

## Physical and biochemical regulation of integrin release during rear detachment of migrating cells

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Accepted 15 January; published on WWW 9 March 1998

### SUMMARY

Cell migration can be considered as a repeated cycle of membrane protrusion and attachment, cytoskeletal contraction and rear detachment. At intermediate and high levels of cell-substratum adhesiveness, cell speed appears to be rate-limited by rear detachment, specifically by the disruption of cytoskeleton-adhesion receptor-extracellular matrix (ECM) linkages. Often, cytoskeletal linkages fracture to release integrin adhesion receptors from the cell. Cell-extracellular matrix bonds may also dissociate, allowing the integrins to remain with the cell. To investigate molecular mechanisms involved in fracturing these linkages and regulating cell speed, we have developed an experimental system to track integrins during the process of rear retraction in Chinese hamster ovary (CHO) cells. Integrin expression level was varied by transfecting CHO B2 cells, which express very little endogenous  $\alpha 5$  integrin, with a plasmid containing human  $\alpha 5$  integrin cDNA and sorting the cells into three populations with different  $\alpha 5$  expression levels. Receptor/ligand affinity was varied using CHO cells transfected with either  $\alpha \text{IIb}\beta 3$  or  $\alpha \text{IIb}\beta 3(\beta 1-2)$ , a high affinity variant.  $\alpha \text{IIb}\beta 3(\beta 1-2)$  is activated to a higher affinity state with an anti-LIBS2 antibody. Fluorescent probes were conjugated to non-adhesion perturbing anti-integrin antibodies, which label integrins in CHO cells migrating on a matrix-coated glass coverslip. The rear retraction area was determined using phase contrast

microscopy and integrins initially in this area were tracked by fluorescence microscopy and a cooled CCD camera.

We find that rear retraction rate appears to limit cell speed at intermediate and high adhesiveness, but not at low adhesiveness. Upon rear retraction, the amount of integrin released from the cell increases as extracellular matrix concentration, receptor level and receptor-ligand affinity increase. In fact, integrin release is a constant function of cell-substratum adhesiveness and the number of cell-substratum bonds. In the adhesive regime where rear detachment limits the rate of cell migration, cell speed has an inverse relationship to the amount of integrin released at the rear of the cell. At high cell-substratum adhesiveness, calpain, a  $\text{Ca}^{2+}$ -dependent protease, is also involved in release of cytoskeletal linkages during rear retraction. Inhibition of calpain results in decreased integrin release from the cell membrane, and consequently a decrease in cell speed, during migration. These observations suggest a model for rear retraction in which applied tension and calpain-mediated cytoskeletal linkage cleavage are required at high adhesiveness, but only applied tension is required at low adhesiveness.

Key words: Cell migration, Cell adhesion, Rear detachment, Integrin, Calpain, CHO cell

### INTRODUCTION

To migrate, a cell must be able to convert intracellularly generated forces into traction forces to propel itself through its environment. The complex process of migration can be usefully perceived as a dynamic arrangement of a number of distinct events, including membrane protrusion, formation of stable contacts between the cell and extracellular matrix (ECM), cytoskeletal contraction, cell body translocation and release of cell-substratum adhesions at the rear of the cell (Stossel, 1993; Sheetz, 1994; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). In some circumstances,

proteolytic degradation of extracellular matrix is also required (Werb, 1997). Cell migration is not likely to be universally limited over a comprehensive range of movement environments by any single one of these individual motility processes (Lauffenburger and Horwitz, 1996). For instance, in some circumstances migration speed seems to be governed by lamellipod extension and attachment (Wessels et al., 1994), whereas in others rear detachment appears to be limiting (Marks et al., 1991; Jay et al., 1995). In general, quantitative studies elucidating conditions under which various motility processes are rate-limiting for cell migration speed have not yet been pursued.

Cell migration is regulated, at least in part, through cell-substratum adhesive interactions, with maximum speed occurring at an intermediate adhesiveness (Goodman et al., 1989; Duband et al., 1991; DiMilla et al., 1993; Keely et al., 1995; Huttenlocher et al., 1996; Palecek et al., 1997). At low adhesiveness, cytoskeletal forces disrupt cell-substratum attachments so that cells are unable to generate the traction needed for locomotion. At high adhesiveness, cytoskeletal forces are insufficient to break cell-substratum attachments, leaving cells spread but incapable of locomotion. At intermediate adhesiveness, cytoskeletal forces are roughly in balance with adhesion so that traction can be maintained at the cell front while it can be disrupted at the cell rear, permitting net cell body movement (DiMilla et al., 1991; Sheetz, 1994; Huttenlocher et al., 1995).

In environments where rear detachment limits cell migration speed, it can be hypothesized that breakage of cell-substratum attachments is an important local regulatory event. Low-affinity transmembrane glycoprotein adhesion receptors, including members of the heterodimeric integrin family, typically mediate cell adhesion to ECM ligands (Hynes, 1992). In highly adherent cells, integrins and cytoskeletal components can associate into organized focal adhesions, which serve as regions of close contact between the cell and substratum and also transmit signals between the cell and its environment (Burrige et al., 1988). In motile cells integrins also form a linkage between the cytoskeleton and ECM, but these adhesion structures are less organized and more dynamic in migrating cells than in highly adherent, stationary cells (Couchman et al., 1982; Duband et al., 1988; Regen and Horwitz, 1992).

Breakage of cell-substratum attachments needed to allow locomotion can, in principle, occur by either intracellular or extracellular fracture of the cytoskeletal-integrin-ECM linkage. For fibroblasts migrating in vitro, a large fraction of integrins in adhesion structures has been found to be released from the cell and left on the substratum (Regen and Horwitz, 1992; Palecek et al., 1996). The remaining integrins release from the substratum and either move forward along the cell edge as an aggregate to form a new adhesion or disperse into the cell membrane. Interestingly, integrin release from the cell membrane also appears to occur during the migration through extracellular matrices by corneal fibroblasts in vivo (Hay, 1985) and by tumor cell lines in vitro (Niggemann et al., 1997). Mechanisms of rear release include contributions from cytoskeletal contractility. *Dictyostelium* expressing myosin II mutants show inhibited migration on adhesive substrata (Jay et al., 1995) and antibodies against myosin light chain kinase inhibit migration when injected into macrophages (Wilson et al., 1991).

The mechanism that allows intracellular release of cytoskeletal connections at the rear of a migrating cell is also likely to involve a regulated, biochemical process. The addition of ATP to permeabilized fibroblasts (Crowley and Horwitz, 1995) results in the rapid breakdown of focal adhesions, detachment from the substratum, and integrin tracks where cells resided prior to detachment. This is attributed to tyrosine phosphorylation of cytoskeletal components and tension generated by cell contraction. Surges in intracellular  $Ca^{2+}$  levels have been detected during rear release in neutrophils (Marks et al., 1991). These calcium transients have been implicated in adhesive release of neutrophils migrating on

vitronectin through the  $Ca^{2+}$ -calmodulin-dependent phosphatase calcineurin (Maxfield, 1993; Hendey et al., 1992). Calpain, a  $Ca^{2+}$ -dependent protease which localizes to focal adhesions (Beckerle et al., 1987), regulates cell locomotion and rear retraction in CHO cells migrating on fibronectin or fibrinogen by destabilizing cytoskeletal linkages (Huttenlocher et al., 1997). In vitro, calpain cleaves the cytoplasmic domain of the  $\beta$  subunit of integrins as well as other cytoskeletal molecules including focal adhesion kinase (FAK) and talin (Du et al., 1995; Cooray et al., 1996; Inomata et al., 1996).

In the present study we address the issue of how the release of adhesions at the cell rear can regulate the rear detachment rate and cell speed. We track integrins at the rear of migrating CHO cells as we vary cell-substratum adhesiveness through ECM concentration, receptor expression and receptor-ligand affinity. Using a fluorophore conjugated to anti-integrin monoclonal antibodies we can visualize and quantitate the relative fraction of integrin which detaches from the cell upon rear retraction. We find that the fraction of integrins which releases from the cell is quite variable at any condition, ranging from virtually none to virtually all of the integrin in the detachment area. The mean amount of integrin which rips from the cell increases as cell-substratum adhesiveness increases, due to changes in ECM concentration, receptor expression or receptor-ligand affinity. The amount of integrin that rips from the cell does not monotonically correlate with cell speed over the entire range of cell adhesiveness. However, at adhesiveness levels where migration speed is limited by rear detachment, cell speed has an inverse relationship to the amount of integrin released at the cell rear. These results suggest that the cytoskeleton-integrin-ECM linkage is an important locus for the regulation of rear detachment and, consequently, cell migration speed, and that both physical and biochemical mechanisms are involved in release of adhesions at the rear of a migrating cell. A biochemical mechanism of integrin release from migrating cells involves calpain regulation of cytoskeletal linkages. Calpain inhibition diminishes rear detachment rate and causes fewer integrins to release from the rear of migrating cells, suggesting that the fracture occurs mainly at an intra- rather than an extra-cellular locus.

