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Integrin dynamics on the tail region of migrating fibroblasts

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SUMMARY

Cell migration is a complex process that can be considered as a repeated cycle of lamellipod extension and attachment, cytoskeletal contraction, and tail detachment. While lamellipodial and cytoskeletal phenomena are currently the focus of considerable research on cell migration, under many conditions locomotion appears to be rate-limited by events at the cell rear, especially release of cell/substratum adhesions. To study the mechanism of tail detachment, we have developed a novel experimental system that permits observation of integrin dynamics on the ventral surface of migrating fibroblasts. Photoactivatable caged fluorescein is coupled to a non-adhesion-perturbing anti-avian-β_1 integrin subunit antibody, which labels integrins on chicken fibroblasts migrating on a laminin-coated glass coverslip. Ultraviolet light is focused through a pinhole to photoactivate the caged fluorophore in a 10-μm-diameter spot at the rear of a polarized cell. The fate of integrins initially present in this spot is monitored using a cooled CCD camera to follow the movement of fluorescent intensity as a function of time over a 2 to 3 hour period. We find that a substantial fraction of the integrins is left behind on the substratum as the cell detaches and locomotes, while another fraction collects into vesicles which are transported along the cell body as the cell migrates. As aggregates rip from the cell membrane, the integrin-cytoskeletal bonds are preferentially fractured resulting in 81±15% of the integrin remaining attached to the substratum. We additionally find that adhesions sometimes disperse into integrins which can form new adhesions at other locations in the cell. Adhesions along the cell edge can release from the substrate and translocate with the cell. They either disperse in the cell membrane, rip from the cell membrane and remain attached to the substratum, or form a new aggregate. These observations indicate that the behavior of integrins at the cell rear is much more dynamic than previously appreciated, suggesting that an important locus for regulation of motility may reside in this region.

Key words: Integrin, Cell migration, Vesicle

INTRODUCTION

The process of cell migration can be considered as a coordinated cycle that includes: lamellipodial extension, formation of lamellipod/substratum attachments, cytoskeletal contraction, and release of cell/substratum attachments at the rear of the cell. While the rate of locomotion may be proportional to the frequency of lamellipod extension (Wessels et al., 1994), the rate of cell locomotion is generally not limited by the rate of extension (Abercrombie et al., 1970; Felder and Elson, 1990). Either strong lamellipod/substratum attachments may not form efficiently, so that a significant fraction of extensions are futile, or alternatively, release of cell/substratum attachments may be rate-limiting.

In a number of circumstances the latter situation is observed, with cell migration speed consequently governed primarily by uropodal dynamics. Tail retraction in fibroblasts appears to consist of two processes: a fast retraction due to elastic recoil of tension, and a slow, ATP-dependent active retraction (Chen, 1981). This is consistent with more recent findings of both mechanical and biochemical regulation of focal adhesion breakdown (Crowley and Horwitz, 1995). Similarly, inhibition of Ca^{2+} transients in neutrophils results in reduced migration rates on fibronectin or vitronectin; these cells often possess elongated tails (Marks et al., 1991). In *Dictyostelium*, loss of myosin-II-dependent contraction in the uropod correlates with diminished locomotion speed on highly adhesive substrata (Jay et al., 1995).

Cell adhesion to extracellular matrix (ECM) ligands is mediated by low-affinity transmembrane glycoprotein adhesion receptors, including members of the heterodimeric integrin family (Buck and Horwitz, 1987; Ruoslahti and Piersbacher, 1987; Hynes, 1992; Sonnenberg, 1993). In nonmotile fibroblasts, integrins and associated cytoskeletal components associate into highly organized focal adhesions which mediate a close attachment between the cell and its substratum (Burridge et al., 1988). Connections between integrins and cytoskeleton are less well organized in migrating fibroblasts, but integrins interact with the substratum and cytoskeleton through similar linkages (Regen and Horwitz, 1992). Induction

of focal contact disassembly into less organized structures in nonmotile fibroblasts results in cell locomotion (Dunlevy and Couchman, 1993), presumably permitting a more favorable balance between intracellular contractile force and cell/substratum traction forces (DiMilla et al., 1993).

For the cell tail to detach preferentially, a front-vs-rear asymmetry in cell/substratum adhesion strength must exist across cell dimensions (Lauffenburger, 1989). Possible sources of such an asymmetry include differences in adhesion-receptor affinity for substratum ligands, adhesion-receptor number, or adhesion-receptor/cytoskeleton linkage avidity, between lamellipod and tail (DiMilla et al., 1991). The last of these possibilities was experimentally observed: integrin linkages to cytoskeleton in migrating NIH3T3 fibroblasts have been found to be more prevalent in the cell front than at the cell rear (Schmidt et al., 1993).

Along with transmitting force between the cell and substratum, integrins are involved in signal transduction to the cell from extracellular adhesion ligands (Lo and Chen, 1994; Sastry and Horwitz, 1993; Schwartz, 1992) and in 'inside-out' signaling (Ginsberg et al., 1992). The former could regulate adhesion-receptor/cytoskeleton linkage, while the latter could influence adhesion-receptor/ECM-ligand binding. Phosphorylation of the integrin cytoplasmic tail or cytoskeletal proteins likely affects the biophysical and biochemical properties of the focal adhesion. Consistent with this possibility, site-directed mutations of integrins mimicking phosphorylation exhibit weakened β_1 integrin linkages to cytoskeleton in NIH3T3 fibroblasts (Schmidt et al., 1993). Hence, the dynamic behavior of integrins in cell/substratum adhesion structures at the rear of the cell is likely to be important in governing cell migration speed.

Regen and Horwitz (1992) observed integrin macroaggregates on migrating chick fibroblasts using an anti-integrin antibody tagged with fluorescein. They reported that aggregates in migrating cells are dynamic in size and integrin density. Following rear detachment, a portion of the β_1 and α_5 integrin subunits remained behind on the substrate. Integrin-containing vesicles were detected in the cell body. Displacement of integrin staining on the cell edge and dorsal surface was also reported. However, given the high number and density of integrins on the cell surface, labeling the entire cell made tracking and rigorous analysis of individual aggregates virtually impossible.

In this present study, we investigate dynamic integrin behavior in the tail regions of migrating chick fibroblasts by tracking a photoactivatable fluorophore coupled to an anti-β_1 subunit antibody. Employing a caged fluorophore, activated by ultraviolet (UV) light as a label on the anti-integrin antibody, we follow specific integrin macroaggregates within a circumscribed region in cell tails. Computer-aided processing of images obtained with a cooled-CCD camera allows quantitation of the relative amount of integrin left on the substrate as cells advanced. We found four different modes of dynamic behavior of integrin macroaggregates during rear release. Consistent with results of previous studies (Regen and Horwitz, 1992; Altankov and Grinnell, 1995; Lawson and Maxfield, 1995) we measure a substantial fraction of the integrins left behind on the substrate and another significant fraction transported toward the cell body in endocytic vesicles. We also see dispersion of initially-aggregated integrins; sometimes these integrins can reform aggregates elsewhere on the cell surface. Unexpectedly, we observe some integrin-containing adhesion structures moving intact along the cell edge in the direction of cell migration, reattaching to the substrate and forming new adhesions in the cell tail or cell body. The apparent density of integrins within these structures varies with their rate of movement, with slower-moving structures increasing and faster-moving structures decreasing in fluorescence intensity.

MATERIALS AND METHODS

Cell culture

Skeletal muscle fibroblasts were obtained from myoblast preparations from 11-day-old chick breast muscle as described previously (Neff et al., 1982). Muscle fibroblasts were maintained for up to 10 passages in tissue culture treated plates with high glucose DME containing 10% FBS (Hybri-Max; Sigma Chemical Co.).

Microscopy plates were constructed by punching a hole in the bottom of a 35 mm tissue culture dish. Glass coverslips (22 mm^2, no. 1) were acid-washed in 20% HNO_3 for 30 minutes, rinsed with deionized water overnight, and silaned by exposure to hexamethyl-disilazane vapor (Sigma Chemical Co.) for 30 minutes at 200°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 Corp., Midland MI). Prior to use, the plates were sterilized with 70% ethanol and rinsed with sterile deionized water.

For migration experiments, cells in exponential growth phase were removed from 100 mm tissue culture plates with a wash of 1 ml 0.02% EDTA prepared in calcium and magnesium free phosphate buffer solution (CMF-PBS) followed by a 3 minute treatment with 150 μl 0.25% trypsin and 0.02% EDTA prepared in CMF-PBS. The coverslip in the bottom of a microscopy plate was coated with 40 μg/ml laminin for 1 hour at 37°C and then rinsed with sterile deionized water. Cells were resuspended in microscopy medium, high glucose DME containing 20 mM HEPES buffer (Sigma Chemical Co.), 50 units/ml penicillin and 50 μg/ml streptomycin, without phenol red. The cells were seeded at a low density, so they would not interfere with the migration of other cells, on the laminin-coated coverslip.

