Dynamics of $\beta_1$ Integrin-mediated Adhesive Contacts in Motile Fibroblasts

Chester M. Regen and Alan E Horwitz
Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801

Abstract. Motile chick skeletal fibroblasts adhere to a laminin substrate by means of clustered $\beta_1$ integrins. These integrin "macroaggregates" are similar to classic focal contacts but do not appear dark under interference-reflection microscopy. They contain $\alpha_5$ integrin and are associated with extracellular fibronectin. To study their behavior during cell movement, time-lapse, low-light video microscopy was used to image integrins on living cells tagged with a fluorescent anti-$\beta_1$ integrin antibody.

Integrin macroaggregates remain fixed with respect to the substratum, despite the fact that they fluctuate in size, density, and shape over a period of minutes. Upon detachment of the cell rear, as much as 85% of the $\beta_1$ integrin density of a macroaggregate remains behind on the substrate, along with both $\alpha_5$ integrin and fibronectin. Release of the cell rear does not involve cleavage of the $\beta_1$ integrin cytoplasmic domain from the remainder of the protein. These results indicate that cell motility does not require regulated detachment of integrin receptors from the substrate. On the other hand, cytoskeletal components and a variable fraction of the integrins are carried forward with the cell during detachment, suggesting that some type of cortical disassembly process does occur.

Integrin macroaggregate structures are not recycled intact after detachment of the cell rear from the substrate. They do not persist on the cell surface, nor can they be seen to be engulfed by vesicles; yet, some of the individual integrins that make up these macroaggregates are eventually transported forward by both vesicular and cell-surface routes. Antibody-tagged integrins accumulate in dense patches at the lateral edges and dorsal surface of the cell, and move forward on the cell surface. The tagged integrins also enter cytoplasmic vesicles, which move forward within the cytoplasm.

Macroaggregates generally form and grow at the cell front; however, application of fluorescent antibody causes integrins to disappear from the leading edge. Therefore, it has not been possible to directly visualize the recycling of the forward moving tagged integrins into new macroaggregates. The tagged integrins also enter cytoplasmic vesicles, which move forward within the cytoplasm.

FIBROBLAST translocation requires spatial regulation of cell attachment to the substratum. Adhesive interactions must form at the cell front, the site of lamellipodial extension. Once formed, these cell-substrate interactions must be stable enough to transmit force, i.e., to generate traction. On the other hand, the rear of the cell must be able to detach from the substrate to allow the cell to advance. Thus, to understand the process of cell motility, the formation, maintenance, and release of cell-substrate adhesive interactions need to be characterized.

In previous studies, adhesive interactions formed by living cells in vitro have been visualized using interference reflection microscopy (IRM) (Curtis, 1964). Under appropriate conditions, many kinds of cells—fibroblast types in particular—show black focal contacts under IRM (Burridge, et al., 1988; Singer et al., 1987, 1988), which represent areas of very close interaction between the cell and the substrate to which it is attached (Izzard and Lochner, 1976). Of the few types of fibroblasts described to translocate in vitro, the best studied example is the fibroblast explanted from the embryonic chick heart (Abercrombie, 1980; Trinkaus, 1984). These cells move by means of a highly discontinuous cycle. First, the cell advances as the rear stretches out into a long tail, until the cell can go no further. After a time the tail abruptly rips, and retracts into the cell body, allowing the tail to become a long, extended lamellipodium. Therefore, it has not been possible to directly visualize the recycling of the forward moving tagged integrins into new macroaggregates. The tagged integrins also enter cytoplasmic vesicles, which move forward within the cytoplasm.

Please address correspondence to Dr. Alan E Horwitz, University of Illinois, Department of Cell and Structural Biology, 505 South Goodwin Avenue, 506 Morrill Hall, Urbana, Illinois 61801.

Chester M. Regen's present address is Bio-Rad Microscience Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts HP2 7TD, United Kingdom.

1. Abbreviations used in this paper: IRM, interference-reflection microscopy; ECM, extracellular matrix.
cell front to advance rapidly until the cell body is again under tension (Chen, 1981a,b). In these cells, focal contacts appear to form at the leading edge, and diminish in number as they move (in the cell frame of reference) backward toward the cell center. The cell rear is anchored by a small number of focal contacts, often only a single one. The movement of these cells appears to be substantially hindered by the strong attachments at the rear, the strength of which is evident from the observation that the rearmost adhesion persists as a focal contact even after the tail has ripped away (Izzard and Lochner, 1980; Abercrombie, 1980; Chen, 1981b).

The picture provided by IRM studies of chick heart fibroblasts is incomplete and likely not general. Most motile cells do not show focal contacts, and the presence of focal contacts generally appears to be associated with maintenance of a static, spread-cell shape (Couchman and Rees, 1978; Kolega et al., 1979, 1982). Nonetheless, the idea that release of the rear involves ripping of the membrane, and not necessarily release of adhesive interactions, has received important confirmation from in situ studies. Chick corneal fibroblasts migrating through the corneal stroma rip their tails and leave cytoplasmic fragments behind (Bard and Hay, 1975). On the basis of these and other observations, a “fixed cortex” model of cell translocation has been proposed in which cell-surface receptors are irreversibly fixed to the surrounding extracellular matrix (ECM) during motion (Hay, 1985; Bilouzov and Hay, 1989; see also Bray and White, 1988). In this conception, a residue of cortex and plasmalemma remains fixed to the substrate in the cell’s wake, as the inner (subcortical) cytoplasm is extruded forward. New plasmalemma is inserted at the leading edge, rendering the process sustainable.

