Traction Forces Mediated by $\alpha 6\beta 4$ Integrin: Implications for Basement Membrane Organization and Tumor Invasion[□]

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The integrin $\alpha 6\beta 4$, a laminin receptor that stabilizes epithelial cell adhesion to the basement membrane (BM) through its association with cytokeratins, can stimulate the formation and stabilization of actin-rich protrusions in carcinoma cells. An important, unresolved issue, however, is whether this integrin can transmit forces to the substrate generated by the acto-myosin system. Using a traction-force detection assay, we detected forces exerted through $\alpha 6\beta 4$ on either laminin-1 or on an anti- $\alpha 6$ antibody, demonstrating that this integrin can transmit forces without the need to engage other integrins. These $\alpha 6\beta 4$ -dependent traction forces were organized into a compression machine localized to the base of lamellae. We hypothesized that the compression forces generated by $\alpha 6\beta 4$ result in the remodeling of BMs because this integrin plays a major role in the interaction of epithelial and carcinoma cells with such structures. Indeed, we observed that carcinoma cells are able to remodel a reconstituted BM through $\alpha 6\beta 4$ -mediated compression forces by a process that involves the packing of BM material under the cells and the mechanical removal of BM from adjacent areas. The distinct signaling functions of $\alpha 6\beta 4$, which activate phosphoinositide 3-OH kinase and RhoA, also contribute to remodeling. Importantly, we demonstrate remodeling of a native BM by epithelial cells and the involvement of $\alpha 6\beta 4$ in this remodeling. Our findings have important implications for the mechanism of both BM organization and tumor invasion.

INTRODUCTION

The $\alpha 6\beta 4$ integrin, which is expressed primarily on the basal surface of most epithelia and in most carcinoma cells, is a structural and functional anomaly among the integrin family of receptors. This integrin is defined as an adhesion receptor for most of the known basement membrane laminins. The distinguishing structural feature of $\alpha 6\beta 4$ is the atypical cytoplasmic domain of the $\beta 4$ subunit. A primary function of $\alpha 6\beta 4$, revealed by studies on knockout and transgenic mice, is to maintain the integrity of epithelia. This critical role for $\alpha 6\beta 4$ derives from its ability to mediate the formation of stable adhesive structures termed hemidesmosomes on the basal cell surface that link the cytokeratin

network with laminins in the basement membrane (Green and Jones, 1996; Shaw *et al.*, 1997).

Although this involvement of $\alpha 6\beta 4$ in hemidesmosome organization and function has dominated the study of this integrin, recent studies have revealed novel and important functions for this integrin in epithelial wound healing and in the migration and invasion of carcinoma cells. Many studies have observed that expression of $\alpha 6\beta 4$ is maintained or often increased in invasive and metastatic carcinomas and that $\alpha 6\beta 4$ expression levels actually correlate with the progression of these carcinomas (reviewed in Rabinovitz and Mercurio, 1996). Although such studies have provided evidence to implicate $\alpha 6\beta 4$ in the invasive process, they do not explain how an integrin, which associates with intermediate filaments and forms stable adhesive contacts, could promote the dynamic processes of migration and invasion. Indeed, the presence of $\alpha 6\beta 4$ -containing hemidesmosomes would impede invasion. A significant breakthrough, therefore, was our finding that $\alpha 6\beta 4$ actually mediates the migration of carcinoma cells through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of

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Abbreviations used: BM, basement membrane; ECM, extracellular, BDM, butanedione monoxime.

filopodia and lamellae (Rabinovitz and Mercurio, 1997; O'Connor *et al.*, 1998). This finding implied that the function and cytoskeletal association of $\alpha 6 \beta 4$ in invasive carcinoma cells is distinct from its established role of anchoring epithelial cells to the basement membrane (BM) through its association with cytokeratins. A second significant finding that provided a mechanistic basis for the involvement of $\alpha 6 \beta 4$ in invasion was that this integrin stimulates the activity of the enzyme phosphoinositide 3-OH kinase (PI3-K) in invasive carcinoma cells and that PI3-K is essential for migration and invasion (Shaw *et al.*, 1997).

Our current interest is to understand the mechanisms by which the $\alpha 6 \beta 4$ integrin contributes to the migration process and to relate such mechanisms to invasion. The migration of many cells in culture involves actin polymerization that generates protrusions at the edge of the cell and the contraction of the actin network by associated myosin motors (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). It has become apparent from studies on fish keratocytes and fibroblasts that adhesion to the substrate in the lamellar area provides the necessary traction to support the propulsive forces generated by the cytoskeleton to haul the rest of the cell (Lee *et al.*, 1994; Burton *et al.*, 1999). Given this knowledge, one possible mechanism by which $\alpha 6 \beta 4$ stimulates migration is to enhance the generation of traction by lamellae. We have shown that $\alpha 6 \beta 4$ is localized to the base of lamellae in carcinoma cells migrating on laminin (Rabinovitz and Mercurio, 1997). Moreover, the formation of actin bundles parallel to the lamella in such cells, which is considered to be crucial for the generation of traction in the keratocyte and fibroblast models, is dependent on $\alpha 6 \beta 4$ (Rabinovitz and Mercurio, 1997). The ability of $\alpha 6 \beta 4$ to generate traction forces created by the actomyosin cytoskeleton has not been determined, in part, because most studies on this integrin have focused on its association with the cytokeratin cytoskeleton. Moreover, the consequences of $\alpha 6 \beta 4$ -mediated traction forces on BM organization and invasion have not been considered.

In this study, we used a well characterized traction-force detection assay (Dembo and Wang, 1999; Pelham and Wang, 1999) to establish that such forces are exerted through the $\alpha 6 \beta 4$ integrin. These $\alpha 6 \beta 4$ -dependent traction forces are organized into a compression machine localized to the base of lamellae. Moreover, we observed that carcinoma cells are able to remodel a reconstituted BM through $\alpha 6 \beta 4$ -mediated compression forces by a process that involves the packing of BM material under the cells and the mechanical removal of BM from adjacent areas. Importantly, we also demonstrate that the signaling properties of $\alpha 6 \beta 4$ can stimulate BM remodeling. Finally, we provide evidence for the remodeling of a natural BM by epithelial cells and for the involvement of $\alpha 6 \beta 4$ in this process. Our findings have important implications for the mechanism of both BM organization and tumor invasion.