Matrix proteins, antibodies, and caged fluorescein

Laminin and fibronectin were prepared from murine Engelbreth-Holm-Swarm tumors and human plasma, respectively, as described previously (Ruoslahti et al., 1982; Rupert et al., 1982). ES66-8 rat hybridoma cells directed against the extracellular domain of chicken β_1 integrin were kindly provided by Dr Kenneth Yamada. The ES66 antibody was precipitated from the cell suspension supernatant by ammonium sulfate precipitation and purified using anion exchange chromatography (Duband et al., 1988). ES66 was covalently conjugated to water-soluble caged-carboxyfluorescein, generously provided by Dr Timothy Mitchison. ES66 (1.25 mg/ml) diluted in 0.1 M sodium bicarbonate at pH 9 was incubated at room temperature for 4 hours with the caged fluorescein at a dye to protein ratio of 100:1. Conjugated ES66 was separated from free dye by gel filtration. The ends of glass Pasteur pipettes (Fisher Scientific) were broken off and the remaining center cylinder was acid-washed and silaned to reduce protein adsorption to the glass surface. A small piece of cheesecloth was placed in the bottom of the pipette and 1 ml Sephadex G-25 (Pharmacia Inc., Piscataway, NJ), swelled in PBS, was added to the pipette and allowed to settle while the column was rinsed with several volumes of pH 9 $NaHCO_3$; 200 ml of the antibody-dye solution was added to the top of the column and allowed to drain in the column. The column was placed in a microfuge tube and spun five times for 3 minutes at 3,000 rpm in a tabletop centrifuge. The eluate contain-

ing the purified conjugated protein was collected, aliquoted, wrapped in foil, and stored under argon at –80°C. All procedures involving caged fluorescein were performed under red lights to prevent uncaging of the fluorophore.

Cell staining

For migration experiments, cells were plated on laminin coated coverslips in microscopy plates for 2 hours. Cells were incubated for 30 minutes at 37°C with caged-fluorescein-conjugated ES66, diluted to 80 μg/ml, determined as the optimal concentration for staining live cells, in warm microscopy medium. The cells were rinsed 8× with warm microscopy medium, and 3 ml warm microscopy medium was added to the plate.

Microscopy

A Diaphot inverted microscope (Nikon Inc., Tokyo, Japan) with a ×60/1.4 NA phase planapochromat objective was used for immunofluorescence studies. An electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) was used to control 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 to send to a Quadra 950 (Apple Computer, Cupertino, CA) for analysis. BDS-Image software (Oncore Imaging, Rockville, MD) was used to control the camera shutter and process the images.

Stained cells were placed in a temperature regulated, humidified atmospheric chamber, described previously (Regen and Horwitz, 1992), mounted on an inverted microscope stage. Warmed, humidified nitrogen was passed over the cells to reduce photo-oxidation of fluorescein. Cells continued to migrate for at least 24 hours under these conditions.

A fibroblast with a migrating morphology, possessing a wide lamellipod and a narrow tail, was located using phase optics, which did not remove the caging groups from the fluorescein molecules. A 1.0 mm pinhole (Melles Griot, Irvine, CA) in a slider mounted at the microscope epifluorescence field diaphragm plane was focused on the fibroblast tail. The fluorescein in a 10 μm spot was activated with a 100 millisecond burst of UV light from the 100 W mercury lamp through the pinhole, and 10 second exposures of immunofluorescence staining were acquired with the cooled CCD at various times using a fluorescein filter set. Cell position as a function of time was determined by acquiring phase contrast images immediately following fluorescence image acquisition. Images were acquired until the cell migrated off the field of view or photobleaching caused the activated dye to become too dim to observe, usually ten to fifteen 10 second exposures.

Image analysis

A variety of image analysis techniques were employed to enhance edges of adhesion structures and reduce blurring in the images due to out of focus light. A LaPlacian edge detection mask (Prewitt, 1970) which sharpens edges without regard to direction of the edge by approximating the second derivative of the image in the horizontal, vertical, and diagonal directions, was added to the original image. The mask was of the form:

$$\begin{matrix} -1 & -1 & -1 \\ -1 & 8 & -1 \\ -1 & -1 & -1. \end{matrix}$$

This mask creates a new image matrix, B, from the original image, A, according to the following function

$$b_{n,n} = 8a_{n,n} - a_{n-1,n-1} - a_{n-1,n} - a_{n-1,n+1} - a_{n,n-1} - a_{n,n+1} - a_{n+1,n-1} - a_{n+1,n} - a_{n+1,n+1}.$$

Image C (=A+B) has up to an order of magnitude greater adhesion structure to background pixel value ratio than image A, without losing significant detail. Images were contrast stretched to enhance the difference in intensity between the stained integrins and the background.

Cell edge position was added to fluorescent images by manual tracing of the cell edge on the phase image using Photoshop (3.0, Adobe Systems Inc., Mountain View, CA) and applying that layer to the fluorescent image. Movement of intensity was tracked by subtracting sequential images resulting in an image with bright areas where intensity was gained and dark areas where intensity was lost or by superimposing immunofluorescent images, with the earlier image displayed on a red channel and the later image on a green channel.

Quantitation of the amount of integrin in macroaggregates

Since a CCD produces pixel values linearly proportional to the number of photons striking the imager, we can quantitate the amount of integrin left behind on the substrate as the cell detaches. The amount of integrin in macroaggregates was quantitated by summing the pixel intensities of each pixel in the integrin aggregate. The aggregate dimensions were defined using an N-triangulation algorithm in BDS-Image on image C, the sum of the LaPlace transform and the raw data. This mask was applied to image A, the raw data immunofluorescent image. The average background pixel value for the region surrounding the aggregate was measured and subtracted from each pixel in the raw image. If the aggregate was in the photoactivated spot, then part of the photoactivated spot outside the cell was used to calculate the background. If the aggregate was outside the photoactivated spot, then an area outside the spot was used to calculate the background. Using the mask which defined the aggregate dimensions and the raw image with the background subtracted, a measure of the amount of integrin in the aggregate was calculated by summing all of the pixel values comprising the aggregate.

We demonstrated that fluorescence intensity is linearly proportional to antibody concentration over the range of intensities in our study by coating coverslips with various concentrations of fluorescein and exposing the camera for 15 seconds. We also determined that for greater than 90% of the photoactivated spot, activation of the fluorophore was uniform. Aggregates which were on the edge of the spot, where an activation intensity gradient existed, were not used in the quantitative study. To quantitate the integrin on the substrate, the average background value was subtracted from the image and the intensities of all pixels in the aggregate were summed.

RESULTS

Fibroblasts with antibody-labelled integrins are motile

We use a nonadhesion-perturbing ES66 anti-β_1 integrin subunit antibody to track the dynamics of integrins in chick skeletal myofibroblasts. To follow the integrins via immunofluorescence microscopy, we conjugate photo-activatable, caged carboxy-fluorescein molecules to the antibody. UV light passing through a pinhole focused on the desired area of the cell activates the fluorophore in a 10 μm diameter circle. The movement of fluorescent intensity as the cell migrates reveals the fate of integrin macroaggregates initially in the cell tail.

Since the goal of this study is to determine how integrin dynamics affect cell locomotion, we choose only migratory cells for observation. The presence of a long, narrow tail large enough to photoactivate is also an important criterion for selecting cells to observe. Together these features give the observed cells a triangular-shaped morphology with a very thin, wide, ruffled leading edge, a thick cell body, and a thin, narrow, elongated tail: 80% of the cells exhibiting this morphology are motile, compared to about half of the general cell population.

Quantification of a parameter characterizing cell motility is

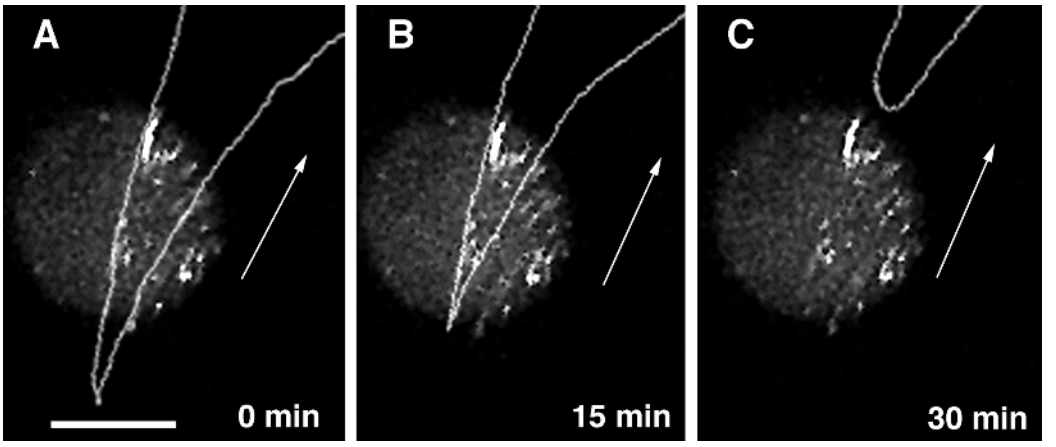

Fig. 1. Integrins remain behind as tracks on the substratum during migration. Anti-β_1 integrin immunofluorescence images show the tail region of a migrating cell at the time the rear is photoactivated (A), 15 minutes after activation (B), and 30 minutes after activation (C). Integrin macroaggregates appear as bright elongated structures within the photoactivated circle. The cell outlines, obtained from the phase images, are traced on the fluorescent images. Net cell translocation as well as tail translocation is toward the top of the images, as indicated by the arrow. As the tail moves off the photoactivated spot, macroaggregate intensity decreases slightly, but most of the integrin is left behind attached to the substrate. Bar, 10 μm.

problematic because different areas of the cells move at different rates resulting in shape changes. The lamellae often extend with no movement of the cell tail. The cell rear can break free from the substratum and leap forward a large distance without a corresponding displacement of the cell front. Additionally, the cells reverse often and greatly vary their motility rates over time, making instantaneous speeds difficult to measure. However, following the distance traveled by the cell centroid gives a good representation of cell motility.

