Title
Modulation of squamous carcinoma cell motility by RhoA and Cdc42-PAK1 signaling

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MODULATION OF SQUAMOUS CARCINOMA CELL MOTILITY BY RHOA AND CDC42-PAK1 SIGNALING

by

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DISSERTATION

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ABSTRACT

Squamous cell carcinoma invasion and dissemination require cell migration through the extracellular matrix as well as remodeling of intercellular adhesions. Rho family proteins control important aspects of locomotion that involve signaling interplay between adhesion receptors and the cytoskeleton. Molecular factors that control head and neck squamous carcinoma cell motility are studied in the present research. Using type I collagen, recombinant laminin-10, and laminin-5 as integrin ligands, α3 integrin signaling is identified to promote cell migration while α2 integrin signaling is not.

Laminin-5-α3 integrin signaling inactivates RhoA, activates Rac1, and specifically activates Cdc42-PAK1 pathway. α2 integrin signaling activates RhoA, Rac1, and Cdc42, but does not activate PAK1. Inhibition of RhoA promotes cell migration on collagen I. PAK1 activation is required for haptotaxis. Furthermore, there is inhibitory cross-talk between RhoA and Cdc42.

In coordinated cell movements, where disruption of cell-cell adhesions is involved, high Rho activity inhibits integrin-mediated decompaction of multicellular aggregates. The effect of inhibition of RhoA on decompaction of multucellular aggregates is dependent on the nature of the extracellular matrix. Unexpectedly, high PAK1 activity impairs decompaction of multicellular aggregates and subsequent scattering. Similarly, high PAK1 activity impairs wound healing.

The data demonstrate that Rho GTPases play a crucial role in regulating squamous carcinoma cell motility, and that ligand-integrin pairs regulate cell motility differentially by

modulating activities of Rho GTPases and their effectors. The motile behavior of cells depends on the interplay between cell-cell adhesions and cell-extracellular matrix adhesions, and the interplay between adhesion receptors and Rho GTPases.

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CHAPTER 1

INTRODUCTION AND OVERVIEW

Squamous cell carcinoma (SCC) is the most common type of head and neck cancer. The prognosis for SCC remains poor due to the propensity for local and distant metastasis and frequent recurrence. Primary therapies including surgery and radiation may miss disseminated tumor masses. While early detection is the most critical process for reducing the morbidity and mortality of oral cancer, it is essential that we gain a better understanding of the basic mechanisms of invasion and metastasis. Adhesion receptors and their ligands are important in modulating not only invasion of oral SCC cells but also their survival and proliferation. Normal oral mucosal epithelial cells use integrins to maintain their anchorage to the basement membrane, whereas the formation of stratifying cell layers depends on intercellular adhesions mediated by cadherins. The process of SCC invasion and dissemination requires active cell migration through the extracellular matrix (ECM) with the simultaneous remodeling of intercellular adhesions. Integrins are clearly important in the invasive process, whereas intercellular adhesion receptors restrain invasion and promote a more differentiated phenotype.

Cell-cell interactions

Alterations in cell-cell adhesion contribute to a transformed phenotype. Epithelial cells form extensive intercellular contacts. Cadherins are Ca^{2+}-dependent transmembrane homophilic receptors that form adherens junctions between epithelial cells. Cadherin-

cadherin interactions are stabilized by the associated catenins (α-, β-, or γ-catenin), which link to the actin cytoskeleton. Disruption of adherens junctions leads to loss of cell-cell contacts and allows cell motility. The downregulation of E-cadherin-mediated cellular adhesions has been correlated with increased invasiveness and metastasis of tumors and a poor prognosis for oral cancers (1-3). Loss of E-cadherin is accompanied by a gain of N-cadherin, and increased motility and invasion (4,5).

In epithelial cells, Rho family proteins play a role in the establishment and maintenance of E-cadherin-mediated cell-cell adhesions. Studies done in MDCK cells using dominant-active or dominant-negative mutants of Rho GTPases show that Rho, Rac, and Cdc42 are involved in cell-cell adhesions (6-10). The effect of Rac1 and Cdc42 on cell-cell adhesion may be through their interaction with IQGAP1 (11). IQGAP1 has a negative role in adherens junctions by competing with α-catenin to interact with β-catenin (12). Rac/Cdc42 may enhance cell-cell adhesion by inhibiting association of IQGAP1 with E-cadherin/β-catenin. Despite having a role in establishment of cell-cell adhesion, Rho GTPases are also found to function in cell scattering. Dysfunction of E-cadherin mediated cell-cell adhesion is believed to be essential for cell scattering. Microinjection of N19RhoA or Nl7Racl inhibits HGF-induced membrane ruffling and subsequent cell scattering of MDCK cells (11). This suggests that RhoA, Cdc42, and Rac1 activity may be necessary to some extent for cell scattering. Thus, Rho small GTPases seem to have dual roles in formation and dissolution of cell-cell adhesions.

Cell-ECM interactions

Normal epithelial cells and SCC cells are known to be highly anchorage-dependent. The basement membrane underlying these cells is rich in ECM proteins such as laminins. The major ECM receptors are the heterodimeric integrins. In head and neck SCC, the major integrin receptors found in cells include $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha6\beta1/\alpha6\beta4$, and the αv complexes (13,14). After binding to ECM ligands, integrins are known to transduce signals that not only mediate adhesion and motility but also regulate cell survival and programmed cell death.

Integrin activation is important for tumor invasion. We have found that intercellular adhesions stabilize cadherin levels, favoring a differentiated phenotype. Engagement of $\alpha3\beta1$ integrin disrupts adherens junctions and remodels cell masses (15). However, the role of $\alpha3\beta1$ integrin signaling in dissolution of cell-cell adhesions and promotion of cell scattering is not fully understood.

Laminins and their receptors

Laminins are the most important and abundant non-collagenous protein of the basement membrane. Laminins are composed of an α, β, and γ chain. To date, 15 laminins have been identified (16). The primary laminins expressed in stratified epithelium and derived carcinomas are laminin-5 and laminin-10/11. Laminin-5 is essential for the anchorage of epidermis to dermis. Lack or mutations in three laminin-5 genes, $\alpha3$, $\beta3$, and $\gamma2$, results in Herlitz-type junctional epidermolysis bullosa (16,17). It is well-established that laminin-5 plays a significant role in SCC tumor biology. Early studies indicated that laminin-5 is synthesized and secreted in mucosal keratinocytes and head and neck SCC (18,19). Laminin-5 is overexpressed primarily at the invasive front of

SCC (18,20). Moreover, immunohistochemical staining indicates that laminin-5 deposition outside the basement membrane is a marker for invasion, and that oral SCC invasion is guided by the laminin-5 matrix (18,21,22). The γ2 chain of laminin-5 is highly expressed in recurrent diseases of head and neck SCC and in SCCs of the skin, colon, esophagus, and larynx (23-25). Overall, increased laminin-5 expression may provide a significant marker for the invasive phenotype and indicate a more serious prognosis for patients with SCC. The mechanisms by which laminin-5 promotes invasion are unclear and need extensive studies.

A number of integrins, including α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, and αvβ3 are known to recognize laminins (24,26,27). Cells utilize multiple integrins to recognize laminin isoforms. In addition, there is overlapping ligand specificity among different laminin isoforms. α3β1, α6β1, and α6β4 integrins bind to laminin-5 and laminin-10/11 (16,24,28). It is unclear whether there is a discrepancy in usage of integrins in cell adhesion and migration on laminins.

Although both α3 and α6β4 integrins are overexpressed in SCC of head and neck (25), they play different roles in SCC tumor biology. Studies of α3-null mice revealed that this receptor is required for the establishment and /or maintenance of epidermal/dermal junctions (29). Moreover, α3 integrin expression level is an important candidate biomarkers for lymph node metastasis in oral SCC (30). α3β1 integrin plays a major role in both cell adhesion and migration on laminin-5 (15,19,31-33). The α3β1 integrin pathway also regulates production of matrix metalloproteinase (MMP) -2 and -9 (34,35). The α6 subunit can form a heterodimer with β1 and β4 in SCC cells. The α6 subunit preferentially combines with the β1 subunit in some SCCs. Thus, the other

potential α6β4 receptor may not be active (36). However, expression of α6β4 integrin is increased markedly in SCC but not in basal cell carcinoma (24,37). Suprabasal expression of α6β4 is no longer engaged in hemidesmosome formation, but promotes tumourigenesis by preventing TGFβ from suppressing clonal expansion of initiated cells in the epidermal basal layer (38,39). Both β4 integrin and laminin-5 genes are required for the genesis of invasive carcinoma upon expression of oncogenic Ras/IκBα (40). In invasive carcinomas, α6β4 integrin cooperates with growth factor receptors to activate phosphoinositide 3-kinase, which is required for α6β4-mediated invasiveness (28).

Alterations in MMPs and proteolysis of laminins

In head and neck cancers as well as in other carcinomas, tumor expansion and spread to distant sites require degradation of the ECM. MMPs are endopeptidases involved in degradation of ECM, activation of specific ligands, and shedding of extracellular fragments of membrane receptors (41). MMP-7, -9, and -12 are suggested as bad prognostic markers for oral carcinomas (42). Localization of MMP-2 or MMP-9 in the invasive front correlates with diminished patient survival times (43,44).

Processing of laminin-5 is observed during tissue repair, morphogenesis, and tumor invasion. Different cleavage gives different physiological responses. The α3 chain of laminin-5 cleaved by plasmin favors hemidesmosome assembly and stability (33,45). Cleavage occurs between the globular modules 3 and 4 (LG3 and LG4) of the laminin α3 chain (45,46). The recombinant LG3 module of the α3 chain supports cell adhesion and migration in an integrin α3β1-dependent manner (47). The LG4 module of laminin α3 chain promotes neurite outgrowth, but LG4-mediated cell adhesion requires cell surface

heparan sulfate proteoglycans (48,49). On the contrary, cleavage of γ2 chain by MMP-2 or membrane type-1 MMP promotes cell migration (50,51). In addition, bone morphogenic protein-1 cleaves both the α3 and γ2 chains of laminin-5 and may have relevance for processing of human laminin-5 in *vivo* (52,53).

Regulation of migration by Rho GTPases

Rho GTPases have been recognized as key regulators that mediate the distinct actin cytoskeleton changes required for cell migration (54,55) and may be involved in modulating motility on different substrates such as laminin and collagen. Rho GTPases act in concert to remodel the cytoskeleton. Rac is essential for lamellipodial protrusions and for forward movement. Cdc42 is required to maintain cell polarity and direction of movement. Rho is required to maintain cell adhesion during movement (56,57).

Studies have shown the different requirements for Rac and Rho in cell motility. Increased Rac activity may promote migration and invasion of T lymphocytes, but may inhibit the motility of epithelial cells owing to an increase in the formation of cadherin-based cell-cell adhesions (58-60). Increased Rho activity is associated with a fibroblastoid phenotype that is characterized by disassembly of cadherin-based cell-cell adhesions and acquired invasive capacity (61,62). It is likely that different effects of Rac and Rho on motility depend on cell types.

Both the expression level of Rho GTPases and the activated Rho GTPases display abnormality in SCC. RhoA, Rac2, and Cdc42 are overexpressed in the premalignant and SCC cell lines of human head and neck origin (63). Whether or not increased protein levels of Rho GTPases lead to their increased activities in tumor cells needs further

investigation. For example, the Rac3 activity increased in human breast cancer cell lines and tumor tissue without a change in the Rac3 protein level (64). Thus, identifying the activation status of Rho GTPases may be important for revealing the involvement of these family members in SCC.

By controlling activities of Rho GTPases, regulators of Rho GTPases may function as tumor suppressors. The activation and inactivation of Rho GTPases are regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). For example, T-lymphoma invasion and metastasis-inducing protein (Tiam1) is a specific GEF for Rac. Tiam1 deficiency decreases Rac activity. Tiam1 knockout mice developed fewer skin tumors induced by Ras. Interestingly, these tumors in Tiam1-/- mice grew more slowly than those in wild-type mice. However, most of these small tumors were malignant (65). Thus, Tiam1 plays an active role in tumor initiation and growth but suppresses malignant conversion. Furthermore, p190 RhoGAP decreases Rho activity. Both the GTPase domain and the GAP domain of p190 RhoGAP act as suppressors in Ras-dependent transformation (66). The region of the p190-A RhoGAP gene in chromosome 19 is frequently rearranged or deleted in a number of human tumors (67).

Rho GTPases participate in cell function by interacting with a diverse array of effectors. The effectors of Rho GTPases are unambiguously involved in cancer progression and invasion. For example, IQGAP1 is one of the downstream effectors of Cdc42/Rac (12). IQGAP1 inhibits the intrinsic GTPase activity of Cdc42 (68), thereby significantly increasing cellular levels of active Cdc42 (69). Invasion mediated by constitutively active Cdc42 is attenuated by IQGAP1ΔGRD, a dominant-negative

IQGAP1 (70). IQGAP1ΔGRD substantially reduces active levels of Cdc42, preventing formation of filopodia (69). In addition, p21-activated kinases (PAKs) are identified as effectors of Cdc42/Rac (71). However, they can be activated in a GTPase-independent manner. The N-terminal scaffold domain of PAK contains the hallmark Cdc42/Rac p21-binding domain and several proline-rich SH3-binding motifs that mediate binding to the SH3-SH2 adaptor NCK (72,73) as well as the Cool/PIX family (74,75). In breast cancer cells, a correlation was found between PAK1 activity and invasiveness (76). Similarly, Rho-associated coiled-coil kinase (ROCK) is a downstream effector of RhoA. The RhoA/ROCK pathway plays an important role in invasion and metastasis in bladder cancer, breast cancer, and melanoma (77-79). However, study on human keratinocytes suggests that ROCK plays a critical role in regulating the balance between proliferation and differentiation (80). Inhibition of ROCK results in an increase in proliferation and an inhibition of terminal differentiation in keratinocytes. Recently, Sahai and Marshall (81) identified two modes of tumor cell motility in three-dimensional matrices that involve different uses of Rho signaling. Rho signaling through ROCK promoted a rounded bleb-associated mode of motility that does not require pericellular proteolysis. In contrast, elongated cell motility did not require Rho, ROCK or ezrin function. Overall, the specific functions of Rho effectors in oral SCC deserve further study.

Overview of the thesis

The goal of the present study is to understand the molecular mechanisms that control head and neck SCC cell migration and invasion. Substantial research has established that Rho GTPases are involved in cell motility. However, the involvement of

Rho GTPases in cell motility shows cell-type specificity. Each cell type may show alterations in motile behavior, depending upon the type of ECM cells encountered and whether the cells form cell-cell adhesions.

In previous studies, I have characterized the deposition of laminins by two oral SCC cell lines and identified the types of integrin receptors involved in adhesion and migration to laminins. SCC cells were found to deposit laminin-5 and laminin-10/11 but not laminin-2 (merosin). Laminin-5 was a more potent adhesive laminin. The α2 integrin was involved in adhesion to recombinant laminin-10 but was not involved in migration to recombinant laminin-10 in either cell line.

Using α2 integrin ligand type I collagen and α3 integrin ligand laminin-5, I focused on the contribution of these two integrin pathways to cell migration. I analyzed the activities of RhoA, Cdc42, and Rac1 when cells were seeded on type I collagen and laminin-5. The collagen-α2 integrin pathway activated RhoA, while the laminin-5-α3 integrin pathway inactivated RhoA and activated the Cdc42-PAK1 pathway. Using pharmacological inhibitors and mutations of interested genes, I next determined functions of Rho GTPases and their effectors in cell motility on collagen I and laminin-5. Inactivation of RhoA pathway enhanced cell migration on collagen I. PAK1 activation enhanced haptotaxis as well. Furthermore, there was an inhibitory cross-talk between RhoA and Cdc42.

Finally, I focused on determining functions of RhoA and PAK1 in coordinated cell movement where disruption of cell-cell adhesions was involved. Expression of V14RhoA blocked decompaction of multicellular aggregates completely. Expression of E423 PAK1 suppressed decompaction of multicellular aggregates.

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CHAPTER 2

LAMININ DEPOSITION IN TWO SQUAMOUS CARCINOMA CELL LINES AND IDENTIFICATION OF INTEGRIN RECEPTORS FOR ADHESION AND MIGRATION TO RECOMBINANT LAMININ-10

Introduction

The various extracellular matrix components in the basement membrane such as laminins, nidogens, collagen IV and proteoglycans act as adhesive scaffolds to anchor epithelial cells and are essential for organizing tissue architecture. Laminins are heterotrimeric glycoproteins with α, β, and γ subunits. Laminins function in adhesion, proliferation, differentiation, and survival of cells (1). Currently, 15 laminins have been identified with restricted distribution (2,3). Laminin α5 chain (laminin-10/11) and γ2 chain (laminin-5) are consistently expressed in basement membrane of normal mucosa, oral epithelial dysplasia and oral SCC. Studies have identified the laminin γ2 chain as a marker of invasiveness in SCC and other epithelial malignancies (4-6). However, expression of other laminin chains shows variations between normal and disease condition. For example, in oral SCC, expression of laminin α2 and β2 chains disappeared in 40-65% of patients. In apparent contradiction, Kosmehl et al reported a re-expression of laminin α2 and β2 chains in adult hyperproliferative, dysplastic, and carcinomatous lesions (5).

A number of integrins, including α1β1, α2β1, α3β1, α6β1, α7β1, αvβ3, and α6β4, are known to recognize laminin isoforms (1,7). Many binding sites for cell-surface receptors are located at the C-terminus of the large globular domain (G domain) of the laminin α chain. α3β1, α6β1, α7β1, and α6β4 integrins bind to the G domain (3,7,8). Cells utilize multiple integrins to recognize the same laminin isoforms. For example, α3β1, α6β1, α7β1, αvβ3, and α6β4 integrins all bind to laminin-10 (9-12). In addition, there is overlapping ligand specificity among different laminin isoforms. For instance, α3β1 and α6β1 integrins can recognize laminin-2, -5, -8, -10, and -11 (1,3,13-15).

In the present study, we examined the deposition of laminins by two head and neck SCC cell lines, HSC-3 cells and UM-SCC-10A cells. Using integrin blocking antibodies, we identified the integrin sets for adhesion and migration to recombinant laminin-10 in these two cell lines.

Experimental procedures

Reagents. Laminin-1 was purchased from Sigma-Aldrich (St. Louis, MO). Purified recombinant laminin-10 was provided by Dr. Masayuki (Okayama University, Okayama, Japan).

Antibodies. Monoclonal antibodies (mAbs) to human integrins included VM1 to α2 (prescribed previously (16)), AIIB2 to β1 (provided by Dr. Caroline Damsky, UCSF), GoH3 to α6 (PharMingen, San Diego, CA), and J143 to α3 (from ATCC). GB3 (anti-laminin γ2 chain) was purchased from Serotec, Inc. (Raleigh, NC). Mouse anti-human laminin α5 mAb (clone 4C7), mouse anti-human laminin α2 mAb (Merosin M chain),

and mouse anti-human laminin γ2 mAb (laminin-5, clone P3H9-2) were purchased from Chemicon International, Inc. (Temecula, CA).

Cell culture. Two human SCC cell lines, HSC-3 and UM-SCC-10A, were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum as described (16). HaCat cells, a human immortalized keratinocyte cell line, were maintained in DMEM supplemented with 10% fetal calf serum.

Immunofluorescence staining. Adherent cells in chamber slides (Nalge Nunc International, Naperville, IL) were fixed for 10 min with 4% freshly prepared paraformaldehyde in phosphate-buffered saline (PBS), or fixed for 10 min with 4% freshly prepared paraformaldehyde and then permeabilized for 5 min with 0.5% NP-40. In some cases, cells were removed with 20 mM NH_4OH for 5 min, followed by PBS washing. Nonspecific reactivity was blocked with 1% normal goat serum in PBS for at least 60 min. The cells were then stained with primary antibodies GB3 and 4C7, and counterstained by fluorescein-labeled secondary antibodies. Slides were mounted with Vectashield (Vector, Burlingame, CA) and viewed using a Nikon fluorescence microscope.

Matrix preparation and immunoblotting. HSC-3, SCC-10A, and HaCat cells were allowed to reach confluency on plates. The cells were removed with 20 mM NH_4OH for 5 min, followed by PBS washing according to the method of Gospodarowicz (17). The matrix was collected by a buffer containing 8 M urea, 1% sodium dodecyl sulfate (SDS), and 15% β-mercaptoethanol in 10 mM Tris-HCl, pH 6.8. Cells grown to confluency were collected by lysis buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM $MgCl_2$, and cocktail of protease inhibitors (Cat.

No. 1873580, Roche Molecular Biochemicals, Indianapolis, IN). 30 μg of cell extracts and matrix extracts were processed for SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) on a 6% or 7.5% gel. After transfer to nitrocellulose membranes (Millipore Corp., Bedford, MA), proteins were probed with primary antibodies and secondary horseradish peroxidase-coupled antibody. Blots were developed by chemiluminescence using the ECL system (Amersham Biosciences Corp., Piscataway, NJ).

Immunoprecipitation of laminin α5 chain from culture medium. Cells were metabolically labeled with [^{35}S] methionine (11 μCi/ml, Amersham) in minimal essential medium (without methionine) for 20 h. The conditioned media were then collected, and protease inhibitors were added. The media were pre-cleared with protein G-Sepharose beads, and then incubated with mouse IgG or 4C7 (anti-laminin α5 chain) for 1 h at 4°C, followed by incubation with protein G at 4°C for 1 h. The beads were collected and washed. The bound protein was eluted by boiling in Laemmli buffer. Samples were processed for SDS-PAGE and fluorography.

Quantification of secreted laminin-10/11 in insoluble matrix. HSC-3 and SCC-10A cells (2×10^4 cells/well) were incubated in a 96-well plate. The culture medium was changed every day. After 48 h, the cells were removed with 20mM NH_4OH for 5 min. The plate was washed with H_2O and PBS. After blocking with 3% BSA, the laminin-10/11 secreted into the matrix was estimated by an enzyme-linked immunosorbant assay (ELISA) using 4C7 mAb (1:1000) for 60 min. Detection was performed with a rabbit anti-mouse secondary antibody conjugated with horseradish peroxidase and 2,2'-azino-di (3-ethyl-benzothiazoline-6 sulfonic acid) (Zymed Laboratories, South San Francisco,

CA) as a substrate. The optimal density was measured at 405 nm. The data are expressed as the mean of triplicate wells ± SD.