In recent years, considerable insight has been gained into the molecular basis of cell adhesion. In many instances, cell adhesion is mediated by integrins, a superfamily of heterodimeric cell-surface receptors (for reviews see Buck and Horwitz, 1987; Ruohola and Pierschbacher, 1987; Albelda and Buck, 1990). In particular, integrins of the β, family play a central role in cell-substrate adhesion, by forming transmembrane links between ECM components, such as fibronectin, laminin, and collagen, and the actin cytoskeleton (Horwitz et al., 1985, 1986). Application of “adhesion-perturbing” anti-β, integrin antibodies prevents the attachment of many types of cells to purified ECM substrata (Buck and Horwitz, 1987). In addition, β, integrins are found in focal contacts when these are present (Burridge et al., 1988). Thus, because we can visualize integrins, a more molecular analysis of adhesive events during cell motility is now possible.

In the present study, we found that chick skeletal fibroblasts are highly motile when plated on laminin. This movement depends on adhesive interactions mediated by β, integrins: when adhesion-perturbing anti-β, integrin antibodies are applied to preattached cells, retraction of the lamellae occurs and movement ceases (our published observations). The β, integrins on chicken skeletal fibroblasts are clustered into organized integrin “macropaggregates,” which are readily visualized by immunofluorescence, and yet do not correspond to IRM-positive focal contacts. Thus labeling of living cells with a fluorescently tagged nonperturbing mAb directed against β, integrin allows the visualization of integrin macropaggregates on living cells. By a number of criteria these structures represent sites of adhesive interaction with the substrate, and so we have been able to observe the regulation of adhesive contacts during cell movement.

We have found that the interaction of β, integrins with the substrate can be reversible, as the macropaggregates grow and shrink over time. Despite the dynamic nature of these contacts, detachment at the rear occurs, in part, by a ripping process, so that an appreciable fraction of β, integrins does in fact remain behind the cell. There does not appear to be a concerted release of receptors from the substrate as the cell rear detaches; yet, some type of disassembly of structure does occur, as cytoskeletal components do not remain behind with the integrin. Integrin does not accumulate at the cell rear and we have identified several modes by which integrin appears to be carried forward by the cell. Interestingly, the mAb causes integrin to clear from the lamella; therefore, we have studied macropaggregate formation indirectly by comparing integrin staining with cell motile history.

**Materials and Methods**

**Reagents**

A rat hybridoma, ES66-8, directed against chicken β, integrin was generously provided by Dr. Kenneth Yamada (National Institutes of Health, Bethesda, MD). The ES66 mAb recognizes the chicken β, integrin chain, without interfering with the integrin’s adhesive function (Duband et al., 1988). ES66 was covalently labeled with lissamine rhodamine sulfonyl chloride (Molecular Probes, Inc., Eugene, OR) as described elsewhere (Scullion et al., 1987). To detect the α5 integrin subunit, a cocktail of three mouse mAbs—ASA2, ASD7, and ASB2—was used as described (Muschler and Horwitz, 1991). To detect the β5 integrin cytoplasmic domain, an antisera was generated by immunizing a rabbit with a synthetic amino acid 20mer conjugated to keyhole limpet hemacyanin. This peptide represents the terminal 20 amino acids (carboxyl end) of the chicken β5 integrin cytoplasmic domain.

Cellular fibronectin was detected using the B3/D6 mouse mAb, which is specific for chick fibronectin (Gardner and Fambrough, 1983). Culture supernant from the B3/D6 hybridoma was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract NOI-HD-6-2915 from the National Institutes of Health). Vinculin and talin were recognized by affinity-purified antisera obtained from rabbits immunized with purified preparations of the proteins obtained from chicken gizzard.

Unconjugated ES66 antibody was visualized with fluorescein-conjugated anti-rat secondary antibodies (Sigma Chemical Co., St. Louis, MO). Mouse mAbs were visualized with fluorescein-conjugated anti–mouse secondary antibodies (Cappel-Organon Teknika, West Chester, PA). Polyclonal primary antibodies were visualized using rhodamine anti–rabbit secondary antibodies. It was confirmed that there was no cross-reaction between the rat, mouse, and rabbit secondarys and their respective primaries, and that there was negligible fluorescence bleed-through between the rhodamine and fluorescein fluorescence channels in dual-label studies.

**Cells**

Cultures of skeletal muscle fibroblasts were obtained from myoblast preparations from 11-d-old chick breast muscle prepared as described previously (Neff et al., 1982). Fibroblasts were purified by preplating on tissue culture plates (Falcon 3003; Becton Dickinson and Co., Lincoln Park, NJ); the cells that had not adhered to tissue culture plates within 1 h were discarded. Breast muscle fibroblasts were maintained for up to 10 passages in tissue culture plates with DME containing 5% FBS (Hybri-Max, Sigma Chemical Co.). For experiments, cells were replated in DME containing 10% FBS in microscopy plates coated with mouse laminin at a concentration of 40 μg/ml, and purified as described by Timpl et al. (1979). Cells were plated at low density so that the majority of cells were not contacting any neighbors.