MATERIALS AND METHODS

Cells and Antibodies

The clone A cell line was isolated from a human, poorly differentiated colon adenocarcinoma (Lotz *et al.*, 1990). The clone A integrin receptors have been described previously (Lotz *et al.*, 1990). Stable subclones of MDA-MB-435 human breast carcinoma cells were used

that had been transfected with either the expression vector alone (mock transfectants) or a full-length $\beta 4$ cDNA (MDA/ $\beta 4$ transfectants). The characterization of these transfectants has been described previously (Shaw *et al.*, 1997).

The following three monoclonal antibodies (mAbs) were used in this study: GoH3, a rat mAb specific for the integrin $\alpha 6$ subunit (Immunotech, Westbrook, ME); MC-13, a mouse mAb specific for the integrin $\beta 1$ subunit (provided by Dr. Steven Akiyama); and anti-LDL receptor mAb (Oncogene Science, Cambridge, MA).

Reagents

Laminin-1, prepared from the EHS sarcoma, was provided by Dr. Hynda Kleinman (National Institutes of Health, Bethesda, MD). Fluorescein isothiocyanate (FITC)-labeled laminin was provided by Dr. Peter D. Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ). Matrigel was purchased from BD Biosciences (San Jose, CA). Cytochalasin B, nocodazole, butanedione monoxime (BDM), and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma (St. Louis, MO). The Rho kinase inhibitor Y27632 was purchased from U.S. Biochemical (Cleveland, OH) and the PI3-K inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). Fluorescent beads (2- μ m fluospheres) were obtained from Molecular Probes (Eugene, OR), and gold colloid particles (5 nm) were obtained from Ted Pella (Redding, CA).

Traction-Detection System

A flexible, polyacrylamide gel containing fluorescent beads and coated with either laminin (100 μ g/ml) or goat anti-rat/mouse antibodies (100 μ g/ml) was prepared following a published protocol (Dembo and Wang, 1999; Pelham and Wang, 1999). Cells were plated on the laminin substrate and incubated for 1 h at 37°C in a CO₂ incubator. Before plating the cells on the antibody substrates, the cells were incubated for 20 min in the presence of either the rat GoH3 antibody or anti-LDL receptor antibody, and the unbound antibody was removed by two cycles of centrifugation and resuspension in medium. The dishes were sealed with Parafilm (American National Can, Menasha, WI). Random cells were filmed digitally using time lapse video-microscopy on a Nikon Diaphot 300 inverted microscope equipped with a heated stage, by using both phase contrast and fluorescence optics. This microscope was connected to a charge-coupled device camera (Dage-MTI, Michigan City, IN), a frame-grabber (Scion, Frederick, MD), and a G3 Power Macintosh computer to capture the images. Images were collected and analyzed with IPlab Spectrum image analysis software (Scanalytics, Fairfax, VA). Both phase contrast and fluorescence images were registered for each point in time. The phase contrast and fluorescent images were merged and frame sequences were animated using the image analysis software.

Bead displacement was quantified as described (Wang and Pelham, 1998; Pelham and Wang, 1999). Briefly, cells and fluorescent beads were photographed after a 1-h incubation, and the cells were then treated for 15 min with either trypsin/EDTA (for cells on laminin substrate) or for 1 h with proteinase K (1 mg/ml phosphate-buffered saline; for cells on antibody substrate) to relax the elastic substrate. A second photograph was taken at this time point and compared with the first photograph. In this way, vector maps indicating the magnitude and direction of bead displacement were built and analyzed using image analysis software. For the comparative purposes of our study, we estimated traction forces only in terms of relative bead displacement, because displacement is related proportionally to the traction force according to Hooke's law. It was apparent that groups of beads in defined regions were being displaced coherently in a similar direction. To facilitate quantification of the total area displaced by each cell as an estimate of traction, regions of coherent bead displacement were delineated. The displacement of individual beads inside each of these regions was calculated and the values obtained were averaged. The average

displacement of beads was then multiplied by the area of the region containing the beads to obtain the total region displacement. The total area displaced per cell was obtained by the sum of all region displacement. A total of 10 cells was analyzed for each assay.

Remodeling of Reconstituted BM

Matrigel was mixed with fluorescent beads and reconstituted on coverslips that had been glued to "punched" plastic Petri dishes. Clone A or MDA-MB-435 cells were plated on the top of the gel and incubated for 1–8 h inside a CO₂ incubator, the time depending on the nature of experiment. In some experiments, the dishes were sealed with parafilm, and analyzed using time-lapse videomicroscopy as described above. For each point in time, both cells and fluorescent beads were photographed using phase contrast or fluorescence microscopy, respectively. Function-blocking antibodies, PMA, or cytoskeleton-related inhibitors were added at the concentrations and times described in the corresponding figures. In some experiments, the cells were assayed on Matrigel prepared without fluorescent beads, and then the preparations were fixed with methanol and stained with Coomassie Blue. In other experiments, FITC-labeled laminin (instead of fluorescent beads) was mixed with Matrigel and reconstituted as described above before plating the cells. A semiquantitative analysis of Matrigel compression was done by determining the distance between specific pairs of beads filmed before, during, and after they were traversed by lamellae. Ten pairs of beads were tracked for each assay. In some experiments, a digital analysis of the fluorescence intensity produced by the clustered beads around the cells was done to assess Matrigel compression. As the beads concentrate in small areas, they also increase the net fluorescence of the area. The background produced by nonclustered beads was removed by thresholding the images above a certain intensity value.