Adhesion assay. Cell adhesion was measured by a microcolorimetric assay (18). Briefly, 96-well flat-bottom microtiter plates were coated with ligands and incubated at room temperature for 1 h. The cells were pre-incubated with or without blocking mAbs to integrin receptors for 30 min at room temperature. Cells were allowed to attach at 2×10^4 cells per well for 15 min at 37°C followed by fixation with 1% formaldehyde and staining with 2% crystal violet blue. Finally, the optical density was measured at 562 nm. The data are expressed as the mean of triplicate wells ± SD using the total value of input cells as 100%.

Migration assay. For a transwell migration assay, the underside of the transwell (8-μm pore size; Costar) was precoated with recombinant laminin-10 (5 μg/ml). Detached cells were pre-incubated with mouse IgG or blocking mAbs to integrin receptors for 30 min at room temperature. 2×10^5 cells were loaded onto the upper chamber, and the lower chamber was filled with serum-free medium. Cells were incubated for 3 h at 37°C. Cells on both sides were fixed and stained with crystal violet. Cells retained on the upper side were wiped off with Q-tips. Cells migrating through the filter were counted and averaged from ten randomly chosen high-power fields (a 20X objective). Migration was 100% for cells incubated in control IgG. Each column represents the mean ± SD of triplicate assays.

Results

HSC-3 and SCC-10A cells deposit laminin-5 (α3β3γ2) and laminin-10 (α5β1γ1) /11 (α5β2γ1)

We first investigated the deposition of specific laminins by HSC-3 cells. HSC-3 cells produced strong positive staining for laminin γ2 chain and laminin α5 chain inside and outside of cells (Fig. 1). Deposition of γ2 chain of laminin-5 in the matrix was further confirmed by Western blotting. The γ2 chain of laminin-5 was processed in the matrix of HSC-3 and SCC-10A cells, generating cleaved products with molecular weight 80kDa and 105kDa (Fig. 2 A). Deposition of the laminin α5 chain in the matrix by HSC-3 and SCC-10A was further confirmed by ELISA (Fig. 2 B). In another approach, both cell lines were metabolically labeled with ^{35}S-methionine, and the laminin α5 chain was immunoprecipitated from the conditioned medium (Fig. 2 C). In addition to the bands corresponding to 380 kDa α5 and 200 kDa β1, γ1, or β2, we also found a thick band with a lower molecular weight which might be the processed laminin α5 chain. HSC-3 and SCC-10A cells expressed α2 chain-containing merosin inside the cells, however, the matrix deposited by those cell lines did not contain merosin (Fig. 2 D). In contrast, the matrix deposited by a non-transformed HaCat cells contained merosin.

Adhesion of HSC-3 cells to laminins

Substrate concentration-dependent adhesion analysis showed that HSC-3 cells adhered well to laminin-5 with a maximum adhesion at 5 μg/ml. Cells adhered well to recombinant laminin-10 with a maximum adhesion at 10 μg/ml. Cells rarely adhered to laminin-1 even at a high coating concentration (Fig. 3).

Antibody perturbation experiment of HSC-3 cells to recombinant laminin-10

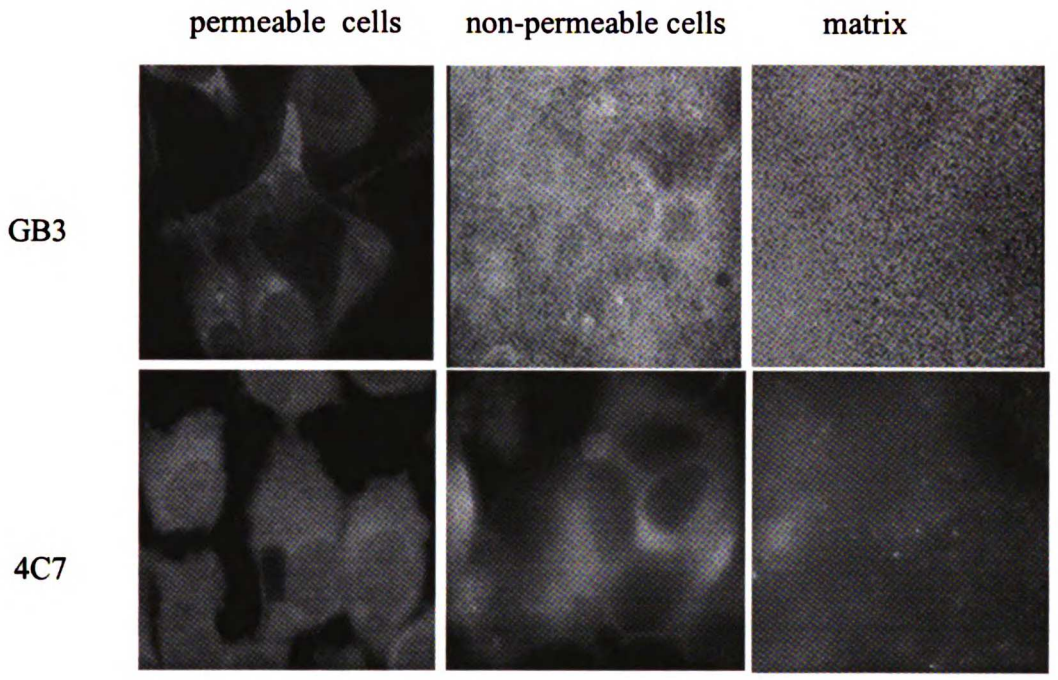
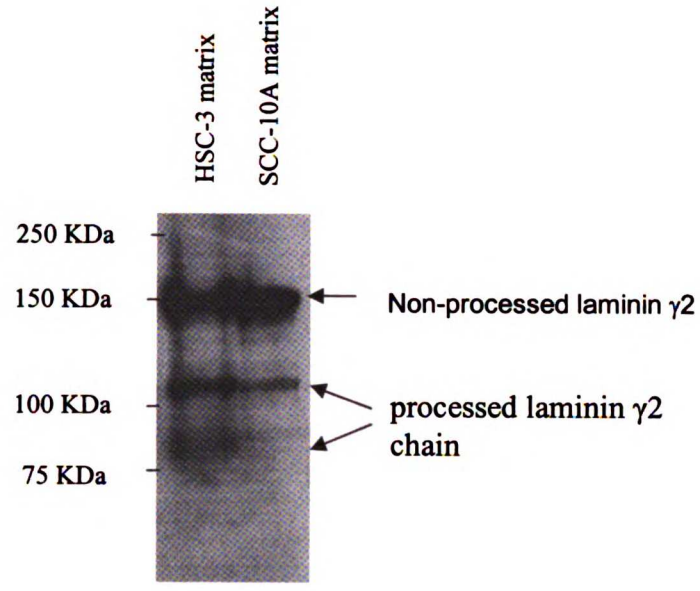
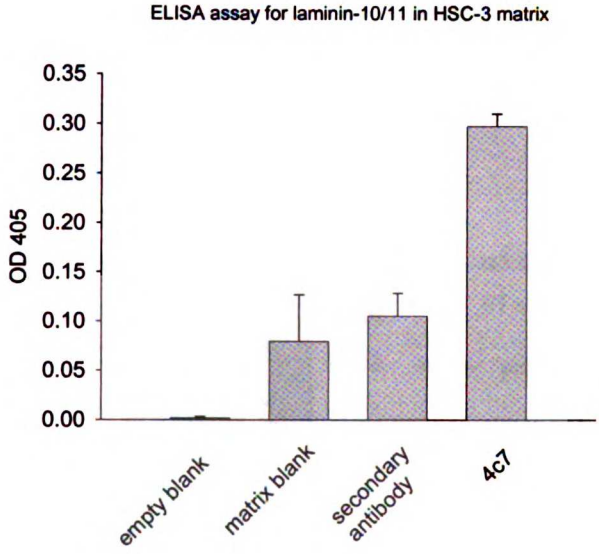


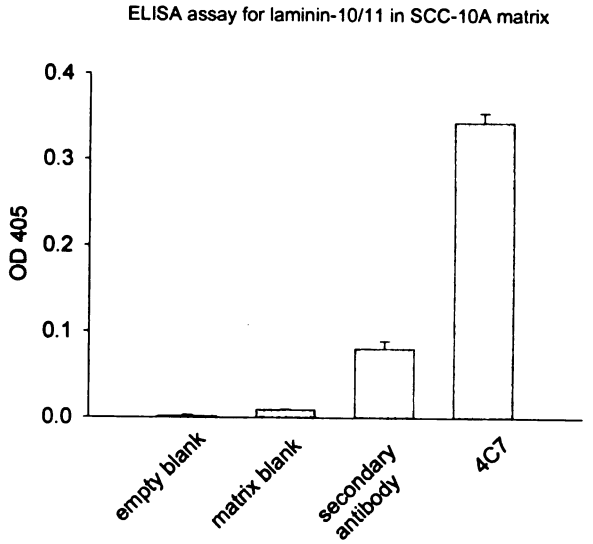
Figure 1. staining of laminin subunits. Cells grown in chamber slides for several days were fixed in the presence/absence of 0.5% NP-40. Or cells were removed with 20 mM NH_4OH. The slides were then stained for laminin γ2 chain with GB3 antibody, or stained for laminin α5 chain with 4C7 antibody.

A

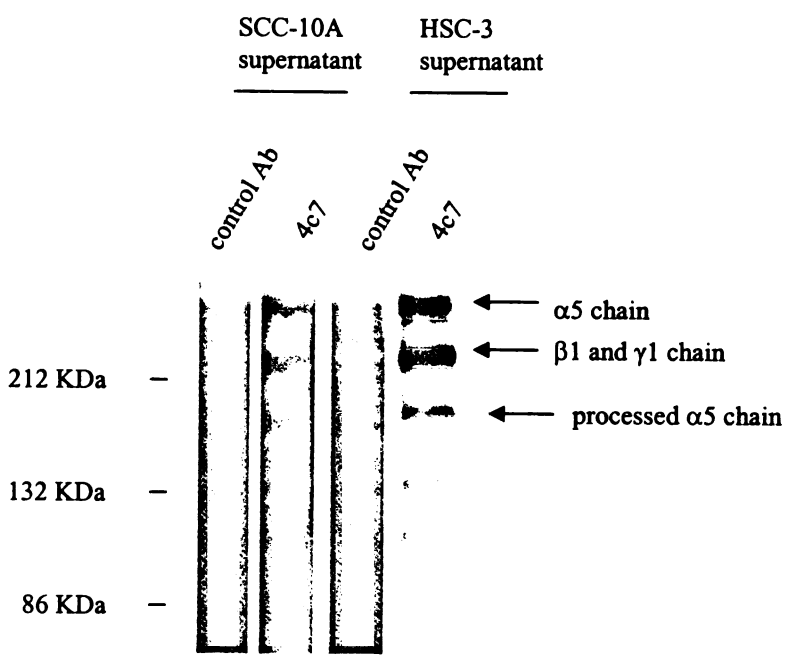

HSC-3 matrix
SCC-10A matrix
250 KDa
150 KDa
100 KDa
75 KDa
Non-processed laminin γ2
processed laminin γ2 chain

B


ELISA assay for laminin-10/11 in HSC-3 matrix
OD 405
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
empty blank
matrix blank
secondary antibody
4C7


ELISA assay for laminin-10/11 in SCC-10A matrix
OD 405
0.4
0.3
0.2
0.1
0.0
empty blank
matrix blank
secondary antibody
4C7

C


SCC-10A supernatant
HSC-3 supernatant
control Ab
4c7
control Ab
4c7
α5 chain
β1 and γ1 chain
processed α5 chain
212 KDa
132 KDa
86 KDa

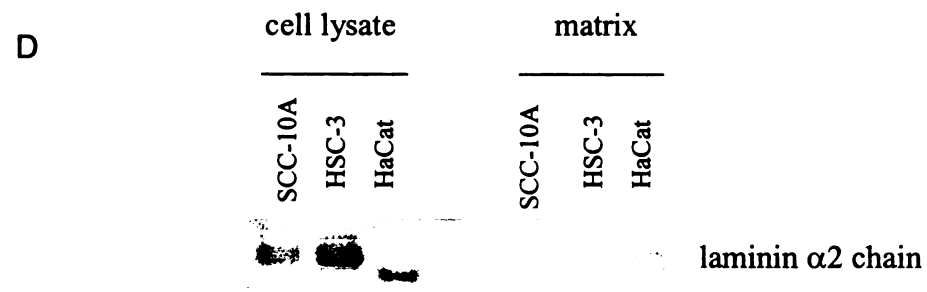


Figure 2. Analysis of laminin deposition in the matrix. (A) HSC-3 and SCC-10A cells grown on plates for several days were removed. Matrix lysates were collected and subjected to immunoblotting with anti-human laminin γ2 antibody. Arrows point to laminin γ2 chain. (B) HSC-3 and SCC-10A cells grown on a 96-well plate for two days were removed. ELISA assays using 4C7 antibody to recognize laminin α5 chain were performed as described in Experimental procedures. (C) HSC-3 cells and SCC-10A cells were pulse-labeled with ^{35}S-methionine. The condition media from HSC-3 cell culture and SCC-10A cell culture were collected and incubated with mouse control IgG and 4C7 antibody. Following incubation with protein G-Sepharose beads, the immunocomplexes were collected, washed, eluted with Laemmli buffer, and subjected to SDS-PAGE and fluorography. (D) Cell lysates and matrix lysates from HaCat cells, SCC-10A cells, and HSC-3 cells were subjected to immunoblotting with anti-human laminin α2 chain mAb in a reduced form.

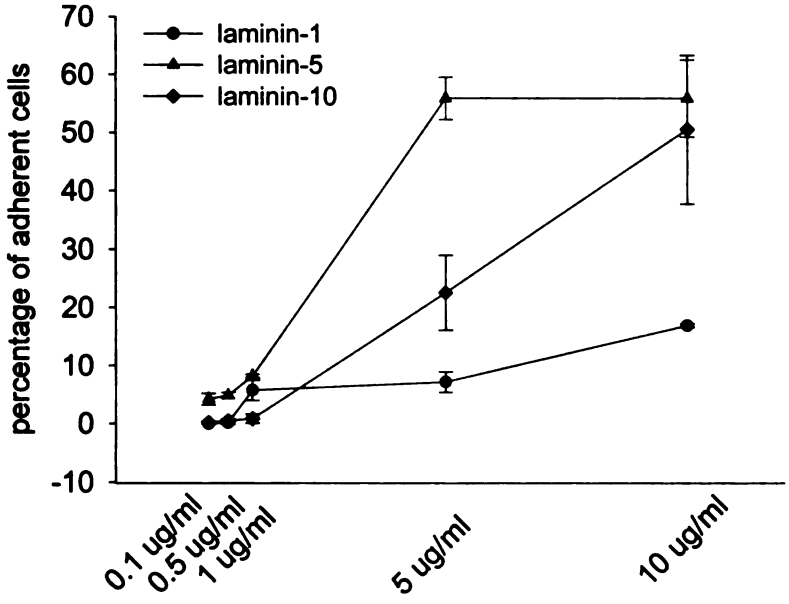


Figure 3. Adhesion of HSC-3 cells to different laminin isoforms. A 96-well plate was coated with different concentrations of laminin-1, -5, or -10 for 1 h. HSC-3 cells were seeded onto the 96-well plate for 15 min. Cells were then fixed and stained with 2% crystal violet. The absorbance was measured at 562 nm. The values were calculated as described in Experimental procedures.

We tested whether or not antibodies against known laminin-10/11 integrin receptors perturb HSC-3 cells adhesion and migration to recombinant laminin-10. Adhesion to recombinant laminin-10 was drastically inhibited by the anti-β1 integrin antibody, moderately inhibited by anti-α3 antibody or antibodies to α2 and α6 integrins (Fig. 4 A). Antibodies to α2, α3, and α6 integrins had the same effect as the anti-β1 integrin antibody alone. In transwell migration assays, migration was almost completely inhibited by anti-β1 integrin antibody and moderately inhibited by anti-α3 or anti-α6 integrin antibody. Anti-α2 integrin antibody had no effect on migration (Fig. 4 B).

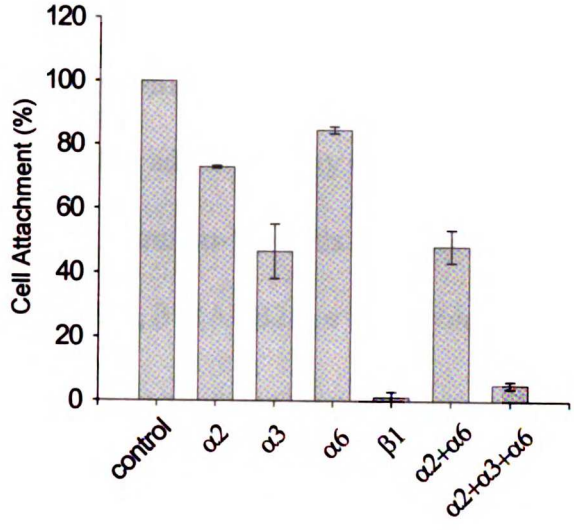
Antibody perturbation experiment of SCC-10A cells to recombinant laminin-10

We tested whether or not antibodies against known laminin-10/11 integrin receptors perturb SCC-10A cells adhesion and migration to recombinant laminin-10. Adhesion to recombinant laminin-10 was drastically inhibited by the anti-β1 integrin antibody, and moderately inhibited by antibodies to α2 and α3 integrins. Antibody to α6 integrin had no effect on adhesion (Fig. 5 A). The data suggest that other integrins except α2, α3, and α6 or non-integrin receptors are possibly involved in the recognition as well. In transwell migration assays, migration was almost completely inhibited by anti-α3 or anti-β1 integrin antibody. Antibodies to α2 or α6 integrin had no effect on migration (Fig. 5 B).

Discussion

Integrin-binding specificity of laminins differs according to cell type, due to distinct repertories of integrins. For example, although HSC-3 cells and SCC-10A cells possess similar integrin profiles, they utilize different combinations of integrins to

A


Cell Attachment (%)
120
100
80
60
40
20
0
control
α2
α3
α6
β1
α2+α6
α2+α3+α6

B

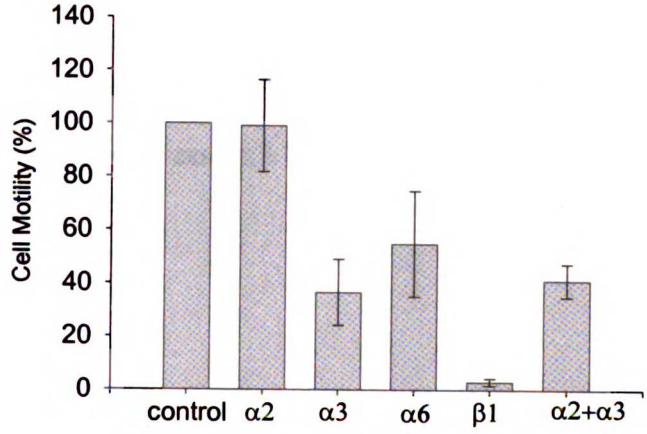
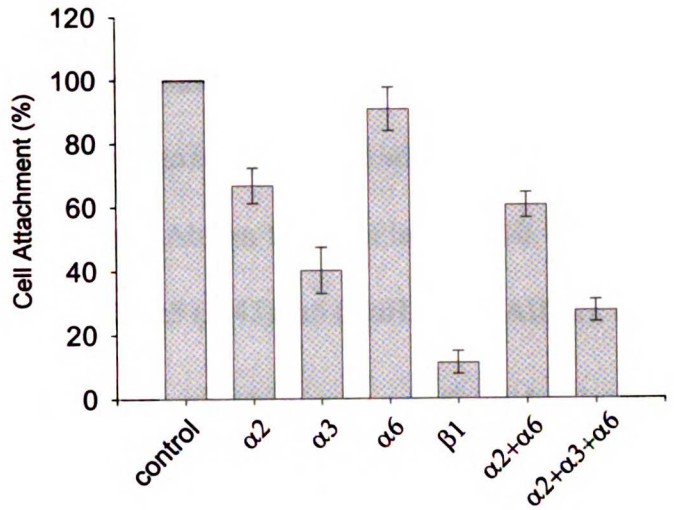

Cell Motility (%)
140
120
100
80
60
40
20
0
control
α2
α3
α6
β1
α2+α3

Figure 4. Effects of anti-integrin antibodies on the adhesion and migration of HSC-3 cells to recombinant laminin-10. (A) Inhibition of adhesion by function-perturbing mAbs to integrins. The adhesion assay was performed in the presence of anti-integrin mAbs on 10 μg/ml laminin-10. Cells were pre-incubated with mAbs to α2 (VM1), α3 (J143), α6 (GoH3), β1 (AIIB2), or in combinations. The concentration of each antibody was 10 μg/ml. Each column represents the mean of triplicate assays ± SD. (B) The transwell inserts were precoated with 5 μg/ml laminin-10. Transwell migration assays were performed in the presence of control IgG, anti-α2 (VM1), anti-α3 (J143), anti-α6 (GoH3), anti-β1 (AIIB2), or in combinations. The concentration of each antibody was 10 μg/ml. Each column represents the mean of triplicate assays ± SD.

A


Cell Attachment (%)
120
100
80
60
40
20
0
control
α2
α3
α6
β1
α2+α6
α2+α3+α6

B

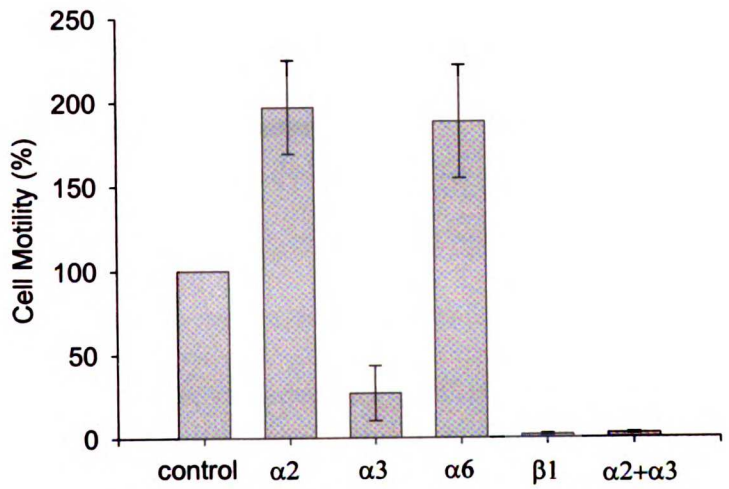

Cell Motility (%)
250
200
150
100
50
0
control
α2
α3
α6
β1
α2+α3

Figure 5. Effects of anti-integrin antibodies on the adhesion and migration of SCC-10Acells to recombinant laminin-10. (A) Inhibition of adhesion by function-perturbing mAbs to integrins. The adhesion assay was performed in the presence of anti-integrin mAbs on 10 μg/ml laminin-10. Cells were pre-incubated with mAbs to α2 (VM1), α3 (J143), α6 (GoH3), β1 (AIIB2), or in combinations. The concentration of each antibody was 10 μg/ml. Each column represents the mean of triplicate assays ± SD. (B) The transwell inserts were precoated with 5 μg/ml laminin-10. Transwell migration assays were performed in the presence of control IgG, anti-α2 (VM1), anti-α3 (J143), anti-α6 (GoH3), anti-β1 (AIIB2), or in combinations. The concentration of each antibody was 10 μg/ml. Each column represents the mean of triplicate assays ± SD.

recognize recombinant laminin-10. Furthermore, cells utilize different combination of integrins to adhere and migrate to recombinant laminin-10.

