$\alpha_6\beta_4$ Integrin Regulates the Collective Migration of Epithelial Cells

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Abstract

$\alpha_6\beta_4$ integrin is localized in a unique punctate distribution at the cell–substratum interface along the leading front of single, front–rear–polarized A549 cells. These puncta are interspersed between focal adhesions and lack association with the actin cytoskeleton. Knockdown of β_4 integrin in A549 cells inhibits their directed migration, with knockdown cells exhibiting large focal adhesions and reduced actin dynamics. Despite these changes, the speed of knockdown cells is equivalent to control cells. Interestingly, in such cells, α_6 integrin retains its punctate distribution. Moreover, in β_4 integrin knockdown cells, we observe a loss of β_1 integrin from focal adhesions and an enhanced association with α_6 integrin. We confirmed the switch in the β integrin binding partner of α_6 integrin in the knockdown cells by immunoprecipitation. We next investigated the role of β_4 integrin in collective cell migration. Wounded monolayers of β_4 integrin knockdown cells exhibit reduced collective migration compared with controls. When we forced expression of β_4 integrin in the leader cells of wounded monolayers, collective migration was restored. Similarly, forced

expression of β_4 integrin in primary rat alveolar epithelial cells also promotes collective cell migration. In addition, we interrogated the pathway by which β_4 integrin regulates A549 cell–directed migration. Constitutively active Ras-related C3 botulinum toxin substrate 1 rescues motility defects resulting from β_4 integrin deficiency. Together, our results support the hypothesis that $\alpha_6\beta_4$ integrin is a positive regulator of collective cell migration of A549 cells through influence on signal pathways in leader cells.

Keywords: cell motility; cell adhesion; cytoskeleton; directed motility; matrix adhesions

Clinical Relevance

The impact of this research relates to the role of an important integrin expressed by progenitor cells in the lung and lung tumor cells. We demonstrate for the first time that integrin β_4 is a regulator of sheets of epithelial cells.

β_4 integrin is the most unusual of all the β integrin subunits, because its cytoplasmic domain is over 1,000 amino acids long (1). It is best known as a component of the hemidesmosome, a stable adhesion device that anchors epithelial cells to the basement membrane in a variety of tissues (2).

α_6 integrin is an obligate partner of β_4 integrin and $\alpha_6\beta_4$ integrin heterodimers bind to laminin in the extracellular matrix (3). $\alpha_6\beta_4$ integrin is found in a variety of

normal cell types that lack bona fide hemidesmosomes both *in vitro* and *in vivo* (4, 5). In such cells, the functions of $\alpha_6\beta_4$ integrin are controversial, at least with respect to its role in motility versus stable attachment (6–11). For example, we have reported that, in single epidermal cells, $\alpha_6\beta_4$ integrin supports motile behavior, whereas others have reported that loss of β_4 integrin promotes migration (7, 12). Nonetheless, it has been well established

that $\alpha_6\beta_4$ integrin regulates tumor cell migration (1, 13). This helps explain the correlation between $\alpha_6\beta_4$ integrin expression levels and poor prognosis in several tumor types. For example, high expression of β_4 integrin is associated with aggressiveness of lung tumors (14–16). In this regard, the widely used chemotherapeutic agent, all-*trans* retinoic acid (ATRA), reduces β_4 integrin expression, while at the same time

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impeding the collective migration of the Lewis lung cancer cell line C87 (17). Together, these findings lead us to hypothesize that β_4 integrin not only regulates single, but also collective, epithelial cell motility. Here, we tested this hypothesis using A549 cells.

