to this latter possibility, phosphorylation of cell surface receptors can organize complexes of signaling constituents, which might also serve to recruit cytoskeletal components.

How do alterations in receptor affinity and cytoskeletal organization affect migration rates? Our data showed that a shift in the substrate concentration optimum was a major contributor to the observed changes in migration rates. This is consistent with the notion that adhesive strength can regulate migration since both affinity and substrate concentration contribute to the strength of adhesion (DiMilla et al., 1991). However, it is also clear that shifts in substrate concentration optima account for only some of the inhibitions since some mutations, e.g., α L Δ , showed greatly inhibited migration, even when the rates were compared at their respective concentration optima. While some decrease is predicted by the model of DiMilla et al. (1991), the shifts for LIBS6-treated cells and α L Δ -expressing cells were similar, yet the maximum migration rates differed significantly. These results suggest that many observed inhibitions in migration may in fact be shifts in curves for optimal migration, and that the observed alterations in migration rate may in fact only be seen at specific substrate concentrations. Thus, the mechanisms of migratory regulation are likely very complex and involve inter-related parameters (Table III). For example, ligation of integrin receptors stimulates signal transduction events that affect many downstream processes including organization of adhesive complexes and molecules involved in integrin-actin linkages. The substrate density can also affect the organization of adhesive complexes since the density determines the degree of clustering, which in turn determines which molecules associate with the complex, as well as the fraction of integrins that are ligated. Finally, adhesive interactions and cytoskeletal integrin connections occur via highly organized and potentially stable clusters that likely are much stronger than those mediated by a similar number of dispersed, single molecules.

In summary, our results suggest that modulation of the

Table III. Summary of Integrin-mediated Affinity, Cytoskeletal Linkages, and Migration in CHO Cells Expressing α IIB β 3 Integrin and Mutants

Mutant	Affinity	Focal contact	Morphology (spreading)	Migration
α IIB β 3	low	+	++	NL
α L Δ β 3	high	++	++++	DEC
α L β 3	low	+	++	NL
α IIB Δ 996 β 3	low	+++	++	DEC
α IIB β Δ 724	low	0	-	*
α IIB β Δ 717	high	0	+	DEC

High affinity refers to presence of PAC1 binding.

*The β Δ 724-expressing cells showed increased migration by random assay and decreased migration by haptotactic assay on 10 μ g/ml fibrinogen.

NL, normal; DEC, decreased.

strength of integrin–ligand or –cytoskeletal interactions plays an important role in regulating cell migration. Our results point to several mechanisms for altering cell–substrate adhesiveness and thus modulating migration rate. For example, changes in integrin affinity and focal adhesion organization, in combination with alterations in substrate concentration, have major effects on the cell migration rate. Furthermore, we show, in particular, that freezing the integrin receptor in the high affinity state has a dramatic effect on cell migration and provides a potential mechanism to rapidly alter the migratory characteristics of cells. Further understanding of the basic mechanisms by which integrins mediate cell migration should facilitate the design of therapeutic interventions for control of pathologic cell migration, such as tumor metastasis or chronic inflammation.

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