Analysis of Corneal BMs

Rabbit corneas were used to obtain stroma with preserved BMs as well as sheets of corneal epithelium. The method to remove epithelia from their underlying stroma and to obtain viable epithelial sheets has been described previously (Gipson and Grill, 1982; Gipson *et al.*, 1983). The separated stroma containing the BM was transferred to a gold colloid suspension (5-nm particles; adjusted to pH 9) and incubated at room temperature for 30 min. The gold particles bound to the stroma were stabilized by removing the gold colloid suspension and incubating the samples in Hanks' balanced salt solution containing 1% bovine serum albumin for 10 min. The tissue was rinsed extensively using the bovine serum albumin/Hanks' balanced salt solution buffer. This corneal substrate was cut in quarters. The epithelial sheets obtained from other corneas were cut smaller than the portion of gold labeled stroma to which it was to be applied to provide a leading edge because it has been shown previously that epithelia in these circumstances can migrate along the substrate and retard the formation of hemidesmosomes (Gipson *et al.*, 1983). Before recombining with the substrate, the epithelial sheet was incubated or not with GoH3 or anti-rabbit major histocompatibility complex (MHC)-I antibody (10 µg/ml) for 20 min. The GoH3 antibody has been previously shown to recognize the α6 integrin of rabbit corneas (Stepp *et al.*, 1990). MHC-I is also present in cornea (Gipson, unpublished data). The epithelial sheet was recombined with the substrate and incubated for 3–6 h in minimal essential medium (supplemented as described previously; Gipson *et al.*, 1983). The tissues were fixed and processed for electron microscopy analysis by using standard techniques (Gipson *et al.*, 1983). Five to 10 fields were photographed for each block sectioned at two different depths.

The photographic negatives were scanned into G3 PowerMac. The distribution of gold particles was performed by counting and sizing clusters of gold particles by using IPLab spectrum image analysis software.

Figure 1.

Online Supplemental Material

All videos are merged composites of phase contrast (red: cells) and fluorescent images (green: beads). Frame intervals: 2 min. Figure 1b.mov: Formation of a lamella (arrow) produces bead displacement on a laminin-coated elastic substrate. Figure 1c.mov: Filopodium (arrow) produces the displacement of a few beads. Figure 6.mov: Filopodia (arrows) efficiently pull Matrigel/beads toward

positions of each bead at the beginning and end of the frame sequence; the arrows indicate the direction of displacement and the thick-hatched arrow indicates the region where opposing forces are focused. (C) Frame sequence of discrete traction forces produced by filopodia. The columns of frames in the left and middle panels were photographed using phase contrast and fluorescence optics, respectively. The graphic on the right column represents the displacement of a small number of beads produced by the filopodia in the left columns. Grid lines are spatial references. **Video Figure 1c.mov** contains the corresponding frame sequence. (D and E) Cells were incubated 1 h at 37°C in the presence of GoH3 antibody or rat IgG control, and photographed using phase contrast and fluorescence illumination. The cells were treated with trypsin/EDTA to produce a relaxation of the substrate. The position of beads before and after trypsin/EDTA treatment was registered and a map of vectors representing the magnitude and direction of displacement was built for every cell. (D) Example of a vector map of such bead displacement. In E, the average bead displacement/cell (μm^2) was calculated for cells in the presence or absence of the GoH3 antibody (see MATERIALS AND METHODS for calculation procedure).

the cell body. Figure 7.mov: clone A cell stimulated with PMA compresses Matrigel/beads (gray scale).

RESULTS

$\alpha 6 \beta 4$ Integrin Can Mediate Traction Forces onto a Laminin Substrate

A well-characterized force-detection system was used to assess the ability of the $\alpha 6 \beta 4$ integrin to mediate traction forces (Wang and Pelham, 1998; Pelham and Wang, 1999). The system consisted of an elastic polyacrylamide sheet that was embedded with fluorescent beads and coated with laminin-1 on its surface. Elastic deformation of the gel by the cell produces displacement of the beads, proportional to the forces generated by the acto-myosin system and transmitted to the substrate via adhesive interactions (Wang and Pelham, 1998; Pelham and Wang, 1999). For these initial studies, we used either clone A colon or A431 squamous carcinoma cells because we and others have established that these cells couple $\alpha 6$ exclusively with $\beta 4$, and that $\alpha 6 \beta 4$ mediates the migration of these cells on laminin (Falcioni *et*

al., 1988; Lotz *et al.*, 1990; Lee *et al.*, 1992; Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999). The cells plated on these elastic substrates formed small lamellae and filopodia (Figure 1A, arrows).

Traction forces were analyzed indirectly by measuring the deformation of the elastic substrate produced by the cells. Bead positions were registered before and after relaxing the substrate by using trypsin, which abolishes cell traction by eliminating adhesion (Pelham and Wang, 1999). The arrows in Figure 1D represent the direction and distance a bead is displaced before and after treatment with trypsin. Subsequently, we assessed the importance of $\alpha 6 \beta 4$ in the transmission of traction forces by quantifying the average bead displacement in the presence or absence of an $\alpha 6$ function-blocking antibody (Figure 1E). This antibody eliminated most of the traction on the substrate generated by the cells. These data provide evidence that $\alpha 6 \beta 4$ can transmit traction forces onto a laminin substrate.

In previous work studying the behavior of clone A cells and other carcinoma cells on laminin (Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999), we demonstrated that

$\alpha 6 \beta 4$ is present in lamellae and that their formation depends on this integrin. We had also observed that $\alpha 6 \beta 4$ is present at sites of filopodia anchorage (Rabinovitz and Mercurio, 1997). Consistent with a role of $\alpha 6 \beta 4$ in the transmission of traction forces at these sites, the analysis of bead displacement by time-lapse videomicroscopy revealed that small lamellae produced the strongest deformation (Figure 1, A and B, and Figure 1a.mov). Vector maps of bead displacement, which were built by connecting the initial and final positions of each bead at the beginning and end of the frame sequence (Figure 1B, middle), revealed that these small lamellae frequently compressed the substrate. These vectors focused their compressive action to the base of the lamellae (Figure 1b.mov, and Figure 1, B and D), an area where $\alpha 6 \beta 4$ expression is concentrated (Rabinovitz and Mercurio, 1997). Filopodia exerted discrete, small forces on the substrate, as indicated by limited displacement of one or a few beads in areas where filopodia attachment was apparent (Figure 1C and Figure 1c.mov). This was observed frequently in cells that presented filopodia. Other cell areas, such as the nuclear area, showed little traction activity. These data suggest that $\alpha 6 \beta 4$ mediates transmission of traction forces at cell protrusions and that these forces comprise a substrate-compression machine.