Materials and Methods

Cell Culture

A549 cells, purchased from American Type Culture Collection (ATCC), and 804G (rat bladder) cells were cultured as described elsewhere (18, 19). Scrambled (SCR) and β_4 integrin knockdown (β_4 KD) A549 cells were generated by transduction of wild-type (WT) cells with a lentiviral vector, encoding scrambled short hairpin RNA (shRNA) or β_4 integrin-specific shRNA, respectively (Sigma-Aldrich, St. Louis, MO). Primary rat alveolar type II cells were isolated as detailed previously (18). Adenovirus encoding green fluorescent protein (GFP), GFP-tagged β_4 integrin (GFP β_4), shRNA against α_6 integrin, GFP-tagged full-length α_6 integrin,

dominant-negative Ras-related C3 botulinum toxin substrate 1 (Rac1), or constitutively active Rac1 were described previously (7, 20). Adenovirus encoding red fluorescent protein-tagged LifeAct was purchased (Ibidi USA, Inc., Madison, WI).

Quantitative PCR

Quantitative PCR reactions were performed as detailed elsewhere (20).

Drug Treatments

ATRA, mitomycin C, and epidermal growth factor (EGF) were purchased from Sigma-Aldrich.

Immunocytochemistry and Flow Cytometry

Immunocytochemistry and flow cytometry were undertaken as detailed previously (20). Mouse monoclonal antibodies against β_1 and β_4 integrin (6S6 and 450-11A) were purchased from EMD Millipore (Billerica, MA) and BD Biosciences (San Jose, CA). Monoclonal rabbit anti-paxillin antibody (Y113) was purchased from Abcam (Cambridge, MA), and the rat antibody

against α_6 integrin (J1b5) was described previously (20). Actin was localized using rhodamine-conjugated phalloidin (Thermo Fisher, Rockford, IL). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Images were captured using a TCS SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Immunoprecipitation, SDS-PAGE, and Immunoblotting

Western blotting and immunoprecipitation experiments were performed as outlined elsewhere (8).

Rac1 Activity Assay

Rac1 activity was assayed using a Rac1 G-LISA kit (Cytoskeleton Inc., Denver, CO).

Actin Dynamics Assay

Kymographs were generated from images of live cells infected with adenovirus encoding red fluorescent protein-LifeAct (Ibidi USA, Inc.) and used to determine the speed of actin arc movement (21).

Figure 1. $\alpha_6\beta_4$ integrin exhibits a polarized distribution in A549 cells. (A) Confocal images of β_4 integrin (green), α_6 integrin (red), and the focal adhesion protein paxillin (pink). Scale bars, 10 μ m. Lower panel, high magnification of the region in the inset. (B) Confocal images of β_4 integrin (green), rhodamine-phalloidin as a marker of F-actin (red), and paxillin (pink). Scale bars, 10 μ m. Right panel, high magnification of the region in the inset.

Single Cell Motility and Conventional Scratch Assays

These assays were performed using previously described procedures (7). Laminin-332-coated surfaces were generated as detailed elsewhere (7).

Hybrid Sheet Scratch Assays

A published protocol was modified to generate scratched hybrid sheets (22). β 4KD2 cells were infected with adenovirus encoding GFP β 4 and, 24 hours later, were plated into a single well. Cells on the right half of the well were scraped away 6 hours later and then β 4KD2 cells were plated on top. After 21 hours, the media were supplemented with 4',6-diamidino-2-phenylindole (Thermo Fisher) and mitomycin C to inhibit proliferation. At 3 hours later, a second scrape was made at a shallow angle relative to the first, leaving two regions of interest. The first region had β 4KD2 leader and follower cells, whereas the second region had β 4KD2 leader cells

expressing GFP β 4 and nonexpressing β 4KD2 follower cells. Phase images and fluorescent images were captured of the scrape area every 10 minutes for 12 hours. The positions of cells at the front of the scratch were determined using the TrackMate plugin (<http://fiji.sc/trackmate>) for Fiji (21). Scratch displacement was defined as the average displacement of these cells in the direction perpendicular to the leading front.

Statistical Analysis

Statistical significance was determined by ANOVA or *t* test as indicated. *P* less than 0.05 was considered significant.