The labeling of integrins with the nonadhesion perturbing ES66 monoclonal antibody does not significantly affect the rate of migration or morphology of skeletal muscle fibroblasts on laminin. Measurement of cell centroid movements indicates that unlabelled fibroblasts, chosen using the criteria mentioned above, migrate at a rate of 70 ± 50 μm/hour (± s.d., n=20) while fibroblasts labelled with ES66 coupled to inactivated caged fluorescein molecules migrate at 60 ± 60 μm/hour. In both cases about one quarter of the cells exhibit the motile triangular morphology described above, while the rest either are motile with very small tails or are nonmotile.

To ensure that exposure to UV light during photoactivation does not affect migration, we photoactivated several entire caged-fluorescein/ES-66 labelled fibroblasts with a bursts of UV light and measured migration speed. A 100 millisecond burst of UV light does not affect migration speed, but exposures above 1 second decrease motility.

Skeletal muscle fibroblasts plated on a laminin substrate form into organized structures, similar to, but finer and less organized than focal adhesions formed when the cells are plated on fibronectin. These structures, referred to as macroaggregates, contain the $\alpha_5\beta_1$ fibronectin receptor, vinculin, actin, and talin (Regen and Horwitz, 1992).

The majority of integrins in macroaggregates remain fixed to the substrate as cells advance

As the cells move forward, the majority of the photoactivated macroaggregates remain fixed with respect to the substrate. Even as the tail detaches and moves out of the original photoactivated region, most of the integrin is left behind attached to the laminin-coated substrate. Fig. 1 shows an example of integrin trails which remain substantially intact upon detaching from the cell membrane. In all cells which move out of the original photoactivated region and which possess detectable macroaggregates, some integrin trails are left behind on the substratum. Although the cell edge at the cell rear can be very thin, the phase contrast microscopy gives a clear definition of the cell edges. The fraction of integrins left behind on the substrate is variable, even among different macroaggregates in the same cell. Once outside the cell, integrin trails are very stable and remain detectable for up to five hours. Photoactivation of a region behind a motile cell often reveals a meshwork of integrin trails covering the substratum.

Under phase contrast microscopy, pieces of membrane are often seen attached to the substratum at the same location where the macroaggregates were torn from the cell. The presence of integrin trails indicates that the integrin-cytoskeleton bond or other cytoskeletal linkages generally sever at a lower force than the integrin-substratum bond.

Fig. 2 shows a histogram of the fraction of integrin left behind on the substrate as the tail detaches from a stationary macroaggregate for 29 aggregates. An average of 81±15% of the integrin is left on the substrate from adhesions which remain stationary as the cell detaches, indicating that aggregate ripping from the cell membrane results in a major loss of integrins for migrating fibroblasts.

Intracellular vesicles transport integrin from the cell rear to the cell body

In addition to the fairly dim, elongated integrin macroaggregates which generally remain fixed with respect to the substratum, bright round integrin-containing cytoplasmic vesicles are evident in many of the cells (Fig. 3). The intensity of vesicles fluctuates as they move within the cell. Focusing on the vesicles and observing the corresponding focal plane on phase contrast images demonstrates that the vesicles move vertically as well as laterally, indicating that they are not attached

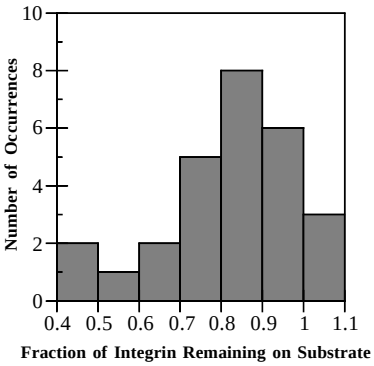

Fig. 2. Histogram of the fraction of integrin left in macroaggregates upon rear detachment. The amount of integrin in 29 stationary integrin macroaggregates is compared before and after rear detachment from the macroaggregate. Integrin amounts are calculated by subtracting an average background from the image and then summing the pixel values in the aggregate. The average fraction remaining adhered to the substrate is 0.81±0.15.

to the cell membrane. These vesicles move in the same direction as the cell, often faster than the tip of the tail, until they reach the perinuclear region where they move at approximately the same rate as the cell nucleus. The ultimate fate of the vesicles after reaching the cell nuclear region cannot be determined since the cells generally move out of the field of view. However, photoactivation of the entire cell at the conclusion of the experiment reveals the presence of the antibody in large vacuole-like structures in addition to a large number of vesicles in the perinuclear region. Photoactivation of a 10 μm spot in the cell body illuminates vesicles which move about the perinuclear region but never enter the lamellipod or tail. Photoactivation in the lamellipod never reveals a vesicle. Therefore, these vesicles do not transport integrins to the leading edge of the cell, necessitating an additional transport mechanism if these integrins are recycled to the lamellipod.

Of the 26 motile cells observed, 21 have at least one vesicle initially in the photoactivated region on the tail. Up to 10 photoactivated vesicles are detected from a single photoactivated spot. Vesicles move in the same direction as the cell cortex