## MATERIALS AND METHODS

### Antibodies and reagents

Fibronectin was prepared from human plasma as described previously (Ruoslahti et al., 1982). Fibrinogen was obtained from Sigma Chemical Company. The  $\alpha$ IIb $\beta$ 3 activating monoclonal antibody mAb62, which recognizes an epitope on  $\beta$ 3, and the non-adhesion perturbing  $\alpha$ IIb $\beta$ 3 monoclonal antibody D57, were used as described previously (O'Toole et al., 1990, 1994; Frelinger et al., 1991). The anti-human  $\alpha$ 5 monoclonal antibody 6F4 was a gift of Dr Ralph Isberg (Tufts University).

Stock solutions of calpain inhibitor I (Boehringer Mannheim, Indianapolis, IN, USA) were prepared at a concentration of 10 mg/ml in ethanol. Calpain inhibitor I was used at a concentration of 50  $\mu$ g/ml. Cells were preincubated with calpain inhibitor for 30 minutes prior to the experiments and maintained in medium containing calpain inhibitor throughout the experiments. These inhibitors have no cell toxicity effects up to concentrations of 100  $\mu$ g/ml.

D57 mAb was conjugated to Oregon Green 488 carboxylic acid, succinimidyl ester (Molecular Probes) and 6F4 mAb was conjugated

to carboxy-X-rhodamine, succinimidyl ester (Molecular Probes). 180  $\mu$ l of mAb diluted to 1.25 mg/ml in 0.1 M sodium bicarbonate at pH 8.3 was stirred for 1 hour at room temperature with 10  $\mu$ l of the fluorophore dissolved in DMSO at 10 mg/ml. 10 ml of 1.5 M hydroxylamine, pH 8.5, was stirred with the reaction mixture for 1 hour at room temperature to stop the reaction and remove dye from unstable conjugates. Conjugated mAb was separated from free dye by gel filtration on Sephadex G-25 minicolumns, prepared as follows. The ends of glass Pasteur pipettes were broken off and the remaining center cylinder was acid-washed and silanated to reduce protein adsorption to the glass surface. A small piece of cotton was placed in the bottom of the pipette and 1 ml Sephadex G-25 (Pharmacia Inc.), swelled in phosphate-buffered saline (PBS), was added to the pipette and allowed to settle while the column was rinsed with several volumes of  $\text{NaHCO}_3$  (pH 8.3). The mAb-dye solution was added to the top of the column and allowed to drain into the column.  $\text{NaHCO}_3$  (pH 8.3), was added to the column and the first fluorescent peak was collected, divided into samples, wrapped in foil and stored under argon at  $-80^\circ\text{C}$ .

The human  $\alpha 5$  cDNA (Argraves et al., 1987) was a gift of Dr Louis Reichardt and was subcloned into the eukaryotic expression vector pRSVneo (Reszka et al., 1992; Sastry et al., 1996).

### Cell culture and transfection

$\alpha 5$ -deficient CHO B2 cells were provided by Dr Rudolph Juliano (University of North Carolina, Chapel Hill). Generation of  $\alpha \text{IIb}\beta 3$  and  $\alpha \text{IIb}\beta 3(\beta 1-2)$  expressing CHO cells is described elsewhere (O'Toole et al., 1989; Bajt et al., 1992). The cells were grown in DMEM containing 10% FBS, 2 mM glutamine and 1% nonessential amino acids.

CHO B2 cells were transfected with human  $\alpha 5$ -cDNA using Lipofectamine (GIBCO-BRL) according to the manufacturer's protocols. The transfected cells were selected in DME containing 100  $\mu\text{g/ml}$  G418 and maintained in DME containing 50  $\mu\text{g/ml}$  G418. Cells expressing the human  $\alpha 5$  integrin subunit were selected by flow cytometry.

Microscopy plates were constructed by punching a hole in the bottom of a 35 mm tissue culture dish. Glass coverslips (22 mm<sup>2</sup>, No. 1) were acid-washed in 20%  $\text{HNO}_3$  for 30 minutes, rinsed with deionized water overnight, and silanated by exposure to hexamethyldisilazane vapor (Sigma) for 30 minutes at  $200^\circ\text{C}$  (Regen and Horwitz, 1992) to block hydrophilic charged groups and reduce nonspecific cell adhesion to the glass surface. The coverslips were attached to the bottoms of the dishes with clear silicone rubber sealant (Dow Corning). Prior to use, the plates were sterilized with 70% ethanol and rinsed with sterile deionized water.

During migration experiments, cells in exponential growth phase were removed from tissue culture plates with a 1 ml wash consisting of 0.02% EDTA in calcium- and magnesium-free phosphate buffer solution (CMF-PBS) followed by a 3 minute treatment with 150  $\mu\text{l}$  of 0.25% trypsin CMF-PBS containing 0.02% EDTA. The coverslip in the bottom of a microscope plate was coated with ECM protein, diluted to the desired concentration in PBS, for 1 hour at  $37^\circ\text{C}$  and then blocked with 1% BSA for 30 minutes at  $37^\circ\text{C}$ . Cells were resuspended in microscopy medium, CCM1 (Hyclone Laboratories, Logan, UT) containing 50 units/ml penicillin and 50  $\mu\text{g/ml}$  streptomycin. The cells were seeded at low density so very few cells were touching other cells.

### Flow cytometry

Quantitative cell surface expression of integrins was determined by flow cytometry as described (Loftus et al., 1990) using the 6F4 mAb at 1:4 dilution of hybridoma supernatant to assay  $\alpha 5$  expression and D57 mAb at 1:200 dilution of mouse ascites to assay  $\alpha \text{IIb}\beta 3$  expression. The cells were washed with PBS and detached from plates with 0.02% EDTA in calcium- and magnesium-free Hepes-Hanks buffer (CMF-HH). Cells were incubated at  $4^\circ\text{C}$  for 30 minutes with

primary antibody and then washed twice with blocking buffer (2% BSA in CMF-HH). Cells were then incubated at  $4^\circ\text{C}$  with the fluorescein-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) for 30 minutes, washed twice with blocking buffer and suspended in CMF-HH. A FACS-STAR (Becton-Dickson) was used to sort  $\alpha 5$ -expressing cells into populations with different relative expression levels and to sort  $\alpha \text{IIb}\beta 3(\beta 1-2)$ -expressing cells into similar surface expression profiles as  $\alpha \text{IIb}\beta 3$  transfected cells. Surface expression of  $\alpha 5$  transfected cells remained constant for more than 3 weeks while expression in  $\alpha \text{IIb}\beta 3(\beta 1-2)$  transfected cells began to fluctuate after 5 days, so these cells were used within 5 days of sorting.

### Immunofluorescence and phase microscopy

Cells were allowed to attach to the microscope plate for 2 hours prior to staining. The cells were then incubated for 30 minutes at  $37^\circ\text{C}$  with Oregon Green-conjugated mAb D57 or rhodamine-conjugated mAb 6F4, diluted to 40  $\mu\text{g/ml}$  in warm CCM1. The cells were rinsed 5 $\times$  with warm CCM1 and 3 ml of warm CCM1 was added to the plate. Stained cells were placed in a temperature regulated, humidified chamber, described previously (Regen and Horwitz, 1992), mounted on the inverted microscope stage. Warmed, humidified 10%  $\text{CO}_2$  was passed over the cells to maintain correct pH. A field with 3-5 cells which were stained brightly with respect to the background was selected. Fluorescence images of the cells were acquired every 30 minutes for 2 hours using a 3-second exposure. Cell position as a function of time was determined by acquiring phase contrast images immediately after the fluorescence images.

A Nikon Diaphot inverted microscope with a  $\times 60/1.4$  NA phase planapochromat objective was used for immunofluorescence studies. An electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) controlled the fluorescence illumination by a 100 W mercury lamp. Phase contrast images were illuminated using a 50 W halogen lamp. A cooled CCD camera (CE200A, Photometrics, Tucson, AZ) acquired and digitized images, which were sent to a Quadra 950 (Apple Computer) for analysis. Oncor Image software (Oncor Imaging, Rockville, MD) was used to control the camera shutter and process the images.