Cover slips were cleaned by soaking in concentrated HNO3, followed by rinsing overnight in water, and drying with acetone. They were silanized by
exposure to hexamethyldisilazane vapor (Sigma Chemical Co.) for 30 min at 200°C to enhance protein adsorption to the glass. Microscopy plates were fashioned by punching a hole in the bottom of a 35-mm tissue culture plate (Falcon 3000), attaching a silanized coverslip with clear silicone rubber sealant (Dow Corning Corp., Midland, MI), and, after the sealant had cured, sterilizing by spraying with 70% ethanol and rinsing with sterile water.

**Time-Lapse Direct Immunofluorescence Studies**

Cells growing on laminin-coated microscopy plates were labeled with lissamine rhodamine-ES66 at a concentration of 20 μg/ml, diluted in the cells’ own medium for 25 min at 37°C, and then washed three times with cell medium. This medium was replaced by microscopy medium consisting of DME prepared without phenol red and containing only 1% FBS, to reduce background fluorescence. Cells continued to translocate for many hours in this medium. Within a few minutes after addition of the microscopy medium, the microscopy plate was placed in an atmospheric chamber on top of the microscope stage, and the experiment was initiated.

The atmospheric chamber consisted of a water jacket-heated stage mounted on the inverted microscope. A mixture of 10% CO₂ in air was humidified by bubbling through a water-filled bottle in the attached water bath. The warm moist gas was pipetted to a transparent plastic hood resting on top of the heated stage. This hood was thin and did not interfere with the extra-long working distance phase condenser above it. A curtain of warm air blowing across the top of the hood prevented condensation. The oil immersion objective was heated by passing AC current through a nylon-coated 0.001 in. steel wire wrapped around the barrel. This arrangement kept the temperature at 37 ± 1°C.

To reduce photodamage, the 100 W mercury lamp output was reduced by a factor of 100 with neutral density filters, and the exposure duration limited to a few seconds during each 4-6-min time interval. Increased exposure resulted in retraction of the lamella and halting of motility; these effects were observed both with and without application of the fluorescent antibody. To achieve adequate image quality, 128 video frames were averaged per image.

**Dual-Label Immunofluorescence**

Dual labeling of β₁ integrin and vinculin was performed as follows. Living cells growing on a laminin substrate were labeled with plain ES66 as described above for rhodamine-ES66. They were permeabilized with 0.01% digitonin (Sigma Chemical Co.) in PBS for 20 s, fixed with fresh 2% formaldehyde in PBS for 10 min, quenched twice with 0.15 M glycine in PBS, blocked with 5% goat serum in PBS for 1 h, and labeled with the anti-vinculin antiserum for 20 min. Finally, a mixture of fluorescent anti-rat and anti-rabbit secondary antibodies was applied. Talin was visualized in a similar way. For dual labeling of the extracellular and cytoplasmic domains of integrin, the digitonin permeabilization was omitted; instead, cells were permeabilized with 0.2% Triton X-100 for 4 min after quenching. For dual labeling of β₁ integrin and cellular fibronectin, living cells were labeled with a mixture of rhodamine-ES66 and untagged B3/D6 antibodies. After fixation and permeabilization, fluorescent anti-mouse secondary antibodies were applied.

**Video Microscopy**

Quantitative immunofluorescence studies were performed using a 60×/1.4 NA phase planapochromat objective on a Diaphot inverted microscope (Nikon, Inc., Tokyo, Japan) equipped for epifluorescent illumination, with an ultra-low light video system consisting of a microchannel plate image intensifier (KS-1381, Optical Elements Co., Washington, DC) mounted in front of a SIT camera (Hamamatsu C2400-08 Photonic Microscopy, Inc., Oak Brook, IL). Illumination by a 100 W mercury lamp was controlled by an electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) mounted on the epifluorescence attachment. We used a custom filter set for lissamine rhodamine, which consisted of an excitation filter, a dichroic mirror, and an emission filter (models 572 DF 10, 580 DRLP, and 590 EFLP, respectively, Omega Optical, Inc., Brattleboro, VT). Images were acquired, analyzed, and stored initially with an image processing system (Model IC-100, Inovision Corp., Research Triangle Park, NC), and later with TCL-Image.

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**Figure 1.** Vinculin is associated with cellular integrin macroaggregates but not with the integrin trails left behind the cell. (A) Anti-β₁ integrin, and (B) antivinculin immunofluorescence staining. Note trails, indicated by arrows. The cell contours obtained from near-simultaneous, phase-contrast images of the cell have been traced on each fluorescent image. Bar, 10 μm.
Figure 2. Time-lapse, anti-β₁ integrin immunofluorescent images demonstrating that integrin macroaggregates remain fixed as the cell translocates. The cell contours obtained from near-simultaneous, phase-contrast images have been traced on each fluorescent image. The cells are moving from lower left to upper right. Note the faint macroaggregates left behind on the substrate as the cell advances. Ovals are fixed in the microscope frame of reference. 500 s elapsed between successive images. Bar, 10 μm.

software (Biological Detection Systems, Pittsburgh, PA) running on a Macintosh IIIfx (Apple Computer, Cupertino, CA). The magnification of the entire system was such that the pixel diameter was 0.22 μm.

Before each fluorescence experiment, the microscope was focused on a uniform planar fluorescent sample consisting of a stripe of indelible red marking pen drawn on a coverslip. The SIT camera gain and offset were adjusted so that, in the digitized video image, pixels in the nonilluminated periphery of the field had a gray level of zero and those in the brightest region of the illuminated field a level near 255 (just saturating), and a reference image saved. Each experimental image was divided by this reference image so that the brightness across the entire field was uniform.