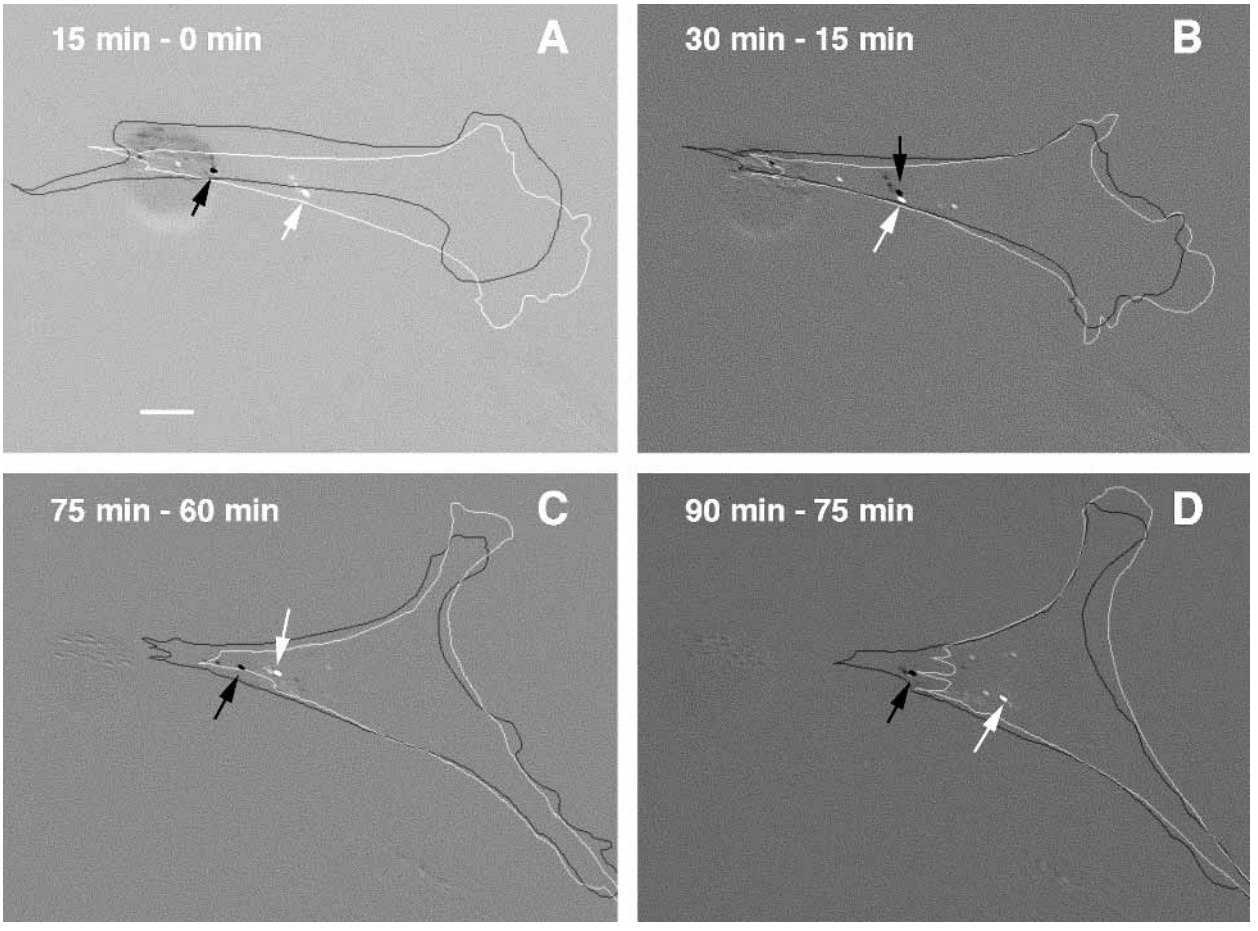

Fig. 3. Movement of integrin vesicles in the tail is toward the cell body. Subtraction of anti-β_1 integrin immunofluorescence image from earlier image highlights the movement of integrin-containing vesicles from the cell rear to the cell body. Black represents loss of intensity and white represents gain, with the background gray representing no change in intensity. The initial image is subtracted from the 15 minutes postactivation image (A), 15 minutes from 30 minutes (B), 60 minutes from 75 minutes (C), and 75 minutes from 90 minutes (D). The cell outline from the earlier time is traced on the fluorescent image in dark gray and the later time in light gray. Vesicles in the tail move into the cell body as the cell migrates forward (left to right) and eventually reside in the perinuclear region. The movement of one particular vesicle is indicated by black and white arrows. Bar, 10 μm.

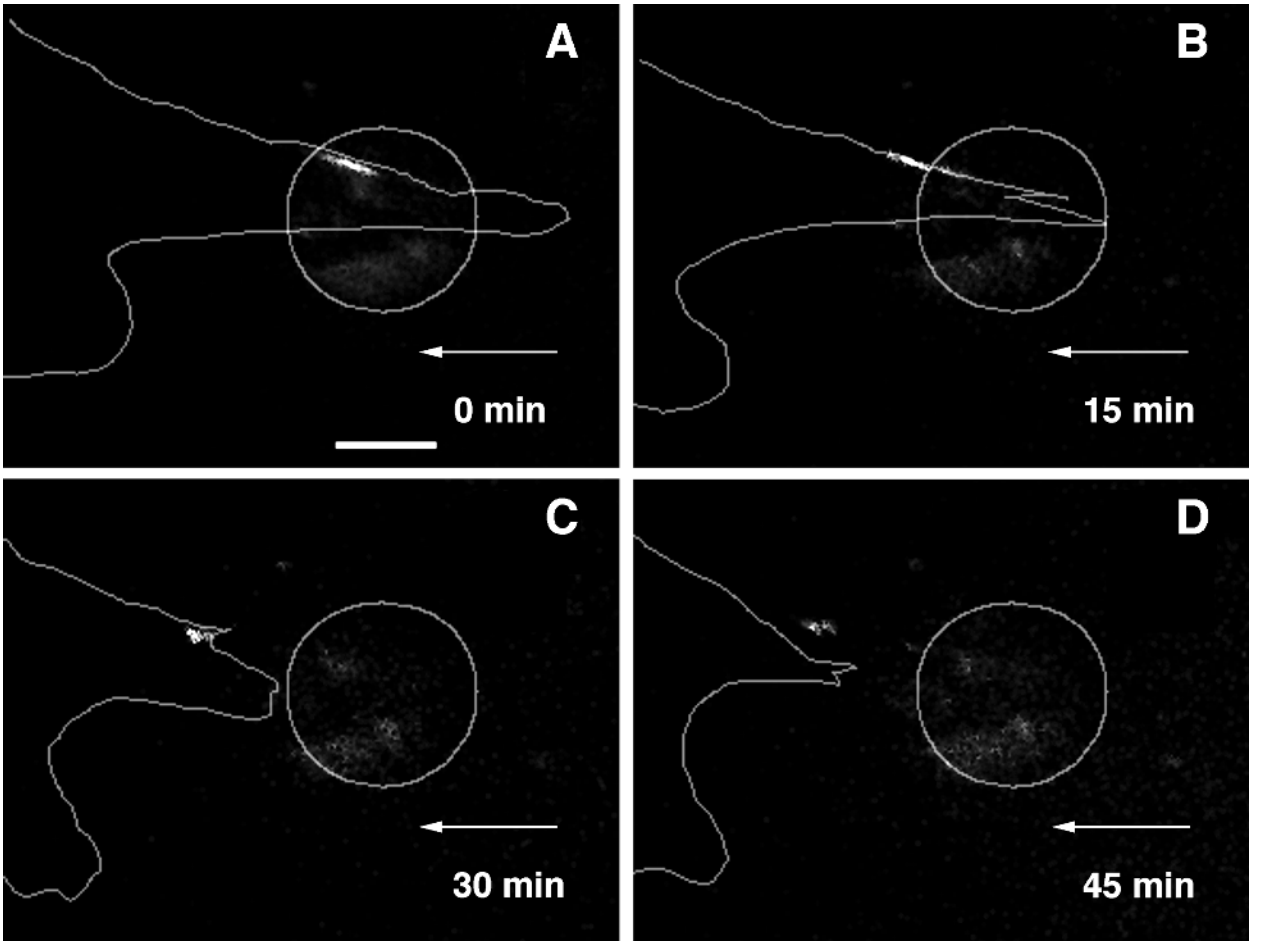

Fig. 4. Integrin macroaggregates can undergo detachment and reattachment as the cell migrates. After photoactivation (A) one aggregate along the cell edge is illuminated. By 15 minutes (B) and 30 minutes (C) the macroaggregate moves out of the photoactivated region, staying along the cell edge. By 45 minutes (D) the macroaggregate reattaches to the substrate and is left behind the cell as an integrin trail with a significantly lower integrin density than the macroaggregate in the cell (A-C). The cell outlines and the photoactivated region are traced on the immunofluorescent images. The cell moves from right to left throughout the experiment. Bar, 10 μm.

moves. In 5 cases, the cell under observation reverses direction and in each case the vesicles in the cell also reverse direction simultaneously. When a cell extends lamellipodia in different directions, vesicles move toward the closer extension. In some cases, vesicles which cannot be accounted for at previous time points are detected in the photoactivated region, likely due to the formation of new vesicles. The definite formation of a new vesicle is never conclusively identified perhaps because vesicles travel in and out of the observed focal plane, but they presumably may form in the tail of the cell since they are present at the very rear of the cell and only move toward the cell body. It is unclear if vesicle formation occurs preferentially in the tail or also occurs in the cell body.

Integrin macroaggregates can translocate

While most macroaggregates in a migrating fibroblast remain attached to the substratum and detach from the cell as it moves out of the photoactivated area, occasionally a macroaggregate will move forward out of the photoactivated area with the cell. Fig. 4 displays an example of a macroaggregate which is pulled outside of the photoactivated area along the edge of a migrating cell. A large macroaggregate moves forward along the edge of the cell. During this movement it changes shape, reattaches to the substrate, and rips from the cell membrane, remaining attached to the substrate as a stable integrin trail. We discounted the possibility that this structure may be a vesicle since it possesses the characteristic shape of integrin macroaggregates and remains attached to the substrate as an integrin trail. Aggregate translocation is fairly common and presumably employed by all of the cells. However, we only photoactivate about 10% of the total uropodal area and see macroaggregate movement along the cell edge in the direction of cell migration in 12 of 20 motile cells. In 5 of these cells the translocated macroaggregates are left on the substrate as integrin trails. In the remaining cells the adhesions either disperse (3 cases) or reattach to the substrate and remain within the cell throughout the experiment (4 cases). Macroaggregates always move along the cell edge, indicating that adhesions along the cell edge may have a greater affinity for the cytoskeleton or a lesser affinity for substrate proteins than adhesions not along the cell edge.