The $\alpha 6 \beta 4$ integrin can stimulate intracellular signaling (Shaw *et al.*, 1997; O'Connor *et al.*, 2000). The possibility existed, therefore, that the inhibition of traction forces by $\alpha 6 \beta 4$ function blocking antibodies resulted from the inhibition of $\alpha 6 \beta 4$ -dependent signaling events that impact the mechanical transmission of traction by laminin receptors other than $\alpha 6 \beta 4$. To establish more definitively that $\alpha 6 \beta 4$ can transmit traction forces onto the substrate directly, we modified the traction-detection system substrate so that it would engage cells exclusively through the $\alpha 6 \beta 4$ integrin. For this purpose, we cross-linked the polyacrylamide gel with an anti-rat antibody to link $\alpha 6 \beta 4$ from A431 cells that had been coated with the rat GoH3 antibody and analyzed the traction generated. Any traction observed in this system must be transmitted through $\alpha 6 \beta 4$. As a specificity control, we coated the cells with a mouse anti-LDL receptor mAb and cross-linked the polyacrylamide with an anti-mouse antibody. The GoH3-coated cells partially spread on the polyacrylamide gel and produced a substantial number of filopodia and lamellar protrusions. Importantly, a significant amount of bead displacement was observed under these conditions (Figure 2). In contrast, the anti-LDL mAb-coated cells did not spread and they produced no protrusions or bead displacement. These data establish that $\alpha 6 \beta 4$ can mechanically transmit traction forces onto a laminin substrate.

Traction Forces Mediated by $\alpha 6 \beta 4$ Integrin Remodel Reconstituted BM

Our finding that the $\alpha 6 \beta 4$ integrin can mediate traction forces on laminin and deform this matrix raised the possibility that cells that express this integrin could also deform or remodel BMs. To examine this possibility, fluorescent beads (0.2 μ m) were embedded in reconstituted BM (Matrigel) and allowed to solidify into a thick gel (40 μ m thick). Clone A cells were plated on the Matrigel and the system was videotaped using both phase contrast and fluorescence microscopy. This video analysis revealed that clone A cells rapidly pull in the adjacent Matrigel, as indicated by the

inward (centripetal) movement and increasing concentration of beads around the cells (Figure 3, A and B, and videos; Figure 6.mov; and Figure 7.mov).

To demonstrate that the observed concentration of beads around the cells reflected a concentration of Matrigel protein, cells were stained with Coomassie Blue to detect protein concentration. The intense Coomassie Blue staining around the cells and diminished staining in adjacent areas (Figure 4A) indicate that clone A cells induce condensation of Matrigel protein around them by sequestering Matrigel from adjacent areas that are devoid of cells. A similar pattern was observed by indirect immunofluorescence by using an anti-laminin antibody (our unpublished data). The possibility that these observations reflect the *de novo* secretion of laminin by clone A cells was excluded by incorporating FITC-laminin in the Matrigel. As shown in Figure 4D, the FITC-laminin was concentrated around the cell in a manner consistent with Coomassie and laminin stains, as well as the observed bead movement. Together, these data demonstrate that clone A cells can remodel reconstituted BM by concentrating BM proteins around them.

Next, we assessed the contribution of the $\alpha 6 \beta 4$ integrin to BM remodeling by using initially a function-blocking antibody. Treatment of clone A cells with this antibody significantly reduced the centripetal movement of beads toward cells (Figure 3C) and the condensation of Matrigel proteins around cells (Figure 4B). Importantly, antibody inhibition of $\alpha 6 \beta 4$ did not detach cells from Matrigel; it only prevented their ability to remodel this matrix. The use of a function-blocking $\beta 1$ integrin antibody, in contrast, did perturb clone A attachment to Matrigel (our unpublished data). Thus, it is likely that remodeling involves the concerted action of both $\alpha 6 \beta 4$ and $\beta 1$ integrins and that these integrins mediate distinct functions, a scenario that we postulated for clone A migration on laminin (Rabinovitz and Mercurio, 1997).

Stimulation of Breast Carcinoma Invasion by $\alpha 6 \beta 4$ Expression Is Coincident with Increased BM Remodeling

Additional evidence to establish a distinct role for $\alpha 6 \beta 4$ in BM remodeling was obtained using a different cell system. MDA-MB-435 breast carcinoma cells do not express this integrin (Shaw *et al.*, 1997). Stable transfectants of these cells that express $\alpha 6 \beta 4$ exhibit a marked increase in their ability to invade Matrigel (Shaw *et al.*, 1997). As shown in Figure 5, the MDA-MB-435/ $\alpha 6 \beta 4$ transfectants were able to remodel Matrigel to a much greater extent than the mock transfectants as evidenced by bead compression around the cells.

Remodeling of Reconstituted BM Results from Compressive Forces Exerted by Lamellae and Filopodia

The dynamics of Matrigel remodeling by clone A cells and the cell structures involved in this process were studied using time-lapse videomicroscopy. At early times after plating on Matrigel, cells extended small lamellae and filopodia in all directions and formed a ring of Matrigel condensation around them (Figure 4D), suggesting that compression forces occur mostly at the cell edges and exclude the central area of the cell. Using the Matrigel/fluorescent bead method described above to monitor movement of BM material, we

Figure 2. $\alpha 6 \beta 4$ integrin can relay forces directly onto the substrate. A polyacrylamide-flexible substrate containing fluorescent beads (2 μm diameter) was cross-linked with either anti-rat or anti-mouse antibodies. A431 cells were coated with the rat GoH3 mAb or the mouse anti-LDL receptor antibody, plated on the gel, and incubated for 1 h at 37°C. (A) Average bead displacement/cell (μm^2) was analyzed by time-lapse video-microscopy by using phase contrast and fluorescent illumination as described in MATERIALS AND METHODS. The cells coated with the GoH3 antibody spread on the gel and produced numerous filopodia and lamellae (B), in contrast to the cell coated with the anti-LDL receptor antibody (C).

observed that the beads moved toward cells in areas where lamellae were protruded. At distal sites, much of the bead pulling was done by filopodia (Figure 6 and Figure 6.mov).