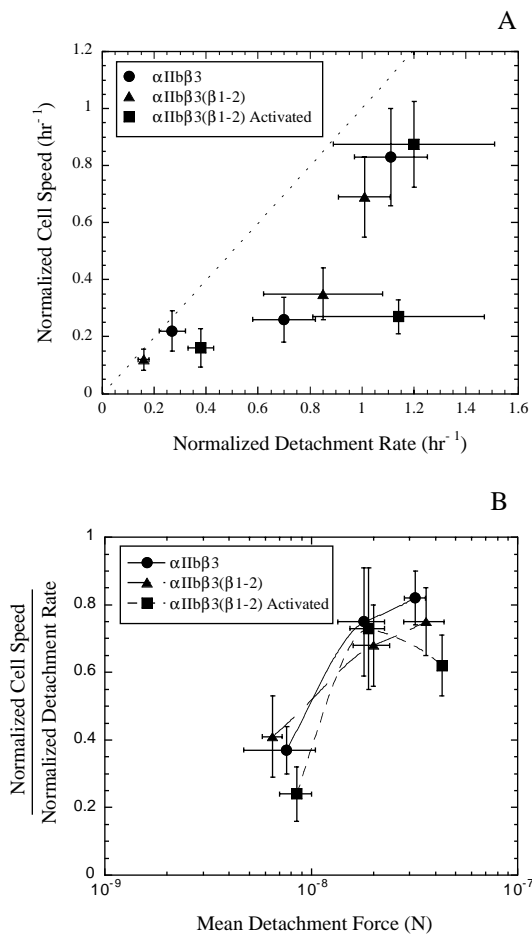
### Image analysis

Cell perimeter at each time point was determined by manually tracing cell edges on the phase contrast images. The cell area which retracted between time 1 ( $t_1$ ) and time 2 ( $t_2$ ) was determined by subtracting a mask of the cell area at time 2 ( $t_2$ ) from the mask at  $t_1$ . The fraction of integrin which ripped from the cell upon rear retraction between  $t_1$  and  $t_2$  was calculated as the ratio of mean fluorescence intensity in the retraction area after detachment to the mean fluorescence intensity in the retraction area before detachment. Average intensity outside the cell was subtracted from the mean intensities to correct for background fluorescence. Retractions of lamellipodia were neglected and retraction areas less than 2  $\mu\text{m}$  wide were also not used. If a cell retracted from the same area more than once, only the first retraction was used. 75-150 different detachments in at least eight different cells were measured at each condition. Fluorescence diminishment due to photobleaching was negligible for the exposure times used. Intensity of integrin patches which ripped from the cell remained constant for several hours after detachment.

## RESULTS

### Rear detachment is rate-limiting for cells migrating at intermediate and high adhesiveness

Intuitively, one expects cell speed to be limited by the formation of adhesions and the generation of traction forces at low cell-substratum adhesiveness, and by release of adhesions



**Fig. 1.** The rate of rear release limits cell migration speed at intermediate and high, but not at low, adhesiveness. (A) Cell speed, normalized to cell diameter, is plotted as a function of rear detachment rate, normalized to cell area, for CHO cells expressing  $\alpha$ IIb $\beta$ 3 and  $\alpha$ IIb $\beta$ 3( $\beta$ 1-2) integrins on different concentrations of fibrinogen. Mean cell diameter and mean cell area were calculated from phase contrast images of more than 50 cells at each fibrinogen concentration. The dotted line indicates where speed equals detachment rate. The ratio of speed to detachment rate indicates how efficiently the detachments move the cell centroid. (B) Speed to detachment ratio is plotted as a function of cell-substratum adhesiveness. Short-term mean detachment force was measured by shear flow detachment of cells after a 20 minute incubation on the surface (Palecek et al., 1997). At adhesiveness values below  $10^{-8}$  N, where the cells are not adherent enough to migrate well, the ratio of speed to detachment rate is below 0.4. At adhesiveness above  $2 \times 10^{-8}$  N, where the cells migrate at maximum speed, the ratio of speed to detachment rate is above 0.6. These results suggest that at intermediate and high adhesiveness, cell detachment rate limits overall migration speed. Error bars on detachment force represent 95% confidence intervals on the mean, and error bars on speed and detachment rates represent s.e.m.

at high adhesiveness. We addressed the issue of release at high adhesiveness by comparing the rate of cell-substratum detachment to cell speed. Cell-substratum detachment rate was measured by outlining areas of a cell which detached from the substrate during a 30 minute time interval, and cell speed was assayed by measuring cell centroid displacement at 15 minute

time intervals and fitting the path to a persistent random walk model.

If rear detachment were the rate-limiting step for migration speed, the rate of detachment should roughly equal the cell speed. Cell speed, normalized to cell diameter, is plotted as a function of detachment rate in Fig. 1A. Mean cell diameter and area were calculated from phase contrast images of more than 50 cells at each ECM concentration. At very low adhesiveness, detachment rates are significantly higher than cell speed. This means that the cell is detaching from the substratum in multiple directions simultaneously, resulting in little movement of the cell centroid. Very low detachment rates correspond to high adhesiveness. In this regime, cell speed is about equal to detachment rate. At intermediate adhesiveness, the detachment rate and migration speed are both high.

The ratio of migration speed to detachment rate provides a quantitative measure of how efficiently the cell detaches. A high ratio of speed to detachment rate indicates that most of the detachments lead to a change in cell centroid position and suggests that cell speed is limited by the rate of detachment (Fig. 1B). A low ratio indicates that the detachments do not effectively produce movement of the cell centroid and that speed is limited by another aspect of motility. At low adhesiveness ( $< 10^{-8}$  N) the ratio of speed to detachment rate ranges from 0.2-0.4, suggesting that cell speed is not limited by rear detachment under these conditions. However, at adhesiveness of  $2 \times 10^{-8}$  N, where the cells migrate at maximum speed, and above, the ratio of speed to detachment rate is 0.6-0.8. Under these conditions cell speed is limited by rear detachment rate.

When locomotion occurs at essentially a steady state, the rate of detachment must equal the rate of formation of new attachments to maintain an approximately constant cell area. Time-lapse videomicroscopy indicates that at high ECM concentrations these cells extend membranes at rates significantly greater than they locomote or retract stable adhesions (data not shown), indicating that membrane protrusion does not limit cell speed at high adhesiveness. The probability of these protrusions forming stable attachments to the substratum may also limit cell speed. However, the probability of forming stable adhesions is likely to increase as cell adhesiveness increases, so adhesion formation is not consistent with limiting cell speed at high adhesiveness.

### Integrin release from the rear of migrating cells increases with ECM concentration, integrin expression and integrin-ECM affinity

Since the detachment rate limits cell speed at high adhesiveness, molecular parameters that affect cell detachment will also limit cell speed at high adhesiveness. One expects that the rate of rear detachment is primarily a function of the contractile force generated at the cell rear and the strength of integrin-mediated linkages between the cell and substrate. At high adhesiveness, for example, more cell-substratum bonds exist, inhibiting rear retraction. We explored the locus of cell-substratum linkage fracture in regulating cell detachment, and the consequential effects on cell speed, as a function of adhesiveness. If the integrin-ECM linkage were to fracture, the integrins would detach from the substratum and remain in the cell, but if integrin-cytoskeleton or other cytoskeletal bonds were to fracture, the integrins would rip from the cell membrane and

remain attached to the substratum. Thus the amount of integrin which rips from the cell is a measure of the relative strength of integrin-ligand bond compared to other bonds in the linkage, with the weaker bonds preferentially fracturing. A mechanism of active linkage release would probably act intracellularly and lead to an increase in the amount of integrin which rips from the cell during rear retraction.