Results

$\alpha_6\beta_4$ Integrin Localization in A549 Cells

In A549 cells, β_4 integrin codistributes with α_6 integrin along their basal surface in punctate arrays near the membrane edge

(Figure 1A). These arrays are spatially distinct from focal adhesions and are distributed more extensively between actin bundles at the edges of cells, rather than at their termination points (Figures 1A and 1B). Consistent with the work of others using lung cancer cell types, the retinoid ATRA reduces both expression of β_4 integrin and the motility of A549 cells (Figure 2) (17). Specifically, ATRA-treated cells exhibit a 45% reduction of β_4 integrin surface expression and a 25% reduction of β_4 integrin message level (Figures 2A–2C). In addition, the ATRA-treated cells also exhibit reduced processivity, a measure of directed migration (Figure 2D). Processivity was defined as: maximum displacement from the cell's origin divided by the length of the path traveled (8). However, because ATRA treatment also alters the expression of the integrins α_3 , α_6 , and β_1 (Figures 2A and 2B), these studies do not allow us to determine whether effects of ATRA on motility are due to β_4 integrin loss or loss of

Figure 2. All-*trans* retinoic acid (ATRA) reduces integrin expression in A549 cells. Cells were treated with 100 nM ATRA or vehicle every 24 hours for 48 hours and then prepared for (A and B) flow cytometry, (C) quantitative PCR (qPCR; three experiments, three replicates per experiment), and (D) 2-hour motility assays. (A) Flow cytometry for integrins α_3 , α_6 , β_1 , and β_4 . (B) The relative average surface expression of the indicated integrins was calculated from flow cytometry data (3 experiments, 4×10^3 events per sample). (D) Quantification of the speed and processivity of ATRA- and vehicle-treated cells (3 experiments, >140 cells). Results are presented as means ($\pm$ SEM). Statistical analyses: *t* test. NS, not significant; ***P* < 0.01, ****P* < 0.001.

another integrin. To resolve this issue, we selectively knocked down β_4 integrin in A549 cells and assayed the motility of the knockdown cells.

Knockdown of β_4 Integrin Reduces Directed Migration

Three stable β_4 KD A549 clones (β_4 KD1–3) and a scrambled shRNA-expressing control (SCR) were generated by lentiviral means and selected for experimentation (Figure 3). All β_4 KDs exhibit knockdown of β_4 integrin by greater than 70% at the message

level and greater than 75% at the protein level (Figures 3A–3C). In addition, compared with WT and SCR controls, they exhibit reduced β_4 integrin surface expression and no differences in the surface expression of α_3 , α_6 , and β_1 integrins (Figure 3D). Moreover, β_4 KD cells migrated less processively than controls (Figure 3E). Furthermore, this phenotype is rescued upon infection with an adenovirus encoding GFP β_4 , the message of which is refractory to the shRNA used for knockdown (Figure 3E).

Because the preferred ligand of β_4 integrin is laminin-332, we also compared the motility of control and β_4 KD cells plated onto dishes coated with laminin-332 (Figure 3F). Although both speed and processivity are increased compared with cells plated onto tissue culture plastic, our findings, that β_4 KD results in a reduction in processivity, are recapitulated when cells move on a laminin-332 matrix (Figure 3F). Together, these data indicate that β_4 integrin supports the motility of A549 cells.

Figure 3. β_4 integrin knockdown (β_4 KD) inhibits the directed migration of A549 cells. (A) β_4 integrin mRNA levels in wild-type (WT) and scrambled shRNA-expressing (SCR) controls and β_4 KD cells by qPCR (three experiments, three replicates per experiment). (B) Protein extracts were made from control and β_4 KD cells and processed for immunoblotting. Immunoblotting was performed using antibodies against β_4 integrin and laminin A/C (loading control). A representative blot from three experiments is shown. (C) Quantification of results presented in B. (D) Surface expression of α_3 , α_6 , β_1 , and β_4 integrin subunits (three experiments, 10^5 events). (E) Speed and processivity of WT, SCR, β_4 KD, and β_4 KD cells expressing green fluorescent protein (GFP)-tagged β_4 integrin (GFP β_4 ; 3 experiments, >70 cells). (F) Speed and processivity of WT, SCR, and β_4 KD cells on laminin-332-rich matrix (3 experiments, >200 cells). Results are presented as means ($\pm$ SEM). Statistical analyses: *t* test (A and C); ANOVA (E and F). * $P < 0.05$, ** $P < 0.001$. shRNA, short hairpin RNA.