The laminin α5 chain is evolutionarily most related to the α3 chain (19). Thus, similarity shared in amino acid levels determines that α5 chain-containing laminins (laminin-10/11) and α3 chain-containing laminins (laminin-5/6) share similar integrin receptors. Laminin-10/11 have been shown to be recognized by α3β1 (9,10,20), α6β1 (11,12), α6β4 (10), and αVβ3 integrins (21). In head and neck SCC cells, α2, α3, and α6 integrins bind recombinant laminin-10. However, in SCC-10A cells, other integrins beside α2, α3, and α6 integrins or non-integrin molecules might recognize recombinant laminin-10 as well. The specific binding sites on the laminin α5 chain have not been mapped yet. Site-directed mutagenesis study showed that cell binding to domain VI of the laminin α5 chain required α3β1 and heparin/heparin sulfate (22).

Although laminin-10/11 and laminin-5 share similarity in adhesion receptors, they are not equivalently important biologically. First, the expression of laminin-5 and laminin-10/11 are different. A decrease in the level of the laminin α5 chain (laminin-10/11) in the basement membrane underlying adult epidermis was observed compared with neonatal skin. However, the level of laminin-5 remained high irrespective of the age (23). The expression of the laminin γ2 chain was also higher than that of the laminin α5 chain in the basement membrane of head and neck SCCs. Second, laminin-5 can't compensate for the lack of laminin-10/11. Laminin-10/11 are important components in embryonic basement membranes. Mice lacking the laminin α5 chain were embryonically lethal (24). However, laminin-10/11 may provide some degree of compensation for the absence of laminin-5. Laminin α3 chain knockout mice were viable but showed

epidermal blistering (25). Third, signaling pathways initiated by laminin-5 and laminin-10/11 may be different. Laminin-5 provided much more potent adhesion than laminin-10/11 (Fig. 3).

The laminin α chain undergoes proteolysis. G4-5 modules of the α3 chain are cleaved off after secretion of laminin-5, and the remaining G3 module plays an essential role in the potent promotion of cell adhesion and motility (26,27). G4-5 modules of the laminin α3 chain mediate deposition of precursor laminin-5 (unpublished data from Carter). G4-5 modules of the laminin α2 chain are required for basement membrane assembly (28,29). It is likely that cleavage of the laminin α5 chain observed in the SCC conditioned medium is related to the basement membrane assembly.

Interestingly, integrins that recognize laminins are not necessarily involved in migration on laminins. The α2 integrin was involved in adhesion to recombinant laminin-10 for both HSC-3 and SCC-10A cells. But the anti-α2 integrin antibody had no effect on migration in either cell lines (Figs. 4 and 5). Why is the α2 integrin utilized for adhesion but not for migration? Adhesion and migration are different biological events, although they are closely related. During migration, focal adhesions are highly turned over. Strong adhesions could limit cell migration. Therefore, it is possible that certain integrins provide strong adhesion, and are not involved in migration.

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CHAPTER 3

THE ROLE OF RHO GTPASES IN INTEGRIN-MEDIATED CELL MOTILITY

Introduction

Cell migration is essential for a number of biological and pathological processes including normal development, angiogenesis, wound repair, and tumor invasion and metastasis. The process of cell spreading and migration on ECM involves integrin receptors and dynamic changes in the cytoskeleton. Migration is viewed as a multistep cycle including generation of protrusions at the leading edge, adhesion formation near the leading edge of the protrusion, movement of the cell body, release of adhesions and retraction at the cell rear (1). The interplay between integrins and the cytoskeleton is regulated by specific signaling pathways that are not completely understood.

Important regulators of the cytoskeletal organization, the Rho family GTPases particularly Rho, Rac, and Cdc42, modulate many facets of cytoskeletal dynamics that underline changes during migration (2,3) (4-6). For most cell types, Rac seems to be essential for the protrusion of lamellipodia at the leading edge and for forward cell movement. RhoA is required to maintain substrate adhesion during cell movement and to generate contractile force as the cell is pushed forward. Cdc42 is required to maintain cell polarity, which induces the localization of lamellipodial activity at the leading edge and the reorientation of the microtubule-organizing center and Golgi apparatus toward the direction of the movement.

The importance of individual Rho GTPases in migration may depend on cell types. Most studies on the role of Rho proteins in migration have been done using fibroblasts (7,8). Studies show that increased Rac activity promotes migration and invasion of T lymphocytes (9,10) and lung adenocarcinoma cells (11), but inhibits motility of epithelial cells due to an increase in the formation of cadherin-based cell-cell adhesions (12). The positive role of RhoA in motility has been found in specific cell types including colon carcinoma cells, hepatoma cells, and lymphoma cells (10,13,14). In contrast, high RhoA activity also inhibits movement in fibroblasts and lung adenocarcinoma cells (11,15). Thus, it is likely that the balance among the activities of RhoA, Rac, and Cdc42 determines whether or not a cell will migrate or become immobilized.

Rho GTPases and integrins are intimately connected. ECM-integrin interaction regulates Rho GTPases. The type of ECM also appears to have dramatic effects on a cell's migratory response. For MDCK epithelial cells, Rac activation enhanced migration on collagen but suppressed migration on laminin 1 or fibronectin substrates (16). During the initial phase of spreading on fibronectin, RhoA activity is reduced through activation of p190RhoGAP as a result of Src and FAK signaling in fibroblasts (15,17,18). Subsequently, RhoA activity increases markedly (17). Rac1 and Cdc42 activities are high during spreading and membrane protrusion (19,20). Other ECM proteins, such as laminin-10/11 (11) and laminin-8 (21), activate Rac1 to promote cell migration. However, $\alpha 4\beta 1$ integrin/ligand interaction downregulated RhoA activity and induced melanoma cell migration (22). It is well established that ECM proteins can induce cell spreading and motility through integrin-dependent regulation of Rho family members.

Previously, we have shown that for SCC cells, laminin-5 ligand promoted rapid cell scattering whereas fibronectin and collagen I did not (23). In the present study, we analyze the integrin-mediated regulation of Rho GTPases and their downstream effectors resulting from adhesion to laminin-5 and type I collagen substrates in SCC cells. On laminin-5 substrate, $\alpha 3\beta 1$ integrin preferentially inactivates RhoA and induces activation of Cdc42 and PAK1, thereby promoting migration of oral SCC cells. In contrast, on type I collagen, $\alpha 2\beta 1$ integrin strongly activates RhoA, leading to enhanced focal contact formation, thereby hindering cell migration. These results suggest that aberrant Rho signaling in SCC may be important in defined cell phenotype.

Experimental procedures

Reagents and antibodies. Y-27632 and C3 transferase were purchased from Calbiochem (San Diego, CA); myelin basic protein (MBP), from Sigma-Aldrich (St. Louis, MO); type -I collagen, from Cohesion Technologies (Palo Alto, CA). mAbs against Rac1, Cdc42, or paxillin (clone 165) were purchased from BD Transduction Laboratories (Lexington, KY). Anti-RhoA mAb and anti-αPAK (N-20) polyclonal antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-hemagglutinin (HA) mAb was purchased from Covance (Richmond, CA). Anti-laminin 5 blocking antibody (clone P3H9-2) and anti-vinculin mAb were obtained from Chemicon International, Inc.(Temecula, CA); anti-phospho-PAK1 (Thr423) polyclonal antibody and anti-Myc mAb, purchased from Cell Signaling Technology Inc. (Beverly, MA); VC5 (mouse anti-$\alpha 5$ integrin) and GoH3 (rat anti-$\alpha 6$ integrin), purchased from Pharmingen (San Diego, CA). J143 (anti-human $\alpha 3$ integrin) was obtained from ATCC. VM1 and VM2, anti-$\alpha 2$

and α3 subunits respectively, were described previously (Zhang and Kramer, 1996). AIIB2 (rat anti-human β1 integrin) was provided by Dr. Caroline Damsky (UCSF). Anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), conjugated to HRP for immunoblotting, or conjugated to fluorescein isothiocyanate (FITC) for immunofluorescence microscopy. Rhodamine-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR).

Cell culture. The human squamous cell carcinoma cell lines, HSC-3 and UM-SCC-10A cells, were maintained as previously described (23) in DMEM supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells used for experiments were cultured without serum overnight.

Adenoviral infection. Adenovirus encoding constitutively active RhoA (V14RhoA) or dominant-negative RhoA (N19RhoA) was provided by A. Hassid (University of Tennessee, Memphis). Adenovirus encoding GFP or dominant-negative Cdc42 (N17Cdc42) was provided by G. E. Davis (Texas A & M University System, College Station). Adenovirus encoding wild type (wt) PAK1 or constitutively active PAK1 (E423 PAK1) was provided by W. T. Gerthoffer (University of Nevada, Reno). HSC-3 cells were infected with adenovirus at a multiplicity of infection of 500 in 1 ml culture medium for 6-well plates or in 4 ml culture medium for 10-cm plates. After 2 h, 2 ml or 6 ml of DMEM was added. After 24 h of culture, cells were used for experiments.

Inhibitor treatments. For treatment with the Rho inhibitor C3 transferase, cells were pre-incubated with 5 μg/ml C3 transferase overnight. For treatment with the ROCK

inhibitor Y-27632, cells were pre-incubated with 25 μM Y-27632 for 30 min prior to and during the experiments.

Laminin-5 matrix preparation. Purified laminin-5 was used as previously described for adhesion and motility assays (24). Laminin-5 substrate was derived from HSC-3 cells or SCC-10A cells as previously described (23). In brief, cells were grown to confluency on plates. Dishes were washed with PBS and cells were removed by treating with 20 mM NH_4OH for 5 min according to the method previously described (25,26). The matrix was then extensively washed with PBS prior to use.

Cell spreading measurements. Suspended cells were seeded onto plates coated with 10 μg/ml collagen I or laminin-5 substrate for the indicated time at 37 °C in serum-free medium. Cells were fixed and stained with solution containing 2% Brilliant Blue (Sigma-Aldrich), 45% methanol, and 10% acetic acid for 10 min as described (15). The relative areas in pixels of individual cells (more than 20) were generated using Metamorph and NIH image software. The average of the relative areas of cells plated on laminin-5 substrate for 30 min was chosen as the maximal cell area. The ratio of the cell area at the indicated time point to the maximal cell area was then determined.

Immunofluorescence staining. Cells were seeded onto chamber slides (Nalge Nunc International, Naperville, IL) coated with 10 μg/ml collagen I or laminin-5 substrate and incubated at 37°C for 1 h. Following fixation with 4% paraformaldehyde for 10 min and permeabilization with 0.5% NP-40 in PBS for 5 min, cells were then incubated with primary antibodies (anti-paxillin mAb or anti-vinculin mAb) for 1 h and followed by goat anti-mouse FITC-conjugated secondary antibodies. Rhodamine-cojugated phalloidin was used to co-stain polymerized actin filaments. Slides were mounted with Vectashield

(Vector, Burlingame, CA) and viewed using a Nikon fluorescence microscope or a Bio-Rad Laboratories laser scanning confocal microscope (model MRC-1024).

Immunoblotting. Cells were solubilized in lysis buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM $MgCl_2$, complete™ protease inhibitor cocktail (Cat. No. 1873580, Roche Molecular Biochemicals, Indianapolis, IN) and processed for SDS-PAGE after adjusting for equal protein loading estimated by the BCA protein assay kit (Pierce, Rockford, IL). After transfer to nitrocellulose membranes (Millipore Corp., Bedford, MA), proteins were probed with primary antibodies and secondary horseradish peroxidase-coupled antibody. Blots were developed by chemiluminescence using the ECL system (Amersham Biosciences Corp., Piscataway, NJ). The band intensities were measured by densitometry using NIH Image software (Scion).

Adhesion assay. Cell adhesion was measured as previously described (27). Briefly, 96-well plates were coated with collagen (10 μg/ml) and laminin-5 (2.5 μg/ml) at 37 °C for 1 h, followed by blocking with 0.1% BSA. HSC-3 cells were incubated with or without blocking mAbs to integrin subunits for 30 min at 4°C, and 2×10^4 cells were added to each well and allowed to attach for 20 min at 37°C. Adherent cells were then quantified by a microcolorimetric assay.

Migration assays. Time-lapse video microscopy was performed as described with a modification (28). Briefly, cells were seeded onto 6-well plates (Falcon, Becton Dickinson Labware) coated with different substrates (10 μg/ml collagen I, laminin-5, or 0.1mg/ml poly-L-lysine (PLL)) for 30 min. Plates were then examined in a Zeiss Axiovert inverted microscope with an X-Y scanning motorized stage (Carl Zeiss

MicroImaging, Inc., Thornwood, NY) and maintained on the stage at 37°C and 5% CO_2. Images were collected at indicated time intervals using a SPOT-RT CCD camera (Molecular Dynamics) and analyzed with Openlab software system (Improvision Inc., Lexington, MA). The positions of individual nuclei were tracked to determine the relative migration rates.

For a transwell migration assay, the underside of the transwell (8-μm pore size; Corning Costar Corp., Cambridge, MA) was precoated with collagen (10 μg/ml) and laminin-5 (1.25 μg/ml or 0.5 μg/ml). Next, 2×10^5 cells were loaded onto the upper chamber of the transwell and the lower chamber was filled with serum-free medium. Cells were incubated for 3 h at 37°C in the serum-free medium. Cells on both sides were fixed and stained with crystal violet. Cells retained on the upper side were wiped off with Q-tips. Cells migrating through the filter were counted and averaged from ten randomly chosen high-power fields (a 20X objective).

Invasion assay. For invasion of type I collagen gels to the upper chamber of each transwell, 30 μl of the DMEM containing 2.4 mg/ml type I collagen was added and allowed to gel at 37°C for 1 h. Next, cells (2×10^5) in 200 μl of serum-free DMEM were added to the upper chamber. Serum-free medium was added to the lower chamber. After 24 h incubation at 37°C, cells on both sides were fixed and stained with crystal violet. Cells retained on the upper side and collagen gel were wiped off with Q-tips. Cells that had penetrated the collagen I gel and reached the underside of the filter membrane were counted in 10 randomly chosen microscopic fields (a 20X objective). For each experimental condition, three invasion chambers were used. The mean and SD were determined.

RhoA, Rac1, and Cdc42 activity assays. Construct expressing the Rho binding domain of ROCK fused to GST was provided by M. A. Woodrow (University of California, San Francisco). Construct expressing PAK 75-132 fused to GST was provided by P.N. Lowe (Medicines Research center, GlaxoSmithKline, U.K.). Proteins were expressed in *E. coli* BL21 and purified as described (17).

Cells were plated on collagen I (10 μg/ml), laminin-5, or 0.1 mg/ml PLL for indicated time, and were lysed in 500 μl of 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM $MgCl_2$, 1 mM sodium vanadate, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, and 2.5 μg/ml leupeptin. The lysates were clarified by centrifugation. For estimating RhoA activity, 40 μg of cell lysates were used to measure total RhoA and 1.5 mg of cell lysates were mixed with 30 μg of GST-ROCK and 90 μl of glutathione-agrose beads (Sigma-Aldrich) for 60 min at 4°C. For estimating activity of Rac1 or Cdc42, 40 μg of cell lysates were used to measure total Rac1 or Cdc42 and 1.5 mg of cell lysates were mixed with 30 μg of GST-PAK and 90 μl of glutathione-agrose beads for 60 min at 4°C. Beads were washed, and bound protein was eluted by boiling in Laemmli buffer. Samples were separated on 12% SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with anti-RhoA mAb, anti-Rac1 mAb, or anti-Cdc42 mAb respectively.

Protein kinase assay. Serum-starved HSC-3 cells or wt PAK1-transduced cells were plated on 10 μg/ml collagen I or laminin-5 substrate for 30 min. Cell lysates were collected using lysis buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.6% Triton X-100, 20 mM β-glycerophosphate, 10% glycerol, 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin) as described by Royal

(29). Cell lysates (800 μg) were immunoprecipitated with anti-αPAK1 (N-20) antibody for 1 h followed by incubation with 40 μl of protein G-Sepharose (40% suspension) for a second 1 h incubation at 4°C. The immune complex was washed twice with the lysis buffer and once with the kinase buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM DTT, 5 mM sodium fluoride, 0.1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin). The immunoprecipitated PAK1 activity was assayed using MBP as a substrate. The kinase reaction was performed for 30 min at 30°C in 50 μl of kinase buffer containing 5 μg of MBP, 100 μM cold ATP, and 6 μCi of [γ-^{32}P] ATP. The reaction was terminated by adding 6 X Laemmli sample buffer followed by boiling for 5 min. Proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel. Dried gels were exposed to X-ray sensitive film and intensifying screen at -80°C. The band intensity was measured by densitometry. PAK1 expression levels were evaluated by Western blotting with the anti-αPAK1 (N-20) antibody.

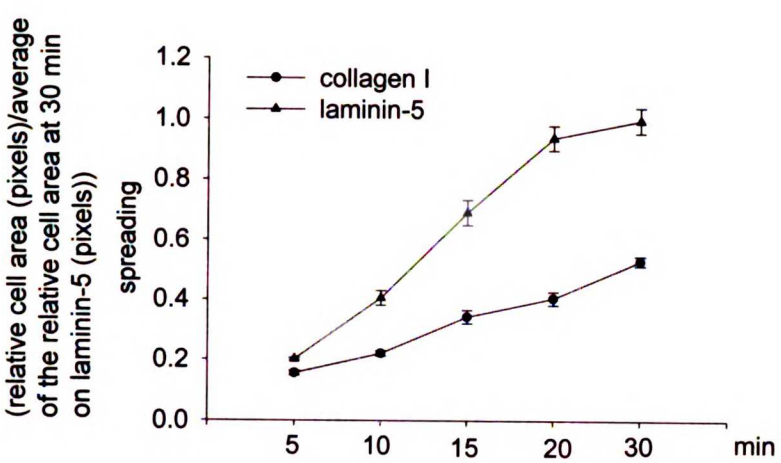
Results

ECM proteins regulate cell spreading and motility differently through integrin receptors

Previous studies using cell aggregates had revealed that compared to collagen, fibronectin and laminin-1, laminin-5 had the most potential to disrupt cell-cell adhesions and promote cell scattering (23). In this study, the effects of laminin-5 and type I collagen on single cell spreading and migration were assessed. Cells were able to attach but showed a slow spreading on type I collagen compared to laminin-5 substrate (Fig. 1 A). Cells

spread almost to a full extent on laminin-5 substrate at 20 min after seeding, whereas cells displayed 1/3 of the full spreading on type I collagen at 20 min. In addition, cells displayed microspikes on the laminin-5 substrate but not on type I collagen. To determine whether adhesion efficiency was different during spreading on type I collagen and laminin-5 substrate, adhesion assays were performed in a 30-min time course. The percentage of adherent cells on type I collagen and laminin-5 was indistinguishable 15 min after plating (Fig 1 B). Next, the integrin receptors for adhesion were determined using anti-integrin blocking antibodies. HSC-3 cells have significant levels of α2, α3, and α6 integrins and low levels of α5 and αv integrins. Treatment of cells with anti-α2 integrin antibody blocked cell adhesion to type I collagen. Treatment of cells with anti-α3 integrin antibody inhibited cell adhesion to laminin-5 (Fig. 1 C). This suggests that signaling from type I collagen and α2 integrin retards cell spreading. Furthermore, the ability of laminin-5 and type I collagen to promote single cell migration was assessed using time-lapse video microscopy. Tracking individual cells showed that HSC-3 cells adhered to collagen and PLL, but migrated poorly even in the presence of serum. Cells plated on laminin-5 substrate showed a random course of migration, and migrated almost 3-fold more rapidly than cells plated on type I collagen (Fig. 1 D). Without serum, both HSC-3 cells and SCC-10A cells migrated 2-3 times faster on laminin-5 substrate than on type I collagen (Fig. 1 E). To evaluate the role of integrin receptors in cell migration, we performed motility assays in the presence of inhibitory antibodies. Blocking antibodies against integrin α3 and β1 inhibited cell movement on laminin-5 most effectively, while blocking antibodies against integrin α2 and α6 as the control antibody had negligible effect (Fig. 1 F). Although α6β4 integrin can act as a receptor for laminin-5, it is not