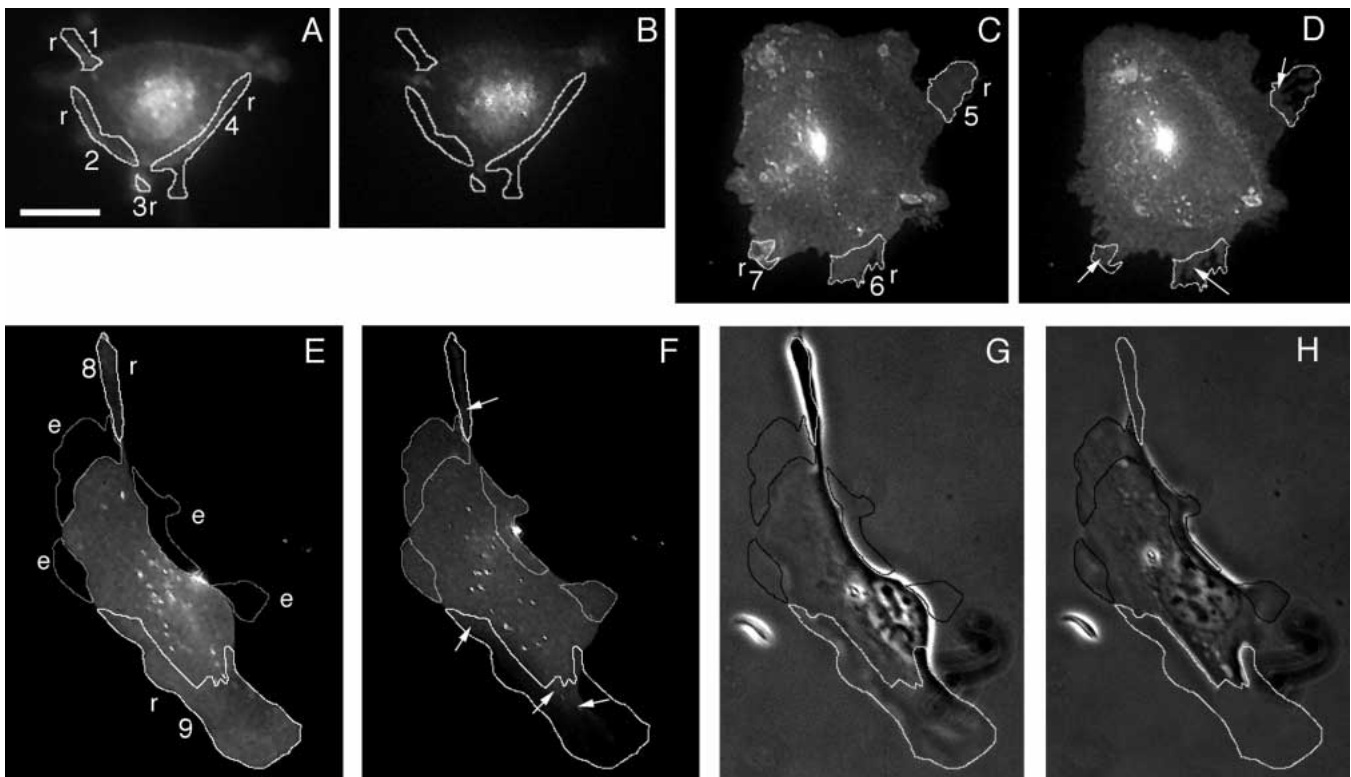
We determined the locus of fracture of the cytoskeleton-ECM-integrin linkage by tracking integrins at the rear of migrating cells. We used nonadhesion-perturbing anti-integrin mAbs conjugated with fluorescent dyes to determine the fate of integrin adhesion receptors at the rear of migrating CHO cells. We used phase contrast imaging to determine the cell retraction area and quantitative fluorescence microscopy to measure the fraction of integrin which ripped from the cell in this area upon rear retraction. Cell-substratum adhesiveness was varied by altering the number of cell-substratum bonds through three variables: ECM protein concentration, receptor expression level and receptor ligand affinity.  $\alpha 5$ -deficient CHO B2 cells were transfected with a human  $\alpha 5$  cDNA and sorted by flow cytometry into populations with three different relative  $\alpha 5$  expression levels (0.17 $\times$ , 0.47 $\times$  and 1 $\times$ ) to vary  $\alpha 5\beta 1$

**Table 1. Soluble  $^{125}\text{I}$ -fibrinogen binding affinities ( $K_d$ ) to recombinant integrins\***

	$\alpha\text{IIb}\beta 3$	$\alpha\text{IIb}\beta 3(\beta 1-2)$
Resting	$<1.4 \times 10^4 \text{ M}^{-1}$ ( $K_d > 7 \text{ mM}$ )	$4.85 \pm 0.84 \times 10^6 \text{ M}^{-1}$ ( $K_d = 206 \text{ nM}$ )
mAb 62-activated	$1.66 \pm 0.33 \times 10^7 \text{ M}^{-1}$ ( $K_d = 60 \text{ nM}$ )	$4.55 \pm 0.77 \times 10^7 \text{ M}^{-1}$ ( $K_d = 22 \text{ nM}$ )

\*From Bajt et al., 1992.

fibronectin receptor expression. To vary integrin-ECM affinity we used CHO cells transfected with the  $\alpha\text{IIb}\beta 3$  fibrinogen receptor, an extracellular domain mutation of the  $\beta 3$  subunit in which 6 amino acids of the ligand-binding domain (Bajt et al., 1992) are replaced with sequences derived from the  $\beta 1$  integrin subunit. This mutation increases the affinity of the integrin for fibrinogen. An even higher affinity state occurs when  $\alpha\text{IIb}\beta 3(\beta 1-2)$  is activated with anti-LIBS2 antibody (Frelinger et al., 1991). Soluble fibrinogen binding affinities for  $\alpha\text{IIb}\beta 3$  and  $\alpha\text{IIb}\beta 3(\beta 1-2)$  in resting and mAb 62-activated state are shown in Table 1.



**Fig. 2.** Integrins release from the rear of CHO cells during migration. Anti- $\alpha\text{IIb}\beta 3$  integrin immunofluorescence images of single living cells labeled with the non-adhesion perturbing mAb D57 conjugated to Oregon Green fluorophor show the movement of integrins during rear retraction on 0.6  $\mu\text{g/ml}$  (A,B), 5  $\mu\text{g/ml}$  (C,D) and 2  $\mu\text{g/ml}$  (E,F) fibrinogen. The area of the cell which retracts between 0 minutes (A,C,E) and 30 minutes (B,D,F) was determined from phase contrast images and is traced on the fluorescence images in light gray (labeled r). Areas of the cell which extend are traced in dark gray (labeled e). Retraction areas are numbered and average pixel intensities before and after detachment are shown in Table 2. Phase contrast images for the cells at 2 mg/ml fibrinogen (G,H) correspond to the fluorescence images in E and F. Detachment areas between G and H are traced in black and attachment areas are traced in white. At 0.6  $\mu\text{g/ml}$  fibrinogen (A,B), cells are quite round and when they retract, most of the integrins dissociate from the substratum and remain with the cell. At 5  $\mu\text{g/ml}$  fibrinogen (C,D), cells are spread and release most of their integrin onto the substratum upon rear retraction. At 2  $\mu\text{g/ml}$  fibrinogen (E,F) an intermediate level, the cells release an intermediate amount of integrin on the substratum. Arrows in D and F indicate examples of patches of integrin which have detached from the cell. Bar, 10  $\mu\text{m}$ .



**Table 2. Quantitative analysis of integrin release during rear retraction**

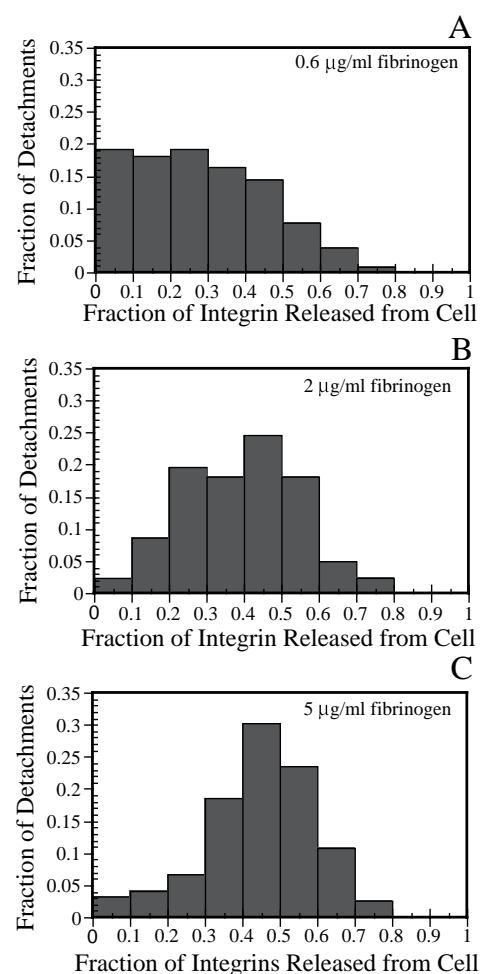
Retraction number*	Fibrinogen concentration (µg/ml)	Average pixel intensity†			Fraction intensity detached F†
		Before detachment I <sub>1</sub>	After detachment I <sub>2</sub>	Background I <sub>B</sub>	
1	0.6	2371	1327	1058	0.205
2	0.6	1623	1131	1003	0.207
3	0.6	2577	1067	1040	0.011
4	0.6	1804	1262	1044	0.352
5	2	1612	1414	945	0.703
6	2	1472	1363	930	0.799
7	2	1530	1485	927	0.926
8	5	1189	934	658	0.528
9	5	1423	1026	669	0.474

\*See Fig. 2 for explanation.  
†F=(I<sub>1</sub>-I<sub>2</sub>)/(I<sub>2</sub>-I<sub>B</sub>).

