

Regulation of Cell Migration by the Calcium-dependent Protease Calpain*

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Integrin receptors play an important role during cell migration by mediating linkages and transmitting forces between the extracellular matrix and the actin cytoskeleton. The mechanisms by which these linkages are regulated and released during migration are not well understood. We show here that cell-permeable inhibitors of the calcium-dependent protease calpain inhibit both $\beta 1$ and $\beta 3$ integrin-mediated cell migration. Calpain inhibition specifically stabilizes peripheral focal adhesions, increases adhesiveness, and decreases the rate of cell detachment. Furthermore, these inhibitors alter the fate of integrin receptors at the rear of the cell during migration. A Chinese hamster ovary cell line expressing low levels of calpain I also shows reduced migration rates with similar morphological changes, further implicating calpain in this process. Taken together, the data suggest that calpain inhibition modulates cell migration by stabilizing cytoskeletal linkages and decreasing the rate of retraction of the cell's rear. Inhibiting calpain-mediated proteolysis may therefore be a potential therapeutic approach to control pathological cell migration such as tumor metastasis.

Cell migration requires a dynamic interaction between a cell, its substratum, and the actin cytoskeleton. Integrin receptors, which are $\alpha\beta$ heterodimers present on the cell surface, play an important role during cell migration by mediating these interactions and transmitting forces between the extracellular matrix and the actin cytoskeleton (1, 2). The mechanisms by which these linkages are regulated and released at the cell's rear during migration are not well understood. Previous studies

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implicate calcium transients in adhesive release in neutrophils migrating on both fibronectin and vitronectin. However, calcineurin mediates the calcium-dependent release of adhesions at the cell's rear in neutrophils migrating on vitronectin but not on fibronectin (3, 4). The specificity of the calcineurin effect for vitronectin and the $\alpha v\beta 3$ integrin suggests that other calcium-dependent mechanisms are also likely to contribute to detachment during migration.

The calcium-dependent protease calpain is an attractive candidate to be a calcium-responsive regulator of cell migration. It localizes to focal adhesions and cleaves many focal adhesion-related proteins including integrin receptors, focal adhesion kinase, and talin (5–8). Calpain is a cysteine protease with two characterized isoforms, calpain I (μ -calpain) and II (m-calpain). Both contain an 80-kDa catalytic subunit and a 30-kDa regulatory domain. Activation requires calcium concentrations in the micromolar range and millimolar range for calpain I and II, respectively (9, 10). The increases in calcium seen in migrating cells appear to be within the range to support activation of calpain (3). In this study we use genetic and inhibitor studies to show that inhibition of the calcium-dependent protease calpain reduces both $\beta 1$ and $\beta 3$ integrin-mediated migration.

EXPERIMENTAL PROCEDURES

Cells and Reagents—CHO¹ KI cells were obtained from American Type Culture Collection (Rockville, MD). CHO cells expressing $\alpha I\text{Ib}\beta 3$ integrin were prepared as described (11). The CHO cell clone SH1, which expresses low levels of calpain I, was isolated as described previously (12). The A4 clone was prepared by cotransfecting SH1 cells with pSBC-muL plasmid containing human calpain I large subunit and a neo selection vector, pMC1Neo using LipofectAMINE (13). A4 cells express approximately four times the amount of calpain I as SH1 cells, as determined by activity in immunoprecipitates.² Western blots using a human-specific antibody confirm expression of the cDNA. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and 1% nonessential amino acids as described previously. Stock solutions of calpain inhibitors I and II (Boehringer Mannheim) were prepared at a concentration of 10 mg/ml (26 mM) in ethanol. Calpain inhibitor I and II were used at concentrations ranging from 1 (2.6 μM) to 100 $\mu\text{g}/\text{ml}$ (260 μM). Benzyloxycarbonyl-Leu-leu-Tyr diazomethyl ketone, ZLLY-CHN2, (Enzyme System Products, Dublin, CA), was prepared in Me₂SO at a concentration of 35 mM and used at a final concentration of 50 μM . Fibronectin and fibrinogen were prepared as described previously (11). Purified D57, a nonadhesion perturbing mouse monoclonal antibody, directed against human $\alpha I\text{Ib}\beta 3$, was conjugated to Oregon Green dye (Molecular Probes) as per manufacturer's protocols. Conjugated D57 was separated from free dye by G-25 Sephadex gel filtration.