A


(relative cell area (pixels)/average of the relative cell area at 30 min on laminin-5 (pixels))
spreading
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1.0
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0.4
0.2
0.0
collagen I
laminin-5
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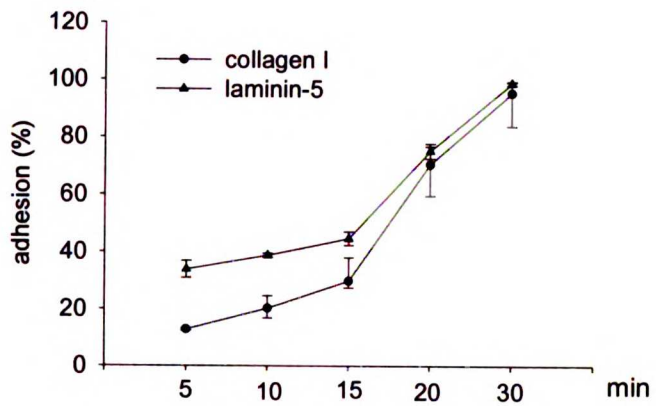
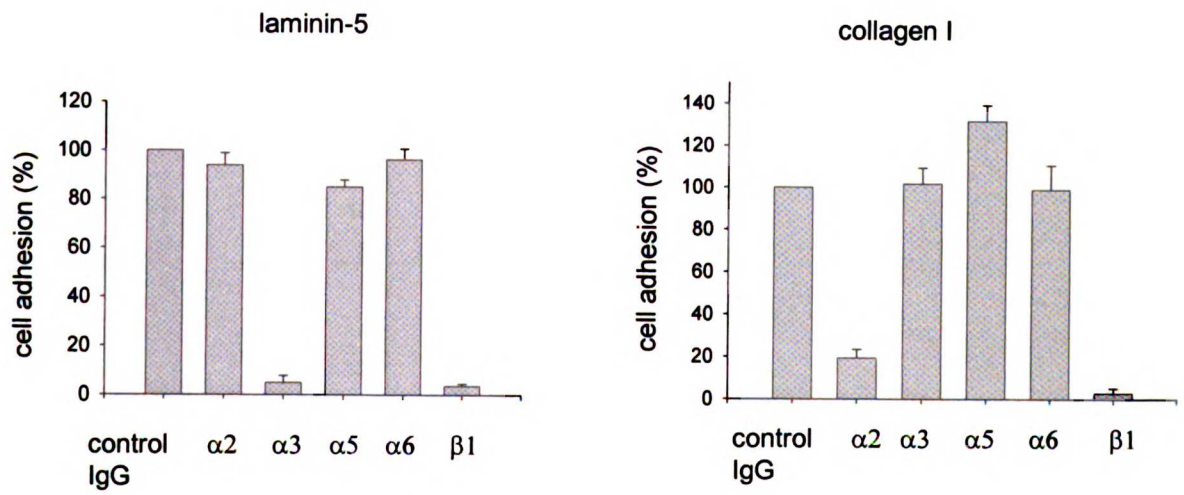
20
30
min

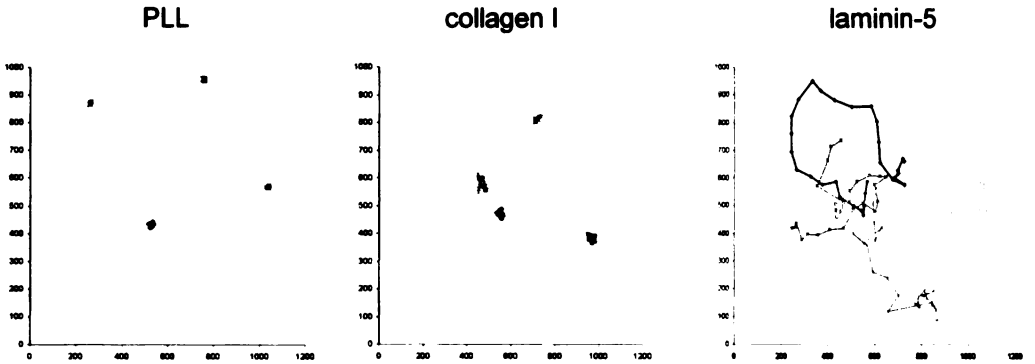
B


adhesion (%)
120
100
80
60
40
20
0
collagen I
laminin-5
5
10
15
20
30
min

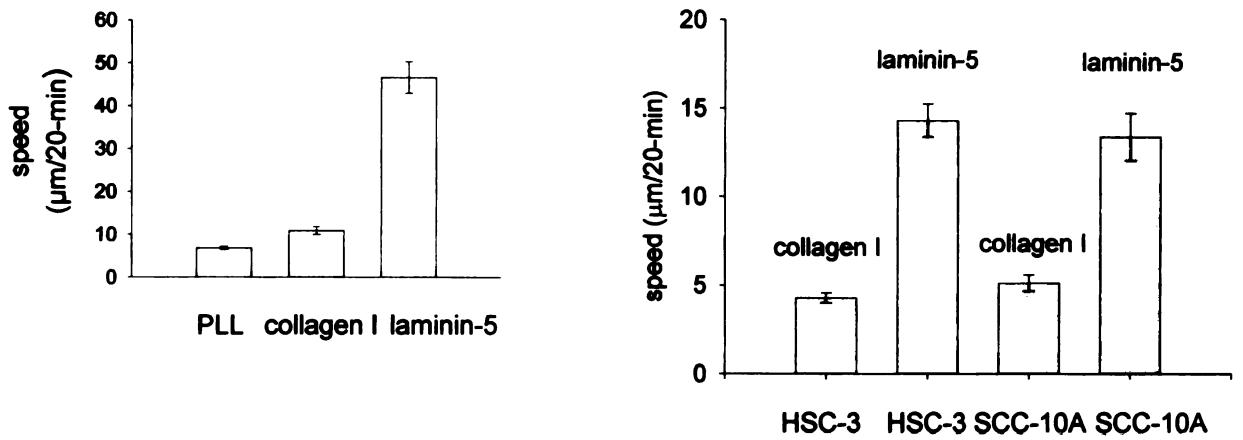
C


laminin-5
collagen I
cell adhesion (%)
120
100
80
60
40
20
0
140
control IgG
α2
α3
α5
α6
β1

D


PLL
collagen I
laminin-5

E


speed (µm/20-min)
60
50
40
30
20
10
0
PLL
collagen I
laminin-5
speed (µm/20-min)
20
15
10
5
0
laminin-5
laminin-5
collagen I
collagen I
HSC-3
HSC-3
SCC-10A
SCC-10A

F

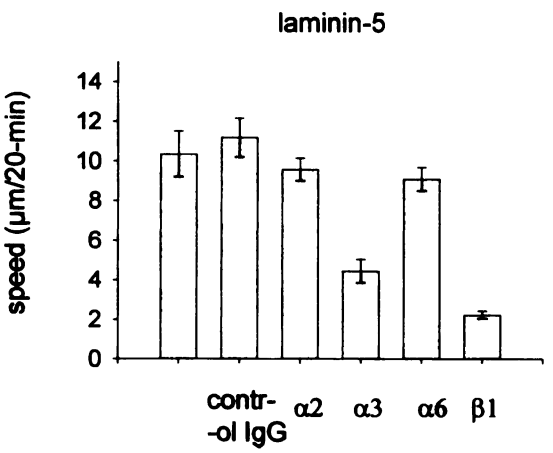

laminin-5
speed (µm/20-min)
14
12
10
8
6
4
2
0
contr-ol IgG
α2
α3
α6
β1

Figure 1. Cell spreading and motility on different ECM substrates. (A) For cell spreading assays, HSC-3 cells were plated on type I collagen (10 μg/ml) and laminin-5 substrates. At the indicated time intervals, cells were fixed and stained with 2% brilliant blue. The areas of more than 20 cells were measured as described in Experimental procedures. The average of the cell areas determined in cells plated on laminin-5 substrate for 30 min was set as the maximal cell area. Data represent the mean of ratios between cell areas at the indicated time and the maximal cell area ± SEM. (B) Cell adhesion to 10 μg/ml collagen I and 2.5 μg/ml laminin-5 was assessed in adhesion assay as described in Experimental procedures. (C) Inhibition of adhesion by function-perturbing mAbs to integrins. The adhesion assay was performed in the presence of anti-integrin antibodies on purified laminin-5 and type I collagen. Cells were pre-incubated with mAbs to α2 (VM1), α3 (J143), α5 (VC5), α6 (GoH3), and β1 (AIIB2) at 4°C for 30 min. The concentration of each antibody was 10 μg/ml. (D) HSC-3 cells were plated on type I collagen (10 μg/ml), laminin-5 substrate, and PLL (0.1 mg/ml) with 10% serum in the medium and time-lapse images on different substrates were taken at a 20-min interval for 8 h. Individual cell tracks are shown. Cell speed was estimated as detailed in Experimental procedures. (E) HSC-3 cells and SCC-10A cells in serum-free medium were plated on type I collagen and laminin-5 substrates. Time-lapse images were taken at a 20-min interval for 3 h. Cell speeds of eight tracked cells in each condition are shown. (F) Inhibition of cell migration on laminin-5 substrate by function perturbing mAbs to integrin receptors. Cells were incubated with mAbs to α2 (VM1), α3 (J143), α6 (GoH3), or β1 (AIIB2) at 10 μg/ml for 30 min at 4°C. Cells were then plated on laminin-5

crucial for cell migration and adhesion in HSC-3 cells. This suggests that α3β1 integrin is the primary receptor for adhesion as well as migration to laminin-5.

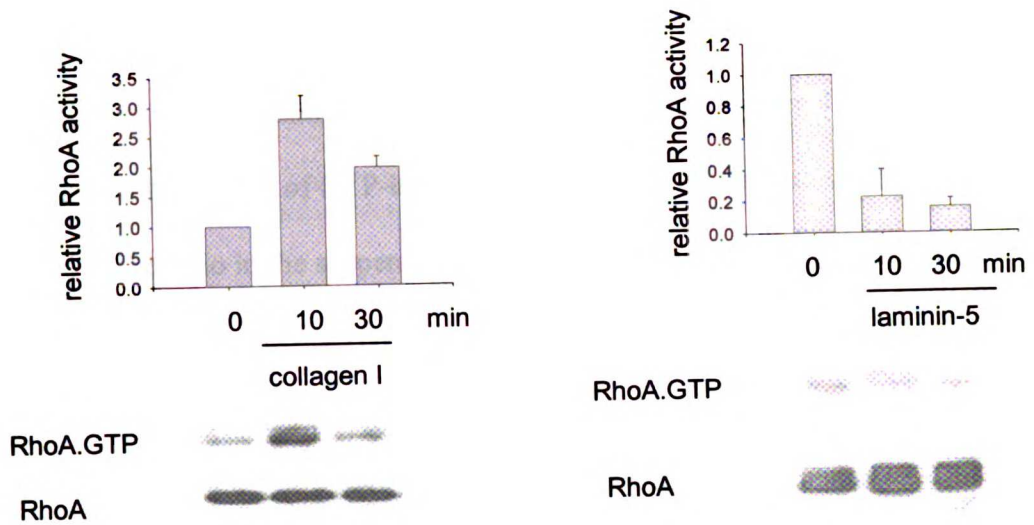
Type I collagen/α2 integrin-mediated RhoA activation inhibits cell spreading

Rho GTPases regulate reorganization of actin cytoskeleton associated with alterations in cell morphology (2,30). Therefore, we examined the activation of RhoA on type I collagen and laminin-5 substrate. RhoA activity was prominently increased in cells plated on type I collagen at 10 min, then decreased but still remained higher at 30 min than the suspension control. However, RhoA activity was decreased at 10 min and 30 min in cells plated on laminin-5 substrate (Fig. 2 A). Poor spreading on type I collagen correlated with an increase in RhoA activity. To determine whether inhibition of the RhoA pathway enhanced cell spreading, we transduced HSC-3 cells with adenovirus encoding N19RhoA. Expression of N19RhoA enhanced cell spreading compared to the control at 20 min, but could not improve the spreading on type I collagen to the same extent as on laminin-5 substrate (Fig. 2 B).

Signaling from laminin-5 and α3 integrin inactivate RhoA, whereas α2 integrin signaling activates RhoA

We examined RhoA activity on laminin-5 substrate over a 3-h time period. RhoA activity was constantly lower compared to the suspension control (Fig. 3 A). To determine whether laminin-5 was responsible for the inactivation of RhoA, the blocking laminin-5 antibody was included during adhesion to laminin-5 substrate. RhoA was inactivated in cells with or without the control antibody treatment, but blocking laminin-5 antibody released the suppression of RhoA activity (Fig. 3 B). Next, using anti-integrin antibodies on immobilized plate surface or in suspension, we examined the effects of integrin

A


relative RhoA activity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
30
min
collagen I
RhoA.GTP
RhoA
relative RhoA activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
30
min
laminin-5
RhoA.GTP
RhoA

B

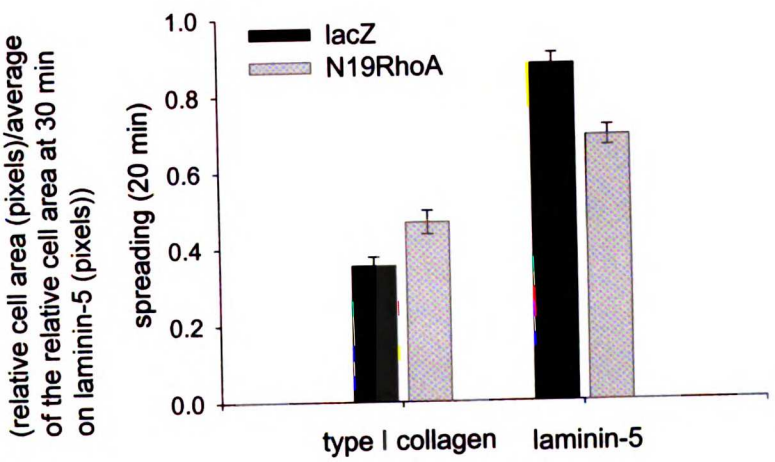

(relative cell area (pixels)/average of the relative cell area at 30 min on laminin-5 (pixels))
spreading (20 min)
1.0
0.8
0.6
0.4
0.2
0.0
lacZ
N19RhoA
type I collagen
laminin-5

Figure 2. RhoA regulates cell spreading. (A) For assays of RhoA activity, cells were plated on type I collagen or laminin-5 substrate for 10 min and 30 min and cell lysates were collected. RhoA activity was measured as described in Experimental procedures. The abundance of GTP-bound and total RhoA was measured by densitometric analysis. A ratio between the values of GTP-bound and total RhoA was calculated. Results are normalized to the ratio in the suspension lane (=1.0). Values represent mean ± SEM of three independent experiments. (B) Effect of RhoA on cell spreading. Cells infected with adenovirus encoding lacZ or N19RhoA were plated on type I collagen and laminin-5 substrates. After 20 min, cells were fixed and stained with 2% brilliant blue. The average of the cell areas determined in cells plated on laminin-5 substrate for 30 min was set as the maximal cell area. Data represent the mean of the ratios between cell areas at 20 min to the maximal cell area ± SEM. 30 cells were evaluated at each time point.

engagement on RhoA activity. Engaging α2 integrin enhanced RhoA activation. In contrast, engaging α3 integrin failed to induce an increase in RhoA activity (Fig. 3 C and D). These data suggest that laminin-5/α3 integrin signaling inactivates RhoA, whereas α2 integrin signaling activates RhoA.

Type I collagen/α2 integrin-mediated RhoA activation inhibits cell migration

To determine whether elevated RhoA activity is associated with the poor migration observed on type I collagen, cells were transduced with adenovirus encoding V14RhoA or N19RhoA. The expression of exogenous RhoA was recognized by the HA-tag (Fig. 4 A). Inhibiting RhoA by expression of N19RhoA caused a 200% increase in haptotaxic migration toward type I collagen compared with a 30% reduction caused by expression of V14RhoA (Fig. 4 B). Moreover, time-lapse video microscopy revealed that exoenzyme C3, N19RhoA, and Y-27632 all increased cell migrating speed by 2-3 times on type I collagen compared to controls (Fig. 4 C). Thus, treatments that inhibit RhoA pathway uniformly stimulated motility. Next, we evaluated the effects of inhibiting Rho on invasion of collagen gel. Expression of N19RhoA increased invasion, whereas expression of V14RhoA inhibited invasion (Fig. 4 D). The results were consistent with what was observed for migration on collagen substrate.

Formation of focal adhesions on type I collagen and laminin-5

To establish the role of adhesions in migration, cells were seeded onto different substrates and evaluated for focal adhesion formation by immunofluorescent staining. We found that denser, larger and more focal adhesions were stained by paxillin on type I collagen. Paxillin positive focal adhesions formed on laminin-5 substrate were poorly stained (Fig. 5 A). However, by immunoblotting, paxillin expression level was similar under both

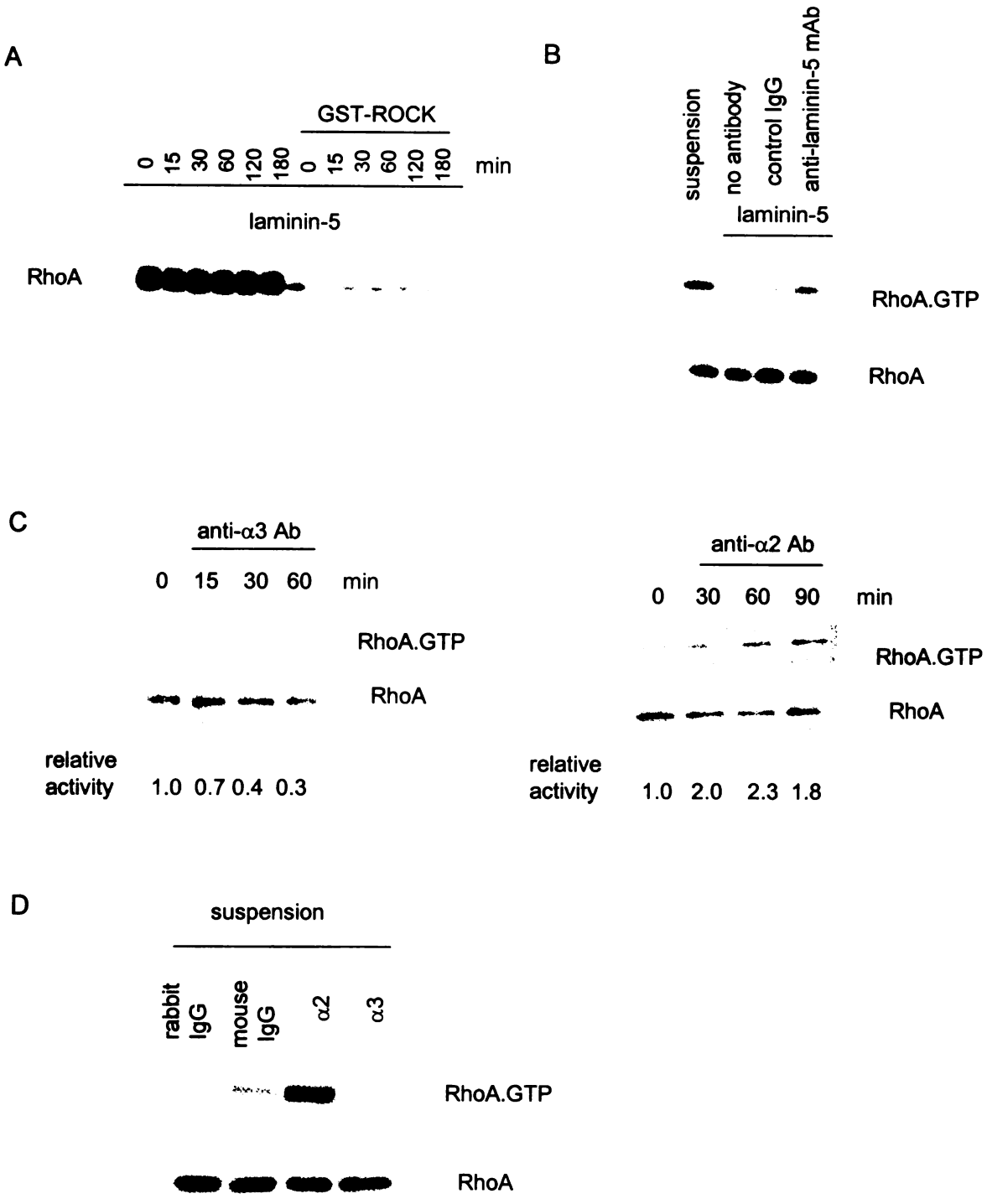

A
GST-ROCK
0 15 30 60 120 180 0 15 30 60 120 180 min
laminin-5
RhoA
B
suspension
no antibody
control IgG
anti-laminin-5 mAb
laminin-5
RhoA.GTP
RhoA
C
anti-α3 Ab
0 15 30 60 min
RhoA.GTP
RhoA
relative activity 1.0 0.7 0.4 0.3
anti-α2 Ab
0 30 60 90 min
RhoA.GTP
RhoA
relative activity 1.0 2.0 2.3 1.8
D
suspension
rabbit IgG
mouse IgG
α2
α3
RhoA.GTP
RhoA

Figure 3. α3 integrin and laminin-5 inactivate RhoA, whereas α2 integrin activates RhoA. (A) Cells were plated on laminin-5 substrate for indicated time intervals. Cell lysates were collected and RhoA activity was measured as described in Experimental procedures. (B) The effect of anti-laminin-5 mAb on RhoA activity was accessed as following. Cells were plated on laminin-5 substrate for 1 h with or without 10 μg/ml mouse control IgG or anti-laminin-5 mAb. Cell lysates were collected and RhoA activity assays were then performed. (C) Cells were seeded onto plates coated with anti-α2 mAb (VM1) or anti-α3 antibody (VM2) for the indicated time. Cell lysates were then collected and subjected to RhoA activity assays. The ratios between densitometric values of GTP-bound and total RhoA were determined. Results are normalized to the ratio in the suspension lane (=1.0). (D) Cells in suspension were incubated with anti-α2 integrin mAb or anti-α3 integrin mAb for 1 h at 4°C. Rabbit anti-mouse IgG was then added and incubated for 45 min. The concentration of each antibody was 1 μg/ml. Cell lysates were then collected and subjected to RhoA activity assays.