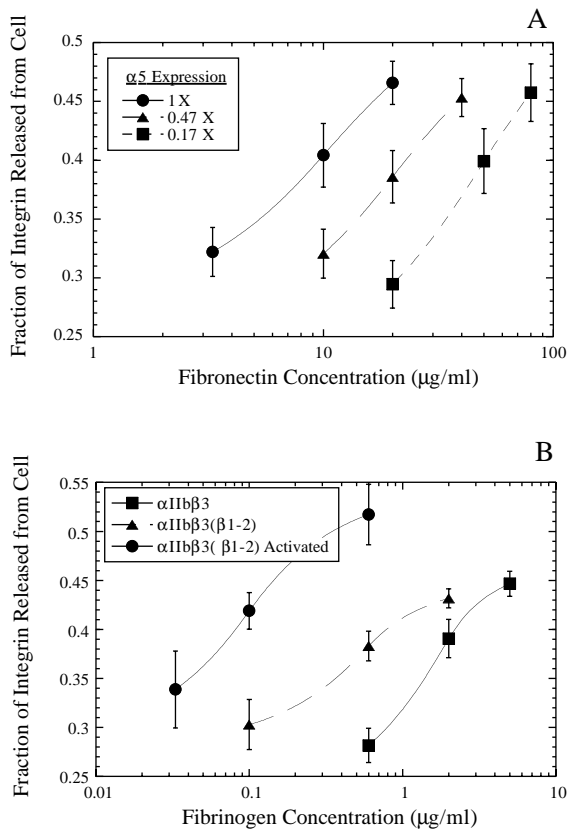
Migrating CHO cells organize integrins into smaller, less concentrated clusters than migrating fibroblasts. Integrins form larger clusters as ECM concentration, integrin expression level and integrin-ECM affinity increase. The resolution of individual adhesion structures was too low to accurately determine their sizes and intensities, however, so we focused on comparing the amount of integrin in the entire membrane retraction area pre- and post-retraction. Endocytic vesicles containing integrins tend to accumulate in the perinuclear region of CHO cells, as in fibroblasts (Regen and Horwitz, 1992; Palecek et al., 1996). Fig. 2 shows wild-type  $\alpha$ Ib $\beta$ 3 integrin movement in CHO cells migrating on different concentrations of fibrinogen. At low fibrinogen concentrations (Fig. 2A,B) the majority of the integrin in the rear retraction area detaches from the substratum and moves with the cell. At high fibrinogen concentrations the majority of the integrin in the retraction area rips from the cell and remains attached to the surface as an integrin trail (Fig. 2C,D). At intermediate fibrinogen concentrations, an intermediate amount of integrin tends to detach from the cell (Fig. 2E,F). Average pixel intensities before (I<sub>1</sub>) and after (I<sub>2</sub>) detachment and average background intensities (I<sub>B</sub>) are shown in Table 2. The fraction of fluorescence intensity (F) remaining in the detachment area after rear retraction is calculated as:  $F=(I_1-I_B)/(I_2-I_B)$ .

Fig. 3 shows histograms of the fraction of  $\alpha$ Ib $\beta$ 3 integrin that is left in the retraction area from each detachment event after the cell rear detaches from substrates coated with different amounts of fibrinogen. At each fibrinogen concentration a wide range of fractions exists, ranging from virtually no integrin to virtually all of the integrin ripping from the cell in the retraction area. At low fibrinogen concentrations less integrin tends to rip from the cells than at higher concentrations, which is consistent with visual observations (Fig. 2). A small fraction of integrin (<20%) is commonly left on the surface at 0.6 µg/ml fibrinogen, when the cells are not adherent enough to migrate well. At least some integrin is almost always left when the cells are on fibrinogen concentrations where they migrate optimally (2 µg/ml) or are too adherent to migrate optimally (5 µg/ml). The mean fraction of integrin deposited on the surface increases from  $0.27\pm 0.18$  at 0.6 µg/ml fibrinogen to  $0.46\pm 0.15$  at 5 µg/ml fibrinogen.

Integrins become more likely to release from the cell as ECM concentration increases (Fig. 3). To determine whether this is generally due to an increase in cell-substratum adhesiveness and a corresponding increase in the number of



**Fig. 3.** Histograms showing the amount of integrin released from CHO cells migrating on 0.6 µg/ml (A), 2 µg/ml (B) and 5 µg/ml (C) fibrinogen for different rear retraction events. The amount of integrin which releases from the cell in the detachment area at the rear of a migrating cell was measured by quantitative immunofluorescence of anti-integrin mAbs. At each fibrinogen concentration a variable range of integrin, ranging from 0-80%, can be released from the cell. The distribution is different at each fibrinogen concentration, however. At 0.6 µg/ml fibrinogen (A) 0-30% of the integrin in the detachment area is usually released from the cell. At 2 (B) and 5 (C) µg/ml fibrinogen, 30-60% of the integrin in the detachment area is typically released from the cell.  $n=104$  (A), 81 (B), 119 (C).

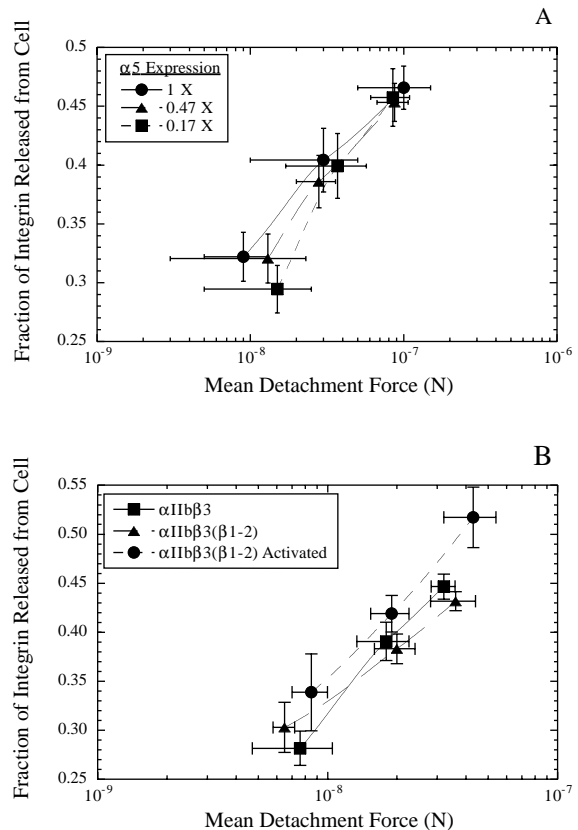


**Fig. 4.** Integrin release increases as receptor number, ligand affinity and substrate concentration increases. The amount of integrin which releases from the detachment area of migrating cells was measured in CHO cells expressing different levels of  $\alpha 5$  integrin (A) and CHO cells expressing  $\alpha\text{IIb}\beta 3$  integrins with different affinities for fibrinogen (B). As ECM concentration, integrin expression or integrin-ligand affinity increases, the amount of integrin released from the rear of migrating CHO cells increases. Error bars represent s.e.m.

cell-substratum bonds, we measured integrin release in CHO cells expressing different levels of  $\alpha 5$  integrin or  $\alpha\text{IIb}\beta 3$  integrins with different affinities for fibrinogen. In CHO cells expressing different levels of  $\alpha 5$ , integrin release increases with fibronectin concentration at each of the expression levels (Fig. 4A). At each fibronectin concentration, integrin release increases as receptor expression increases. Receptor clustering may also increase as ECM concentration increases, so that integrin clustering could correlate with increased integrin release onto the substratum. The linkage fracture point is a function of integrin-ECM and intracellular avidities, so as integrin-ECM affinity increases we expect an increase in integrin release. Our observations verify this idea; at each fibrinogen concentration, integrin release increases as receptor-ligand affinity increases (Fig. 4B). Integrin release also increases as fibrinogen concentration increases for each  $\alpha\text{IIb}\beta 3$ -fibrinogen affinity state.