Quantitation of the integrin in aggregates before and after ripping from the cell membrane, in the five cells which exhibit moving aggregates that eventually rip from the cell and are deposited on the substrate, reveals that 80±18% of the integrin

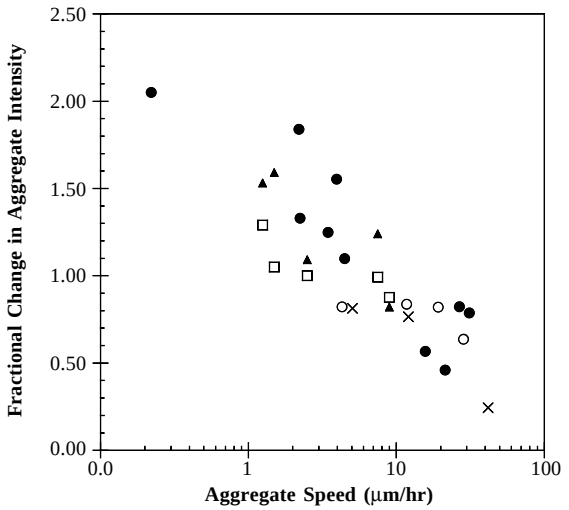

Fig. 5. Integrin macroaggregate intensity decreases as macroaggregate speed increases in fibroblasts with aggregates which move along the cell edge. Macroaggregate intensity and centroid speed were measured every 15 minutes throughout the course of the experiment. Fractional change in intensity is the ratio of intensity at one time to the intensity at the previous time point. Aggregate intensity is calculated by subtracting an average background pixel value from the image and summing the pixel values in the aggregate. Each point shape represents a different macroaggregate.

remains on the substrate after the moving aggregate rips from the membrane, the same amount as remains when stationary aggregates rip. Calculation of aggregate fluorescence intensity as the macroaggregates translocate indicates that aggregate intensity tends to decrease as macroaggregate speed increases (Fig. 5). The aggregate intensity indicates the amount of integrin initially from the photoactivated spot in an aggregate, accounting for both aggregate size and density. Neither size nor density alone correlates with aggregate speed. Measured intensity does not necessarily indicate total integrin density since unlabelled integrin can also associate with the aggregates. Macroaggregate speed correlates with cell speed as measured at the very posterior tip of the cell (data not shown), although the aggregate usually moves more slowly than the cell. During the experiment, cell speed and aggregate intensity are very dynamic, increasing and decreasing often. At low macroaggregate speeds (below 5 μm/hour), aggregates tend to remain at the same intensity or gain intensity, sometimes increasing intensity by as much as 100% during 15 minutes. At higher aggregate speeds (above 10 μm/hour), aggregate intensities decrease exclusively, losing as much as 70% of their intensity over 15 minutes.

Integrin macroaggregates break up by dispersion of integrins on the cell membrane

Integrin which is not left behind on the substrate must be transported out of the cell tail since integrin does not accumulate in the rear of the cell (Regen and Horwitz, 1992). Integrin-containing vesicles are attractive candidates for returning integrin to the cell body, but we have not been able to observe directly the formation of a vesicle from an integrin macroaggregate. Macroaggregates within the cell often disappear during the 15 minutes between immunofluorescence observations, so we decreased the time between image acquisitions to 3 minutes to try to identify the nature of aggregate disassembly.

Fig. 6 shows anti-β_1 immunofluorescence images at 3 minute time intervals for an integrin macroaggregate which moves out of the photoactivated region along the cell edge as the cell moves toward the lower left hand corner of the image. At 15 minutes post activation the integrin staining changes from an intense, well defined macroaggregate on the cell edge to a faint, disperse staining. Observing several focal planes of the cell reveals that the disperse staining is not due to out of focus light caused by the macroaggregate moving to the dorsal surface of the cell. The diffuse integrins continue to disperse away from the cell edge and are no longer detectable 6 minutes after macroaggregate dispersion. One method of macroaggregate degradation appears to be through dispersion of the constituent integrins, although the subsequent fate of these dispersed integrins is unknown since the fluorescence of individual integrins is too dim to be detected by fluorescence microscopy.

New integrin macroaggregates form outside the tail from integrins initially in the tail

Macroaggregates found outside the photoactivated region do not always originate from a single structure moving ahead with the cell. Fig. 7 shows a new macroaggregate form along the cell edge outside the photoactivated region by several integrin-containing structures which translocate along the cell edge and possibly diffuse integrins. These structures do not behave consistently with vesicles since they do not move into the cell body and generally translocate along the cell edge. Since this cell is moving very slowly, these structures are likely to be moving aggregates which are able to remain intact by shrinking and growing according to their interactions with the substrate (see Fig. 5) rather than disperse as in Fig. 6. The aggregates vary in size and intensity as they move along the cell edge and congregate to form a new macroaggregate in the cell body (Fig. 7D and E). Disperse staining near the aggregates is likely involved in the formation of the new macroaggregate, but may only disperse in the membrane. The new macroaggregate remains relatively stationary with respect to the substrate as the cell advances. As the macroaggregate reaches the very rear of the cell (Fig. 7F), the cell reverses direction again and the aggregate does not rip out of the membrane. Aggregate formation at the cell rear ahead of the photoactivated spot also occurs. Thus, cells reuse integrins by forming new adhesions from integrins which were previously in another adhesion structure in the tail.

Frequency of integrin fates

We observe four different fates for integrins in the cell tail: (1) ripping from the cell and remaining attached to the substrate as an integrin trail; (2) transport to the cell body in vesicles; (3) translocation of intact aggregates; and (4) dispersion of aggregates. In each cell followed during this experiment, multiple integrin-containing structures were identified. The image enhancement techniques used to illustrate different integrin fates often mask other fates in the same cell (Figs 1, 3, and 5). Figs 6 and 7 show macroaggregates which rip from the cell as well as vesicles and macroaggregates which translocate along the cell edge. The frequency of each of these events

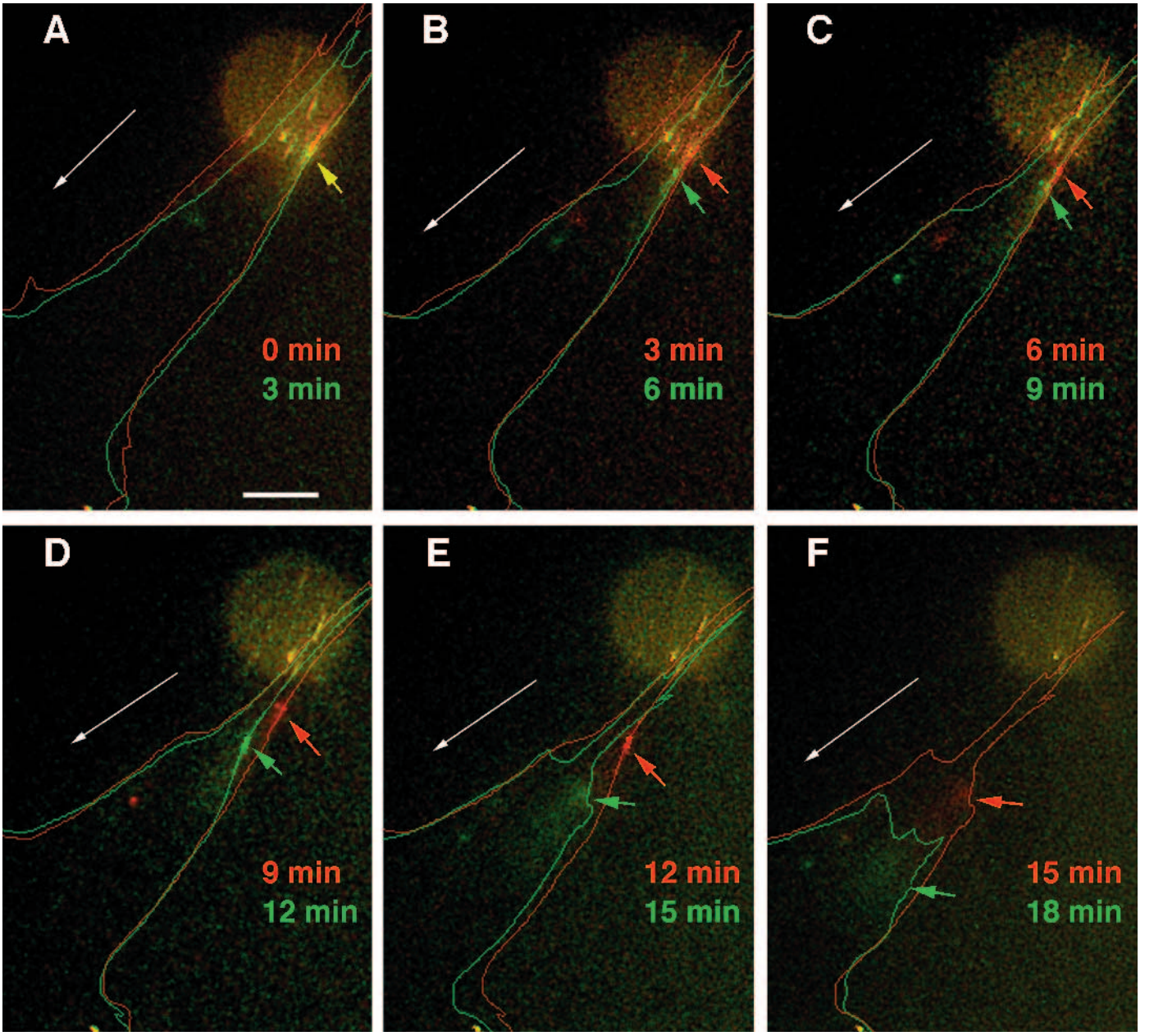

Fig. 6. Integrin macroaggregates disperse into diffuse integrin. Anti-β_1 immunofluorescence images are superimposed, earlier time in red and later time in green, at photoactivation and 3 minutes (A), 3 and 6 minutes (B), 6 and 9 minutes (C), 9 and 12 minutes (D), 12 and 15 minutes (E), and 15 and 18 minutes (F). Cell edges are traced on the immunofluorescent images in corresponding colors and the arrows indicate that the cell moves from the upper right to the lower left throughout the experiment. The yellow arrow (A) indicates the macroaggregate which disperses. Red and green arrows indicate the movement of a macroaggregate with the cell edge for 9 minutes (B-D) and then the dispersion of the macroaggregate (E and F). Bar, 10 μm.

is shown in Table 1. All cells which migrate out of the photoactivated spot leave integrin trails on the substrate. Vesicular transport of integrins to the cell body and translocation of integrin aggregates along the cell edge are also fairly common events, occurring in about three-quarters and half of the motile cells, respectively. Dispersion of integrin aggregates on the cell edge is less common, occurring in about 20% of motile cells. Formation of new macroaggregates outside the photoactivated spot is a relatively rare event, occurring in less than 10% of the cells. Note that the values reported here are only relative frequencies, not probabilities of occurrence, since only a very small region on the cell is photoactivated.