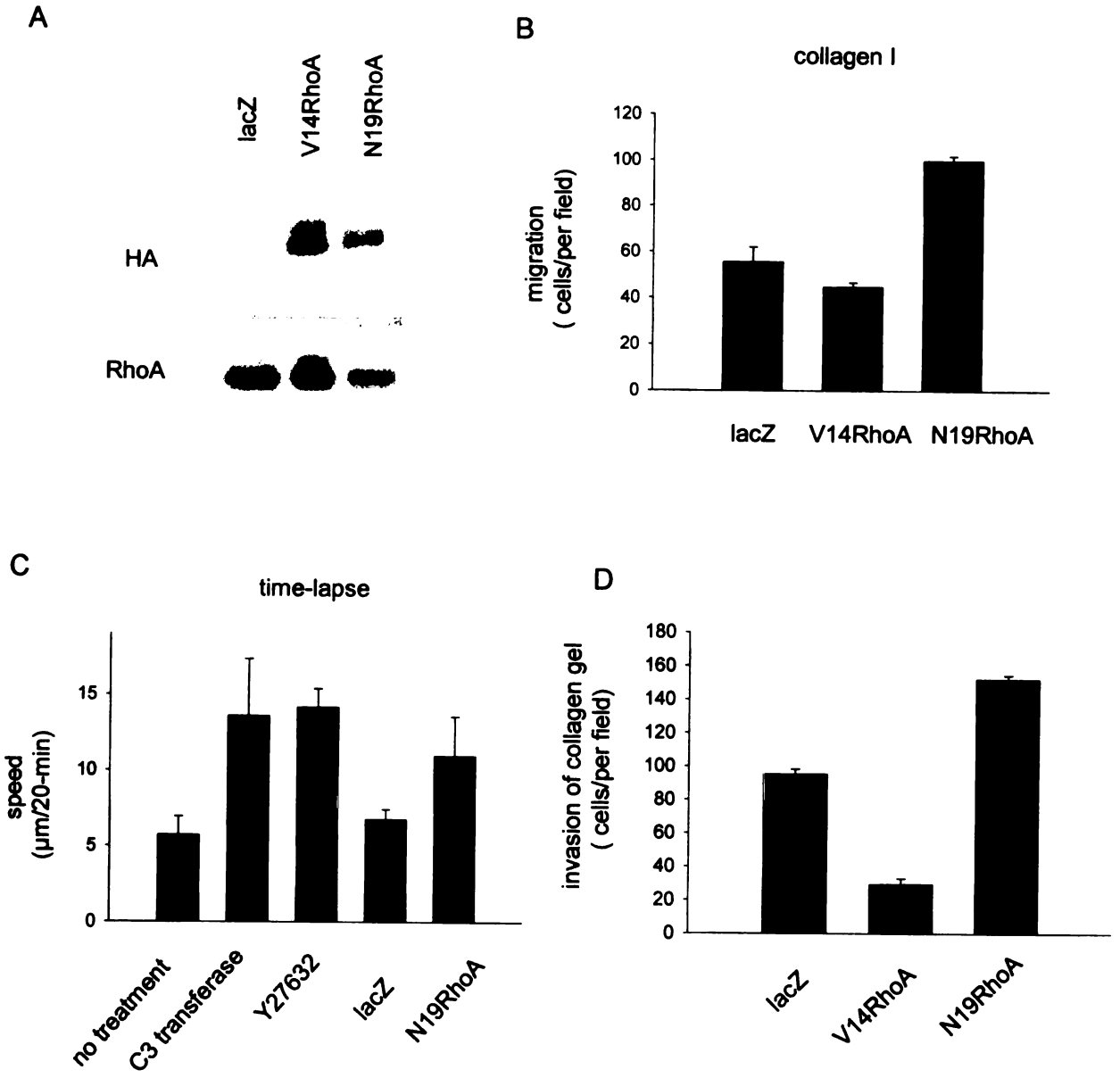
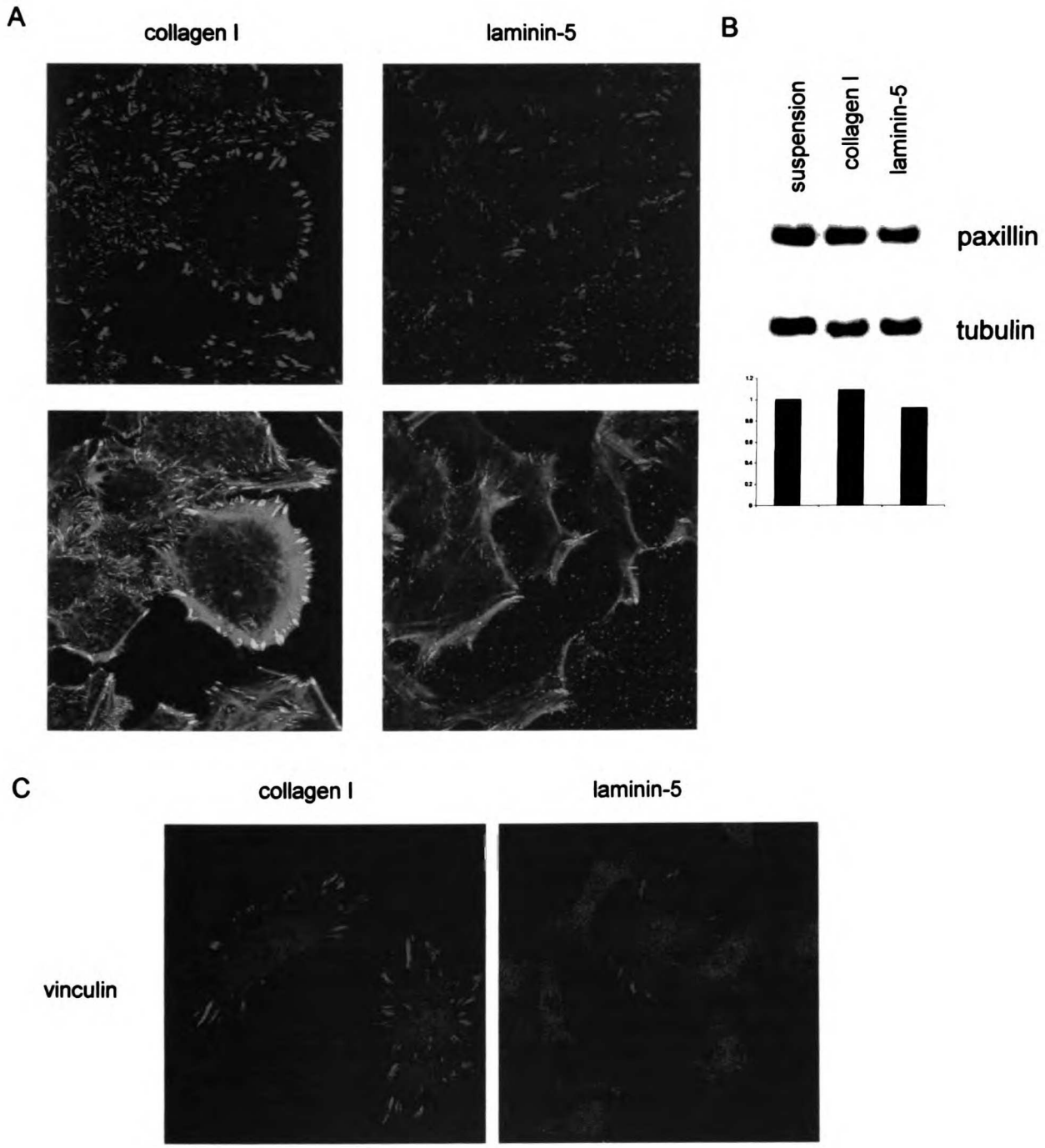

A
lacZ
V14RhoA
N19RhoA
HA
RhoA
B
collagen I
migration
(cells/per field)
120
100
80
60
40
20
0
lacZ
V14RhoA
N19RhoA
C
time-lapse
speed
(μm/20-min)
15
10
5
0
no treatment
C3 transferase
Y27632
lacZ
N19RhoA
D
invasion of collagen gel
(cells/per field)
180
160
140
120
100
80
60
40
20
0
lacZ
V14RhoA
N19RhoA

Figure 4. Inactivating RhoA promotes cell migration on type I collagen. Cells were infected with adenovirus encoding lacZ, V14RhoA, or N19RhoA for 24 h. In (A), cell lysates were collected and subjected to immunoblotting using antibodies against HA and RhoA. (B) Cells were assessed for migration using the transwell assay on type I collagen. Data represent mean of the number of migrating cells ± SEM of triplicate assays. (C) Cells were treated with 5 μg/ml exoenzyme C3 overnight or treated with 25 μM Y-27632 for 30 min. Untreated, treated cells, lacZ-expressing cells, or N19RhoA-expressing cells were plated on 10 μg/ml type I collagen and assessed for migration using time-lapse video microscopy as described in Experimental procedures. Data represent the mean of the migration speed ± SEM of seven tracked cells. (D) Cells were assessed for invasion using the collagen gel. Data represent mean of the number of invaded cells ± SEM of triplicate assays.

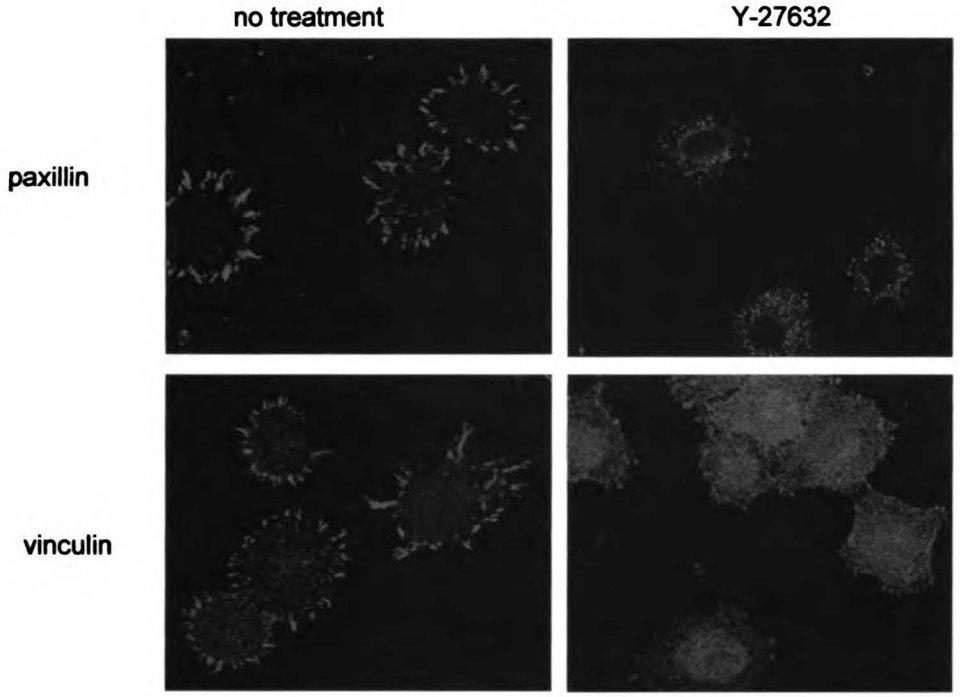
conditions (Fig. 5 B). Staining with anti-vinculin antibody showed a similar pattern to the paxilling staining (Fig. 5 C). Next, we tested the effect of Y-27632, a ROCK inhibitor, on focal adhesion formation. We found that paxillin and vinculin staining at focal adhesions decreased dramatically in the presence of Y-27632 (Fig. 5 D). Moreover, cells treated with Y-27632 on average displayed a more elongated morphology compared to untreated cells (Fig. 5 E). Time-lapse microscopy revealed that cells treated with Y-27632 extended membrane protrusions persistently at the leading edge. However, the retraction of the attaching tails was delayed or defective, leaving long cytoplasmic segments behind. Eventually, the cells reversed direction and retrieved their cellular processes. Some cells showed pseudopods in multiple directions. Therefore, inhibiting RhoA and ROCK activity decreased focal adhesion assembly, and, in the presence of the ROCK inhibitor, retraction of the trailing edge was also defective.

Activation of Cdc42-PAK1 promotes cell migration on laminin-5

RhoA activity assays have shown that laminin-5 substrate inactivates RhoA. To determine the effects of RhoA on migration on laminin-5 substrate, cells were infected with adenovirus encoding V14RhoA or N19RhoA and subjected to a transwell migration assay. Neither expression of N19RhoA nor expression of V14RhoA enhanced cell migration toward immobilized laminin-5 (Fig. 6 A). To test the involvement of Rac1 and Cdc42 in cell migration, we examined the activation of Rac1 and Cdc42 in cells adherent to laminin-5 or type I collagen. Assays revealed that type I collagen induced Rac1 activation nearly 50% more than laminin-5 substrate. Cdc42 activity specifically was 50% more stimulated in cells adherent to laminin-5 substrate than in cells adherent to type I collagen (Fig. 6 B). Furthermore, time course experiments showed that Cdc42


A
collagen I
laminin-5
B
suspension
collagen I
laminin-5
paxillin
tubulin
C
collagen I
laminin-5
vinculin

D



E

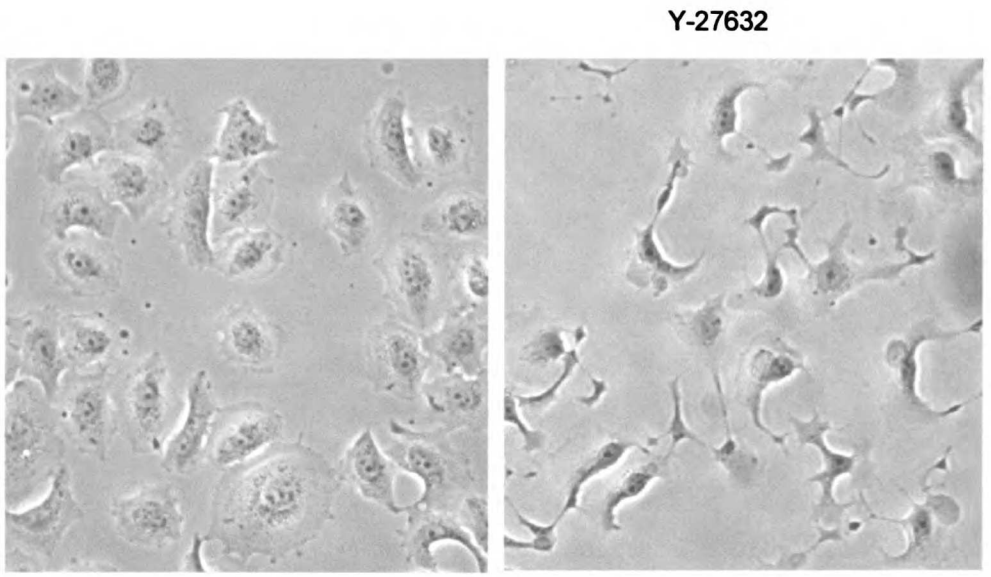
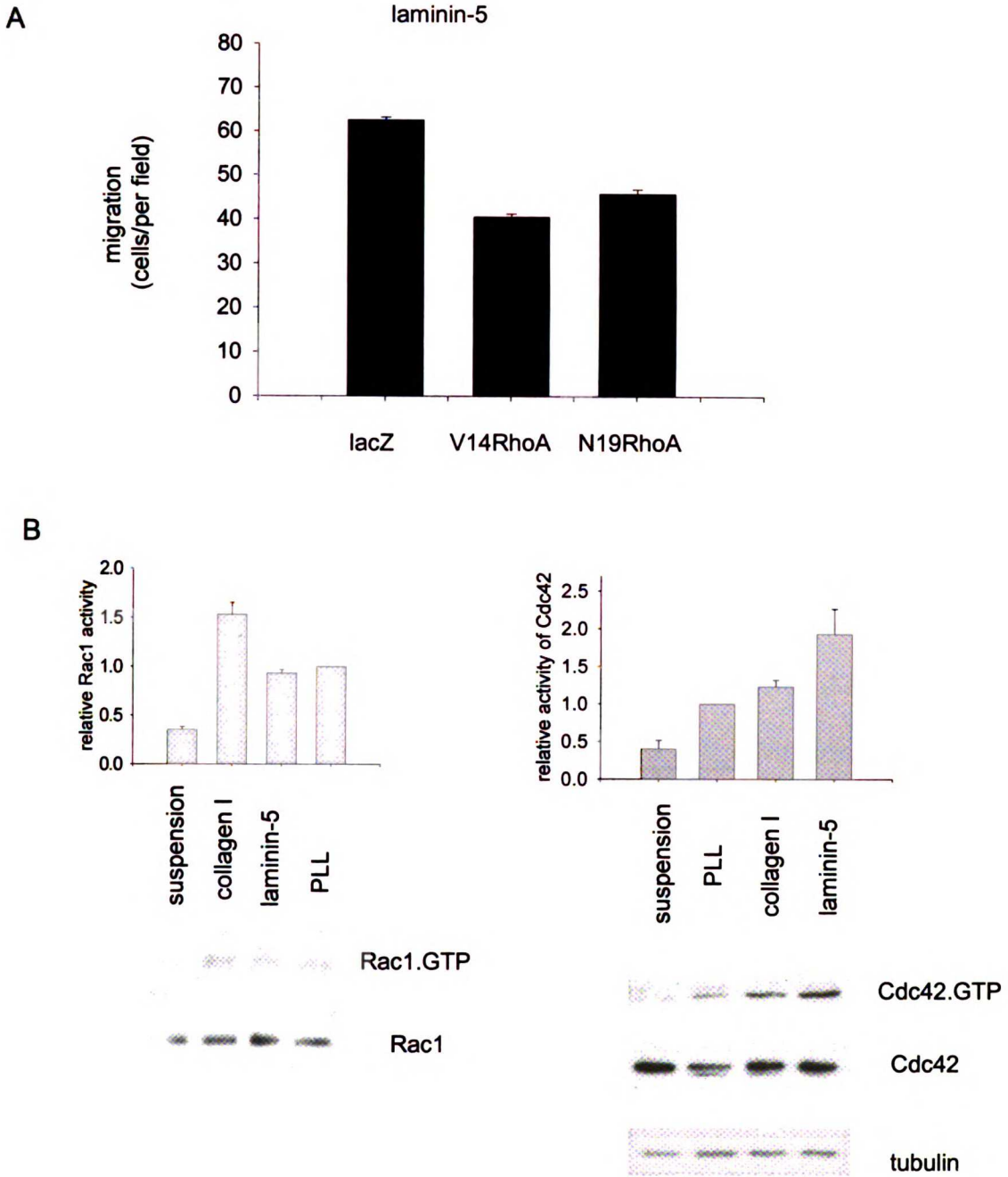
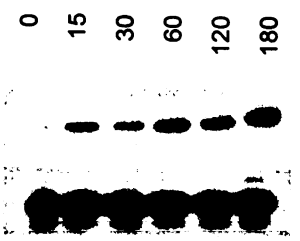


Figure 5. Focal adhesion formation on type I collagen and laminin-5 substrate. Cells were plated on coverslips coated with type I collagen and laminin-5 substrate. After 2 h, cells were fixed and stained for paxillin and F-actin in (A), vinculin in (C). (B) Cell lysates were collected and subjected to immunoblotting using anti-paxillin and anti-tubulin antibodies. (D) Cells plated on type I collagen for more than 1 h with or without Y-27632 treatment were fixed and stained for vinculin and paxillin. (E) Cells plated on collagen I for 3 h with or without Y-27632 treatment were photographed with a 20x objective with phase contrast optics.


A
laminin-5
migration (cells/per field)
80
70
60
50
40
30
20
10
0
lacZ
V14RhoA
N19RhoA
B
relative Rac1 activity
2.0
1.5
1.0
0.5
0.0
suspension
collagen I
laminin-5
PLL
Rac1.GTP
Rac1
relative activity of Cdc42
2.5
2.0
1.5
1.0
0.5
0.0
suspension
PLL
collagen I
laminin-5
Cdc42.GTP
Cdc42
tubulin

C



relative activity 1.0 3.8 3.0 6.6 5.6 7.6

D

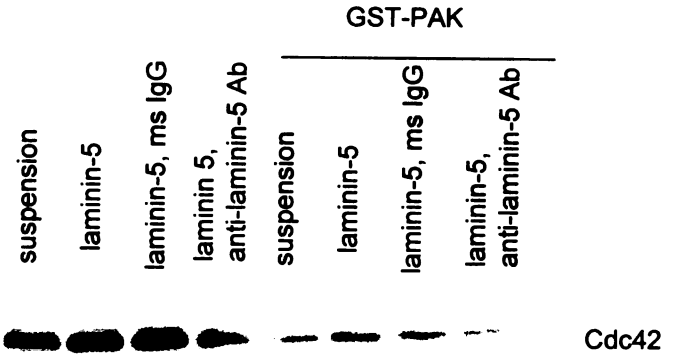
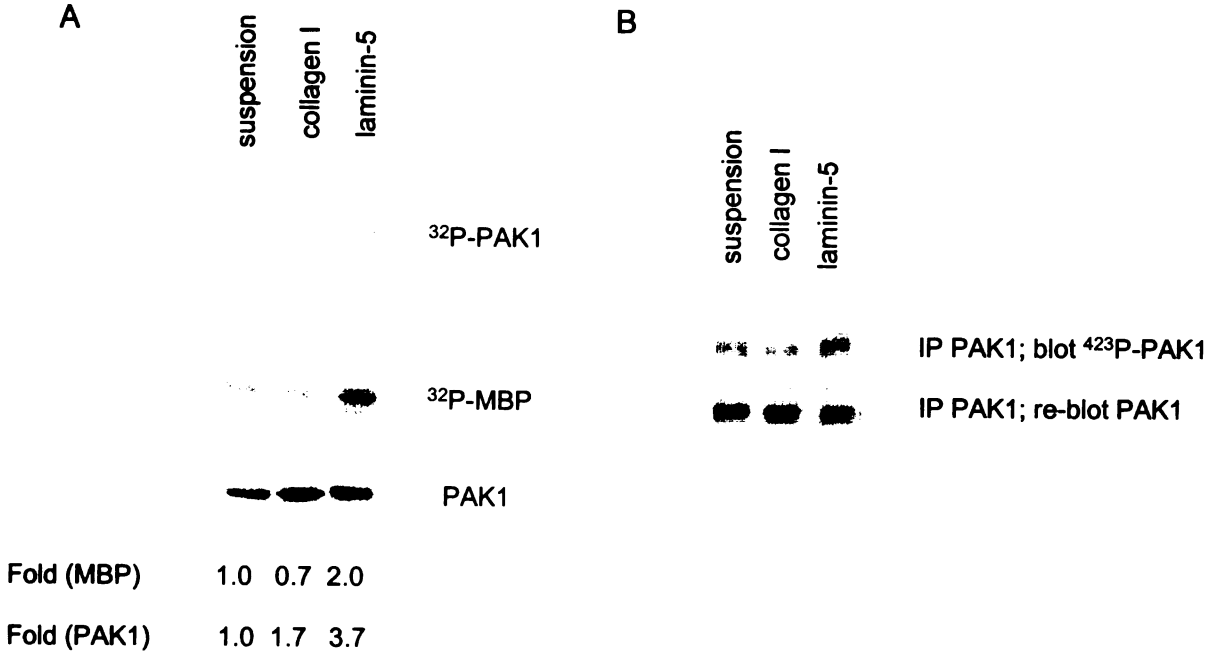


Figure 6. Laminin-5 specifically activates Cdc42. (A) Cells were infected with adenovirus encoding lacZ, V14RhoA, or N19RhoA for 24 h. Cells were then used for transwell migration assays on 1.25 μg/ml laminin-5 for 3 h. (B) Cells were detached and plated on type I collagen, laminin-5 substrate, or PLL for 30 min and cell lysates were collected. Rac1 and Cdc42 activity assays were then performed. Immunoblots show the result of a representative experiment. Graphs show the mean of the ratios between densitometric values of GTP-bound and total Rac1 or Cdc42 ± SEM. Results are normalized to the ratio in PLL lane (=1.0). (C) Cells were plated on laminin-5 substrate for different time periods. Cell lysates were then collected and subjected to Cdc42 activity assay. (D) The plates coated with laminin-5 substrate were pretreated with control IgG or anti-laminin-5 blocking antibody for 30 min. Detached cells were then added to the plates in the presence of anti-laminin-5 blocking antibody (10 μg/ml) or control IgG for another 30 min. Cell lysates were collected and subjected to Cdc42 activity assays.

activity intensified with increasing seeding-time on laminin-5 substrate (Fig. 6 C). When cells were seeded onto laminin-5 substrate in the presence of blocking laminin-5 antibody, active Cdc42 level decreased (Fig. 6 D).