#### Integrin release from the rear of migrating cells correlates with cell-substratum adhesiveness

Integrin release onto the substratum increases as cell-substratum adhesiveness increases, due to changes in ECM



**Fig. 5.** Integrin release correlates with cell-substratum adhesiveness. The amount of integrin which releases from migrating CHO cells during rear retraction was measured for cells expressing different levels of  $\alpha 5$  integrin and CHO cells expressing  $\alpha\text{IIb}\beta 3$  integrins with different affinities for fibrinogen. Adhesiveness was also altered by varying substrate fibronectin or fibrinogen concentrations. Short-term mean detachment force was measured by shear-flow detachment of cells after a 20 minute incubation on the surface (Palecek et al., 1997). As cell-substratum adhesiveness increases in cells expressing different levels of  $\alpha 5$  integrins (A) or  $\alpha\text{IIb}\beta 3$  integrins in different affinity states (B), more integrin is released from the cell. Error bars on mean detachment force represent 95% confidence intervals on the mean and error bars on fraction of integrin released represent s.e.m.

concentration, receptor number and receptor-ligand affinity. Cell speed is also a constant function of adhesiveness as these three variables change (Palecek et al., 1997). Therefore, if integrin release were to limit cell speed, one would expect that integrin release would also be a constant function of cell-substratum adhesiveness. Short-term cell-substratum adhesiveness is reported for each of these cell populations at the same experimental conditions we used to measure measured integrin release (Palecek et al., 1997). Cell-substratum adhesiveness was determined by shear-flow detachment of cells after a 20 minute incubation. During this incubation period cells attach to the surface but do not organize integrins into focal adhesions or begin spreading. The reported mean detachment force is the shear force required to remove 50% of the cells from the substratum. Cell-substratum adhesiveness increases as ECM concentration, receptor expression and receptor-ligand affinity increase, and is linearly proportional to the number of cell-substratum bonds. To relate

integrin release to adhesiveness, we plotted the mean fraction of integrin released from the cell during rear detachment as a function of short-term cell-substratum adhesiveness, which we measured previously (Palecek et al., 1997). A constant, monotonically increasing relationship exists between the amount of integrin which releases from the cell and the adhesiveness as either receptor expression (Fig. 5A) or receptor ligand affinity (Fig. 5B) changes. This suggests that the mechanism of release of adhesions at the rear of migrating cells depends upon the adhesiveness, and thus the number of bonds, which exist between the cell and substratum. If release of adhesions at the rear of the cell was purely a biochemical mechanism we would expect the amount of integrin which releases from a cell to be independent of adhesiveness. Since there is a correlation between adhesiveness and integrin release, it appears that physical forces also play a role in the release of adhesions.

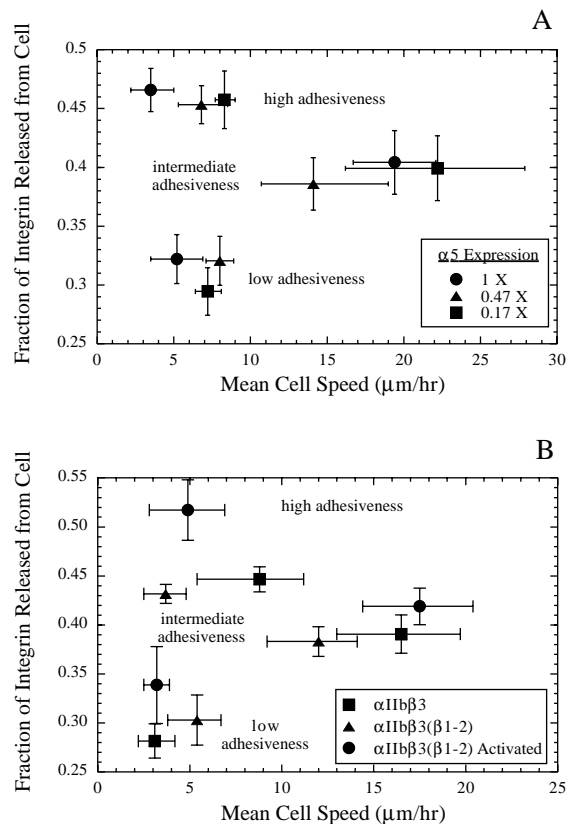
#### Cell speed is inversely related to the amount of integrin released from the rear of migrating cells when rear detachment limits the cell locomotion rate

Since speed and integrin release both correlate with cell-substratum adhesiveness, we plotted integrin release as a function of cell speed to determine whether integrin release limits cell speed. At high adhesiveness, where rear detachment limits cell speed, one expects stronger adhesions, and the integrin-ECM bond is less likely to fracture because intracellular release becomes limiting for rear detachment.

Mean cell speed is also reported for each of the CHO cell populations we used to study integrin release (Palecek et al., 1997). Speed was measured by videomicroscopic tracking of centroids of individual cells. Cell speed exhibits a biphasic dependence on ECM protein concentration at each receptor expression level and receptor-ligand affinity state. Speed is also a constant, biphasic function of cell-substratum adhesiveness. Plotting the fraction of integrin released as a function of mean cell speed, we find that a monotonic relationship does not exist between integrin release and mean cell speed (Fig. 6) over the entire range of speeds. At maximum cell speed an intermediate amount of integrin releases from the cell. As cell speed decreases from the maximum due to increased adhesiveness, more integrin releases from the cell, while as cell speed decreases due to decreased adhesiveness, less integrin releases from the cell. However, at intermediate to high adhesiveness where rear detachment limits cell speed, speed decreases as integrin release increases. These results indicate that cytoskeletal release of the integrins at the cell rear is likely to limit rear detachment rate, and thus cell migration speed, at intermediate and high cell-substratum adhesiveness.

#### Calpain inhibition hinders rear release by strengthening cytoskeletal linkages

Release of adhesions at the cell rear clearly involves a physical mechanism reliant upon cytoskeletal contraction. The tension forces presumably fracture the linkage at the weakest point. It is likely that a biochemical mechanism exists to facilitate rear release by weakening this linkage intracellularly. To search for such a mechanism, we investigated the role of the  $Ca^{2+}$ -dependent protease, calpain, in regulating this linkage. Calpain inhibition by pharmacological or genetic means inhibits rear retraction by stabilizing cytoskeletal structures (Huttenlocher

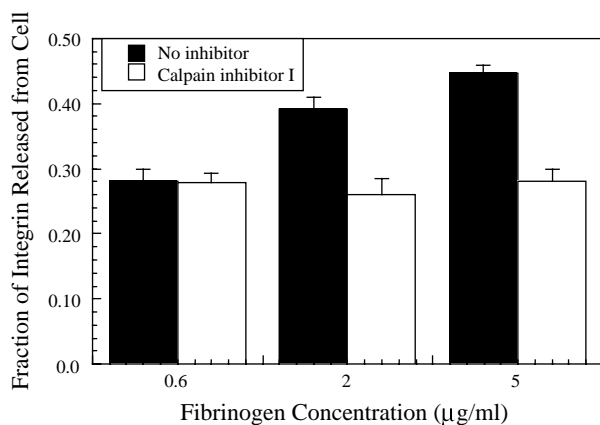


**Fig. 6.** Integrin release correlates inversely with cell speed when rear detachment limits migration speed. The amount of integrin which releases from migrating CHO cells during rear retraction was measured for cells expressing different levels of  $\alpha 5$  integrin (A) and CHO cells expressing  $\alpha IIb\beta 3$  integrins with different affinities for fibrinogen (B). Mean cell migration speed was determined using image analysis to track the centroids of individual cells (Palecek et al., 1997). The amount of integrin which releases from the rear of migrating cells is not a monotonic function of cell speed. As cell speed increases from low adhesiveness to the intermediate adhesiveness, which promotes maximum cell speed, integrin release increases. Over the range of adhesiveness where cell speed is limited by rear detachment (intermediate to high adhesiveness), integrin release has an inverse relationship with cell speed. Error bars on mean cell speed represent 95% confidence intervals on the mean and error bars on fraction of integrin released represent s.e.m.

et al., 1997). Presumably, calpain acts by severing cytoskeletal linkages, allowing the integrins to release from the cell. We measured the fraction of integrin which releases from the rear of migrating CHO cells in which calpain has been inhibited by calpain inhibitor I.