Additionally, edge aggregates which translocate with the cell are susceptible to either ripping out of the membrane in the tail or dispersing on the cell edge. Table 2 shows that the frequency of ripping from the membrane is about 75% greater than the dispersion rate. Four of the twelve aggregates do not disperse or rip from the membrane either because the cell becomes immotile or reverses direction, keeping the aggregate with the cell for the duration of the experiment. Presumably,

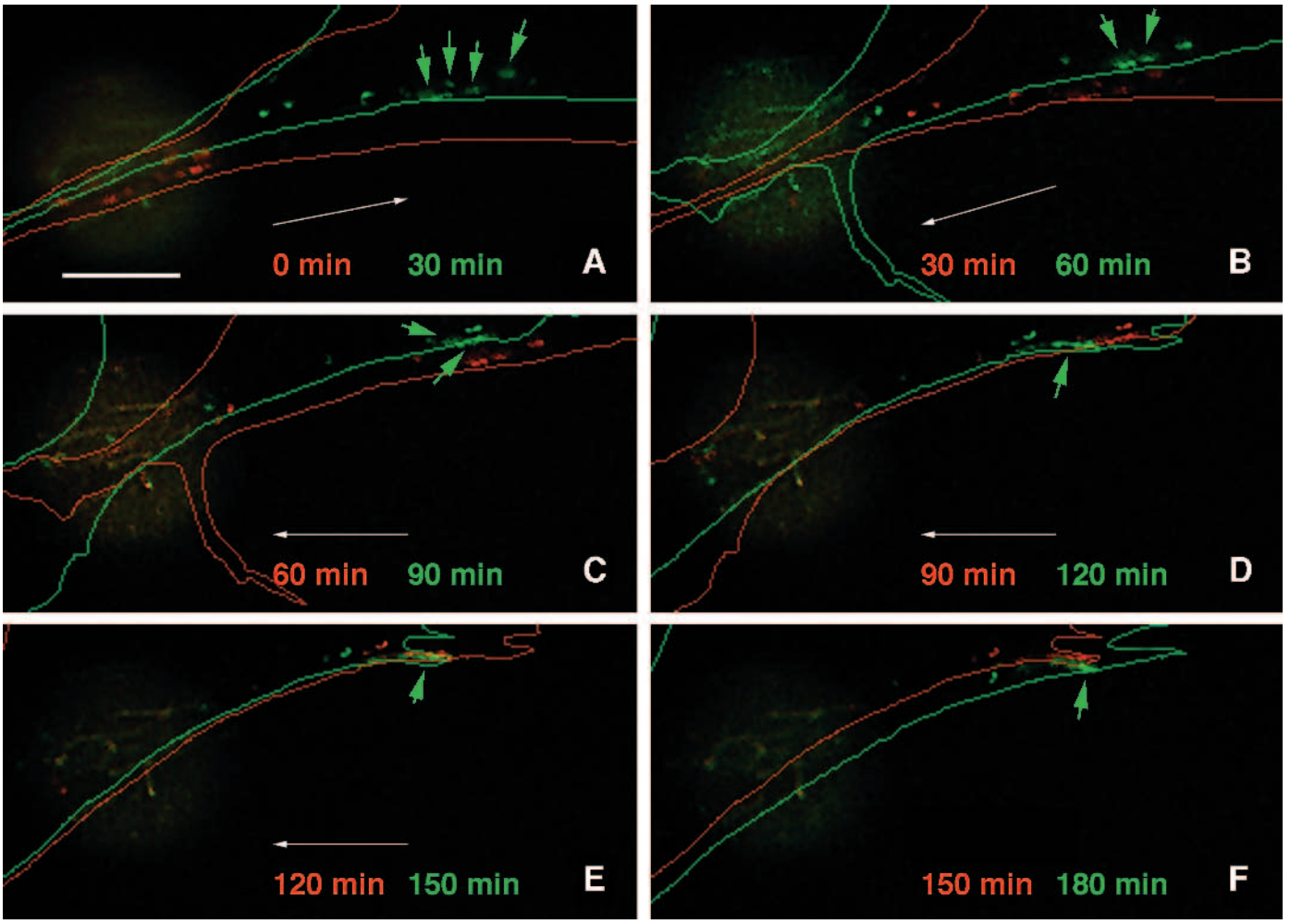

Fig. 7. Integrin macroaggregate forms outside the photoactivated area from diffuse integrin initially within the photoactivated area. Anti-β_1 immunofluorescence images are superimposed, earlier time in red and later time in green, at photoactivation and 30 minutes (A), 30 and 60 minutes (B), 60 and 90 minutes (C), 90 and 120 minutes (D), 120 and 150 minutes (E), and 150 and 180 minutes (F). Cell edges are traced on the immunofluorescent images in corresponding colors and the arrows show the direction of net cell centroid movement. Green arrowheads indicate integrin macroaggregates and diffuse staining which coalesce to form a new macroaggregate. Integrin moves forward with the cell in the form of vesicles and macroaggregates moving along the cell edge (A-D). Some of the integrin forms into a new macroaggregate which remains fixed with respect to the substrate as the cell continues to migrate (E and F). Bar, 10 μm.

eventually these aggregates also either rip from the membrane or disperse.

DISCUSSION

Understanding integrin dynamics and the nature of the cytoskeleton-integrin-substratum linkage in a migrating cell is an essential part of understanding and manipulating the mechanisms responsible for cell migration. To investigate these mechanisms and determine the fate of integrins from released adhesions in the cell we devised a system to track integrin macroaggregates from the cell rear. Photoactivatable, fluorescently labelled anti-β_1-integrin-subunit antibodies are used to track integrin macroaggregates in a chick skeletal muscle fibroblast tail as the cell migrates. In order to determine how a migrating cell regulates rear release we ascertain the fate of integrins in these macroaggregates as the cell rear releases from the substratum. We detect four fates of the integrin macroaggregates: (1) integrin macroaggregates remain attached to the substrate as the cell migrates; (2) integrin-containing endocytic vesicles are transported to the cell body; (3) integrin macroaggregates are translocated along the cell edge or reform outside the photoactivated spot from integrins initially within the photoactivated spot; and (4) integrin macroaggregates disperse in the cell membrane along the cell edge. Vesicle transport and integrin trails on the substrate are very common, occurring in almost all migrating fibroblasts, while translocating and dispersing macroaggregates are rarer, occurring in the photoactivated region in about 50% and 20% of the cells observed, respectively. A previous study by Regen and Horwitz (1992) identified macroaggregate detachment from the cell and vesicle transport, and postulated macroaggregate movement and dispersion. However, the use of photoactivatable fluorescein allows us to reduce background fluorescence and track indi-

Table 1. Relative frequencies of the fates of integrin macroaggregates in the tail of a migrating fibroblast

Macroaggregate fate	Proportion of cells exhibiting fate*	Number of cells observed
Aggregate rips from cell membrane	1.00±0.00	20
Integrin moves to cell body in vesicles	0.73±0.09	26
Edge aggregate moves forward with cell	0.46±0.10	26
Edge aggregate disperses	0.19±0.07	26
Dispersed integrins are reused in a new aggregate	0.08±0.05	26

The integrin macroaggregates in each migrating cell are inspected for ripping from the cell membrane, moving with the cell, or dispersing in the cell membrane. The presence of integrin-containing vesicles and the formation of new aggregates from dispersed integrins is also determined. A cell is deemed to exhibit the fate if the events is detected one or more times.
* ± s.e.m.