Since PAK1 is the best characterized effector protein of Rac1 and Cdc42 among the PAK family of serine/threonine kinases, we then measured the activation of PAK1 using an *in vitro* kinase assay. Compared to type I collagen, laminin-5 substrate doubled PAK1 activity. Also, PAK1 was heavily autophosphorylated in cells seeded onto laminin-5 substrate (Fig. 7 A). Using specific phospho-antibody, PAK1 was phosphorylated more specifically at threonine 423 in cells seeded on laminin-5 substrate (Fig. 7 B). Phosphorylation at threonine 423 has been shown to be strongly correlated with PAK1 activities (31). To determine whether Cdc42 was required for PAK1 activation in cells seeded on laminin-5, cells were infected with adenovirus encoding N17Cdc42 and used for an *in vitro* kinase assay. Expression of N17Cdc42 inhibited both kinase activity and autophosphorylation of PAK1 (Fig. 7 C). These data indicate that laminin-5 via Cdc42 specifically induces PAK1 activation.

To determine whether kinase activity of PAK1 was required for haptotaxis, cells were infected with adenovirus encoding GFP, wt PAK1, or E423 PAK1 and subjected to transwell migration assays. Expression levels of Myc-tagged transgenes are shown in Fig. 8 A. Expression of E423 PAK1 increased migration toward immobilized type I collagen two and a half-fold compared to the control (GFP), and increased migration toward immobilized laminin-5 three-fold compared to the control. However, expression of wt PAK1 did not increase the number of cells migrating toward type I collagen compared to the control. However, expression of wt PAK1 increased migration toward laminin-5 two-


A
suspension
collagen I
laminin-5
32P-PAK1
32P-MBP
PAK1
Fold (MBP) 1.0 0.7 2.0
Fold (PAK1) 1.0 1.7 3.7
B
suspension
collagen I
laminin-5
IP PAK1; blot 423P-PAK1
IP PAK1; re-blot PAK1

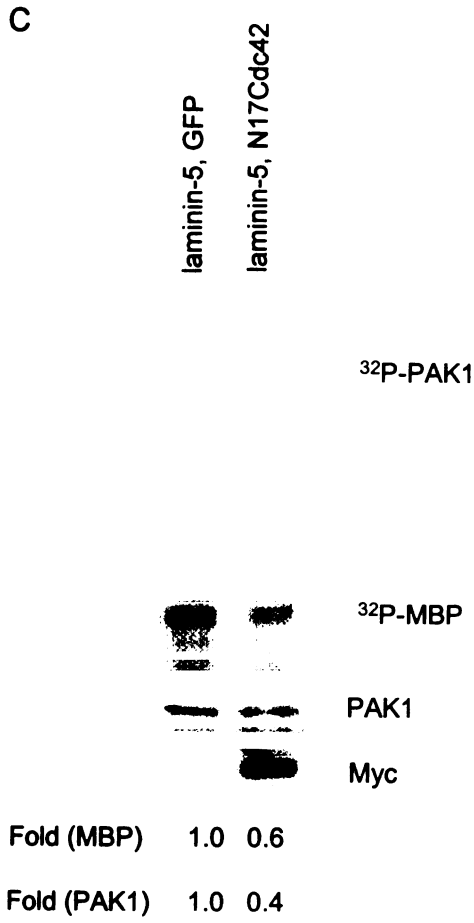

C
laminin-5, GFP
laminin-5, N17Cdc42
32P-PAK1
32P-MBP
PAK1
Myc
Fold (MBP) 1.0 0.6
Fold (PAK1) 1.0 0.4

Figure 7. Laminin-5 induces PAK1 activation, which requires Cdc42. (A) PAK1 was precipitated from cell lysates from cells plated on type I collagen and laminin-5 substrate. The immunocomplexes were subjected to *in vitro* PAK1 kinase assay. Results are normalized to the density of MBP band or PAK1 band in suspension lane. (B) In a different experiment, the immunocomplexes were blotted with anti-phosphoPAK1 antibody and anti-PAK1 antibody. (C) GFP- or N17Cd42-expressing cells were plated on laminin-5 substrate for 30 min. Cell lysates were then collected and used for *in vitro* kinase assay. Results are normalized to the density of MBP band or PAK1 band in GFP lane. Exogenous N17Cdc42 was recognized with anti-Myc antibody.

fold compared to the control (Fig. 8 B). Adhesion to laminin-5 substrate but not type I collagen resulted in autophosphorylation of PAK1 and elevated kinase activity of PAK1 specifically in wt PAK1-expressing cells (Fig. 8 C). These data suggest that autophosphorylation and kinase activity of PAK1 are required for haptotaxis.
In summary, activation of Cdc42 is related to activation of PAK1, and PAK1 kinase activity is required for haptotaxis.

RhoA downregulates Cdc42 activity

RhoA was inactivated when cells were seeded on laminin-5 substrate, whereas Cdc42 was activated. This suggests that there may be a cross-talk between Cdc42 and RhoA. To test this potential cross-talk, cells were infected with adenovirus encoding lacZ (control) or N19RhoA. The forced expression of N19RhoA resulted in two-fold increase in Cdc42 activity when cells were plated on laminin-5 substrate (Fig. 9 A). In contrast, forced expression of N17Cdc42 did not change much the activity of RhoA compared to controls (Fig. 9 B). These findings suggest that there was an intervention between RhoA and Cdc42.

Discussion

ECM proteins are able to stimulate or constrain cell movement dependent on cell substratum adhesiveness (32-34). Recent studies have demonstrated that ECM regulates the activities of Rho GTPases (2,17,19,20,35). We provide evidence that Rho GTPases serve as a controlling platform for migration regulated by ECM-integrin signaling. Importantly, our results demonstrate that specific ligand-integrin binding results in

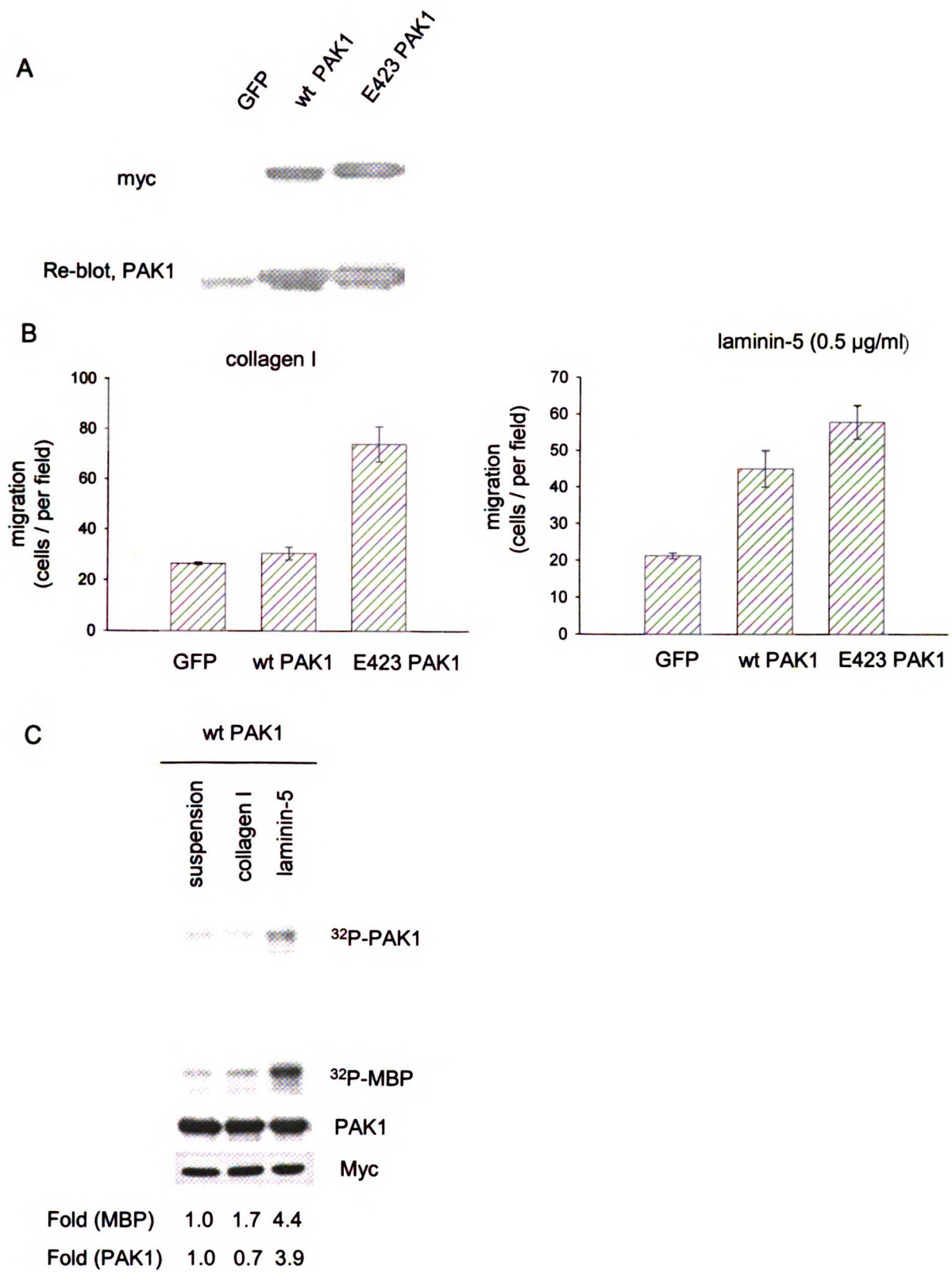

A
GFP
wt PAK1
E423 PAK1
myc
Re-blot, PAK1
B
collagen I
migration (cells / per field)
100
80
60
40
20
0
GFP
wt PAK1
E423 PAK1
laminin-5 (0.5 µg/ml)
migration (cells / per field)
70
60
50
40
30
20
10
0
GFP
wt PAK1
E423 PAK1
C
wt PAK1
suspension
collagen I
laminin-5
32P-PAK1
32P-MBP
PAK1
Myc
Fold (MBP) 1.0 1.7 4.4
Fold (PAK1) 1.0 0.7 3.9

Figure 8. Kinase activity of PAK1 is required for haptotaxis. (A) Cells were infected with GFP (control), wt PAK1, or E423 PAK1 for 24 h. Cell lysates were collected and immunoblotted with anti-Myc antibody and anti-PAK1 antibody. (B) Cells were then processed for transwell migration assays on 10μg/ml type I collagen or 0.5 μg/ml laminin-5. Data represent mean of the number of migrating cells ± SEM of triplicate assays. (C) Cells were infected with adenovirus encoding wt PAK1 for 24 h. Cells were then detached and re-plated on laminin-5 substrate and type I collagen for 30 min. Cell lysates were collected and subjected to *in vitro* kinase assay. Results are normalized to the density of MBP band or PAK1 band in the suspension lane.

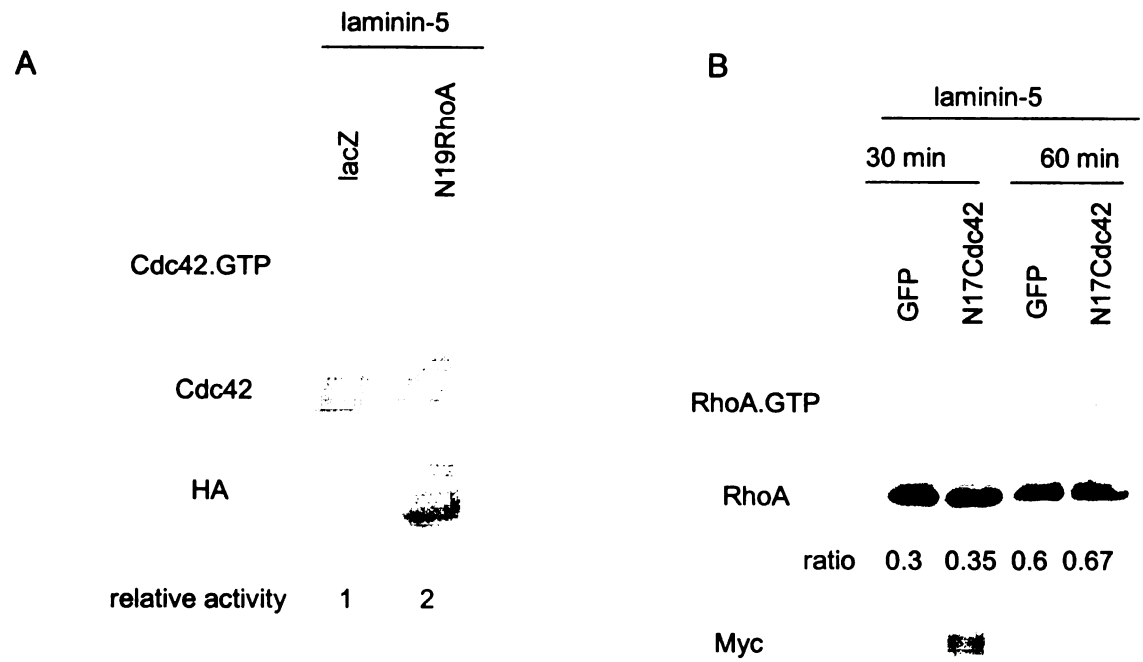


Figure 9. Cross-talk between RhoA and Cdc42. (A) Cells were infected with adenovirus encoding lacZ (control) or N19RhoA for 24 h. Cells were detached and plated on laminin-5 substrate for 30 min. Cell lysates were then collected and used for Cdc42 activity assay. The ratios between densitometric values of GTP-bound and total Cdc42 were calculated. Results are normalized to the ratio in lacZ lane. The exogenous N19RhoA was identified with anti-HA antibody. (B) Cells were infected with adenovirus encoding GFP (control) or N17Cdc42 for 24 h. Cells were detached and plated on laminin-5 substrate for 30 min and 60 min. Cell lysates were then collected and used for RhoA activity assay. The ratios of the densitometric values between GTP-bound and total RhoA in each lane were calculated. The exogenous N17Cdc42 was identified with anti-Myc antibody.

unique patterns of active Rho GTPases. RhoA and Cdc42-PAK1 are the key mediators in the signaling pathways that control cell motility in oral SCC cells.

Previous studies have suggested that high levels of adhesiveness inhibit speed of cell migration (32-34). Congruently, we show that collagen I-α2 integrin adhesive signaling inhibits cell spreading and migration via activation of RhoA/ROCK pathway. RhoA/ROCK is involved in cell migration through regulation of formation of focal adhesions. High RhoA activity promoted focal adhesion formation (Fig. 5 A and C). De-adhesion occured by lowering ROCK activity (Fig. 5 D). De-adhesion is the rate-limiting step to cell migration speed under a condition of high cell-substratum adhesiveness (1,36,37). Therefore, more and larger adhesions formed on type I collagen halted motility. Similarly, cells lacking the tyrosine kinases FAK or Src form more and larger adhesions, and these cells migrate poorly (38-41).

In addition, RhoA/ROCK is negatively involved in membrane protrusive activities. V14RhoA-infected cells lacked protrusive membrane structures. Furthermore, ROCK inhibitor Y-27632 promoted formation of membrane protrusions (Fig. 5 E). Similarly, RhoA and ROCK limited membrane protrusions in monocytic THP-1 cells (42) and in leukocytes (43). RhoA and ROCK regulate myosin-dependent contractile force. Actin-myosin II-mediated contraction may inhibit membrane protrusion. Reducing myosin II activity increases the rate of spreading (44), suggesting that increasing cell stiffness by myosin activation represses cell extension driven by actin polymerization. However, in cell spreading, a process analogous to the forward extension of lamellipodia and filopodia, N19RhoA and Y-27632 were not sufficient to make cells spread on type I collagen to the same extent as on laminin-5, although both enhanced cell spreading on

type I collagen compared to the controls. Enhanced cell spreading has also been observed by inhibiting RhoA via p190RhoGAP in fibroblasts (15) or truncated RhoA in BAE cells (45,46).

It is known that RhoA is inactivated by signaling from ECM proteins such as tenascin (47) and ECM receptors such as β1 integrin (13,48). We provide evidence that laminin-5-α3 integrin signaling inactivates RhoA. Unlike transient inactivation of RhoA by fibronectin (17), laminin-5-α3 integrin signaling maintained the inactivation of RhoA for a very long period of time (Fig. 3 A). However, our results contradict an earlier study by Nguyen et al. where laminin-5 or binding of α3 integrin to immobilized anti-α3 integrin Ab activated RhoA in primary skin keratinocytes derived from confluent cultures (49). Differences in these two studies may be due to the use of different cell lines or different mAb to engage integrin.

Cdc42 and Rac1 play a critical role in membrane protrusive activity (50,51). We found that activity of Cdc42 but not Rac1 was correlated to activity of PAK1 in SCC cells. We propose that the Cdc42/PAK1 pathway is more important in membrane protrusive activities. First, cells attached to type I collagen with high active Rac1 and low active Cdc42/PAK1 spread slowly. By contrast, cells attached to laminin-5 with moderate Rac1 activity and high Cdc42/PAK1 activities spread quickly. It is reported that inhibiting PAK1 and Cdc42 but not Rac1 inhibits spreading of fibroblasts (19). Second, constitutively active PAK1 was sufficient to enhance cell movement toward immobilized type I collagen. Both morphological polarity and directional sensing (compass) allow efficient motility. Cdc42 is required for the establishment of cell polarity. Inhibiting Cdc42 does not inhibit actin polymerization and lamellae formation, but impairs polarity

and motility (3,52). In addition, like the PI3Kγ pathway, the PAK1/Cdc42 pathway is part of the molecular compass required to sense direction (53). Thus, Cdc42/PAK1 appears to be important in "sampling" external environmental cues, initiating polarized actin polymerization, and controlling direction of movement.

Previous study on α3 integrin using laminin-10/11 as a ligand demonstrate that laminin-10/11-α3β1 integrin-dependent pathway preferentially activates Rac through a p130-CrkII-DOCK180 complex, thereby strongly promoting cell migration (11). We demonstrate that Rac1 is not a signaling molecule responsible for the difference in regulation of cell motility by α2β1 and α3β1 integrins. Type I collagen activates Rac1 more strongly than laminin-5. However, cells move poorly on type I collagen. Similarly, in α3β1-dependent EGF-induced keratinocyte migration, Rac1 activation is tampered (54). But Rac1 plays a dominant role in the PI (3,4,5) P3-dependent positive feedback loop required for forming a leading edge (52). It appears that Rac1 works cooperatively with Cdc42 to promote spatially defined protrusive activity needed to drive movement.

Cross-talk between RhoA, Cdc42, and Rac1 varies according to cell types. We show that N19RhoA increases active Cdc42 levels, suggesting that RhoA inhibits activation of Cdc42 in oral SCC cells. This inhibition has been reported in CHO cells (20) and neuronal cells (55,56) previously. However, in fibroblasts Rac inactivates Rho (57). Consistently, downregulation of Rac activity by depletion of Tiam1 increases RhoA activity (58). The mechanism underlying these inhibitory cross-talks is currently unknown. Certainly, the inhibitory cross-talk has its advantages. First, the inhibitory cross-talk can regulate coordinately activation of RhoA and Cdc42/Rac. Second, the inhibitory cross-talk between RhoA and Cdc42/Rac may make only one member of Rho

GTPases dominant in signaling, ensuring the controlled activation of each member of Rho GTPases temporally and spatially. The exclusive localization of active RhoA and Cdc42 has been observed in the trailing tail and leading edge in leukocytes (59). An alternative mechanism to control spatially distributed active RhoA is through ubiquitin-mediated degradation (60). Compartmentalization of active RhoA, Rac1, and Cdc42 and their cooperation are needed to drive cells forward.

A number of integrins including α2β1, α3β1, α6β1, and α6β4 are known to recognize laminin-5 (61,62). However, these integrin receptors elicit different biological events. For example, α6β4 integrin has been shown to be involved in cell adhesion (hemidesmosome formation), cell growth (63,64), tumorigenesis (65), and invasion (66,67). α3β1 integrin plays a major role in both cell adhesion and migration on laminin-5 (23,24,68-70). Our study is consistent with these previous reports. Unlike α3β1 integrin, α6-containing integrins play a less important role in oral SCC cell migration on laminin-5. However, α6β4 integrin stimulates chemotaxis, but has no influence on haptotaxis in breast carcinoma cells (71). It is possible that α6β4 integrin is not important for ECM-dependent motility. α6β4 integrin can elicit ECM-independent signaling (72), which may be crucial for tumor growth and invasion.

Studies have shown that the RhoA/ROCK pathway plays an important role in invasion and metastasis in bladder cancer, breast cancer, hepatoma, and melanoma (73-76). Thus, ROCK inhibitors Y-27632 (14,77) and Wf-536 (74) have been implicated in treatments of these cancers. However, our findings indicate that inactivating ROCK promotes oral SCC cell migration and invasion (Fig. 4), suggesting that ROCK inhibitors may be not useful in treating head and neck SCCs. Similarly, the importance of RhoA in

migration has been shown to vary among cell types. Active RhoA mutant was shown to inhibit motility in fibroblasts and lung adenocarcinoma cells (11,15), whereas motility of lymphoma cells, colon carcinoma cells, and hepatoma cells was enhanced (10,13,14). A recent report sheds light on understanding differences in the requirement of RhoA for cell motility. Sahai et al. demonstrated that elongated cells do not require Rho/ROCK signaling and ezrin function for motility, but a rounded bleb-associated mode of motility requires Rho/ROCK signaling (78). Therefore, anti-metastatic drug selection should target specific Rho signaling in specific cancers.