Cell Migration Assays—Time lapse videomicroscopy and modified Boyden chamber transwell assays were performed as described previously (11). Briefly, cells were washed and resuspended in serum-free hybridoma medium CCM1 (Hyclone Laboratories Inc., Logan, UT) and pretreated with inhibitors for 20 min prior to plating for time lapse videomicroscopy and transwell assays. Plates were coated with substrate, fibronectin, or fibrinogen and blocked with 2% bovine serum albumin for 30 min prior to use. Cells were tracked by time lapse videomicroscopy for 3–6 h. Random transwell assays were performed using membranes coated with substrate on both sides (CoStar Corp., Cambridge, MA). Assays were run for 3 h, and cells were then fixed with methanol and stained with methylene blue (leukostat staining kit, Sigma). Each experiment was performed a minimum of three times.

Immunofluorescence—Coverslips were coated with fibrinogen at 10 $\mu\text{g}/\text{ml}$ after acid washing and ethanol treatment. After 24 h in serum-

¹ The abbreviations used are: CHO, Chinese hamster ovary; BDK, benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone.

² R. Mellgren, unpublished observations.

free CCM1 with or without calpain inhibitor I at 50 $\mu\text{g/ml}$ (130 μM) the cells were fixed in phosphate-buffered saline containing 3% formaldehyde for 15 min and quenched with glycine (0.1 M). They were then treated with 1.0% Triton X-100, blocked in 5% goat serum, and incubated with primary and secondary antibodies (11). The coverslips were mounted and observed using a fluorescence microscope with a 63 \times objective (Axioplan, Carl Zeiss, Inc., Thornwood, NY). For live fluorescence microscopy CHO cells were plated on silanated, fibrinogen-coated glass coverslips for 90 min in serum-free CCM1 (14). Cells were then incubated for 30 min with Oregon Green-conjugated D57, diluted to 40 $\mu\text{g/ml}$ in CCM1, and rinsed five times with CCM1. Stained cells were placed in a temperature-regulated humidified stage mounted on a Nikon Diaphot inverted microscope. Phase contrast and fluorescence images of migrating cells were acquired every 30 min for 2 h using a 60 \times /1.4 NA objective and a cooled CCD camera (CE200A, Photometrics). Oncor Image software (Gaithersburg, MD) was used to control the camera shutter and to process the images. Cell perimeter was determined by manually tracing cell edges on phase contrast images. The cell areas that retracted between t_1 and t_2 was determined by subtracting a mask of the cell area at t_2 from the mask at t_1 . Retraction area was normalized to total cell area to determine the rear retraction rate. Retraction of lamellipodia was neglected. The amount of integrin that ripped from the cell upon rear retraction was measured as the ratio of mean fluorescent intensity in the retraction area after detachment to the mean fluorescent intensity in the retraction area before detachment. Average fluorescent intensity outside of the cell was subtracted from the mean fluorescent intensities to correct for background fluorescence. If a cell detached from the same area more than once during the course of the experiment, only the first detachment was used. Detachment of lamellipodia was neglected. Detachment areas and amount of integrin ripping from cells was measured in at least 12 different cells and four different experiments.

RESULTS AND DISCUSSION

To determine whether calpain plays a role during cell migration, we characterized the migration of cells treated with two different classes of cell-permeable calpain inhibitors as well as the migration of a CHO cell clone that expresses low levels of calpain I. Cell migration was studied using transwell assays and time lapse videomicroscopy. Calpain inhibitors I and II are cell-permeable peptide aldehydes that reversibly inhibit calpains by binding to their active site (15). Benzylloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone (BDK), another type of calpain inhibitor, is an irreversible inhibitor of cysteine proteases (16). We assayed the effects of these inhibitors on both $\beta 1$ and $\beta 3$ integrin-mediated migration in CHO cells. $\alpha 5\beta 1$ -mediated migration was studied using CHO cells on a fibronectin substrate. CHO cells transfected with the $\alpha \text{IIb}\beta 3$ integrin were used to study $\beta 3$ integrin-mediated migration on fibrinogen (11). The migration of a CHO cell clone (SHI) that expresses low levels of calpain I (12) and a clone of SHI cells expressing ectopic calpain I was also characterized. Control CHO cells contain 10% calpain I and 90% calpain II by assay of DEAE column fractions. In contrast, SHI cells contain only 3% calpain I (12), and SHI cells expressing ectopic calpain I (A4 cells) contain 12% calpain I as determined by assay of immunoprecipitates from cell lysates (data not shown).

