$\alpha 4\beta 1$ Integrin Regulates Lamellipodia Protrusion via a Focal Complex/Focal Adhesion-independent Mechanism[□]

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Submitted May 30, 2002; Revised May 30, 2002; Accepted June 20, 2002

Monitoring Editor: Mary C. Beckerle

$\alpha 4\beta 1$ integrin plays an important role in cell migration. We show that when ectopically expressed in Chinese hamster ovary cells, $\alpha 4\beta 1$ is sufficient and required for promoting protrusion of broad lamellipodia in response to scratch-wounding, whereas $\alpha 5\beta 1$ does not have this effect. By time-lapse microscopy of cells expressing an $\alpha 4$ /green fluorescent protein fusion protein, we show that $\alpha 4\beta 1$ forms transient puncta at the leading edge of cells that begin to protrude lamellipodia in response to scratch-wounding. The cells expressing a mutant $\alpha 4$ /green fluorescent protein that binds paxillin at a reduced level had a faster response to scratch-wounding, forming $\alpha 4$ -positive puncta and protruding lamellipodia much earlier. While enhancing lamellipodia protrusion, this mutation reduces random motility of the cells in Transwell assays, indicating that lamellipodia protrusion and random motility are distinct types of motile activities that are differentially regulated by interactions between $\alpha 4\beta 1$ and paxillin. Finally, we show that, at the leading edge, $\alpha 4$ -positive puncta and paxillin-positive focal complexes/adhesions do not colocalize, but $\alpha 4\beta 1$ and paxillin colocalize partially in ruffles. These findings provide evidence for a specific role of $\alpha 4\beta 1$ in lamellipodia protrusion that is distinct from the motility-promoting functions of $\alpha 5\beta 1$ and other integrins that mediate cell adhesion and signaling events through focal complexes and focal adhesions.

INTRODUCTION

Cell migration is essential for a variety of biological events, including embryonic development, wound healing, inflammation, and metastasis of malignant cells. Cell migration along a substratum is regulated by extracellular signals transduced into cells partly through adhesive interactions between the cell and its surrounding extracellular matrix (ECM). Integrins, the major receptors that mediate cell–ECM interactions (Hynes, 1992), play important roles in regulating cell motility.

Integrins are a large family of heterodimeric cell adhesion receptors. Many integrins, including $\alpha 5\beta 1$ and $\alpha V\beta 3$, mediate cell–ECM adhesion by forming junctional complexes

called focal adhesions, which bind extracellularly to specific ECM components and intracellularly to cytoskeletal proteins and signaling molecules. In cultured adherent cells, such as fibroblasts, focal adhesions play key roles in regulating motility (Lauffenburger and Horwitz, 1996; Horwitz and Parsons, 1999). When fibroblasts begin to migrate on an ECM substratum, small nascent focal complexes assemble in plasma membrane protrusions at the leading edge of the cell. These complexes grow larger and subsequently recruit $\alpha 5\beta 1$ and other integrins as they evolve into highly organized focal adhesions (Laukaitis *et al.*, 2001). As a cell moves forward, focal adhesions not only act as anchors but also function as nucleation and activation sites for signaling proteins, which in turn activate an intracellular signaling network, leading to actin cytoskeletal reorganization and generation of cell motility (Lauffenburger and Horwitz, 1996).

$\alpha 4\beta 1$, a member of the integrin family, is not localized in focal adhesions in most cell types, yet this integrin also plays important roles in cell migration. $\alpha 4\beta 1$ binds to an alternatively spliced V25 (also called CS-1) region of fibronectin (FN) (Wayner *et al.*, 1989; Guan and Hynes, 1990) instead of the RGD sequence that is recognized by $\alpha 5\beta 1$ and other integrins localized to focal adhesions (Pytela *et al.*, 1985). $\alpha 4\beta 1$ also binds to vascular cell adhesion molecule-1

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.02–05–0086. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.02–05–0086.

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□ Online version of this article contains video material for some figures. Online version available at www.molbiolcell.org.

Abbreviations used: CHO, Chinese hamster ovary; ECM, extracellular matrix; FN, fibronectin; GFP, green fluorescent protein; VCAM-1, vascular cell adhesion molecule-1.