In summary, our findings demonstrate that different substrate can initiate "go" or "stop" signals to cells through integrin-mediated signaling pathways via regulating Rho GTPases. It is possible that cell migration in *vivo* may be highly dependent on specific substrates and specific integrins.

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CHAPTER 4

THE ROLE OF RHOA AND PAK1 IN COORDINATED CELL MOVEMENT

Introduction

Coordinated cell movement plays a crucial role in biological processes such as embryonic development and wound healing. Coordinated cell movement involves migration of a complex epithelium or collection of associated cells. When cells move in sheets or associated groups, cell-cell adhesions are involved. Cadherin-based adherens junctions and other adhesions mediate junctional formation that keeps cells tightly associated in normal and malignant epithelia. Cell-cell adhesions tend to restrain movement of cell sheets. Whether an epithelial cell migrates or not is determined by the integration of migration-inhibitory cell-cell interactions and migration-promoting cell-substrate interactions. These interactions are affected by numerous external and intrinsic factors, including the nature of the ECM and the relative active level of Rho GTPases. It is known that downregulation of cell-cell adhesion in tumor cells favor their dissemination (1). However, E-cadherin mediated adhesion is neither necessary nor sufficient for suppressing tumor cell invasion. Rather, the invasion suppression signal is mediated through the β-catenin-binding domain of E-cadherin cytoplasmic tail (2). Interactions between ECM and their integrin receptors are capable of remodeling cell-cell adhesions and promoting scattering (3,4).

In epithelial cells, Rho GTPases regulate the formation and maintenance of specialized junctional adhesive complexes, tight junctions and adherens junctions, required for the barrier function of epithelial layers and to establish apical-basolateral polarity (5,6). It has been established that Rho and Rac are necessary for the formation and maintenance of cadherin-dependent cell-cell contacts (5). The underlying mechanisms are not known. It is possible that cadherin endocytosis is inhibited by RhoA and Rac, since active Rho and Rac inhibit endocytic traffic in both polarized and non-polarized cells (7-9). However, a number of studies have implicated RhoA and Rac1 in disassembly of cell-cell contacts (10-13). Thus the involvement of Rho GTPases in cell-cell contacts is complex.

Studies have shown that Rho GTPases are key regulators of coordinated cell movement. For example, during the wound healing processes discrete steps are involved (14-16). Two distinct mechanisms, the pulse-string and lamellipodial crawling, underline the movement of epithelial cell sheets during wound healing (17). The two types of epithelial motility may take place simultaneously or at different stages of the wound healing processes. RhoA is involved in wound healing by means of a contractile string (18,19). Rac1 and Cdc42 are required for wound closure through either lamellipodial crawling or microtubule-dependent polarization of the leading edge (19-21).

$\alpha 3\beta 1$ integrin is required for the migration of oral SCC cells, and integrin $\alpha 3\beta 1$ integrin engagement disrupts cadherin-mediated cell-cell adhesion (4,22). $\alpha 3\beta 1$ integrin signaling activates Cdc42 and the PAK1 pathway but inactivates RhoA, which leads to elevating haptotaxis to laminin-5 (from my work in Chapter 3). We also investigated

whether or not inactivation of RhoA and activation of PAK1 promote coordinated cell movement.

The artificial wound assay, commonly used as an *in vitro* assay for cell motility, was adopted for current studies to evaluate coordinated cell movement involving cell-cell rearrangement. The multicellular aggregate decompaction assay depicts the dissolution of multicellular aggregates after attachment to substrates, and suggests an approach to study how ECM-adhesion receptor interactions modulate cell-cell adhesions (4). In the present study, we used these two approaches to study the role of RhoA and PAK1 in coordinated cell movements.

Experimental procedures

Antibody and reagents. Anti-αPAK1 (N-20) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Y-27632 was purchased from Calbiochem (San Diego, CA). MBP was obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture. Human squamous cell carcinoma cell line HSC-3 was maintained in DMEM supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Adenoviral Infection. Adenovirus encoding constitutively active RhoA (V14RhoA) or dominant-negative RhoA (N19RhoA) was provided by A. Hassid, (University of Tennessee, Memphis). Adenovirus encoding wild type (wt) PAK1 or constitutively active PAK1 (E423 PAK1) was provide by W. T. Gerthoffer (University of Nevada, Reno). HSC-3 cells grown on 6-well plates were infected with adenovirus at a multiplicity of infection of 100 in 1 ml cell culture medium for 2 h. 2 ml of culture medium was then

added. Cells were cultured for 24 h, and then used for assays of MCA scattering and wound healing.

Inhibitor treatment. For treatment with the ROCK inhibitor Y-27632, cells were incubated with 25 μM Y-27632 for 30 min prior to and during the experiments.

Laminin-5 matrix preparation. Laminin-5 substrate was derived from HSC-3 cells or SCC-10A cells as previously described (4). In brief, cells were grown to confluence on plates. Dishes were washed with PBS and cells were removed by treating with 20 mM NH_4OH for 5 min according to the method previously described (23,24). The matrix was then extensively washed with PBS prior to use.

Immunofluorescence staining. Cells grown in chamber slides (Nunc) were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% NP-40 in PBS for 5 min. Cells were then incubated with Rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 40 min to stain polymerized actin filaments. Chamber slides were mounted with Vectashield (Vector, Burlingame, CA) and viewed using a Nikon fluorescence microscope.

Multicellular aggregate (MCA) formation. MCA was formed as previously described (4). Briefly, subconfluent HSC-3 cells were treated with trypsin-EDTA to prepare single-cell suspension (5×10^3 cells/ml). 100 μl of single-cell suspension was plated in the polyHEME-coated 96-well plates and incubated for 16 h in 5% CO_2 at 37°C.

MCA decompaction and scattering assay. This assay was performed as described (4). In brief, single MCA was transferred to 10 μg/ml collagen I- or laminin-5 substrate-coated 24-well plates containing 250 μl per well of serum-free DMEM. Photos were taken at 0, 7, and 24 h with a phase-contrast microscope. The areas of MCA outlines

were measured using NIH Image software. The extent of MCA decompaction and scattering is represented as the ratios between the areas of MCA outlines at the indicated incubation times and the areas of initial MCA outlines at 0 h.

Wound healing assay. The confluent monolayer of cells in multi-well plates were wounded by manually scratching with a pipette tip and washed three times extensively with PBS. The wound margins were marked by scratching the underside of plates with a needle. The cells were then incubated for the indicated times in serum-free DMEM. At 0 and 20 h, cells were photographed.

Protein kinase assay. Unwounded and wounded monolayers of HSC-3 cells were collected using lysis buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.6% Triton X-100, 20 mM β-glycerophosphate, 10% glycerol, 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin) as described by Royal (25). Cell lysates (800 μg) were immunoprecipitated with anti-αPAK1 (N-20) antibody for 1 h followed by incubation with 40 μl of protein G-Sepharose (40% suspension) for 1 h at 4°C. The immune complex was washed twice with the lysis buffer and once with the kinase buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM DTT, 5 mM sodium fluoride, 0.1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin). The immunoprecipitated PAK1 activity was assayed using MBP as a substrate. The kinase reaction was performed for 30 min at 30°C in 50 μl of kinase buffer containing 5 μg of MBP, 100 μM cold ATP, and 6 μCi of [γ-^{32}P] ATP. The reaction was terminated by adding 6 X Laemmli sample buffer followed by boiling for 5 min. Proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel. Dried gels were exposed to X-ray sensitive film and

intensifying screen at -80°C. The band intensity was measured by densitometry. PAK1 expression levels were evaluated with the anti-αPAK1 (N-20) antibody.

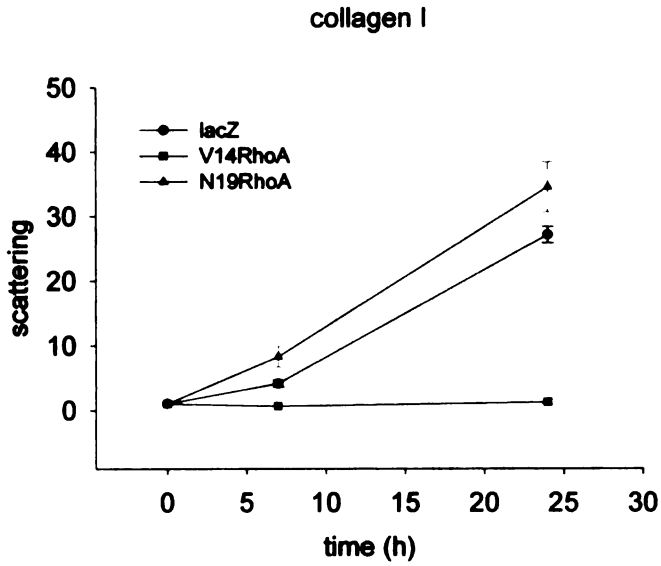
Results

RhoA in coordinated cell movement

To determine the effects of RhoA on coordinated cell movement, we performed MCA decompaction and wound healing assays using V14RhoA- and N19RhoA-expressing HSC-3 cells. Collagen I and laminin-5 substrates were tested by MCA decompaction assay. Expression of V14RhoA blocked MCA decompaction completely on both collagen I and laminin-5 substrates (Figs. 1 and 2). The LacZ-expressing control MCA remodeled into monolayer following seeding on both substrates over 24 h, but more single cells scattered on laminin-5 than on collagen I. Collagen I and laminin-5 caused different effects on decompaction of N19RhoA-expressing MCA. Expression of N19RhoA enhanced MCA decompaction compared to the lacZ control on type I collagen. Expression of N19RhoA did not enhance MCA decompaction on laminin-5 substrate. Furthermore, treatment with Y-27632 enhanced MCA decompaction for V14RhoA-expressing cells on both type I collagen and laminin-5 (Figs. 1 and 2). Treatment with Y-27632 enhanced MCA decompaction for control cells on collagen I but not on laminin-5 substrate (Figs. 1 and 2).

In the wound healing assay, 20 h after wounding, more healing was observed in N19RhoA-expessing cells than in V14RhoA-expressing cells and control cells (Fig. 3). These results suggest that high RhoA activity impairs coordinated cell movement.

PAK1 in coordinated cell movements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50
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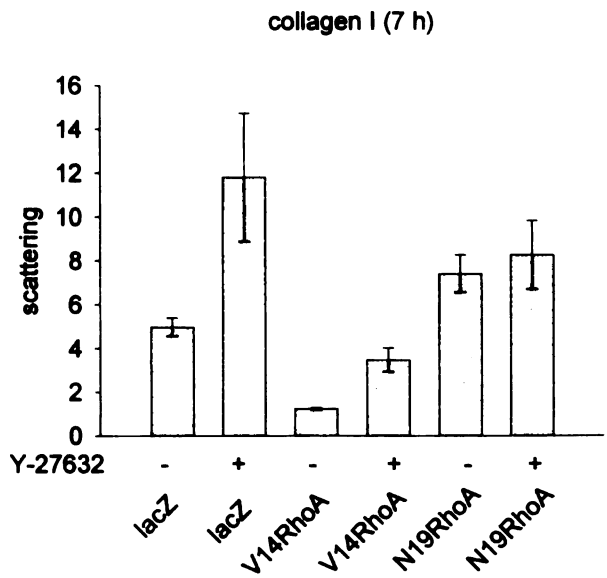
time (h)


collagen I (7 h)
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V14RhoA
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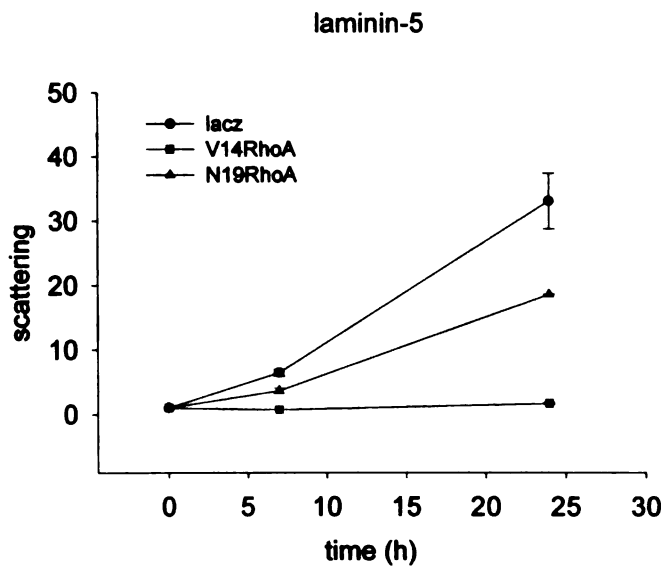
N19RhoA

Figure 1. Effects of RhoA and ROCK on MCA scattering on collagen I. MCAs were formed using lacZ-, V14RhoA-, or N19RhoA-expressing cells. MCAs were then seeded onto collagen I-coated 24-well plates in the presence/absence of 25 μM Y-27632, and were followed over time by a phase-contrast microscope at 0, 7, and 24 h. MCA decompaction and scattering was measured as described in Experimental procedures. Scattering is represented as the ratios between the areas of MCA outlines at the indicated times (7 or 24 h) and the areas of initial MCA outlines at 0 h. Values are mean ± SD of triplicate wells.


laminin-5
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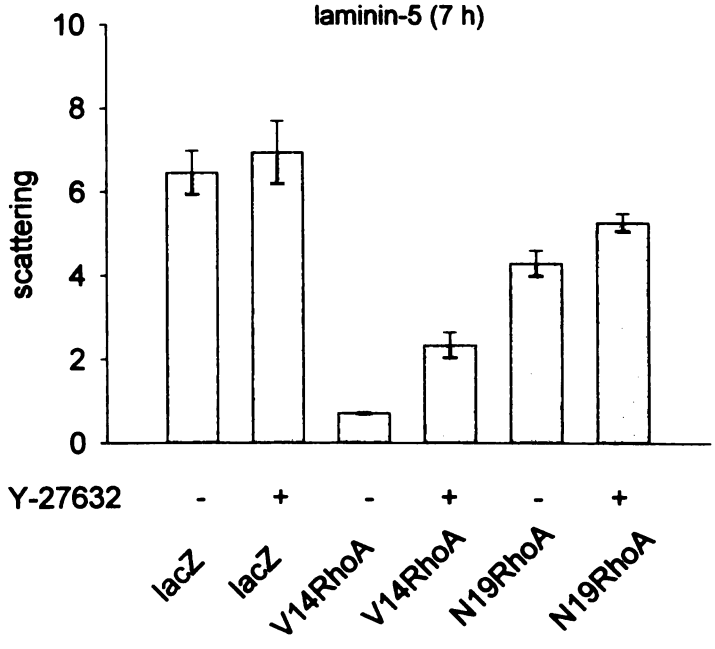
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N19RhoA

Figure 2. Effects of RhoA and ROCK on MCA scattering on laminin-5. MCAs were formed using lacZ-, V14RhoA-, or N19RhoA-expressing cells. MCAs were then seeded on laminin-5 substrate-coated 24-well plates in the presence/absence of 25 μM Y-27632, and were followed over time by a phase-contrast microscope at 0, 7, and 24 h. MCA decompaction and scattering was measured as described in Experimental procedures. Scattering is represented as the ratios between the areas of MCA outlines at the indicated times (7 or 24 h) and the areas of initial MCA outlines at 0 h. Values are mean ± SD of triplicate wells.

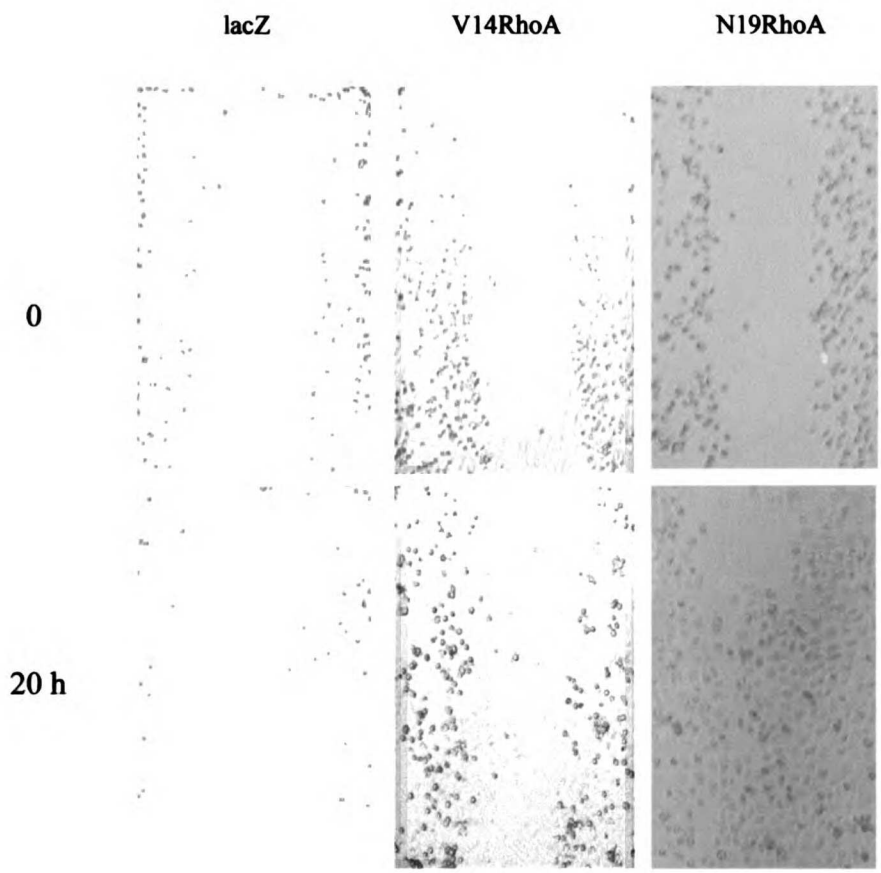


Figure 3. Inhibition of Rho activity accelerates the healing of the wound. Cells were infected with adenovirus encoding lacZ,V14RhoA, or N19RhoA on a 6-well plate for 24 h. The monolayer cells were then scratched. The healing of the wounds was monitored by taking photos at 0 and 20 h after wounding with a 10X phase-contrast objective.

To determine the involvement of PAK1 in coordinated cell movement, MCA decompaction and wound healing assays were performed using E423 PAK1- and wt PAK1-expressing cells. GFP-expressing cells were used as a control. Unexpectedly, MCA decompaction was much slower for E423 PAK1-expressing cells. At only 7 h after re-plating on collagen I, MCA showed limited rearrangement into the monolayer. In contrast, wt PAK1 slightly enhanced MCA decompaction and scattering on collagen I substrate after 24 h incubation (Fig. 4). Similarly, expression of E423 PAK1 prolonged wound healing (Fig. 5).

Staining of cells at the margin of the wound for F-actin showed that E423 PAK1-expressing cells assembled cortical actin cables across cell borders, and formed less membrane protrusion, whereas control cells and wt PAK1-expressing cells formed outgrown leader cells which exhibited extensive membrane protrusions (Fig. 6 B). However, individual E423 PAK1-expressing cells showed extensive membrane protrusions (Fig. 6 A). These results suggest that the detachment from cell groups is not dependent on high level of active PAK1. *In vitro* kinase assay showed that a wounded monolayer had less PAK1 activity than an unwounded monolayer (Fig. 7 upper panel). PAK1 kinase activity fluctuated during wound healing process. At 30 min after wounding, PAK1 activity increased. Following healing of the wound for 3 h, PAK1 activity was found to decrease. PAK1 activity was slightly higher in a closed wound (20 h) than in a healing wound (3 h) (Fig 7. lower panel).

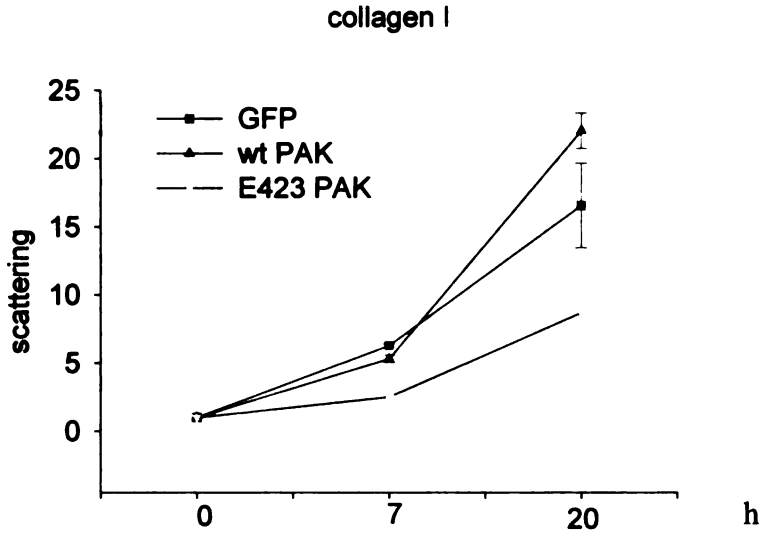


Figure 4. E423 PAK1 suppresses MCA decompaction and scattering. Cells were infected with adenoviruses encoding GFP, wt PAK1, or E423 PAK1 for 24 h. These cells were then used to form MCAs. MCAs were transferred to collagen I (10 μg/ml)-coated 24-well plates. MCA decompaction and scattering was monitored by taking photos at 0, 7, and 20 h with a 10X phase-contrast objective. Scattering is represented as the ratios between the areas of MCA outlines at the indicated time (7 or 24 h) and the areas of initial MCA outlines at 0 h. Data are mean ± SD of triplicate wells.