β_1 Integrin Associates with α_6 Integrin in β_4 KD Cells

Remarkably, even in β_4 KDs, α_6 integrin localizes in the same distinct punctate arrays along the substratum-attached surface of the cells as it does in controls (Figure 4A). In control cells, β_1 integrin localizes to a subset of focal adhesions,

primarily at the leading front of single cells (Figure 4B). Surprisingly, β_1 integrin appears to be lost from the focal adhesions of β_4 KDs (Figure 4B). Rather, in β_4 KDs, α_6 and β_1 integrin are found together and group around actin bundles, rather than localize at their endpoints (Figure 4C). We quantified β_1 integrin association with focal

adhesions by comparing the number of β_1 integrin-positive focal adhesions to the total number of focal adhesions in 11 control and 11 knockdown cells, in two separate experiments in which cells were immunostained for β_1 integrin and paxillin 24 hours after plating onto coverslips. β_1 integrin localized to 53% of 750 paxillin-positive focal

Figure 4. β_1 integrin associates with α_6 integrin in β_4 KD cells. (A) Confocal images of a β_4 KD2 cell expressing GFP-tagged α_6 integrin (green) and immunostained for β_1 integrin (red). Right panel, high magnification of the region in the inset. Scale bars, 10 μ m. (B) Confocal images of WT and β_4 KD2 cells immunostained for β_1 integrin (green) and paxillin (red) show that β_1 integrin colocalizes with paxillin in a subset of focal adhesions in WT cells but not in β_4 KD cells. Right panels, high magnification of the regions in the insets. Scale bars, 10 μ m. (C) Confocal images of WT and β_4 KD2 cells immunostained for α_6 integrin (green) and rhodamine-phalloidin to mark F-actin (red). Lower panels, high magnification of the regions in the insets. Scale bars, 10 μ m. (D) SCR and β_4 KD2 cells were infected with adenovirus encoding GFP-tagged α_6 integrin. At 2 days after infection, cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitated with anti-GFP antibodies conjugated to agarose beads. Samples were then processed via SDS-PAGE and probed with antibodies against β_1 and β_4 integrin. IB, immunoblot; IP, immunoprecipitation. (E) Protein extracts were prepared from WT cells infected with adenovirus encoding GFP or α_6 shRNA. After SDS-PAGE and transfer to a nitrocellulose membrane, extracts were immunoblotted with antibodies against the indicated proteins (three experiments). (F) Quantification of speed and processivity of GFP and α_6 shRNA-expressing cells (3 experiments, >130 cells). Results are presented as means ($\pm$ SEM). Statistical analyses: *t* test; ***P* < 0.001.

Figure 5. β_4 integrin expression regulates Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling and actin dynamics. (A) After a 1-hour incubation in vehicle or 10 ng/ml epidermal growth factor (EGF), the motility of SCR and β_4 KD2 cells was assayed. (B) A Rac1 G-LISA activation assay was performed on cells 2 days after plating (two experiments). (C) Actin arc bundle retrograde movement was visualized in cells expressing red fluorescent protein-tagged LifeAct. Kymographs (right panels) were generated along the 5-pixel-wide line (red) shown in the left panels. Scale bar, 10 μ m. (D) Quantification of kymographs, two examples of which are shown in C (3 experiments, >10 cells per experiment). (E) Quantification of cellular aspect ratio (3 experiments, >100 cells). (F) Quantification of the number of cells with exactly one lamellipodium (3 experiments, >100 cells per experiment). (G) A Rac1 G-LISA activation assay was performed on cells 2 days after infection with adenovirus encoding the indicated protein (two experiments). (H) Quantification of the speed and processivity of GFP or dominant-negative Rac1 (DN Rac1)-expressing A549 cells (3 experiments, >25 cells per experiment). (I) Quantification of the speed and processivity of WT, SCR, β_4 KD cells, and both WT and β_4 KD cells expressing constitutively active Rac1 (CA Rac1; 3 experiments, >25 cells per experiment). Results are presented as means ($\pm$ SEM). Statistical analyses: *t* test (H); ANOVA (B, D–F, and I). Because ANOVA indicated differences between groups in A and G, specific groups were compared by *t* test, as indicated. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

adhesions in control cells, whereas β_1 integrin localized with only 7% of 605 focal adhesions in β_4 KD2 cells (Figure 4B).