IRM was performed using a 100×/1.3 N.A. phase neofluor objective and a filter cube consisting of a 546-nm excitation filter, a 50% beamsplitter, and a pair of polarizers (Omega Optical) (Izzard and Lochner, 1976).

We used 0.1-μm fluorescent latex microspheres (Polysciences, Inc., Warrington, PA) to investigate both the depth-of-field of the objective and the thickness of the cells. Vertical displacement was determined from the markings on the microscope fine-focus knob. Defocusing by 1 μm caused the beads to be slightly blurred; at 3 μm, they were invisible. Microspheres were added to a microscopy plate of cells growing on laminin. By focusing on spheres resting on the substrate, and then on ones atop the cell dorsal surfaces, we found that (a) cell bodies were 3–5-μm thick, and (b) lamellae can be as thin as 1 μm. Thus, we concluded that this objective could be used to distinguish between the dorsal and ventral surfaces of the bodies of well-spread cells, but not of their lamellae. This conclusion was borne out during the immunofluorescence experiments. When we focused on integrin debris on the substrate, integrin on the cell ventral surface was in focus, whereas the fluorescence from integrin patches on the dorsal surface was diffuse.

Comparison of Immunofluorescent Staining to Cell Motile History

Working with a 20× objective so that ~10 cells were visible at once, still video images were acquired after typical intervals of 0, 40, 60, and 70 min. After the last image was acquired, the cells were fixed at 25°C with 2% formaldehyde in PBS for 10 min, quenched twice with 0.15 M glycine in PBS, blocked with 5% goat serum in PBS for 30 min, and labeled with 20 μg/ml rhodamine-ES66 in the blocking solution for 30 min. Less than 1 min elapsed between the final image acquisition and contact with the cells by the fixative.

Spatial variation in the distribution of integrin across the leading edge was analyzed quantitatively by comparing the most recently extended region of the lamella to the adjacent older region. To identify these regions, traverses from the final two or three 20× phase images were scaled by a factor of 3 (to match the magnification of the 60× fluorescence lens), positioned by eye, and overlaid on the fluorescence image.

Distribution parameters were calculated as follows. A small background value was subtracted from each image so that the brightness of clean regions on the substrate was just zero. Next, a binary mask was formed, in which those pixels whose brightness exceeded a threshold value were set. The threshold brightness was chosen so that, over a particular region of interest of the cell, the structure of the mask closely resembled the integrin organization. Thus, the threshold value represents the minimum brightness of pixels present in macroaggregates. The mean brightness of the set pixels was calculated, which corresponded to the density of the macroaggregates. The ratio of the number of set pixels to the total number of pixels in the region was taken to represent the fraction of area devoted to macroaggregates. Finally, the mean size, in pixels, of the macroaggregates was computed.

Results

Integrin on Motile Skeletal Muscle Fibroblasts

We used chicken skeletal muscle fibroblasts as a model sys-
Integrin macroaggregates are associated with extracellular, cell-derived fibronectin. (A) Anti-β1 integrin and (B) anti-chicken fibronectin immunofluorescence images of the same double-labeled cell. Integrin and fibronectin staining patterns match closely. Note in particular that integrin trails are associated with fibronectin fibrils (boxes). The cell contour has been traced on the two images. Bar, 10 μm.

Figure 4. Simultaneous rhodamine anti-β1 integrin-direct (A) and fluorescein anti-α5 integrin-indirect (B) immunofluorescence of a single cell. Cells were stained with the anti-β1 antibody and then fixed, permeabilized, and stained with the anti-α5 antibody cocktail. Because of this procedure, (A) shows surface labeling only, while (B) shows both surface and cytoplasmic labeling. Bar, 10 μm.

Figure 3. Integrin macroaggregates are associated with extracellular, cell-derived fibronectin. (A) Anti-β1 integrin and (B) anti-chicken fibronectin immunofluorescence images of the same double-labeled cell. Integrin and fibronectin staining patterns match closely. Note in particular that integrin trails are associated with fibronectin fibrils. The cell contour has been traced on the two images. Bar, 10 μm.

tem for the microscopic study of the role of integrins in motility. These cells are large and provide high-quality epifluorescence images. The cultures contain both motile cells that have a pronounced lamella, and static cells that lack the lamella and instead have a "pinned-out" appearance. While the proportion of static cells varies from one culture to the next, we found that by plating cells on laminin in medium containing 10% FBS, approximately one-half of the cells appeared motile. In contrast, fibronectin substrata and other types of sera decreased the motile fraction at least twofold.

The migration of the motile cells was quite variable. They reorient themselves often, and they stop and start. It was not uncommon to observe a lamella to split in two, with the two halves pulling in opposing directions and stretching the cell as a result. Consequently, quantification of motility was difficult. However, the migration rates of those cells that (a)
displayed an obvious single lamella at one end and a thin tail at the other, and (b) moved at roughly constant velocity over two successive time periods could be measured meaningfully. These cells translocated their nuclei with a mean net velocity of $57 \pm 40 \mu m/h$ ($\pm SD$, $n = 17$).