To examine the involvement of lamellae in remodeling in more detail, we stimulated clone A cells on Matrigel with PMA, which results in the formation of large, fan-shaped lamellae. PMA stimulation increased the remodeling of Matrigel significantly as evidenced by an increase in bead concentration under the cells compared with unstimulated cells (Figure 7 and Figure 7.mov). By analyzing bead compression, which is defined as the distance between specific beads before, during, and after the cell passes over them, we noted that Matrigel is compressed to 50% of its original dimension and recovers to only 65% of its original size (our unpublished data). Importantly, compression occurs specifically under lamellae and terminates behind the flat portion of lamellae (Figure 8 and Figure 8.mov). No bead movement was observed at the rear of the cell or at the rear of the "wings" of the lamellae, suggesting that most of the force is compressive only at the sites where Matrigel is in contact with lamellae. This phenomenon was particularly evident at the wings of lamellae, where there is a small distance between the lamella and the rear of the cell (Figure 7.mov). Soon after the wing of a lamella passed over an area of Matrigel, a modest, but significant relaxation of the Matrigel was seen (Figure 8). Thus, it is clear from these images that most of the compression is achieved, not between the front and rear of the cell, but in the area beneath the lamellae, before the bulk of cytoplasm begins.

Our results indicate that BM remodeling is clearly a phenomenon driven by traction. The massive movements of Matrigel associated with cell protrusions that occur in such short times support this conclusion. Furthermore, remodeling requires the acto-myosin system because it is inhibited by either cytochalasin B or BDM (our unpublished data).

Signaling Pathways Stimulated by $\alpha 6 \beta 4$ Are Involved in BM Remodeling

Based on the findings that $\alpha 6 \beta 4$ stimulates the activity of signaling molecules involved in regulating actin dynamics in carcinoma cells (Shaw *et al.*, 1997; O'Connor *et al.*, 2000), it was important to assess whether these signaling pathways are involved in BM remodeling. Specifically, $\alpha 6 \beta 4$ has been shown to stimulate the activity of both PI3-K and RhoA. We assessed the participation of these molecules in the remodeling activity of clone A cells by using pharmacological inhibitors. As shown in Figure 9 both LY294002 (PI3-K) and Y27632 (Rho kinase) impeded the remodeling process. These data indicate an important role for PI3-K and RhoA in BM remodeling, and they suggest an important role for $\alpha 6 \beta 4$ -mediated signaling in addition to its ability to mediate traction forces in the remodeling process.

Evidence for Remodeling of Native BMs by $\alpha 6 \beta 4$ Integrin

Given our findings that the $\alpha 6 \beta 4$ integrin can mediate traction forces and remodel reconstituted BMs, it was important to establish whether these events occurred with *native* BMs. For this purpose, we used the BM of the rabbit cornea. The corneal epithelium can be removed to yield a "denuded" BM (Gipson *et al.*, 1983). An intact epithelial-stromal struc-

Figure 3. Mechanical remodeling of reconstituted BM is dependent on the $\alpha 6 \beta 4$ integrin. Clone A cells were plated on a film of Matrigel containing fluorescent beads and incubated in the presence of GoH3 (C and D and G and H) or control antibody (A and B and E and F) for 4 h at 37°C. The cells were photographed at high (A–D) and low (E–H) magnifications by using phase contrast (A and C) or fluorescence (B and D) microscopy. Notice the inhibition of bead concentration around the cells in the presence of the GoH3 antibody. Bars, 10 μ m (A–D), 100 μ m (E–H).

ture can be reconstituted by the addition of a freshly isolated epithelium to the denuded BM. The size of the epithelium that is added back is smaller than the area of the BM, a situation that promotes cell migration in the direction of the free edge. In our experiments, we coated the denuded BM with gold colloid particles (5 nm) that bound to the BM in a homogeneous pattern (Figure 10A). Subsequently, these denuded BMs were recombined with corneal epithelia. Our

assumption was that a mechanical displacement or remodeling of the BM by the epithelium would alter the distribution pattern of the gold particles. For example, a local compression of BM material by the epithelial cells would result in the formation of a more densely packed group of gold particles. Indeed, using this approach, we observed that the epithelium did induce a redistribution of gold particles into more densely packed clusters (Figure 10B). Quantitative

Figure 4. Remodeling of reconstituted BM involves condensation of material gathered from adjacent areas and is dependent on the integrin $\alpha 6 \beta 4$. (A and B) Clone A cells were plated on a film of Matrigel in the presence of either GoH3 (B) or control (A) antibody and incubated for 4 h. The cells were fixed and stained with Coomassie Blue. Notice the condensation of matrix material that forms around the cells and the clearing of material that occurs in adjacent areas. Bar, 75 μm . (C) At a short time of incubation (1 h), the cells have already produced a condensation ring around them. In this photograph, the cells were removed using EDTA to reveal the underlying matrix. (D) Fluorescence image of Clone A cells that were plated on a film of Matrigel containing FITC-conjugated laminin and incubated for 8 h. Notice that the fluorescent laminin condenses around the cells. Some intact Matrigel can be seen as a light veil at the top of this image. Bar, 25 μm .

analysis of this particle redistribution revealed a significant increase in clustering at 3 h and an additional increase at 6 h (Figure 10C). At these time points, mature hemidesmosomes were not apparent, an observation that is consistent with

previous evidence showing that migratory epithelia do not form hemidesmosomes (Gipson *et al.*, 1993).