Treatment with calpain inhibitor I reduces both $\beta 1$ and $\beta 3$ integrin-mediated CHO cell migration as measured by a transwell random migration assay. 130 μM of calpain inhibitor I reduces $\alpha \text{IIb}\beta 3$ -mediated migration by 78% on fibrinogen, and $\alpha 5\beta 1$ mediated migration by 60% on fibronectin (Fig. 1). The decrease in migration rate depends on the concentration of calpain inhibitor, with effects seen at concentrations as low as 2.6 μM . Cell toxicity, with rounding, is seen in some (<20%) cells at high concentrations of calpain inhibitor (260 μM). Although calpain inhibitor II and BDK also inhibit cell migration, these inhibitions are not as dramatic as those seen with calpain inhibitor I (data not shown). Further evidence supporting a role for calpain in this process is demonstrated by a similar (60%) reduction in the migration rates of a CHO cell clone, SHI,

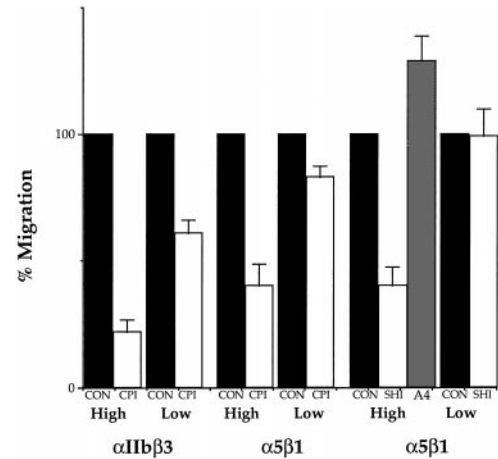


FIG. 1. Calpain inhibitor I reduces both $\beta 1$ and $\beta 3$ integrin-mediated cell migration by random transwell assay. Treatment with calpain inhibitor I (CPI) inhibits both $\alpha \text{IIb}\beta 3$ and $\alpha 5\beta 1$ integrin-mediated cell migration at higher substrate concentrations. The reduction in cell speed is compensated for at low substrate concentrations on both fibronectin and fibrinogen. CHO cell clone, SHI, which expresses low levels of calpain I, also shows reduced migration rates at higher substrate concentrations. Rescue of this phenotype is demonstrated by transfecting SHI cells with human calpain I cDNA (A4). Migration of CHO cells ectopically expressing $\alpha \text{IIb}\beta 3$ and untransfected CHO cells was studied on 10 $\mu\text{g/ml}$ of fibrinogen and 10 $\mu\text{g/ml}$ of fibronectin respectively (High). Migration was also measured at lower substrate concentrations, 1 $\mu\text{g/ml}$ of fibrinogen and 0.1 $\mu\text{g/ml}$ (or 0.5 $\mu\text{g/ml}$ for SHI cells) of fibronectin (Low), respectively, to determine if the effect is substrate concentration-dependent. Migration is expressed as the percentage of untreated control cells (CON) that migrate on both fibrinogen ($\alpha \text{IIb}\beta 3$) and fibronectin ($\alpha 5\beta 1$) over 3 h. Each data point represents the average of a minimum of three separate experiments with error bars showing S.D.

which expresses reduced levels of calpain I but normal levels of calpain II and the cathepsins (12) (Fig. 1). A SHI clone transfected with the human calpain I cDNA rescues this phenotype and shows migration rates equal to or greater than control CHO cells (Fig. 1). Taken together, the data suggest that decreased levels of calpain activity result in reduced migration rates.