Focal adhesion organization is also perturbed in knockdown cells. Specifically, focal adhesions tend to be oriented parallel to the cell edge rather than perpendicular to

it (Figure 4B). Finally, we confirmed that α_6 integrin switches β subunit binding partners as a result of β_4 KD by immunoprecipitation, implying that α_6 integrin acts as a sink for β_1 integrin in the absence of β_4 integrin (Figure 4D). Moreover, because there is no increase in

total β_1 integrin cell surface expression, these data indicate that β_4 KD results in a depletion of β_1 integrin from focal adhesions.

Thus, there are two explanations for the defects in motility that we detail in β_4 KD cells. These are: (1) loss of $\alpha_6\beta_4$ integrin

complexes has a direct negative impact on migration; or (2) formation of $\alpha_6\beta_1$ integrin complexes negatively regulates migration. To distinguish between these possibilities, we disrupted $\alpha_6\beta_4$ integrin complexes by knocking down α_6 integrin in control cells (Figure 4E). α_6 integrin knockdown cells exhibit reduced total and cell surface levels of β_4 integrin, but no change in α_3 or β_1 integrin (Figure 4E and data not shown). Moreover, they exhibit a 25% reduction in speed and processivity (Figure 4F). These data support the notion that $\alpha_6\beta_4$ integrin is a positive regulator of processivity.

β_4 Integrin Modulates EGF-Mediated Motility and Rac1 Activity

β_4 integrin has been reported to be necessary for EGF-induced motility via Rac1 (23). Thus, we tested whether this is the case in A549 cells. Specifically, we assayed the motility of serum-starved A549 WT and β_4 KD cells in the presence or absence of EGF (Figure 5A). Though EGF increased the speed and processivity of WT cells by 60%, it increased the speed and processivity of β_4 KD cells by less than 30%. Thus, our results indicate that β_4 integrin is a positive regulator of EGF-mediated motility, consistent with the work of others (23, 24).

A number of studies indicate that EGF regulates Rac1 activity, and thereby determines directed migration by modifying actin dynamics and cell polarity (23, 25–27). We therefore assayed Rac1 activity in control and β_4 KD cells. Rac1 activity was reduced 55% in β_4 KDs compared with controls (Figure 5B). Consistent with this finding, β_4 KDs also exhibited reduced actin dynamics and polarity (Figures 5C–5E). Kymographic analyses of movies generated from control and β_4 KD cells expressing the fluorescently tagged F-actin marker, LifeAct, revealed that the speed of actin arc bundle retrograde movement in β_4 KDs was 25% of the speed of controls (Figures 5C and 5D). Knockdown cells also exhibited reduced aspect ratio (Figure 5E). In addition, whereas roughly 80% of control cells possessed one lamellipodium, only about 50% of knockdown cells did so (Figure 5F). Together, these data indicate that β_4 KDs are less polarized than controls.