Table 2. Relative frequencies of the fates of integrin macroaggregates which translocate along the cell edge in a migrating fibroblast

Macroaggregate fate	Proportion of aggregates exhibiting fate*	Number of aggregates observed
Aggregate rips from cell membrane	0.42±0.14	12
Aggregate disperses	0.25±0.13	12

Each translocating integrin macroaggregate is observed for eventual ripping from the membrane or dispersion into the cell membrane. The remaining aggregates exhibit neither fate during the course of the experiment.
* ± s.e.m.

vidual macroaggregates to conclusively demonstrate macroaggregate translocation and dispersion as well as observe the formation of macroaggregates from integrin initially in the cell tail. Improvements in fluorescent imaging technology also allow us to reliably measure the fraction of integrin which remains attached to the substrate upon aggregate detachment from the cell as 81±15% and the relative frequencies for the fates of integrin-containing structures in the cell.

Our results indicate that dissociation of the integrin/cytoskeletal or cytoskeletal/cytoskeletal bonds, rather than integrin/substrate detachment, is the rate-limiting step of rear detachment. Virtually all of the integrin macroaggregates rip from the cell membrane and most of the integrins in these aggregates remain attached to the substrate upon detachment. Rear release in migrating fibroblasts appears to be caused in part by tension created by elongation of the tail upon the advance of the cell and active contraction of the cytoskeleton (Chen, 1981; Crowley and Horwitz, 1995). These forces may approach a critical point at which they sever the weakest bond in the cytoskeletal-integrin-substratum linkage, denoting a stronger linkage between the integrins and substratum than between the integrins and cytoskeletal elements. Alternatively, there may be a cellular control mechanism regulating rear release by altering the properties of the integrin-cytoskeletal linkage and facilitating cleavage of the integrin-cytoskeletal element bonds. To this point, no such control mechanism has been described, but surges of intracellular Ca^{2+} levels have been detected in association with rear release in neutrophils (Marks et al., 1991). Potentially, a control system might include region-specific post-translational modifications of integrins or other cytoskeletal proteins in a migrating fibroblast. In addition to tension created by cytoskeletal contraction, tyrosine phosphorylation is involved in the destabilization of focal contacts at the rear of the cell. 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 the cells resided prior to detachment. Adhesion release is attributed to two phenomena, tyrosine phosphorylation of cytoskeletally associated proteins and tension generated by cell contraction. There is evidence that rho, rac, and cdc42 regulate the formation of focal adhesions and focal complexes (Nobes and Hall, 1995). Their action may also be important in focal adhesion destabilization at the cell rear.

Aggregate ripping from the cell membrane in migrating fibroblasts is also a physiologic phenomenon. Observation of migration of corneal fibroblasts in vivo has shown that pieces of cell membrane are left attached to the substrate upon rear detachment (Hay, 1985). The loss of large amounts of integrins from the cell to the substrate upon detachment appears to be a very inefficient method of migration. To sustain migration for prolonged periods of time, the cell must compensate for the loss of cellular integrins with synthesis of new integrins. Physiologically, however, fibroblast migration occurs over relatively short distances. Cells which move more often and over larger distances than fibroblasts, such as neutrophils or keratinocytes, move with a rapid, constant-velocity, gliding motion with extension and retraction occurring perpendicularly to the cell edge (Lee et al., 1993) rather than the slower, discrete extensions and retractions characteristic of fibroblast migration. We speculate that such cells do not expend the vast quantities of integrins used in fibroblast migration because they have a more coordinated migration mechanism or they form different types of adhesion linkages. The slow migration of fibroblasts may result from a regulatory mechanism allowing detachment of strong integrin-cytoskeletal adhesions while the rapid migration of other cell types may result from tension more easily detaching weaker integrin-substratum adhesions.

A portion of the integrin which is not deposited on the substrate behind the migrating fibroblast is transported to the cell body by integrin-containing vesicles. β_1 integrins participate in endocytosis and recycling (Bretscher, 1989; Sczekan and Juliano, 1990; Tawil et al., 1993; Watts and Marsh, 1992; Altankov and Grinnell, 1995). The binding of the antibody to the integrin probably results in a higher level of integrin internalization than normal, with most of this integrin likely degrading in lysosomes. Our observations of numerous integrin-containing vesicles moving from the cell rear to the cell body suggest these vesicles may be an important method actively employed by the cell to prevent integrin accumulation in the cell rear. The transport of integrin-containing vesicles is always toward the cell body and is often more rapid than the overall cell speed, suggesting a directed transport of the vesicles. Neither vesicle formation nor vesicle fate could be ascertained using our method. Blockage of recycling but not endocytosis in CHO cells adhered to fibronectin results in the disruption of adhesion plaques in the cell periphery but not in the cell body (Tranqui et al., 1993), suggesting that integrins in tail macroaggregates are dynamic. Exchange of aggregated integrins with free integrins or the dispersion of integrins may weaken the adhesion, allowing rear detachment. In neutrophil

migration on vitronectin, transient increases in Ca^{2+} concentrations in the cell tail allow the $\alpha_v\beta_3$ integrin to detach from vitronectin and endocytose into vesicles for possible transport to the leading edge of the cell (Lawson and Maxfield, 1995).

Instead of ripping from the cell membrane and remaining attached to the substrate, in some cases integrin macroaggregates can detach from the substratum, move intact along the cell edge, and reattach to the substratum. These aggregates do not leave detectable levels of integrins attached to the substrate as they translocate forward, but they may rip from the membrane and remain behind the cell attached to the substrate virtually intact, identical to the integrin trails mentioned previously. The movement of the integrin macroaggregates is similar to the movement of aggregated Pgp-1, thought to be linked to the cytoskeleton, from the uropod to the perinuclear region of migrating fibroblasts (Holifield and Jacobson, 1991). A balance between regulated rear release and applied tension explains why most macroaggregates detach from the cytoskeleton and remain fixed to the substrate while others detach from the substrate. Rear release in migrating fibroblasts consists of a series of passive elastic recoils and active ATP-dependent retractions (Chen, 1981). The ATP could be necessary for cytoskeletal contraction to detach the rear, or alternatively to phosphorylate proteins in the adhesion linkage, allowing the macroaggregates to detach from the cytoskeleton. The adhesions which detach from the substrate are likely not weakened by the rear release mechanism to the extent the adhesions which detach from the cytoskeleton are weakened. Yet, the tension applied to the moving adhesions is sufficient enough to break the integrin-substratum bonds.

The aggregates only move during rear retraction and move over a wide range of speeds, from 1 to 80 μm/hour. Aggregate speed correlates with cell tail tip speed, but is generally slightly slower. The aggregates change in shape and integrin density as they move with the cell but always remain along the cell edge. As they move, the macroaggregates can either gain or lose intensity and the change in intensity correlates with cell speed. Eventually the moving aggregates either reattach to the substrate and form a new adhesion or disperse their integrins into the cell membrane. The behavior of these aggregates can be explained by analyzing the forces on the aggregates as the cell advances. As the cell migrates, the forces on the macroaggregate increase until they exceed a critical point and cleave the integrin-substratum bonds. The aggregate moves in the direction of the applied force and the tension on the aggregate decreases as the tail retracts. Eventually, the force on the aggregate drops below the critical level and the aggregate stably reattaches to the substrate. Integrin receptor occupancy stabilizes the formation of focal adhesions (Miyamoto et al., 1995). If the aggregate moves slowly, it can generally remain attached to the substrate and stabilize as a result of its interactions with the substrate proteins, allowing the aggregate to grow. If the aggregate moves rapidly, its interactions with the substratum are more transient, perhaps resulting in a greater rate of integrin dispersion from the aggregates or more integrin deposition on the substratum as the aggregate moves forward.

In addition to ripping from the cell membrane, integrin macroaggregates disassemble and disperse their integrins in the cell membrane. Aggregate dispersion is much rarer than ripping from the membrane, and was only observed among aggregates which translocated along the cell edge as the cell migrated. This suggests that integrin dispersion requires aggregate detachment to occur. If the aggregate moves forward in rapid jumps it is unlikely to be detached for several minutes, the time scale of macroaggregate dispersion. However, since the aggregate is on the cell edge, the changing shape of the tail during rear detachment or the forces applied by cytoskeletal contraction may cause the aggregate to move to the dorsal surface of the cell resulting in loss of receptor occupancy and integrin dispersion. Unfortunately, our method cannot distinguish between movement from the ventral to the dorsal surface of the cell and the beginning of aggregate dispersion. The fate of dispersed integrins is unknown. Laser-optical trapping of anti-integrin antibody coated gold beads on the leading-edge dorsal surface of a migrating fibroblast demonstrate directed transport of the integrins to the cell edge (Schmidt et al., 1993). If such a mechanism also exists in the tail, upon reaching the cell edge the integrins may congregate into endocytic vesicles, consolidate into macroaggregates, or translocate to the cell body for the formation of new adhesions.