Previous studies implicate cell-substratum adhesiveness as an important determinant of cell migration speed, with maximum migration demonstrated at intermediate cell-substratum adhesiveness (11, 17, 18). At lower substrate concentrations cell speed is apparently limited by the ability to form attachments at the cell front, whereas at high substrate concentrations cell speed tends to be limited by the rate of cell-substratum detachment. To determine if calpain inhibitors influence cell migration by altering cell-substratum adhesiveness, the dependence of this inhibition on substrate concentration was examined. The reduction in migration rates with calpain inhibition is greatest at higher substrate concentrations, and this inhibition can be quantitatively reduced by lowering the substrate concentration (Fig. 1). SHI cells also show decreased inhibition of migration at lower substrate concentrations (Fig. 1). These results suggest that calpain inhibition reduces cell migration speed by affecting the rate of rear detachment. The substrate concentration dependence of the migration rates also argues against inhibitor-induced cell toxicity because migration is comparable with control cells at lower substrate concentrations.

Observations by time lapse videomicroscopy demonstrate that reduced detachment rates at the rear contribute to the decreased migration rates seen with calpain inhibitor treatment. Cells treated with calpain inhibitor I demonstrate ruffling and lamellipodial projections but have inhibited release at

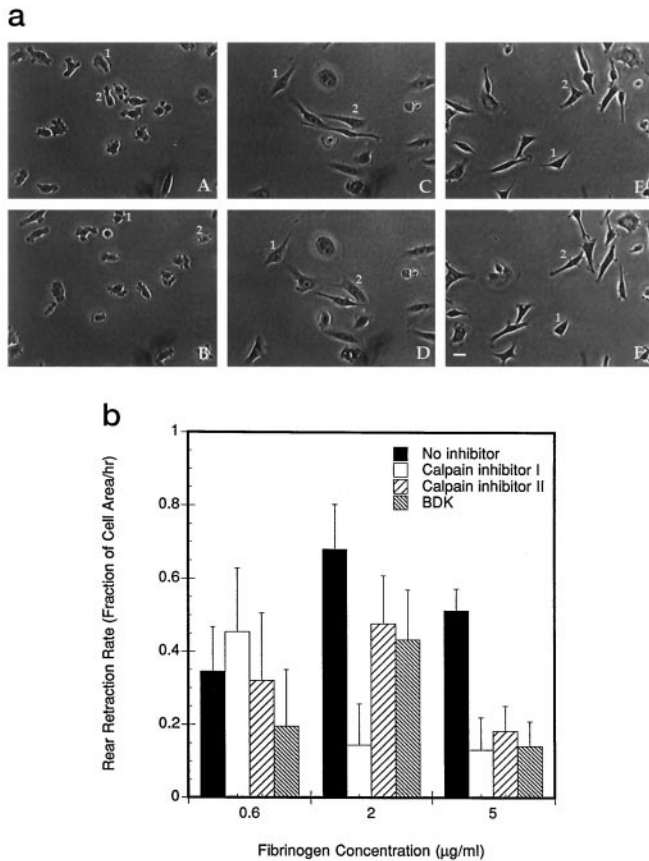


FIG. 2. *a*, effect of calpain inhibitors on morphology and migration of CHO cells on fibronectin. Cells were plated on 1 $\mu\text{g/ml}$ of fibronectin, and migration was observed by time lapse videomicroscopy. The images represent time lapse images at time 0 (A, C, and E) and 30 min later (B, D, and F). Numbers refer to individual cells tracked during the 30-min observation period. Control cells demonstrate a rounded morphology and rapid migration in the majority of cells (A and B). Cells treated with calpain inhibitor I (130 μM) show a more elongated morphology and reduced retraction at the rear of the cell with minimal movement of the cell body after 30 min (C and D). SHI cells expressing low levels of calpain I also show a more elongated morphology with reduced retraction of the cell's rear (E and F). Bar, 20 μm . *b*, calpain inhibitors reduce the rate of retraction of the cell's rear during migration at high fibrinogen concentrations. Detachment rate is measured by tracing cell outlines on phase contrast images and normalizing the area of the cell's rear that detaches to the total cell area. Error bars represent standard deviations of detachment rate measurements. Calpain inhibitors I and II were used at 130 μM , and the BDK was used at 50 μM . At high fibrinogen concentrations (5 $\mu\text{g/ml}$) calpain inhibitor I, calpain inhibitor II, and BDK all inhibit rear retraction rate. At low fibrinogen concentrations (0.6 $\mu\text{g/ml}$) rear retraction is not inhibited by calpain inhibitors.

the rear of the cell. These cells display a range of morphologies with many cells showing a highly elongated tail (Fig. 2*a*). Cells treated with calpain inhibitors often release adhesions with a sudden snapping motion, and after detachment, large pieces of membrane may remain on the substratum. Other cells show minimal movement of the cell body despite normal cell shape and lamellipodial activity. Cells treated with the other calpain inhibitors (calpain inhibitor II and BDK) show morphologic changes similar to those seen with calpain inhibitor I treatment, although they are less extreme (data not shown). The SHI cells also show morphologic changes similar to CHO cells treated with calpain inhibitors including an elongated morphology and reduced detachment (Fig. 2*a*). In contrast, control cells are generally not elongated unless plated on higher substrate concentrations.