A variable fraction of the $\beta_1$ integrins in skeletal fibroblasts migrating on laminin were arranged into multimicron-sized patches. Both linear and punctate patches were present, with linear shapes generally predominating (Figs. 1A and 2). Individual patches on a single cell varied substantially in their brightness, i.e., integrin density. Essentially all of the patches appeared to be closely associated with endogenously synthesized extracellular fibronectin fibrils (Fig. 3). When we performed dual-label immunofluorescence studies using antibodies directed against $\beta_1$ and $\alpha_5$ integrin, integrin patches stained essentially identically for both subunits (Fig. 4), indicating that the patches contained the $\alpha_5\beta_1$-fibronectin receptor. The minor difference in appearance between the two images in Fig. 4 arises from the fact that the $\beta_1$ image shows surface integrins only, while the $\alpha_5$ image also includes molecules within the cell.

Skeletal fibroblasts attached to laminin did not exhibit dark focal contact regions when visualized by IRM (Fig. 5, A and B); instead, faint gray, close contacts could sometimes be discerned. This negative result was confirmed by means of total internal reflection microscopy (our unpublished observations) (Axelrod, 1988). In contrast, the same cells did exhibit focal contacts when attached to a fibronectin substrate (Fig. 5, C and D). Also, the integrin patches of cells on laminin appeared generally finer and more fibrillar than those of cells on fibronectin. Since the integrin patches of cells on laminin did co-localize with the cytoskeletal proteins vinculin (Fig. 1B), actin, and talin (data not shown), these patches share some, but not all, features of focal contacts. To emphasize this difference we refer to them as macroaggregates.

**Ventral Macroaggregates Represent Points of Substrate Attachment**

We were able to distinguish the ventral (lower) surfaces of the cells from the dorsal (upper) surfaces by careful focusing of the microscope (see Materials and Methods). In addition to the finely detailed integrin organization observed on the ventral surface, cells often possessed a few intensely bright ribs or patches on their dorsal surface. Furthermore, labeled integrins were often observed to be present in cytoplasmic vesicles; these were located above the ventral plane. They moved vertically and laterally fast enough so that it was not possible to track individual ones from one exposure to the
next. The rapid movement of these vesicles ensured that they could be easily distinguished from the more static macroaggregates when the video display alternated rapidly back and forth between successive time-lapse frames.

Two types of observations suggest that ventral macroaggregates represent points of attachment to the substrate. First, in time-lapse experiments, the macroaggregates remained positionally fixed on the substrate from one interval to the next. In Fig. 2, several prominent macroaggregates are identified in each frame by ovals, the positions of which are fixed in the substrate frame of reference. These particular macroaggregates remain fixed throughout the entire 1 h duration of the experiment. During this time the cell advances so that the upper three ovals can be seen to pass from the cell front to the rear. While macroaggregates often appeared to fluctuate in density (see below), lateral translation of ventral macroaggregates was extremely uncommon. Only macroaggregates located close to a cell edge moved laterally, and their net displacement was small, typically only 1-2 μm.

Second, as cells advanced, substantial amounts of the integrin density present in the macroaggregates remained behind the cell trailing edge, affixed to the substrate (see below).

**Integrin Macroaggregates Are Dynamic**

Integrin macroaggregates fluctuated in size, density, and shape. This phenomenon was most evident in a careful comparison of a series of time-lapse immunofluorescence images in which the focal plane remained virtually constant, as evidenced by the appearance of fluorescent debris on the substrate. Fig. 6 B shows the evolution, over time, of the four square regions marked in Fig. 6 A, the initial image of the series. Several types of fluctuation can be observed. For example, in region 1, a macroaggregate forms at the former cell edge. In region 2, two vertical macroaggregates are present at time t = 0; the right one has disappeared 6 min later; and the left one then appears to gradually disperse and then disappear over the next 24 min. In region 3, the macroaggregate in the upper right lengthens, partially disperses, and then brightens at the lower left. In region 4, the macroaggregate appears to brighten slowly.

These fluctuations were not due to out-of-focus fluorescence from integrin on the dorsal surface or in the cytoplasm. The macroaggregates visualized on this cell are highly elongate. Blur from dorsal surface structures might add overall brightness to a macroaggregate, but would not cause it to appear to extend or retract. Fluctuations were observed in macroaggregates on the thickest parts of the cell body, where any dorsal contribution to the image would be extremely blurred (see Materials and Methods), as well as on the thinner lamella. Furthermore, most of the changes progressed over a period of several frames. Lateral alignment of an antibody-labeled endocytotic vesicle or dorsal surface structure with the end of a linear macroaggregate on the ventral surface below might contribute intensity transiently, but because of their rapid movements, they did not remain fixed from one time-lapse frame to the next.

We can also rule out changes in microscope focus as the origin of the fluctuations. A change of focal plane sufficient to cause a macroaggregate to form or disappear would be evident as misfocussing of the detritus on the substrate. Conversely, since the depth-of-field of the objective is ~1 μm, a macroaggregate would have to shift vertically well in excess of this distance to blur enough to disappear. Such a shift of one-fourth to one-half of the cell thickness seems unlikely, especially since we saw cases where a macroaggregate disappeared while nearby ones remained visible.