Next, we assessed whether the $\alpha 6 \beta 4$ integrin contributes to the remodeling of the corneal BM. The corneal epithelium

Figure 5. Expression of the $\alpha 6 \beta 4$ integrin in breast carcinoma cells increases their ability to remodel reconstituted BM. MDA-MB-435 transfectants expressing the $\alpha 6 \beta 4$ integrin (C and D) or mock transfectants (A and B) were plated on Matrigel containing fluorescent beads and incubated at 37°C for 4 h. The cells were photographed using phase contrast (A and C) or fluorescence (B and D) microscopy. Note the intense concentration of beads surrounding the $\alpha 6 \beta 4$ transfectants in comparison with the mock transfectants. Bar, 10 μm .

Figure 6. Remodeling of reconstituted BM by filopodia. Clone A cells were plated on Matrigel containing fluorescent beads. The cells were analyzed by video microscopy with both phase contrast and fluorescence illumination. The frame sequence shown was taken at 2-min intervals. Only a few beads (arrowheads) from a small area surrounding a filopodium (arrow) are pulled toward the cell at the bottom. The lines in the second and subsequent frames represent the movement tracks of the beads indicated in the first frame. Beads distal to filopodia show little movement. In the **video** (Figure 6.mov), the arrows point to the area where filopodia activity is occurring.

expresses $\alpha 6 \beta 4$ but not $\alpha 6 \beta 1$ (Stepp *et al.*, 1993). An $\alpha 6 \beta 4$ function-blocking antibody was incubated with isolated epithelium before and after recombining this epithelium with denuded BM. Importantly, this antibody treatment did not perturb the attachment of the epithelium to the BM, an observation consistent with recent findings that BM attachment is mediated by $\beta 1$ integrins (Raghavan *et al.*, 2000). We did observe, however, that this antibody treatment reduced the formation of gold particle aggregates significantly (Figure 10D). As a specificity control, we used an antibody specific for the rabbit MHC-I protein, which showed no inhibitory activity (Figure 10D). These data provide evidence that the $\alpha 6 \beta 4$ integrin participates in the remodeling of a natural BM.

DISCUSSION

The integrin $\alpha 6 \beta 4$, a laminin receptor that stabilizes epithelial cell adhesion to the BM through its association with cytokeratins, can stimulate the formation and stabilization of actin-rich protrusions in carcinoma cells (Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999). An important, unresolved issue, however, was whether this integrin could transmit forces to the substrate generated by the acto-myosin system. Although $\alpha 6 \beta 4$ can associate with the actin cytoskeleton (Rabinovitz and Mercurio, 1997), this association does not imply necessarily that it can transmit forces to a substrate directly. Moreover, the distinct signaling functions of $\alpha 6 \beta 4$ could have a significant impact on actin dynamics and acto-myosin contraction. Using a well-characterized traction-force detection assay, we were able to detect forces

exerted through the $\alpha 6 \beta 4$ integrin on either laminin-1, a matrix ligand, or on an anti- $\alpha 6 \beta 4$ antibody, demonstrating that this integrin can transmit forces without the need to engage other integrins. These $\alpha 6 \beta 4$ -dependent traction forces were organized into a compression machine localized to the base of lamellae. We hypothesized that the compression forces generated by $\alpha 6 \beta 4$ result in the remodeling of BMs because this integrin plays a major role in the interaction of epithelial and carcinoma cells with such structures. Indeed, we observed that carcinoma cells are able to remodel a reconstituted BM through $\alpha 6 \beta 4$ -mediated compression forces by a process that involves the packing of BM material under the cells and the mechanical removal of BM from adjacent areas. Importantly, we also provide evidence for the remodeling of a native BM by epithelial cells and for the involvement of $\alpha 6 \beta 4$ in this remodeling. In addition, we demonstrate that the distinct signaling functions of $\alpha 6 \beta 4$ also stimulate remodeling. Our findings have important implications for the mechanism of both BM organization and tumor invasion.

Traction forces play an important role in cell migration and in remodeling of the extracellular membrane (ECM) (Stopak and Harris, 1982; Lee *et al.*, 1994). These forces are generated by myosin motors, which act on the actin cytoskeleton, to transmit tension to the ECM through cell adhesion receptors (Mitchison and Cramer, 1996). Studies in this area, however, have focused primarily on the ability of $\beta 1$ integrins, which associate only with F-actin, to mediate traction forces (Lee and Jacobson, 1997). The $\alpha 6 \beta 4$ integrin, in contrast, is a unique receptor that was defined originally as the only integrin that associates with cytokeratins and not

Figure 7. Fan-shaped lamellae are powerful compressors of reconstituted BM. Time-lapse analysis of clone A cells stimulated with PMA (25 ng/ml) by using phase contrast (left) or fluorescent (right) illumination. Panels shown represent frames taken at 15-min intervals. Bar, 10 μ m. In Figure 7.mov, the phase contrast and fluorescent images frames were captured 2-min intervals and then merged. Notice the strong compression that occurs under the lamella, relaxing partially after the base of the lamella passes over the compressed region. Also, note that although most of the direction of compression occurs perpendicular to the lamella, strong compression parallel to the lamella is evident at the end of the video.

with F-actin (Green and Jones, 1996). To date, no studies have demonstrated that cytokeratins generate traction forces. Importantly, therefore, our observation that $\alpha 6 \beta 4$ is able to mediate traction forces strengthens our hypothesis that this integrin can interact with F-actin, as well as cytokeratins, and that its association with F-actin can be a significant factor in cell movement. The ability of $\alpha 6 \beta 4$ to mediate traction forces generated by the acto-myosin cytoskeleton provides an important functional consequence of its interaction with F-actin because such forces may play a critical role in cell migration and BM remodeling.