To demonstrate more directly that calpain inhibition affects release at the cell's rear, we quantified the rates of rear retrac-

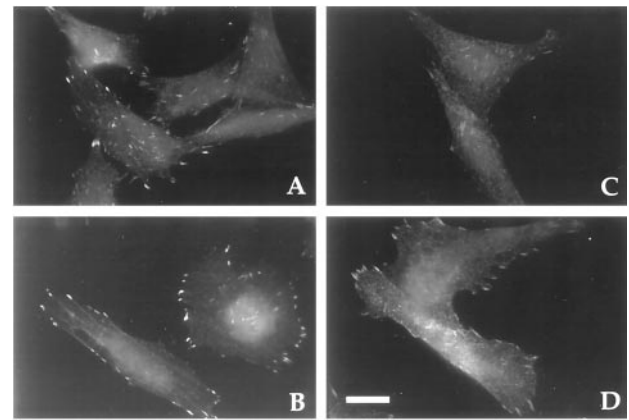


FIG. 3. Effects of calpain inhibitor I on cellular morphology of CHO cells expressing the $\alpha\text{IIb}\beta 3$ integrin receptor. Cells were cultured for 24 h on fibrinogen-coated coverslips (10 $\mu\text{g/ml}$) in serum-free CCM1 medium and immunostained with vinculin (A and B) and the $\alpha\text{IIb}\beta 3$ antibody D57 (C and D). CHO cells treated with calpain inhibitor I (130 μM) demonstrate fewer but larger, more peripheral focal adhesions (B and D) than untreated cells (A and C). Bar, 20 μm .

tion for cells migrating in the presence of calpain inhibitors (Fig. 2*b*). At a low fibrinogen concentration (0.6 $\mu\text{g/ml}$), calpain inhibitors have no detectable effect on the rate of rear retraction. At an intermediate fibrinogen concentration (2 $\mu\text{g/ml}$), which supports maximum migration, calpain inhibitor I shows a 5-fold inhibition in the rate of rear retraction. Significant inhibition of the retraction rate is also seen with calpain inhibitors I and II and diazomethyl ketone as compared with control cells at high substrate concentrations. Our results suggest that treatment with calpain inhibitors blocks migration by specifically inhibiting cell-substratum detachment at the rear of the cell.

Because calpain localizes to focal adhesions and destabilizes many focal adhesion components (5–8), it seems likely that calpain inhibitors may inhibit rear release by stabilizing cytoskeletal linkages (19) and as a result strengthen focal adhesions. We tested this hypothesis by assaying the effects of calpain inhibitors on focal adhesion organization and stability. We stained for focal adhesion components in serum-starved CHO cells ectopically expressing the $\alpha\text{IIb}\beta 3$ integrin plated on fibrinogen. Cells treated with calpain inhibitor I show fewer but more prominent, peripherally located focal adhesions than untreated cells (Fig. 3). Furthermore, SHI cells also show stronger, more peripheral focal adhesions when plated on fibronectin, directly implicating calpain I in these morphological changes (data not shown). SHI cells ectopically expressing human calpain I, on the other hand, show more central focal adhesions than SHI cells. Thus, reducing calpain activity appears to stabilize peripheral focal adhesions.