**Integrin Remains Behind on the Substrate as Cells Advance**

Detachment of the cell rear was generally followed by one
of two scenarios. In the majority of cases, the cell rear advanced gradually from one time-lapse frame to the next, maintaining its tapered shape (Fig. 7A). In a minority of cases, the cell rear was drawn into a thin tail, which detached abruptly. In the example shown in Fig. 7B, detachment occurs between the fourth and fifth frames. The cell rear retracts out of the field of view, having travelled in excess of 50 μm during the 4-min interval. The width of the macroaggregates seems to be correlated with the ability of the tail to release. Cells that advanced smoothly, as did the cell in Fig. 7A, always displayed the extremely fine, fibrillar macroaggregates evident in that figure. In contrast, cells that released abruptly, like the one in Fig. 7B, had one or more thick macroaggregates at the rear.

Anti-β2-integrin immunostaining of fixed motile skeletal fibroblasts revealed, irrespective of detachment scenario, the presence of integrins attached to the substrate behind the rear of most cells. Furthermore, time-lapse experiments showed that there is often a detailed spatial correspondence between the cell-surface macroaggregates on a cell body and the integrin “trails,” which remain behind after the cell has translocated (Fig. 8). Dual-label immunofluorescence of β1 integrin and vinculin showed that vinculin was not left behind in the trails, despite the fact that it was usually highly concentrated in the rear of the cell body proper (Fig. 1). We failed to observe vinculin in trails when cells were fixed before permeabilization, or when these steps were reversed. Similar results were obtained with reagents directed against talin and F-actin (data not shown). By immunostaining cells previously labeled with the fluorescent lipid DiO-C16, we confirmed that small amounts of lipid also remain in the trails (data not shown).

The fraction of total cellular integrin left behind varied greatly. In Fig. 7A, an example is illustrated in which prominent trails were evident; in such cases, the cells left behind roughly one-third of their total labeled integrin at the rear. In contrast, the cell in Fig. 7B left integrin behind at only a few sites (enclosed by boxes in the final frame of the figure). Only a few percent of the total labeled integrin at the rear is left behind in this case. There was also considerable variation in the fraction of integrin left behind by each individual macroaggregate during detachment of the tail. To determine the proportion of a macroaggregate's integrins that were left behind, we looked at macroaggregates located at the end of a fine fiber at the very rear of a cell, so that there was essentially no contribution from diffuse dorsal surface fluorescence to the measurement. We observed as much as 85%, and as little as 10%, of particular macroaggregates to be left behind.

Since the cell rear detaches while surface integrins remain attached to the substrate, we investigated the hypothesis that detachment might be mediated by proteolysis of the short
Integrin Move Forward by Both Cell-Surface and Cytoplasmic Routes

In contrast to vinculin (see above), integrin did not accumulate at the rear of advancing cells. Therefore, the majority of integrins present in the cell rear that do not remain behind on the substrate would either have to have been transported forward by the cell or lost to the medium. In fact, we observed several types of forward transport of integrin structures which were not on the ventral surface. First, the side edges of the cell appear both to "trap" integrins and to move them forward. A dramatic example of this process can be seen in Fig. 7B, where the progress of an integrin-rich part of the cell's left edge can be seen. Second, cells often had a few bright patches of integrin on their dorsal surface. These moved forward as the cell advanced, retaining their integrity, while a substantial displacement occurred (Fig. 9A). Third, cells labeled with rhodamine-ES66 often accumulated fluorescence in cytoplasmic vesicles. These vesicles, which could be quite large, moved forward within the cell (Fig. 9B). The rapid forward movement of these three types of structures is in dramatic contrast to the lack of movement of the integrin macroaggregates located on the ventral surface.

Surprisingly, events at the leading edge were altered by the application of the fluorescent antibody. The lamellae of cells labeled with rhodamine-ES66 dimmed over the duration of a 30-min experiment to become completely undetectable. The depleted region could not be relabeled by subsequent application of antibody. Clearing appeared to be induced by the fluorescent antibody; staining of prefixed cells showed organized surface integrin on the lamella (see below). These unusual effects prevented us from investigating whether integrin present on the cell surface is normally transported forward to the leading edge for recycling during translocation.

On the other hand, we could be quite confident that under these altered conditions, there was no observable forward transport of fluorescent antibody-labeled integrins to the leading edge. The lamella is thin enough that we can be confident that ventral macroaggregates, cytoplasmic vesicles, and dorsal patches would all be in focus. We never saw any of these integrin-containing structures moving forward to the front of a cell. The zone of clearing was confined to the lamella; its margin did not travel rearward to the nucleus and beyond. Whatever the cause of the depletion of integrin, the observation stands that the cells translocate in a seemingly normal way despite the lack of integrin on the lamella.

Individual Macroaggregates Are Not Recycled Intact

As only a fraction of the macroaggregated integrin remained behind on the substrate, we examined time-lapse images of detachment events to see if individual macroaggregates could be observed to move forward on the surface after release. We looked at the behavior of individual rearward mac-
roaggregates as they encountered the forward moving trailing edge of the cell. We never observed an individual released macroaggregate to travel forward as a discrete structure. In fact, released macroaggregates were rarely identifiable by the time of the next time-lapse frame, 4–6 min later. These observations suggest that interaction with the substrate plays an important role in stabilizing the macroaggregate.

Neither did we find any evidence that released macroaggregates can be engulfed intact into vesicles. Although integrin-containing cytoplasmic vesicles were frequently evident in the cytoplasm, we did not observe vesicles in the vicinity of the detaching rear. In these experiments we were careful to focus both on the ventral surface and on a cytoplasmic plane ~2 μm higher.