The organization of the $\alpha 6 \beta 4$ -dependent traction forces in carcinoma cells is consistent with the description of these forces in other extensively studied models, such as fish keratocytes and fibroblasts (Lee *et al.*, 1994; Dembo *et al.*,

1996; Svitkina *et al.*, 1997; Burton *et al.*, 1999; Oliver *et al.*, 1999; Pelham and Wang, 1999). Fish keratocytes have a distinct compressive component that is parallel to the lamella and probably functions to release rear attachments allowing the cell to move forward efficiently (Lee *et al.*, 1994; Oliver *et al.*, 1995). The "dynamic contraction network" model has been proposed to explain this compression pattern (Svitkina *et al.*, 1997; Burton *et al.*, 1999). In this model, compression forces increase gradually from the edge of the cell and achieve a maximum at the base of the lamella. Force vectors from the cell's edge are directed to the base of the lamella, whereas beyond this point the vectors can reverse direction. Interestingly, the dynamic contraction network may account for the $\alpha 6 \beta 4$ -dependent traction forces that we observed. Specifically, the vector maps of bead displacement

Figure 8. Matrigel compression ends at the base of lamellae. Clone A cells were stimulated with PMA (25 ng/ml) and analyzed using time-lapse video microscopy. Both phase contrast and fluorescent images were taken at 2-min intervals and merged digitally. Top panel shows a phase contrast image of the cell and bottom panels show a magnified frame sequence of the region specified by the rectangle in the top panel. The frame sequence shows the compression of a group of beads (arrows) until the base of the lamellae (arrowheads) passes over them, a time at which the beads partially relax (bottom). This observation was commonly seen for other beads undergoing compression beneath the lamella. Asterisk denotes the leading edge. Bar, 10 μ m.

Figure 9. Signaling pathways regulated by the $\alpha 6 \beta 4$ integrin are necessary for BM remodeling. Clone A cells were plated on Matrigel containing fluorescent beads and incubated for 4 h in the presence of either DMSO (A and B); Y27632, a Rho-kinase inhibitor (30 μ M) (C and D); or LY294002, a PI3-K inhibitor (20 μ M). The cells were photographed using either phase contrast (A, C, and E) and fluorescence (B, D, and F) microscopy. Bar, 100 μ m. Notice that the inhibitors inhibit the remodeling process strongly.

we generated suggest a concentration of forces from the edge of the cell toward the base of the lamella that reverses direction after this point. In addition, we noted that cells “pull” Matrigel inward toward the base of the lamella where compression is maximal and that the compressed Matrigel relaxes after this point. Additional evidence in support of this model is provided by our previous finding that a gradient of actin filament bundles is present parallel to the lamella in colon carcinoma cells that concentrate at the base of this structure (Rabinovitz and Mercurio, 1997). The enhanced localization of $\alpha 6 \beta 4$ at the base of lamellae (Rabinovitz and Mercurio, 1997; Rabinovitz *et al.*, 1999) is compatible with the idea that $\alpha 6 \beta 4$ is localized at sites of traction to provide the necessary attachment. Interestingly, however, $\alpha 6 \beta 4$ is also localized in retraction fibers at the rear of migrating cells (Rabinovitz and Mercurio, 1997) but we observed that little traction is generated by these structures. These observations suggest that the ability of $\alpha 6 \beta 4$ to generate traction is a function of its localization within the cell.

A likely possibility is that the $\alpha 6 \beta 4$ -dependent traction forces account for the ability of this integrin to mediate both compression and migration on laminin matrices. Forces that lead to compression may pull the cell body forward if some

Figure 10. $\alpha 6 \beta 4$ integrin functions in the remodeling of corneal BM. Deepithelialized corneas were labeled with gold colloid particles (5 nm) and recombined with fresh cornea epithelium as described in MATERIALS AND METHODS. The recombined corneas were incubated at 37°C for either 3 or 6 h, in the either presence or absence of the GoH3 mAb. (A) Electron micrograph of a recombined

release of rear attachments occurs. Indeed, our observation that the lamella of PMA-stimulated clone A cells produces both compression and forward movement is consistent with the idea that both compression and movement can occur at the same time (Figure 7.mov). The mechanism of rear detachment in clone A cells involves little frictional force, as indicated by the absence of bead movement behind the lamella. This rear detachment may result from lateral forces such as those observed in fish keratocytes, which generate minimal frictional force (Oliver *et al.*, 1998, 1999). These forces probably result from the high concentration and orientation of stress fibers at the base of the lamella. As mentioned above, clone A cells exhibit a concentration of stress fibers parallel to the base of the lamella (Rabinovitz and Mercurio, 1997). Importantly, we observed significant bead movement parallel and toward to the wings of lamellae in PMA-stimulated cells (Figure 7.mov), and relaxation of the gel behind the base of the lamella, in agreement with the mechanism of keratocyte movement.

A key finding in this study is that the compressive component of $\alpha 6 \beta 4$ -mediated traction forces results in the remodeling of BMs. In fact, few studies have focused on the possibility that cells alter BM organization and that this reorganization is an integrin-mediated process. The salient example is the remodeling of Matrigel by endothelial cells to form tubule structures, a process that is dependent on $\beta 1$ integrins (Vernon *et al.*, 1992; Davis and Camarillo, 1995). Our findings are significant not only because they extend this concept of remodeling but also because they link the biophysical properties of an integrin that plays a preeminent role in the interaction of both epithelial and carcinoma cells with BMs in this remodeling function. Moreover, an important and novel aspect of our study is the finding that epithelial cells can remodel native BMs mechanically and that the $\alpha 6 \beta 4$ integrin contributes to this remodeling. Specifically, we demonstrated, using the well-established cornea reconstitution model (Gipson and Grill, 1982; Gipson *et al.*, 1983), that the corneal epithelium is able to remodel corneal BMs that had been coated with gold particles as evidenced by the redistribution of these particles. Supporting the mechanical nature of this redistribution, a function-blocking antibody specific for $\alpha 6 \beta 4$ was able to inhibit the redistribution of gold particles significantly. We conclude from these results that BM remodeling by epithelial and carcinoma cells is not limited to reconstituted basement membranes.