The above results suggest that β_4 integrin regulates A549 cell migration via

an impact on Rac1 activation. To test this hypothesis, we first assayed the motility of A549 cells infected with either an adenovirus encoding a dominant-negative or constitutively active Rac1 construct. We confirmed the efficacy of these constructs

using a Rac1 G-LISA kit (Figure 5G). Cells expressing the dominant-negative Rac1 construct exhibited a less than 15% reduction in speed and a 30% reduction in processivity compared with controls (Figure 5H). The processivity of β_4 KDs

Figure 6. Hybrid monolayers. (A) Schematic of procedure to create hybrid cell monolayers. A scrape (indicated by the gray rectangle) was made in a hybrid monolayer composed of β_4 KD and β_4 KD cells expressing GFP-tagged β_4 integrin (GFP β_4) or CA-Rac1. The angle of the scrape allowed us to analyze healing of sheets of knockdown cells in which all follower cells were β_4 KD cells and the leading cells were β_4 KD cells (upper red circle in A) or either knockdown cells expressing GFP β_4 (GFP β_4 / β_4 KD) or CA-Rac1 (CA-Rac1/ β_4 KD) (lower red circle in A). (B) Fluorescence and phase-contrast images of the two different regions in A. Red lines indicate the edges of the original scratch, whereas the yellow lines indicate the wound edge at 12 hours after wounding. In the upper right panel, several GFP β_4 integrin-positive cells are close to the leading edge. Note that the GFP β_4 / β_4 KD cell combination has begun to move over the wound, whereas the KD cells have not. Scale bar, 300 μ m. (C) Quantification of hybrid sheet experiments (3 experiments, >10 scratches). ANOVA indicated differences between groups. Specific groups were compared by *t* test, as indicated. (D) Western blot of β_4 integrin in rat bladder 804G cells and primary rat alveolar epithelial cells (AECs) shows low expression levels in freshly isolated cells. (E) Quantification of the leading-edge displacement of scratched epithelial sheets of rat AECs infected with adenovirus encoding GFP or GFP β_4 (17 scratches, *t* test). Results are presented as means ($\pm$ SEM). ***P* < 0.001.

Figure 7. Diagram of β_4 integrin-mediated motility. In β_4 integrin-expressing cells, $\alpha_6\beta_4$ integrin induces Rac1 activation, which leads to directed migration. In β_4 integrin-deficient cells, α_6 integrin partners with β_1 integrin and does not activate Rac1. Thus, β_4 integrin-deficient cells do not exhibit directed migration. FA β_1 integrin, focal adhesion β_1 integrin.

infected with an adenovirus encoding constitutively active Rac1 was restored to the same level as controls, whereas speed was unaffected (Figure 5I).

β_4 Integrin Regulates Collective Migration

Thus far, our results have focused on single-cell migration. However, *in vivo*, processes, such as development, wound healing, and metastasis, are all products of collective migration (28–31). In particular, these processes tend to be driven by cells situated at the leading front of a cell sheet (leader cells), a phenotype that has been shown to be mediated by Rac1 (22, 30, 32–36). Therefore, we sought to determine the role of β_4 integrin in leader cells during collective cell migration. To do this, we modified the procedure used by Chapnick and Liu (22) to develop scratched “hybrid sheets” of β_4 KD cells in which the leader cells were either β_4 KD2 cells or β_4 KD2 cells forced to express GFP β_4 (Figures 6A

and 6B). Next, we performed time-lapse microscopy for 12 hours and compared scratch closure (Figure 6B). Scratched sheets of β_4 KD2 cells migrated 20% as far as did scratched sheets of control cells (Figure 6C). Moreover, scratched sheets of β_4 KD2 cells in which the leader cells expressed either GFP β_4 or constitutively active Rac1 migrated nearly as much as controls (Figure 6C).

Finally, we also assayed the role of β_4 integrin in the collective migration of primary rat alveolar epithelial cells. These cells expressed little endogenous β_4 integrin (Figure 6D), consistent with previous reports (18). Intriguingly, there is a dramatic and significant increase in their collective migration after the forced expression of GFP β_4 (Figure 6E).