Macroggregates Form Progressively at the Cell Front

The disappearance of labeled integrin from the lamella precluded study of macroaggregate formation at the front by means of time-lapse immunofluorescence experiments. Therefore, we used an alternate, indirect approach to examine the process. Motility of unlabeled cells was recorded by means of a series of time-lapse, phase-contrast images. Immediately after the final image was recorded, cells were fixed and immunostained with the rhodamine-ES66 antibody. Epifluorescent images of the integrin distribution could then be overlaid with each of the tracings of the cell edge in the phase-contrast image. The result was a fluorescence image with a series of contour lines on it much like a topographic map. The forward-most contour is the leading edge of the cell, and the region between this and the next contour is the most recently extended region of the lamella. Similarly, the region between the second contour and the third is an older region.

Comparison was made between the most recently extended part of the lamella and the older region just proximal to it. Distribution parameters were computed that represented the minimum and mean density of integrins in macroaggregates, the fraction of area occupied by macroaggregates, and mean macroaggregate size. The data are presented in Table I. All four parameters reflected a highly significant difference between the newest and older regions (P < .0001).

The gradients in macroaggregate size were much more striking than those in density (brightness); thus, macroaggregates grow as they mature at the cell front.

We often noted groups of macroaggregates near the cell front which matched a previous contour of the leading edge of the cell. Since successive tracings of the cell shape were spaced 10–20 min apart, it is extremely unlikely that the time-lapse imaging coincidently imaged a cell while it was establishing contacts at the front. More likely, these observations suggest that the extension of the leading edge is punctuated by stationary periods during which macroaggregates are established.
Table 1. Comparison of Integrin Organization Between
Newest and Previous Regions of Advancing Motile Cells

| Parameter                          | Percent | Gradient* | n   | Significance† |
|------------------------------------|---------|-----------|-----|---------------|
| Minimum receptor density in aggregates | 17      | 22        | 2   | P < .0001     |
| Mean receptor density in aggregates | 19      | 22        | 2   | P < .0001     |
| Fraction of area occupied by aggregates | 56      | 22        | 2   | P < .0001     |
| Aggregate mean size in pixels      | 41      | 20        | 2   | P < .0001     |

* Fractional comparison between the most recently added region at the advancing leading edge and a corresponding older region. For each distribution parameter D, gradient = (D_{most} - D_{new})/D_{new}.
† Significance of the differences (D_{most} - D_{new}) evaluated by means of a t-test.

Discussion

Based on observations of the migration of corneal fibroblasts in situ and neural crest cells in vitro, Bilozur and Hay have proposed a “fixed cortex” theory of mesenchymal cell movement in vivo, in which the actin-rich cell cortex remains essentially fixed with respect to the surrounding ECM during cell motion (Bilozur and Hay, 1989; Bard and Hay, 1975; Hay, 1985). This theory is supported by the classic studies of Abercrombie and colleagues on the movement of chick heart fibroblasts plated on coverslips, in which focal contacts were observed to form at the cell front, and visible pieces of cell membrane to be left behind on the substrate after traumatic ripping of a long drawn out tail (Abercrombie, 1980).

However, subsequent observations on these cells suggested that within a few hours after explantation, they begin a transition from a motile to a tightly anchored phenotype. On the basis of studies on these and several other types of explanted embryonic fibroblasts, a number of reports have concluded that focal contacts are more characteristic of the static, not the motile, phenotype (Couchman and Rees, 1979; Kolega et al., 1982; Duband et al., 1988).

We developed the chick skeletal fibroblast model because we sought a tissue culture system in which fibroblasts could be passaged for many generations while retaining their motile phenotype. Thus, uncertainties associated with the changing behavior of primary fibroblasts could be circumvented. Our observations on the behavior of skeletal muscle fibroblasts on laminin suggest that their associations with the substrate differ from the classic description of chick heart fibroblasts (e.g., Trinkaus, 1984). Release at the rear of these cells is generally rather smooth; large, phase-visible membrane pieces are not left behind on the substrate. The cells do not organize their integrin tightly into focal contacts, thought to represent sites of great mechanical strength (Burdidge, 1986); instead, their integrins are aggregated into dynamic structures, which presumably mediate weaker attachments. Presumably, all these differences are associated in some way with the absence in these cells of actin stress fibers (unpublished observations) (Couchman and Rees, 1979; Herman et al., 1981).

Perhaps our most significant observation is that, despite the apparently dynamic character of the substrate attachments, most of them do not release at the rear. Instead, membrane ripping appears to be the major mechanism allowing detachment of the cell rear. Often more than half of the integrin present is left behind on the substrate. Although some of the adhesive interactions in skeletal fibroblasts appear to be reversible, these cells do not require release of integrins from the substrate for translocation. Thus, our studies confirm the idea that rapid fibroblast motility is compatible with components of the cell surface remaining behind on the substrate.

On the other hand, our observations seem to be at variance with the assertion of the fixed cortex model that the interactions between the cell cortex and the ECM are necessarily irreversible. In addition to the integrins remaining on the substratum as trails behind the cells, we also observed integrin release from the substrate, followed by dispersal and forward movement on the cell surface. Thus, it appears that both integrin dissociation from the substrate and ripping out of integrin-containing membrane regions occur during detachment of the cell rear. The relative contributions of these two processes appear quite variable, as the fraction of aggregated integrin carried forward with the cell surface varied greatly.