The distinct signaling properties of $\alpha 6 \beta 4$ also contribute to BM remodeling by this integrin. At least two key signaling molecules, PI3-K and RhoA, are activated by $\alpha 6 \beta 4$ in carcinoma cells (Shaw *et al.*, 1997; O'Connor *et al.*, 2000). Importantly, the $\alpha 6 \beta 4$ -mediated activation of these signaling mol-

cornea showing areas of BM (arrowheads) either denuded (A) or covered with epithelium (B, asterisk). Notice that the distribution of the gold particles on the BM is homogeneous in A and aggregated in B (arrows). Bar, 0.5 μm . (C and D) Quantitative analysis of gold particle distribution. (C) Density of aggregates per unit length of BM (μm), was estimated at 3 and 6 h after recombining the epithelium with the denuded BM. Shown is the distribution of different size aggregates (D) Number of particles present in aggregates (>10 gold particles) per unit area of BM was quantified in recombined corneas that were incubated for 6 h in the presence or absence of the GoH3 mAb or anti-rabbit MHC-I antibody.

ecules has been shown to be essential for the ability of this integrin to promote carcinoma migration and invasion (Shaw *et al.*, 1997; O'Connor *et al.*, 1998). Given our findings in the present study, a link between $\alpha 6 \beta 4$ -mediated compression and $\alpha 6 \beta 4$ -mediated signaling becomes apparent. Specifically, the contractile forces that are converted to traction by $\alpha 6 \beta 4$ under lamellae could arise from $\alpha 6 \beta 4$ -mediated activation of Rho and the consequent Rho stimulation of actin myosin contraction. This $\alpha 6 \beta 4$ -mediated signaling of contraction may also be an important component of invasion that occurs by $\alpha 6 \beta 4$ -dependent BM remodeling as we postulated above. In support of this possibility, we observed that inhibition of Rho kinase activity disrupts the $\alpha 6 \beta 4$ -mediated compression of Matrigel. Along the same lines, $\alpha 6 \beta 4$ -stimulation of PI3-K and consequent changes in actin dynamics may contribute to the formation of filopodial and lamellar protrusions, processes that are also facilitated by the engagement of $\alpha 6 \beta 4$ with laminins in the matrix. Together, the studies on $\alpha 6 \beta 4$ exemplify the fruitfulness of integrating signaling studies with studies on integrin-mediated traction forces and ECM remodeling.

Our findings on BM remodeling by the $\alpha 6 \beta 4$ integrin may have important implications for BM organization, especially in light of recent studies on integrin knockout mice. It is apparent from analysis of the $\beta 4$ knockout mice that the $\alpha 6 \beta 4$ integrin is not necessary for the formation of the BM itself (Dowling *et al.*, 1996; Dipsio *et al.*, 1997). Interestingly, however, we noticed in the published electron micrographs of the epidermis from these mice that the BM is atypically smooth and homogeneous (Dowling *et al.*, 1996). In contrast, similar electron microscopy images obtained from wild-type mice, as well as other BMs, indicate an increased concentration of BM material adjacent to the hemidesmosomes and a relatively "thin" BM in areas between BMs (Gipson *et al.*, 1983; Rousselle *et al.*, 1991). The hypothesis can be derived from these images, in concert with our data, that the $\alpha 6 \beta 4$ integrin "sweeps" or compresses BM as part of the process of hemidesmosome formation. Such localized compression of BM would be consistent with our observation that homogeneously distributed gold particles attached to the cornea BM are gathered into discrete clusters by the epithelium, suggesting that compression occurs toward discrete, small areas. Although $\alpha 6 \beta 4$ may play a distinct role in BM remodeling, other integrins probably contribute to this process. Evidence to support a role for $\beta 1$ integrins, for example, in BM organization comes from the analysis of $\beta 1$ integrin knockout mice. The BMs of these mice are disorganized compared with wild-type mice (Raghavan *et al.*, 2000). Interestingly, although the BMs of the $\alpha 3$ integrin knockout mice appear to be discontinuous, stretches of BMs corresponding to areas of hemidesmosome localization are preserved (Dipsio *et al.*, 1997). One explanation for these observations based on our results is that the $\alpha 6 \beta 4$ integrin compresses BM toward the hemidesmosome and the $\alpha 3 \beta 1$ integrin functions to maintain a continuous structure, perhaps by contributing to BM formation. This notion is consistent with the report that the $\alpha 7 \beta 1$ integrin, a laminin receptor on muscle cells, can nucleate laminin polymerization (Colognato *et al.*, 1999).

A major implication of BM remodeling is tumor invasion. Although remodeling as a mechanism of BM breaching during tumor invasion has not been considered previously, our

findings support such a mechanism. Specifically, compression of BM in certain areas would generate gaps in adjacent areas through which tumor cells could escape. Interestingly, expression of the $\alpha 6 \beta 4$ integrin in MDA-MB-435 breast carcinoma cells increases their ability to invade Matrigel in a standard Boyden chamber assay dramatically (Shaw *et al.*, 1997). In addition, we observed in the present study that $\alpha 6 \beta 4$ expression in these cells enhanced their ability to remodel Matrigel substantially. A likely possibility is that the stimulation of invasion that is coincident with $\alpha 6 \beta 4$ expression derives from an increase in Matrigel remodeling. It is also worth noting that the $\alpha 6 \beta 4$ -mediated remodeling of Matrigel is not blocked by protease inhibitors (our unpublished data). Although these findings do not discount a role for proteases in tumor invasion, they do suggest that force-dependent remodeling should be considered as a mechanism of invasion. In fact, ECM remodeling as a component of tumor progression has been suggested previously. For example, a correlation between ECM remodeling and melanoma invasion has been observed (Klein *et al.*, 1991). Similar studies observed that highly invasive melanoma cells can remodel three-dimensional matrices, although partial matrix degradation was also observed (Friedl *et al.*, 1997). Most likely, both remodeling activity and protease activity are needed to maximize invasion (Silletti *et al.*, 1998).

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