The effect of calpain inhibition on integrin release at the cell rear depends upon substrate concentration. The amount of integrin released from the cell increases as fibrinogen concentration increases in control cells, but is independent of fibrinogen concentration in cells treated with calpain inhibitor I (Fig. 7). At 2 and 5  $\mu g/ml$  fibrinogen, where rear retraction limits cell speed, calpain inhibitor I significantly reduces the amount of integrin which releases at the rear of migrating cells. At 0.6  $\mu g/ml$  fibrinogen, where rear release is not likely to limit migration speed, calpain inhibition has no effect on the cytoskeletal release of integrins during migration. Calpain





**Fig. 7.** Calpain facilitates cytoskeletal linkage release at high cell-substratum adhesiveness. The amount of integrin which releases from the rear of CHO cells migrating on fibrinogen was measured for cells which express  $\alpha$ IIb $\beta$ 3 integrin. At high fibrinogen concentrations (2 or 5  $\mu$ g/ml), calpain inhibitor I causes less integrin to release from the cell upon rear retraction. This reduction in the amount of integrin on the substratum suggests a relative strengthening of the integrin-cytoskeleton bond compared to the integrin-ECM bond. At low fibrinogen concentrations (0.6  $\mu$ g/ml), calpain inhibition has no effect on integrin release during cell migration. Error bars represent s.e.m.

appears to be an important regulatory molecule for controlling release of adhesions at the rear of cells migrating on substrates with high adhesiveness by fracturing cytoskeletal linkages. Thus, calpain activity will allow integrins to preferentially release from within cell rather than from the substratum.

## DISCUSSION

A mathematical model for cell migration (DiMilla et al., 1991), along with compilation of experimental findings (Sheetz, 1994; Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996), suggests that cell migration speed should be governed by motility processes at the cell front or the cell rear under different conditions. Conceptually, detachment of cell-substratum interactions at the cell rear can be predicted to limit cell migration speed in situations of high cell-substratum adhesiveness, with lamellipod extension and attachment at the cell front limiting migration speed in situations of low adhesiveness; in situations of intermediate adhesiveness, both cell front and cell rear processes probably act in coordination.

To our knowledge, no studies to date have explored the conditions under which motility processes occurring at either the cell front or cell rear govern locomotion, although a number of investigations have reported aspects of rate-limitation in specific situations. The rate of locomotion of *Dictyostelium* cells can be governed by lamellipod extension (Wessels et al., 1994), but on highly adhesive substrata their locomotion is limited by their ability to detach at the rear (Jay et al., 1995). The rate of lamellipodial extension in primary cultures of chick and mouse fibroblasts has been found to be approximately fivefold greater than the overall cell speed (Abercrombie et al., 1970; Felder and Elson, 1990). Protrusion rate is relatively

constant in spread fibroblasts while retraction rate governs changes in cell area (Dunn and Zicha, 1995), and speed does not strongly correlate with rate of membrane flow (Condeelis, 1993). Often, lamellipodia are not able to form stable attachments to the substratum and retract (Bard and Hay, 1975). In some cases, rear detachment and lamellipodial extension have been observed to be correlated: protrusion of the leading edge appears to sometimes be required for rear retraction in *Dictyostelium* cell locomotion (Weber et al., 1995), and in chick heart fibroblasts lamellipodial spreading could be found to increase rapidly following retraction at the trailing edge of the cell (Chen, 1979).

Therefore, a first aim of the work described in this paper was to examine the relationship between the rates of cell body translocation and cell rear detachment across a range of cell-substratum adhesiveness conditions, for CHO cells transfected with  $\alpha$ IIb $\beta$ 3 integrins migrating on fibrinogen-coated surfaces. As shown in Fig. 1, we have found that the rate of cell body translocation is approximately equal to the rate of cell rear detachment under conditions of intermediate and high adhesiveness, whereas translocation is substantially slower than rear detachment under conditions of low adhesiveness. These data are consistent with our predictions, and are not contradictory to the literature reports of specific situations cited above. Understanding the regulatory mechanism of adhesion release is crucial to rational manipulation of cell speed at conditions where release is rate-limiting. To investigate these mechanisms, we probed the fate of integrin adhesion receptors at the rear of CHO cells during  $\alpha$ 5 $\beta$ 1 integrin-mediated migration on fibronectin or  $\alpha$ IIb $\beta$ 3 integrin-mediated migration on fibrinogen. We labeled integrins on live cells with a nonadhesion-perturbing mAb conjugated to a fluorescent probe and measured both the fraction of integrins which release from the cell and the fraction which remain with the cell upon rear retraction. This allows us to determine whether the cytoskeletal linkage or extracellular linkage is more stable, and to locate the locus of regulation of adhesion release. We measured integrin release as a function of cell-substratum adhesiveness by varying ECM protein concentration, integrin expression and integrin-ligand affinity. We then correlated integrin release to cell migration speed at adhesive regimes where rear retraction rates limit cell speed. To investigate a mechanism of active release of adhesions at the cell rear we also analyzed the effects of calpain, a  $Ca^{2+}$ -dependent protease implicated in adhesive release, on release of adhesions at the rear of migrating cells. Our results indicate that a significant, though variable, amount of integrin detaches from the rear of migrating CHO cells upon rear retraction. The detachment of integrins at the rear of migrating CHO cells is similar to the detachment of  $\beta$ 1 integrins reported in 3T3 fibroblasts migrating on laminin (Regen et al., 1992; Palecek et al., 1996). The amount of integrin which releases from the cell increases as receptor clustering and integrin-ECM avidity increase, due to changes in ECM protein concentration, receptor expression or receptor-ligand affinity. In fact, integrin release from the cell rear is a constant function of cell-substratum adhesiveness and the number of cell-substratum bonds. We demonstrated that at intermediate and high adhesiveness, where rear detachment rate limits cell speed, cell speed correlates inversely with integrin release. Calpain cleavage of cytoskeletal linkages appears to be an important mechanism for regulation of

adhesion release during migration when speed is limited by rear detachment. Calpain inhibition significantly reduces the amount of integrin which releases from the rear of cells migrating on high ECM concentrations.

The constant, increasing relationship between the amount of integrin released from the cell during rear retraction and the cell-substratum adhesiveness indicates that the avidity of cell-substratum interactions influences cell rear detachment. Short-term cell-substratum adhesiveness measurements are linearly proportional to the number of cell-substratum bonds (Palecek et al., 1997). If relatively few bonds exist, the integrins are more likely to release from the substratum. If many bonds exist the adhesion receptors are more likely to release from the cell and remain attached to the substratum. Increased receptor organization may also play a role in the increased integrin release at high adhesiveness, since integrin clustering increases as integrin or ECM concentration and integrin-ECM affinity increases. Concentration of cytoskeletal proteins may alter the avidity of individual bonds within the linkage. Alternatively, signaling or regulatory molecules for rear release, which weaken the intracellular linkage, may be concentrated at focal adhesions. The relationship between integrin release and bond number suggests that the mechanism for rear release involves a physical component, due to tension at the rear of the cell. If linkage release was purely biochemical, we would expect integrin release to be independent of cell-substratum adhesiveness.