Previous studies using migrating fibroblasts demonstrate that a substantial fraction of integrin receptors can detach from the cell and remain on the substratum during rear retraction (14, 20), suggesting that fracturing of integrin-cytoskeletal bonds may be an important release mechanism at the cell's rear during migration. Because calpain targets and cleaves the integrin cytoplasmic domain as well as other cytoskeletally associated proteins (5–8), we hypothesized that calpain severs the connection between molecules that comprise the integrin-cytoskeletal linkage at the cell's rear. We assayed the fate of integrin at the cell's rear by using fluorescently conjugated antibodies to measure the amount of integrin receptor that detaches from the cell and remains on the substratum (14). This was performed by determining a ratio of mean fluorescence intensity in the retraction area before and after detachment (Fig. 4). We found that the fraction of $\alpha\text{IIb}\beta 3$ integrin

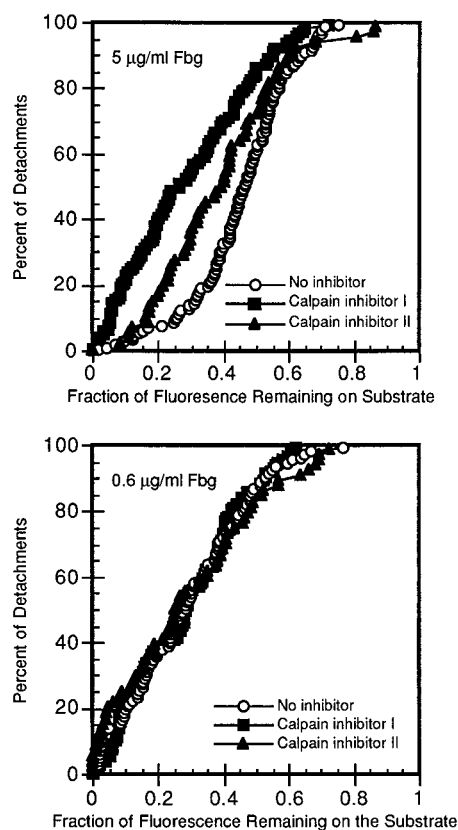


FIG. 4. Calpain inhibitors reduce the amount of integrin that detaches at the rear of a migrating cell at high fibrinogen concentrations. The area of the cell tail that detaches was determined in cells labeled with Oregon Green-conjugated D57 anti- α IIb β 3 antibody by tracing phase-contrast cell outlines. The amount of integrin detaching from the cell is calculated as the ratio of average fluorescent intensities in the retraction area after retraction to before retraction, corrected for background fluorescence. The probability distributions of the fraction of fluorescence remaining on the surface after rear retraction are shown. Calpain inhibitors I and II were used at 130 μ M. At high fibrinogen concentrations, 5 μ g/ml (top), calpain inhibitor I causes less integrin to detach from the cell upon rear retraction. This reduction in the amount of integrin on the substratum suggests a relative strengthening of the integrin-cytoskeletal bond compared with the integrin-extracellular matrix bond. Calpain inhibitor II also inhibits integrin ripping from the cell but less than calpain inhibitor I. At low fibrinogen concentrations (0.6 μ g/ml; bottom), calpain inhibitors have no effect on integrin ripping during cell migration.

receptor remaining on the substratum depends on substrate concentration. The proportion of integrin ripping from the cell and remaining on the substratum increases as the fibrinogen concentration increases in control cells. Treatment with calpain inhibitors significantly reduces the amount of integrin that remains on the substratum at high fibrinogen concentrations. If the integrin-cytoskeletal linkage is strengthened by calpain inhibition, the integrin-ECM bond should be more

likely to fracture during rear retraction. This is consistent with our finding that there is less integrin on the substratum at high cell-substratum adhesiveness in cells treated with calpain inhibitors. At low cell-substratum adhesiveness, where cell speed is not limited by rear retraction, calpain inhibitors have no effect on the amount of integrin that remains on the substratum.

We conclude that a reduction in calpain activity inhibits cell migration by decreasing the rate of cell detachment and stabilizing integrin-cytoskeletal linkages. These observations suggest a novel calcium-dependent mechanism for regulating integrin-cytoskeletal linkages and cell detachment during migration. Interestingly, gradients in calcium concentrations with higher levels found at the cell's rear are seen in migrating eosinophils (21). This asymmetry in calcium concentration along with localized calcium fluctuations may serve to regulate calpain activation. Previous studies also support a dissociation of the integrin-cytoskeletal bond at the rear of a cell as it migrates (22). These observations are consistent with a possible calpain-mediated mechanism for weakening the integrin-cytoskeletal bond at the rear of migrating cells. Our findings suggest that inhibition of calpain activity is a potential approach for the treatment of pathologic cell migration such as tumor metastasis.

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