Discussion

The role of β_4 integrin in motility is controversial, with some published studies

reporting that it is a positive regulator and others indicating that it is a negative regulator (6). For example, in migrating keratinocytes, $\alpha_6\beta_4$ integrin assembles into punctate arrays at their leading fronts, which appear to facilitate cell “steering” (7, 8). However, these complexes have also been reported to inhibit motility when associated with keratins (9). Interestingly, there are also conflicting reports concerning the motility functions of β_4 integrin in the A549 cell line (10, 11). One of the objectives of the present study was to address this controversy. To do so, we investigated the function of β_4 integrin in both single and collective cell migration. Our data indicate that β_4 integrin is a positive regulator of motility by determining directed migration. This is consistent with several reports of the role of β_4 integrin in keratinocytes (25). Moreover, our finding that loss of β_4 integrin impacts processivity, whereas loss of α_6 integrin impacts both processivity and speed, implies that α_6 integrin-containing

heterodimers, regardless of the β integrin binding partner, determines speed.

Remarkably, our study also indicates that the expression of β_4 integrin in leader cells is sufficient to induce migration of an entire epithelial cell sheet. The importance of leader cells in determining collective migration in tissue remodeling has been well established (32, 34). Most notably, work by Wang and colleagues (37) has shown that the selective activation of Rac1 in leader cells is sufficient to induce collective migration. Our finding, that expressing either β_4 integrin or constitutively active Rac1 in the leader cells of sheets of β_4 KDs rescues collective migration, is consistent with both this notion and reports by ourselves and others that β_4 integrin regulates Rac1 activity (27, 32, 36, 38, 39).

A key feature of cell migration is cytoskeletal remodeling (40, 41). Rac1 is known to be a regulator of cytoskeletal remodeling (42). Thus, our finding, that Rac1 activity is reduced in β_4 KD cells, suggested that cytoskeleton dynamics are also reduced in these cells compared with control cells. This is indeed the case with regard to actin dynamics. Actin dynamics could also be modulated via association of β_4 integrin with actin. Previous studies have reported that β_4 integrin physically interacts with actin (13). However, in A549 cells we observe no clear colocalization of

actin with $\alpha_6\beta_4$ integrin. Thus, rather than support a direct role of β_4 integrin in cytoskeletal cell surface anchorage, our findings support the hypothesis that β_4 integrin regulates cytoskeletal dynamics and, hence, motility, indirectly via its effect on Rac1 activation.

In a number of cell types, $\alpha_6\beta_1$ integrin has been observed in focal adhesions (43, 44). Thus, we had expected the loss of β_4 integrin in A549 cells to result in either a concomitant loss of α_6 integrin expression or the association of α_6 integrin with β_1 integrin in focal adhesions. We were therefore surprised to find that loss of β_4 integrin induced β_1 integrin to leave focal adhesions and associate with distinct α_6 integrin puncta near the cell edge. This finding implicates α_6 integrin as the mediator of $\alpha_6\beta_4/\alpha_6\beta_1$ integrin localization. Moreover, our data, showing that α_6 integrin knockdown cells exhibit reduced speed and processivity, support our hypothesis that $\alpha_6\beta_4$ integrin is a positive regulator of migration, rather than $\alpha_6\beta_1$ integrin being a negative regulator.

Together, our results lead us to propose the following model (Figure 7). $\alpha_6\beta_4$ integrin induces Rac1 activation. Rac1, in turn, promotes actin dynamics, front–rear polarization, and, as a result, directed migration. In the absence of β_4 integrin, however, β_1 integrin is recruited from focal adhesions to α_6 integrin. In contrast to

$\alpha_6\beta_4$ integrin, $\alpha_6\beta_1$ integrin fails to induce Rac1 activation. Thus, β_4 KD cells fail to migrate in a directed fashion and exhibit significantly reduced collective migration.

Because A549 cells are tumorigenic and collective migration has been reported to enhance tumor cell invasion, our results are directly applicable to cancer biology (30, 45, 46). In addition, the recent identification of β_4 integrin as a marker for rare alveolar progenitor cells and its up-regulation during the repair of the alveolar epithelium, suggests that β_4 integrin also plays a role in alveolar repair (47). Because β_4 integrin does not affect the proliferation of alveolar epithelial cells, we suspect that the function of β_4 integrin in the alveolar epithelium is to enhance tissue regeneration by promoting collective migration, a view that is supported by our results in which we assayed the migration of primary alveolar epithelial cells forced to express β_4 integrin (47). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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