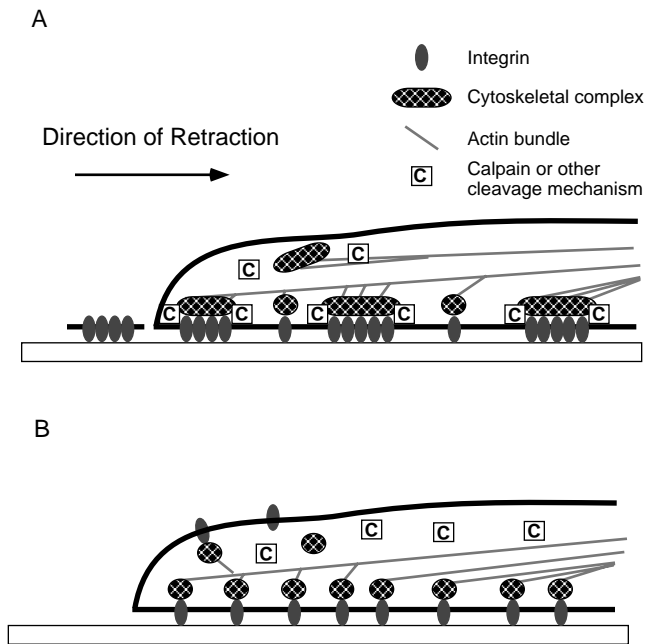
Cell adhesiveness not only determines the amount of integrin which releases from the rear of migrating cells but also regulates cell speed (Palecek et al., 1997), so it is likely that integrin release from cells influences cell speed. Cell migration speed exhibits a biphasic dependence upon cell-substratum adhesiveness. Maximum cell speed is a function of adhesiveness as ECM concentration, integrin expression or integrin-ECM ligand affinity change (Palecek et al., 1997). One might expect integrin release to be inversely proportional to cell speed since fast cells (e.g. neutrophils) tend to release very few integrins during rear retraction (A. Huttenlocher and S. P. Palecek, unpublished) while slow cells (e.g. fibroblasts or CHO cells) release a significant proportion of their integrins. In this scenario, the release of cytoskeletal linkages at the cell rear would be rate-limiting for migration speed. However, integrin release is not a monotonic function of cell speed over the entire range of cell-substratum adhesiveness. At intermediate and high adhesiveness, where rear detachment rate limits migration speed, an inverse relationship exists between integrin release and cell speed. The molecular-level event which governs release of the cell from the substratum, and thus regulates cell speed, appears to be release of linkages between integrins and the cytoskeleton. At low adhesiveness and low migration speed, less integrin releases from the cells than at intermediate adhesiveness and high speed, indicating that release of adhesion factors is not rate-limiting for cell speed at low adhesiveness. Detachment rate data in Fig. 1 show that the cell can detach from the substratum faster than it migrates. At low adhesiveness the cells retract from many directions evenly, causing very little movement in the cell centroid. Lamellipod extension or formation of new adhesions may be rate-limiting under these conditions.

Dissociation of cytoskeletal linkages appears to be regulated by biochemical mechanisms as well as tension forces from

cytoskeletal contraction. Dissociation of integrin-ECM linkages is likely to depend only on physical forces and integrin-ECM bond affinity. An analysis of bonds in series under force (Saterbak and Lauffenburger, 1996) predicts that the probability of linkage fracture at each point is related logarithmically to the affinities of each of the individual bonds. The large amount of variation in the linkage fracture location during rear release suggests that the affinities of the individual bonds in the adhesion complex are quite close, allowing a high probability of both intracellular and extracellular bonds breaking.

Calpain is one mechanism which appears to regulate rear retraction of some cells at intermediate and high adhesiveness. Maximum cell speed decreases when rear release is impeded by inhibition of calpain (Huttenlocher et al., 1997). This raises the question of what governs the maximum speed a cell can attain, and what range of environmental conditions can sustain that maximum speed. A mathematical model of cell migration predicts that a front-versus-rear asymmetry in adhesiveness or contractility is a major parameter that governs maximum cell speed and the range of conditions sustaining it (DiMilla et al., 1991). The greater this asymmetry, the greater the maximum speed and the greater the sustaining range. This asymmetry requirement can be satisfied in a number of ways. The spatial distribution of adhesion receptors due to preferential trafficking or localization into adhesion complexes may lead to higher adhesiveness at the cell front than the cell rear. Asymmetries in adhesion receptor-ECM ligand affinity could also result in an adhesive gradient in the cell. Locking integrins in a high affinity state, thereby eliminating receptor-ligand affinity gradients, inhibits eosinophil, lymphocyte and fibroblast migration (Kuijpers et al., 1993; Dustin et al., 1997; Huttenlocher et al., 1996). Adhesion receptor-cytoskeleton linkage avidity appears to exhibit a spatial asymmetry in the cell; integrin linkages to the cytoskeleton in migrating NIH 3T3 fibroblasts have been found to be more prevalent in the cell front than at the cell rear (Schmidt et al., 1993). Spatial concentrations of signaling molecules may also contribute to the asymmetry. For example, intracellular  $Ca^{2+}$  or phosphoinositide levels may regulate adhesion or contractile forces. In migrating leukocytes, calcium concentration and myosin-II based contractility increases from the front to rear of the cell (Hahn et al., 1992). Also, intracellular calcium transients are required for detachment of the cell rear during neutrophil migration via the phosphatase calcineurin (Maxfield, 1993). Proteases, including calpain, may also be activated by intracellular calcium transients at the rear of migrating cells.

Calpain is likely to aid cell migration by increasing adhesive asymmetry within the cell by weakening cytoskeletal linkages at the cell rear. Inhibition of calpain stabilizes cytoskeletal linkages with respect to integrin-ECM linkages, resulting in preferential fracture of integrin-ECM linkages. Calpain activity appears to regulate rear release by cleavage of cytoskeletal linkages, destabilizing adhesions at the cell rear with respect to adhesions at the cell front, thereby increasing the front-versus-rear asymmetry in adhesiveness. If inhibition of calpain decreases adhesive asymmetry, cells with low calpain activity should migrate over a smaller range of substrate ECM concentrations and not be able to attain as high a maximum cell speed as cells with a higher calpain activity. Experimental



**Fig. 8.** A model for release of cytoskeleton-integrin-ECM linkages at the rear of cells which migrate using (A) adhesion complexes of organized integrins or (B) diffuse, less organized integrins (B). Organized integrins are clustered and bound to cytoskeletal molecules, which link them to the actin cytoskeleton. Calpain or other regulatory molecules for rear release localize to the adhesion structures in an inactive state. Contraction of the actin cytoskeleton applies tension, which is generally insufficient to fracture the linkages, until the calpain is activated and cleaves some of the cytoskeletal linkages. This results in the release of integrins from the rear of the migrating cell. Less organized integrins are similarly linked to the actin cytoskeleton but applied tension from cytoskeletal contraction is strong enough to peel the integrin-ECM bonds without calpain-mediated cytoskeletal bond cleavage. Integrins then dissociate from the substratum and remain within the cell membrane.

measurements by Huttenlocher et al. (1997) quantitatively agree with this prediction. Increasing calpain activity above normal levels may allow us to increase maximum cell speed. However, at a certain point, calpain activity is likely to impede formation of cytoskeletal linkages and reduce maximum speed. Thus, an intermediate calpain activity will theoretically provide maximum cell speed.

Our results suggest a model for rear detachment regulated by both applied tension from cytoskeletal contraction and biochemical modifications of cytoskeletal linkages (Fig. 8). High cell-substratum adhesiveness (Fig. 8A) may represent integrin aggregation into complexes which are under tension due to cytoskeletal contraction. This tension may not be high enough to sever adhesion complex bonds, however. Calpain activity and/or other biochemical mechanisms may weaken bonds within the cytoskeletal linkage enough for the applied tension to fracture the linkage, allowing the integrins in the adhesion complex to rip from the cell. Tyrosine phosphorylation and cytoskeletal tension are involved in ATP-dependent release of adhesions at the rear of migrating fibroblasts (Crowley and Horwitz, 1995). Conversely, low adhesiveness (Fig. 8B) may represent integrins in a less-

aggregated state. In this case, the biochemical signal for rear release may not localize to individual adhesions and rear release will be regulated primarily by applied force, allowing the integrin-ECM bonds to preferentially sever. Alternatively decreased avidity due to lack of integrin clustering at low adhesiveness may not necessitate a biochemical release mechanism – the applied tension at the rear of the cell may be great enough to peel diffuse integrins from the ECM without biochemical cleavage of cytoskeletal linkages. In addition to accounting for migration of cells at different adhesiveness, this model for rear detachment also explains different detachment modes for different cell types. Fibroblasts, which form strong focal adhesions and adhere strongly to the substratum, apparently require integrin release for migration under many conditions. More highly motile cells, in contrast, could exhibit dissociation of the integrin-ECM bonds as the primary detachment mode.

We thank Mark Ginsberg for providing the  $\alpha$ IIB $\beta$ 3 and  $\alpha$ IIB $\beta$ 3( $\beta$ 1-2) cells, Joe Loftus for the MAb62 antibody, Ralph Isberg for the 6F4 antibody, Louis Reichardt for the human  $\alpha$ 5 cDNA and Rudolph Juliano for the CHO B2 cells. This work was supported by NIH grant GM 23244 to A. F. Horwitz and D. A. Lauffenburger, an Arthritis Foundation grant to A. Huttenlocher and a Whitaker Foundation Graduate Fellowship to S. P. Palecek.

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