(VCAM-1), a member of the immunoglobulin superfamily (Osborn *et al.*, 1989; Elices *et al.*, 1990). $\alpha 4\beta 1$ is expressed in many migratory cell types *in vivo*, including neural crest cells and their derivatives (Sheppard *et al.*, 1994; Kil *et al.*, 1998; Pinco *et al.*, 2001), smooth muscle cells of newly formed blood vessels (Sheppard *et al.*, 1994), hematopoietic cell lineages (Neuhaus *et al.*, 1991), and epicardial progenitor cells (Pinco *et al.*, 2001). Furthermore, the migration of neural crest cells and hematopoietic cells on FN can be disrupted in culture by antibodies that specifically inhibit binding between $\alpha 4\beta 1$ and FN (Yednock *et al.*, 1992; Kil *et al.*, 1998; Testaz *et al.*, 1999), and progenitor cells fail to migrate on the heart to form the epicardium in mouse embryos deficient in $\alpha 4\beta 1$ (Sengbusch *et al.*, 2002).

Although an important role for $\alpha 4\beta 1$ in cell migration has been well documented, questions remain as to how $\alpha 4\beta 1$ promotes cell migration. Because $\alpha 4\beta 1$ is not localized in focal adhesions in most cell types and has a ligand-binding specificity different from integrins in focal adhesions, this integrin may promote cell migration by a mechanism distinct from that of $\alpha 5\beta 1$ and other integrins in focal adhesions. This idea is also supported by an observation that the cytoplasmic tails of $\alpha 4$ and $\alpha 5$ subunits confer different cellular activities with the $\alpha 4$ tail conferring migratory activities and the $\alpha 5$ tail conferring adhesive activities (Chan *et al.*, 1992; Kassner *et al.*, 1995).

In this article, we examined the migratory behaviors of Chinese hamster ovary (CHO) cells ectopically expressing $\alpha 4\beta 1$, by using a scratch-wound assay. Our data show that $\alpha 4\beta 1$ plays a unique role in promoting lamellipodia protrusion through a focal complex/focal adhesion-independent mechanism.

MATERIALS AND METHODS

Construction of Plasmids

For expressing green fluorescent protein (GFP)-tagged $\alpha 4$ integrin in CHO cells, we constructed a plasmid pQN4G. To construct this plasmid, upstream human $\alpha 4$ cDNA (Takada *et al.*, 1989; obtained from American Type Culture Collection, Rockville, MD) and downstream mouse $\alpha 4$ cDNA (Neuhaus *et al.*, 1991; a generous gift from Dr. Martin Hemler, Dana-Farber Cancer Institute, Boston, MA) were joined at a unique and conserved *KpnI* site, and the 3' end of this chimeric $\alpha 4$ cDNA was ligated to the 5' end of GFP cDNA by insertion into PGIB25-fN1 GFP plasmid vector (Quantum Biotechnologies, Montreal, Quebec, Canada). The fusion protein's expression was driven by a cytomegalovirus promoter. The pQN4Y991AG plasmid was the same as pQN4G except that the tyrosine at position 1093, equivalent to position 991 in human $\alpha 4$ cDNA product, of the $\alpha 4$ tail region was mutated to alanine by polymerase chain reaction.

Purified Ligands

Mouse plasma FN was purchased from Invitrogen (Carlsbad, CA). A recombinant FN fragment containing FN type III repeats 12–15 and the CS-1 region and recombinant soluble VCAM-1 (Lobb *et al.*, 1991) were provided by Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA) and Roy Lobb (Biogene, Cambridge, MA), respectively.

Cells, Transfections, and Cell Culture

CHO cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), L-glutamine, and antibiotics. CHO- $\alpha 4$ cells (Kassner *et al.*, 1995), provided by Martin

Hemler (Dana-Farber Cancer Institute), were maintained in F-12 (Invitrogen) containing 10% FBS, L-glutamine, antibiotics, and 0.4 mg/ml G418 (Life Invitrogen). CHOB2, CHOB2- $\alpha 4$, and CHOB2- $\alpha 5$ cell lines, provided by Cary Wu (University of Pittsburgh, Pittsburgh, PA) and Michael DiPersio (Albany Medical College, Albany, NY), were maintained in minimal essential medium- α (Invitrogen) containing 10% FBS, L-glutamine, antibiotics, and 0.4 mg/ml G418. CHO cells were transfected with pQN4G and pQN4Y991AG by using Lipofectin/Optimem (Invitrogen) following manufacturer's instructions. CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP clones were selected in F-12 containing 0.8 mg/ml G418 and screened for $\alpha 4$ /GFP and $\alpha 4$ Y991A/GFP expression by using fluorescence-activated cell sorting. Stably transfected cell lines were maintained using the initial concentration of G418.