Integrins from macroaggregates which translocate from the tail are reused in other adhesions. Dispersed integrins are also probably incorporated in these new adhesions. Since we can only track integrins initially in the tail, we cannot tell if the photoactivated integrins add to existing adhesions or form new macroaggregates. Integrin reuse is primarily via translocation

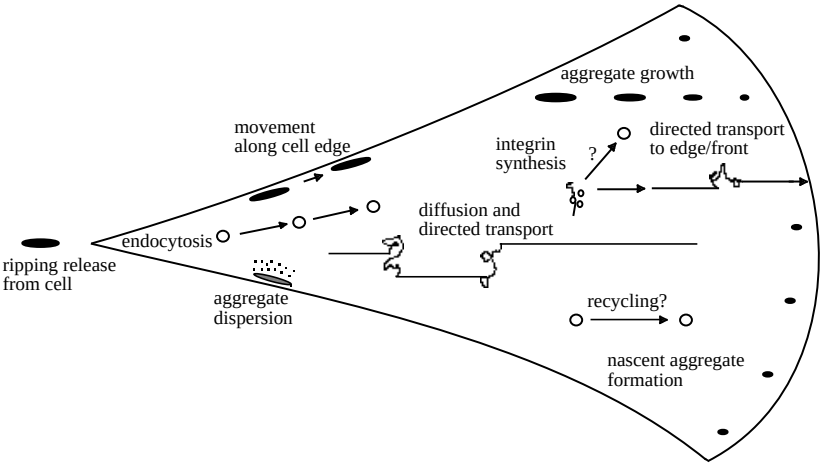

Fig. 8. Schematic diagram of integrin dynamics in migrating fibroblasts. New integrin is synthesized and transported to the lamellipod along cell surface routes or possibly in vesicles. There the integrins form nascent aggregates which grow in size as the cell migrates over them. At the cell rear the aggregates rip from the membrane, translocate along the cell edge or disperse into free integrins. Endocytosis and vesicular transport, as well as diffusion and directed migration, return integrins from the rear to the cell body and lamellipod.

of existing macroaggregates but in 2 of 5 cases where an aggregate dispersed, some of the dispersed integrins appears to reform into a new adhesion structure. In the other 3 cases the integrins are probably also at least partially reused, but the density of fluorophore is not bright enough for us to detect.

Fig. 8 shows a schematic of integrin dynamics in a migrating fibroblast. Nascent integrin progresses to the lamellipod via directed transport along the cell membrane or possibly by vesicular transport. New aggregates form at the leading edge and grow in size as the cell migrates over them. When the aggregates reach the cell rear, they rip from the membrane, translocate along the cell edge, or disperse their integrins into the cell membrane. Endocytosis and vesicular transport, along with diffusion and directed transport, return the free integrins from the rear to the cell body and lamellipod.

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REFERENCES

Abercrombie, M., Heaysman, J. E. M. and Pegrun, S. M. (1970). The locomotion of fibroblasts in culture. I. Movements of the leading edge. *Exp. Cell Res.* **59**, 393-398.

Altankov, G. and Grinnell, F. (1995). Fibronectin receptor internalization and AP-2 complex reorganization in potassium-depleted fibroblasts. *Exp. Cell Res.* **216**, 1-11.

Bretscher, M. S. (1989). Endocytosis and recycling of the fibronectin receptor in CHO cells. *EMBO J.* **8**, 1341-1348.

Buck, C. A. and Horwitz, A. F. (1987). Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* **3**, 179-205.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell. Biol.* **48**, 487-525.

Chen, W.-T. (1981). Mechanism of retraction of the trailing edge during fibroblast movement. *J. Cell Biol.* **90**, 187-200.

Crowley, E. and Horwitz, A. F. (1995). Tyrosine phosphorylation and cytoskeletal tension regulate the release of fibroblast adhesions. *J. Cell Biol.* **131**, 525-537.

DiMilla, P. A., Barbee, K. and Lauffenburger, D. A. (1991). Mathematical model for the effects of adhesion and mechanics on cell migration speed. *Biophys. J.* **60**, 15-37.

DiMilla, P. A., Stone, J. A., Quinn, J. A., Albeda, S. M. and Lauffenburger, D. A. (1993). Maximal migration of human smooth muscle cells on fibronectin and type-IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.* **122**, 729-737.

Duband, J. L., Nuckolls, G. H., Ishihara, A., Hasegawa, T., Yamada, K. M., Thiery, J. P. and Jacobson, K. (1988). Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J. Cell Biol.* **107**, 1385-1396.

Dunlevy, J. R. and Couchman, J. R. (1993). Controlled induction of focal adhesion disassembly and migration in primary fibroblasts. *J. Cell Sci.* **105**, 489-500.

Felder, S. and Elson, E. L. (1990). Mechanics of fibroblast locomotion: quantitative analysis of forces and motions at the leading lamellas of fibroblasts. *J. Cell Biol.* **111**, 2527-2536.

Ginsberg, M. H., Du, X. and Plow, E. F. (1992). Inside-out integrin signaling. *Curr. Opin. Cell Biol.* **4**, 766-771.

Hay, E. D. (1985). Interaction of migrating embryonic cells with extracellular matrix. *Exp. Biol. Med.* **10**, 174-193.

Holifield, B. F. and Jacobson, K. (1991). Mapping trajectories of Pgp-1 membrane protein patches on surfaces of motile fibroblasts reveals a distinct boundary separating capping on the lamella and forward transport of the retracting tail. *J. Cell Sci.* **98**, 191-203.

Hynes, R. O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11-25.

Jay, P. Y., Pham, P. A., Wong, S. A. and Elson, E. L. (1995). A mechanical function of myosin II in cell motility. *J. Cell Sci.* **108**, 387-393.

Lauffenburger, D. A. (1989). A simple model for the effects of receptor-mediated cell-substratum adhesion on cell migration. *Chem. Eng. Sci.* **44**, 1903-1914.

Lawson, M. A. and Maxfield, F. R. (1995). Ca^{2+}- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* **377**, 75-79.

Lee, J., Ishihara, A. and Jacobson, K. (1993). How do cells move along surfaces? *Trends Cell Biol.* **3**, 366-370.

Lo, S. H. and Chen, L. B. (1994). Focal adhesion as a signal transduction organelle. *Cancer Metast. Rev.* **13**, 9-24.

Marks, P. W., Hendey, B. and Maxfield, F. R. (1991). Attachment to fibronectin or vitronectin makes human neutrophil migration sensitive to alterations in cytosolic free calcium concentration. *J. Cell Biol.* **112**, 149-158.

Miyamoto, S., Akiyama, S. K. and Yamada, K. M. (1995). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* **267**, 883-885.

Neff, N. T., Lowrey, C., Decker, C., Tovar, C., Damsky, C., Buck, C. and Horwitz, A. F. (1982). A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. *J. Cell Biol.* **95**, 654-666.

Nobes, C. D. and Hall, A. (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filipodia. *Cell* **81**, 53-62.

Prewitt, J. M. (1970). Object enhancement and extraction. In *Picture Processing and Psychopictonics* (ed. B. S. Lipton and A. Rosenfeld), pp. 75-149. Academic Press, New York.

Regen, C. M. and Horwitz, A. F. (1992). Dynamics of β_1 integrin-mediated adhesive contacts in motile fibroblasts. *J. Cell Biol.* **119**, 1347-1359.

Ruoslahti, E., Hayman, E. G., Pierschbacher, M. and Engvall, E. (1982). Fibronectin: purification, immunochemical properties, and biological activities. *Meth. Enzymol.* **82**, 803-831.

Ruoslahti, E. and Pierschbacher, M. D. (1987). New perspectives in cell adhesion. *Science* **238**, 491-497.

Rupert, T., Rohde, H., Risteli, L., Ott, U., Robey, P. G. and Martin, G. R. (1982). Laminin. *Meth. Enzymol.* **82**, 831-837.

Sastry, S. and Horwitz, A. F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.* **5**, 819-831.

Schmidt, C., Horwitz, A. F., Lauffenburger, D. A. and Sheetz, M. P. (1993). Integrin/cytoskeleton interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol.* **123**, 977-991.

Schwartz, M. A. (1992). Transmembrane signaling by integrins. *Trends Cell Biol.* **2**, 304-308.

Sczekan, M. M. and Juliano, R. L. (1990). Internalization of the fibronectin receptor is a constitutive process. *J. Cell. Physiol.* **142**, 574-580.

Sonnenberg, A. (1993). Integrins and their ligands. *Curr. Top. Microbiol. Immunol.* **184**, 7-26.

Tawil, N., Wilson, P. and Carbonetto, S. (1993). Integrins in point contacts mediate cell spreading - factors that regulate integrin accumulation in point contacts vs. focal contacts. *J. Cell Biol.* **120**, 261-271.

Tranqui, L., Usson, Y., Marie, C. and Block, M. R. (1993). Adhesion of CHO cells to fibronectin is mediated by functionally and structurally distinct adhesion plaques. *J. Cell Sci.* **106**, 377-387.

Watts, C. and Marsh, M. (1992). Endocytosis: what goes in and how? *J. Cell Sci.* **103**, 1-8.

Wessels, D., Vawter-Hugart, H., Murray, J. and Soll, D. R. (1994). Three-dimensional dynamics of pseudopod formation and the regulation of turning during the motility cycle of *Dictyostelium*. *Cell Motil. Cytoskel.* **27**, 1-12.

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