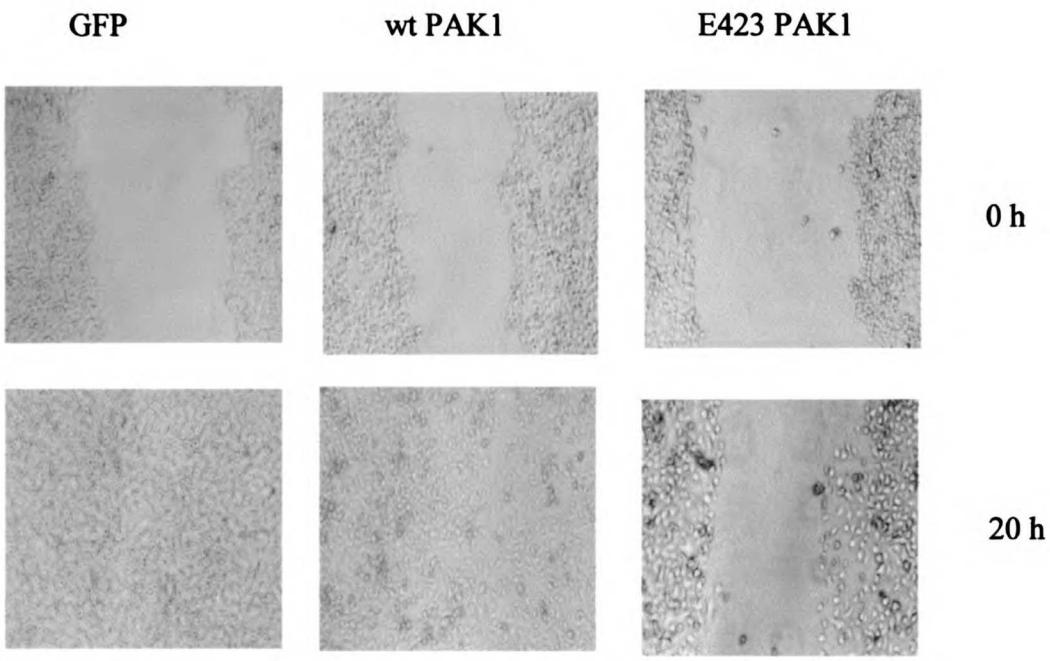


Figure 5. E423 PAK1 suppresses wound healing. Cells were infected with adenoviruses encoding GFP, wt PAK1, or E423 PAK on a 6-well plate for 24 h. The monolayer cells were scratched. The healing of wounds was monitored by taking photos at 0 and 20 h with a 10X phase-contrast objective.

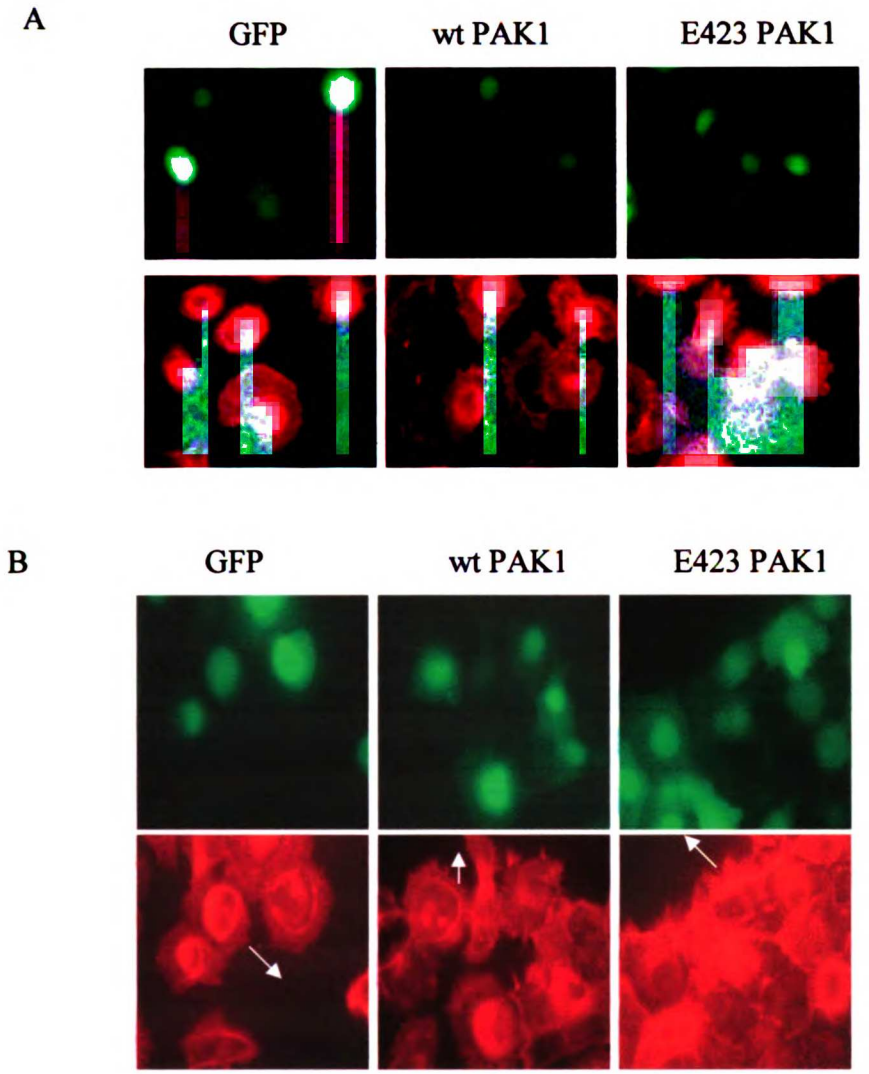
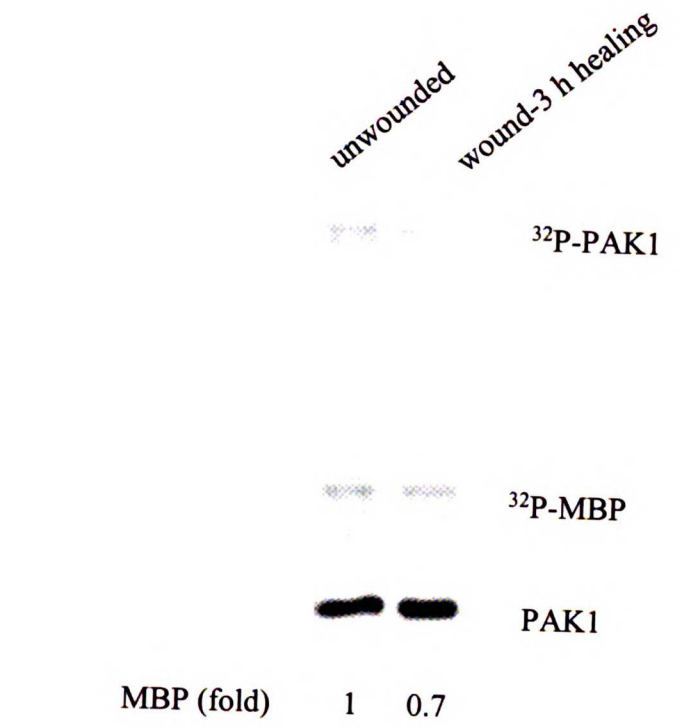


Figure 6. F-actin staining of cells. (A) GFP-, wt PAK1-, or E423 PAK1-expressing cells were plated on chamber slides coated with collagen at a low density. After 3 h, cells were fixed and stained with rhodamine-conjugated phalloidin. Expression of GFP indicated the success of viral infection. (B) GFP-, wt PAK1-, or E423 PAK1-expressing cells in chamber slides were scratched. After 7 h, cells were fixed and stained with rhodamine-conjugated phalloidin. Arrows indicate the moving directions of the cell sheets. Photos were taken with a 60X oil objective.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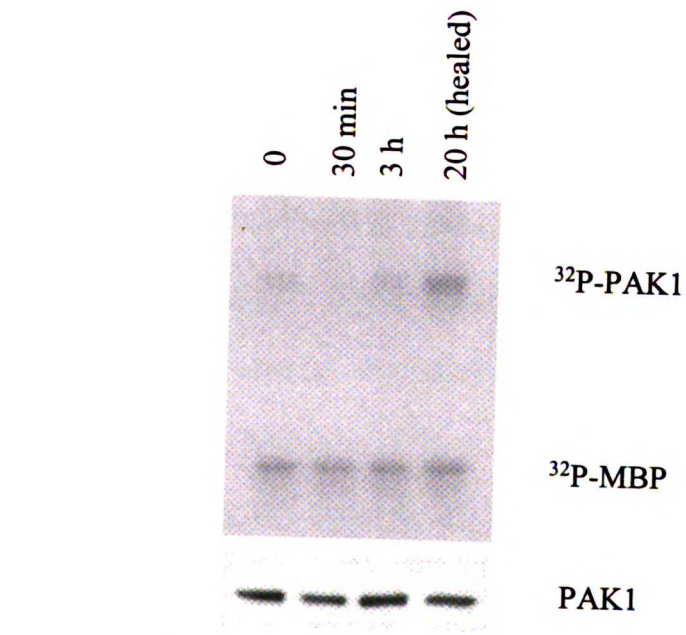
0
30 min
3 h
20 h (healed)
32P-PAK1
32P-MBP
PAK1
MBP (fold) 1 1.1 0.8 1.1
PAK1 (fold) 1 0.5 0.7 4.3

Figure 7. PAK1 kinase activity during wound healing. Confluent monolayer cells were scratched. The wounded monolayer cells were allowed to heal for 0, 30 min, 3 h, and 20 h. The lysates from unwounded and wounded monolayer cells were subjected to *in vitro* PAK1 kinase assays. Results are normalized to the density of MBP band or PAK1 band in unwounded lane (upper panel) or in 0 lane (lower panel).

Discussion

Integrin-mediated adhesion to ECM plays an important role in tissue organization and the behavior of individual cells. In previous study, it was found that the α3β1 integrin effectively induced the remodeling of multicellular aggregates when seeded on laminin-5 substrate (4). Following the spreading on laminin-5, cell-cell junctions were disrupted, leading to rapid cell scattering. In the present study, we show that integrin-induced cellular rearrangement is inhibited by high RhoA activity. Apparently, high levels of active PAK1 are not required in this process.

Coordinated cell movement does not require high RhoA activity. MCAs formed from V14RhoA-expressing cells were stable and unable to remodel or disperse following plating of MCAs on adhesive substrates, even on migration-promoting laminin-5 substrate. This stability is possibly related to RhoA stabilizing cell-cell adhesions. Rho influences the integrity of adherens junctions probably due to the modulation of endocytosis or actin polymerization at cross cell-cell contacts (7). Inhibition of Rho removes cadherin complexes from zonular adherens junctions before significant changes in morphology are observed (26,27). It is also probably due to actomyosin contraction, which prevents the localized polymerization of actin filaments required for cell migration. It was reported that inhibition of RhoA or ROCK converted rat liver epithelial cells at wound edge into leader-like cells (28). Consistently, inhibition of RhoA enhanced wound healing. It suggests that RhoA-dependent contraction does not contribute to wound healing of HSC-3 monolayer cells.

The involvement of PAK1 in coordinated cell movement is unclear. Constitutively active PAK1 is involved in hepatocyte growth factor-induced scattering of

MDCK colonies (25,29). Unexpectedly, constitutively active PAK1 retarded spheroid scattering and wound healing. *In vitro* kinase assay revealed that PAK1 kinase activity fluctuated slightly during the healing process. This may be related to the dynamic nature of the process. It has been reported that in wounded fibroblast monolayer, phosphorylation of PAK1 increased during the first hour after wounding, and then declined. Moreover, phosphorylated PAK1 was localized in cells immediately lining the wound and several cell layers back from the wound edge (30). These suggest that regulated PAK1 activity may be important for coordinated cell movement. The mechanisms by which PAK1 induced suppression of the coordinated cell movements are not fully understood. An important future direction is to determine whether PAK1 is involved in cell-cell adhesions. It is possible that active PAK1 increases actin polymerization at the cell-cell junctions. This may help stabilize newly formed adherens junctions. In MDCK cells, stable expression of low level active Rac, an activator of PAK1, reverted Ras-transformed a mesenchymal phenotype to an epithelial phenotype, with an increase in actin polymerization at the cell-cell junctions (31).

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The altered expression of a variety of genes that regulate the actin cytoskeleton suggests an important role of Rho GTPases for tumor metastasis. This thesis analyzes the role of Rho GTPases and their effectors in integrin-mediated motility. This will lead to a better understanding of how ECM proteins restrain or promote head and neck SCC cell motility *in vitro* via integrin-mediated signals.

Both the ECM and integrins are important regulatory components of tumor cell invasion and migration. Two invasive head and neck SCC cell lines, HSC-3 and SCC-10A, deposit laminin-5 and laminin-10/11 but not laminin-2 (merosin). Using ECM ligands, including type I collagen, laminin-5, and laminin-10/11, α3 integrin signaling is identified to promote cell migration compared to α2 integrin signaling.

It is well known that SCC cell motility relies on laminin-5 and α3 integrins (1-4). We have advanced the study on the laminin-5-α3 integrin signaling pathway, and shown that laminin-5 inactivates the RhoA pathway, activates Rac1, and activates the Cdc42/PAK1 pathway more specifically. PAK1 activation enhances haptotaxis toward immobilized laminin-5.

The collagen I and α2 integrin signaling activates the RhoA pathway, leading to more focal adhesion formation. Inactivating RhoA, or its downstream effector ROCK, decreases focal adhesions and promotes cell motility (random cell movement and

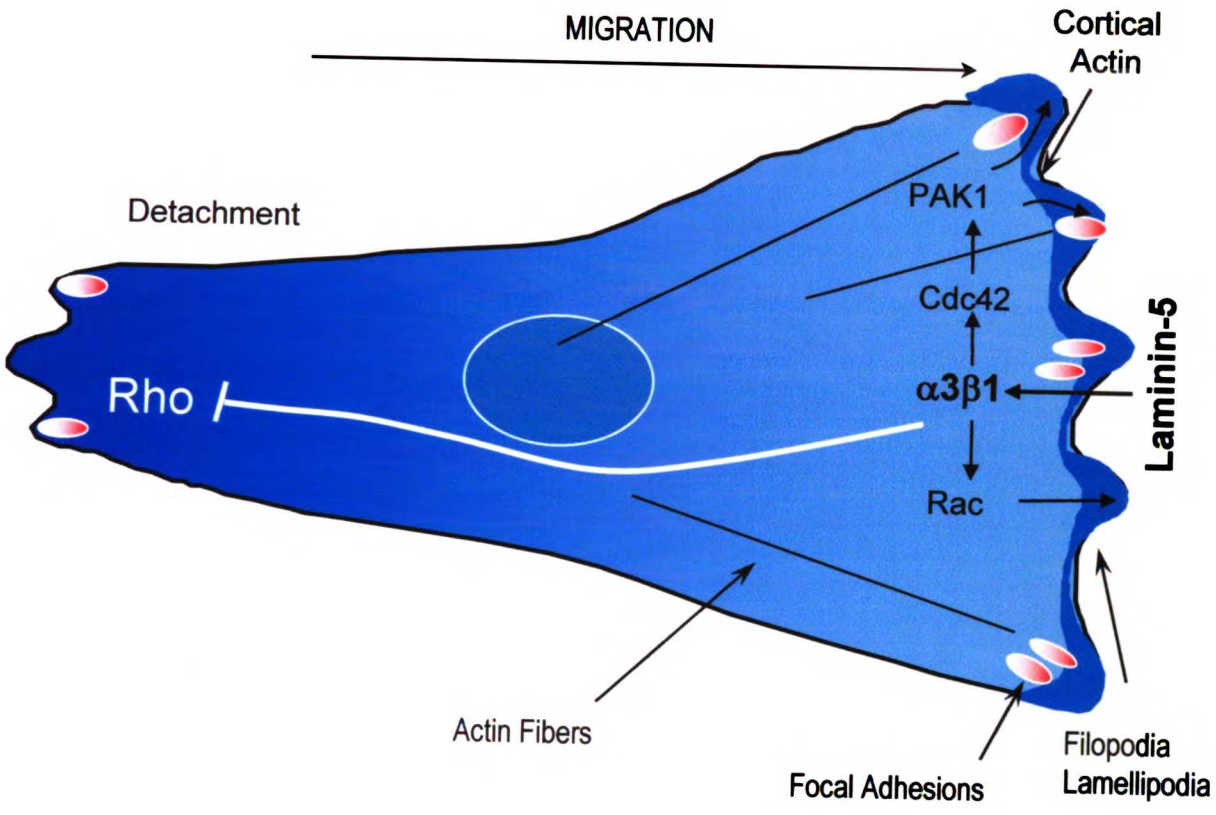
haptotaxis toward immobilized collagen) and invasion of collagen gel. Treatment with ROCK inhibitor Y-27632 causes persistent migration on collagen I but impairs contraction of the cell rear. The collagen I and α2 integrin signaling activates Rac1 and Cdc42 as well. But it does not strongly activate PAK1. Activating PAK1 promotes haptotaxis toward immobilized collagen I.

In a study of coordinated cell movement, where disruption of cell-cell contacts is involved, the decompaction of MCA is completely inhibited by expression of V14RhoA, and is suppressed by expression of E423 PAK1. Similarly, inactivating RhoA by expression of N19RhoA enhances wound healing, whereas expression of E423 PAK1 suppresses wound healing.

In summary, our results suggest mechanisms for restraining or promoting cell locomotion via integrin-mediated regulation of Rho GTPases and their effectors (Fig. 1). Inactivation of RhoA and activation of PAK1 promote cell motility, although PAK1 may have different roles in single cell movement and coordinated cell movement.

Inactivation of RhoA pathway by ROCK inhibitors has been implicated in the treatment of hepatoma (5,6) and melanoma (7). However, our *in vitro* studies suggest that ROCK inhibitors may not be useful to treat head and neck SCCs. Future experiments using mouse models should be performed to explore the *in vivo* role of RhoA in the tumor invasion process.

It is difficult to evaluate the activation status of Rho GTPases in tumor tissue. It has been reported that RhoA, Rac2, and Cdc42 are overexpressed in premalignent and head and neck SCC cells (8). However, increased expression level of Rho GTPases does not necessarily lead to increased activities of Rho GTPases (9). It is necessary to expand


MIGRATION
Cortical Actin
Detachment
PAK1
Cdc42
α3β1
Laminin-5
Rho
Rac
Actin Fibers
Focal Adhesions
Filopodia
Lamellipodia

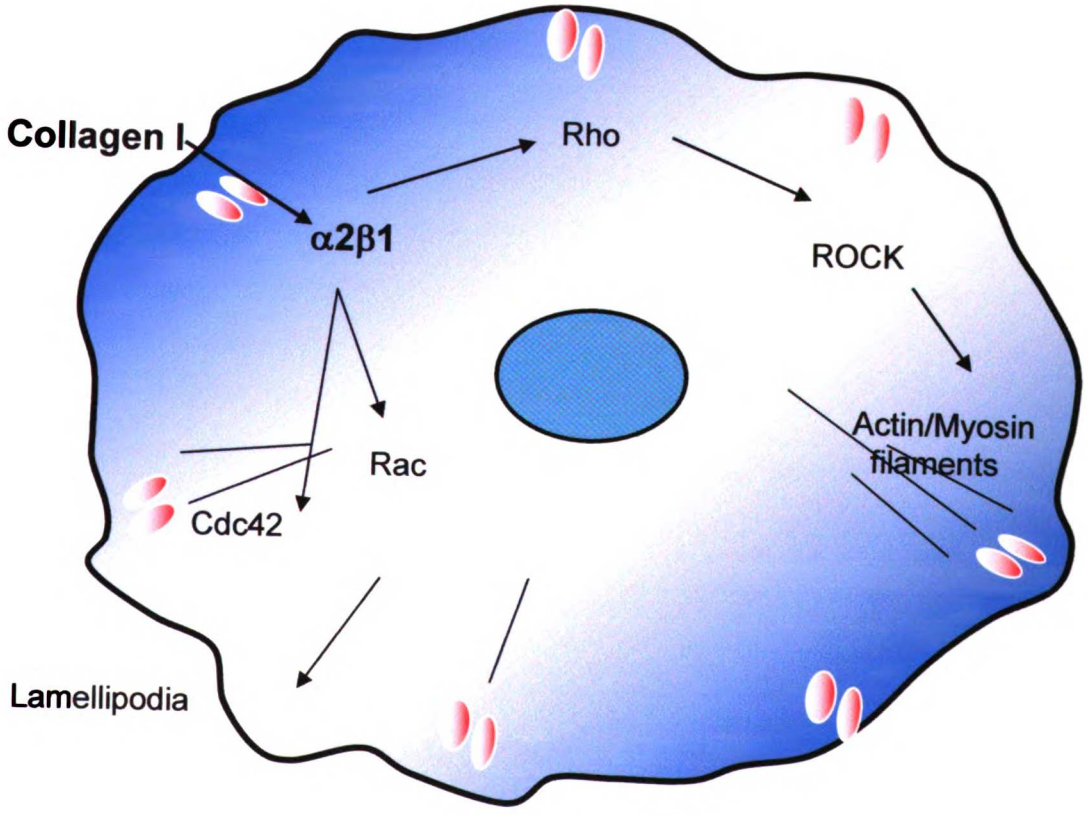

Collagen I
α2β1
Rho
ROCK
Actin/Myosin filaments
Rac
Cdc42
Lamellipodia

Figure 1. Differential signaling events in head and neck SCC cells on laminin-5 and collagen I. Laminin-5/α3β1 integrin interaction leads to inactivation of RhoA but stimulates the Cdc42/PAK1 pathway, which promotes cell migration. In contrast, collagen I/α2β1 interaction activates the RhoA/ROCK pathway, which stabilizes focal adhesions and the cytoskeleton thereby limiting locomotion.

the *in vitro* study of Rho GTPases to a variety of head and neck SCC cell lines in future experiments.

The mechanisms underlying regulation of Rho activities by ECM and integrins have not been well studied to date. Possibly, extracellular signaling could modify Rho GTPases through regulators such as GEFs, GAPs, and GDIs. It has been reported that p130Cas/CrkII/DOCK 180 complex is responsible for Rac activation after laminin-10/11-α3 integrin engagement (10), and that p190 RhoGAP is responsible for RhoA inactivation (11,12). It is not known how Cdc42 is activated after integrin engagements. Studies on PIX, a GEF for Cdc42/Rac, have drawn some attention recently (13,14). Interaction of αPIX with β-parvin suggests an involvement of αPIX in integrin-mediated signaling (15). It will be interesting to see whether PIX is involved in α3 integrin-mediated activation of Cdc42.

The mechanisms by which PAK1-induced suppression of coordinated cell movement are not fully understood. It is possible that PAK1 might be involved in cell-cell adhesions through modulation of the cytoskeleton. More experimentation will be required to answer this question.

Metastasis is perhaps the most challenging aspect for treating progressive cancer. This study helps explain some of the underlying cell-matrix interactions and signaling mechanisms that regulate tumor cell invasion and migration, and may provide potentially useful information for therapeutic interventions.

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