Since a nontrivial fraction of the macroaggregated integrins at the cell rear do dissociate from the substrate, the trailing edge appears to represent a region of cortical disassembly. Local disassembly at the rear is also suggested by the absence of any cytoskeletal components accompanying the integrin and fibronectin trails, despite the fact that vinculin, for one, appears concentrated toward the rear of translocating cells. These observations suggest that cytoskeletal components are released from substrate-associated integrins during detachment of the rear. Thus, the fixed cortex model should be broadened to allow for cytoplasmic components, such as vinculin, and cell-surface components, such as integrin, to be released from the cortex at the cell rear. The factors involved in breakdown of the cortex are not known; however, it seems reasonable to suppose that both physical stresses and biochemical regulation are involved. Recently, Maxfield and co-workers have documented surges in cellular Ca²⁺ associated with release of the rear of neutrophils. Perhaps transient increases in Ca²⁺ levels mediate disruption of the cortex at the rear (Marks et al., 1991).

The fate of the cell-surface integrins that dissociate from the substrate is of some importance. We have no evidence for direct vesicular shuttling from cell rear to leading edge: vesicles were not seen forming or accumulating at the rear of the cell, nor were they present near the front. Although attached rhodamine-labeled antibodies might well alter the fate of endocytosed integrins, cell motility is unaffected by these antibodies, indicating that recycling of integrin to the leading edge is not required for movement.

On the other hand, since the integrin that releases from the substrate does not accumulate at the cell rear, it must be carried forward and some may be degraded. Although macroaggregates are not directly engulfed at the cell rear, we observed that labeled integrin was endocytosed, confirming other reports that β₁ integrin participates in an endocytic cycle (Bretscher, 1989; Szczeklik and Juliano, 1990). Integrins are, in fact, moved forward in cytoplasmic vesicles (Fig. 9 B). Integrins are not moved forward by translation of preformed macroaggregates along the cell surface; instead, we often observed integrins to collect and translate forward in association with a lateral edge or the dorsal surface of the cell. It also seems likely that diffusely distributed surface integrins are carried forward as passive components.
of the cell cortex, particularly as some of the cells had neither vesicles nor struts.

At present the relative contributions of vesicular transport, movement along struts, and diffuse flow to the net forward movement of integrins are not resolved. It is also unclear whether these structures exchange integrins with one another. Hopefully, the recent introduction of photoactivatable fluorophores will allow these questions to be answered in the near future ( Theriot and Mitchison, 1990). Whatever the details of the processes involved, the fixed cortex model needs to be broadened so that its present emphasis on insertion of newly synthesized receptors is broadened to include transport of surface receptors from the rear forward.

Our ability to study the formation of new macroaggregates at the leading edge was limited by the preferential removal of antibody labeled integrins from the lamella. This "clearing" effect reveals a specialization at the front of motile cells that may well be important for translocation. Since this region is known to affect rearward transport of concanavalin A-labeled colloidal gold particles by means of receptors that attach transiently to the cytoskeleton (Kucik et al., 1990), we speculate that the antibody alters the attachment of integrin to actin subunits treadmilling rearward through the lamella (Forscher and Smith, 1988; Fisher et al., 1988; Wang, 1985), and thus, causes net rearward rearrangement of the integrins.

In lieu of visualization of adhesive contacts forming en masse at the leading edge, we compared integrin staining of the lamella to the positional history of the cell, obtained from time-lapse, phase Contrast images. This approach has been used before (Rinnerthaler et al., 1988; Herman et al., 1981), although not to study integrin. Our results confirm the conclusion that adhesive interactions generally form at discrete points at the leading edge and then grow as they mature (Rinnerthaler et al., 1988; DePasquale and Izzard, 1987). However, both these studies describe contact formation during periods of rapid lamellar extension. Our observations suggest that adhesions form cyclicly at the leading edge during periods in which the edge is not advancing.

Duband et al. (1988) have shown by means of FRAP that integrin in patches ("focal contacts") and fibrillar streaks is immobile over a period of several minutes. Since these structures appear in cells that are acquiring a stationary phenotype, it might well be assumed that integrin aggregates are incompatible with cell movement. Our findings argue against this notion. The skeletal muscle fibroblasts described here are clearly motile; the organized integrin and fibronectin on their surfaces do not restrain the cells in any obvious way. In contrast, IRM-dark focal contacts certainly do hinder cell translocation (Chen, 1981b); thus, it will be important to discover the molecular differences between IRM-light macroaggregates and focal contacts. At present, we know that both types of adhesions contain fibronectin, vinculin, talin, and αβ integrin.

It would be useful to know whether our "dynamic" integrin macroaggregates correspond to the "immobile" integrin contacts described by Duband et al. Fig. 6 demonstrates that macroaggregates fluctuate from one time-lapse frame to the next one 6 min later. One would expect such fluctuations to be reflected in a component of the fluorescence recovery with a time constant of a few minutes. No such slow recovery is evident in their data. The apparent discrepancy may well be due to the fact that their studies were performed on cells adherent to substrata adsorbed with comparatively high (50 μg/ml) concentrations of fibronectin, while our substrata were absorbed with laminin. Fibronectin substrata induce our chick skeletal fibroblasts to form integrin aggregates that are more stable, and which represent focal contacts as judged by IRM.

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