Analysis of Lamellipodia Protrusion and $\alpha 4$ -Positive Puncta Formation at Edges of Scratch-Wounds

For the studies using time-lapse microscopy, cells were plated onto glass bottom Microwell dishes (MatTek, Ashland, MA) coated with 10 μ g/ml FN or VCAM-1 for 2 h at 37°C. At confluence, the cell monolayer was scraped with a Pipetman tip to generate scratch-wounds. The wounded surface was washed with phosphate-buffered saline (PBS) and then returned to serum-containing medium. After 2-h incubation, media were replaced with Leibovitz's L-15 medium (Invitrogen) containing 10% FBS. Migration at the wound edge was monitored by phase or fluorescence microscopy by using an Axiovert 135 TV microscope (Carl Zeiss, Thornwood, NY) equipped with a temperature controller (Harvard Apparatus, Holliston, MA). Cell movement was recorded with a charge-coupled device camera (Roper Photometrics, Trenton, NJ) by using IPLab-Spectrum software (Scanalytics, Fairfax, VA). The last frame of each time-lapse movie was analyzed for the percentage of cells at wound edges that protruded broad lamellipodia.

For studies using regular microscopy, cells were plated on tissue culture plates or coverslips coated with 10 mg/ml FN and scratch-wounded as described above. Nonoverlapping fields were photographed at designated time points by phase (250 $\times$) or fluorescence microscopy (630 $\times$). The percentage of cells at wound edges that protruded broad lamellipodia or formed $\alpha 4$ -positive puncta was scored using the phase or fluorescence micrographs, respectively. This method was also used in an antibody perturbation experiment on the fanning activity of CHOB2- $\alpha 4$ cells. In this experiment, an anti- $\alpha 4$ antibody P1H4 (Chemicon International, Temecula, CA), which is identical to a functional blocking antibody, P4C2, was added to the cells at 25 mg/ml. The cells were preincubated with the antibody for 2 h at room temperature, plated on FN and cultured in the presence of the antibody before and after scratch-wounding. At the 2-h time point, the cells at wound edges were photographed and scored for the percentage of cells at wound edges that protruded broad lamellipodia.

Flow Cytometry

Flow cytometry analysis was performed as described by Hildreth *et al.* (1999) with some modifications. Washed cells were resuspended at 2×10^6 cells/ml in PBS, containing 5% normal goat serum (Vector Laboratories, Burlingame, CA) and 1% bovine serum albumin (BSA) (PBS/NGS/BSA), and blocked on ice for 20 min. Cells (100 μ l) were mixed with 100 μ l of one of the following primary antibodies at 20 μ g/ml: mouse anti- $\alpha 4$ ($\alpha 4$ -PUJ1; Upstate Biotechnology, Lake Placid, NY), mouse anti-hamster $\alpha 5$, PB1 (Brown and Juliano, 1985), or mouse anti-hamster $\beta 1$, 7E2 (Brown and Juliano, 1988). PB1 and 7E2 were provided by Rudy Juliano (Department of Pharmacology, University of North Carolina, Chapel Hill, NC). After 45 min on ice, cells were washed with PBS and resuspended in 100 μ l of PBS/normal goat serum/BSA containing 20 μ g/ml of either fluorescein- or R-phycoerythrin-conjugated secondary antibodies (BioSource

International, Camarillo, CA). After 45 min and a final wash with PBS, cells were resuspended in 0.5 ml of 2% paraformaldehyde in PBS and analyzed on a FACStar Plus with an Innova-90 laser (Coherent, Santa Clara, CA) exciting at 488 nm wavelengths and running at 100 mW.

Adhesion Assay

The adhesion assay was performed as in Yang and Hynes (1996) with the following modifications. Triplicate wells of 96-well plates were coated with 10 $\mu\text{g}/\text{ml}$ FN, CS-1, or VCAM-1 at 37°C for 2 h. Then 5×10^4 cells were plated per well and allowed to adhere in a tissue culture incubator. After 15 min, nonadherent cells were removed by submerging the plate in PBS and shaking off the cells. Seven nonoverlapping high-power fields (200 $\times$) along the diameter of each well were photographed, and the number of adherent cells per field was counted.

Migration Assays

For the scratch-wound cell migration assay, the cells were plated on wells of 24-well tissue culture plates coated with 10 mg/ml FN and scratch-wounded as described above to generate scratch-wounds 0.28–0.56 mm in width. Scratch-wounds were allowed to heal in medium containing 10% FBS in a tissue culture incubator. Photographs were taken at designated time points with a phase microscope (Nikon, Melville, NY). By using the photographs, the distance cells migrated was calculated as a percentage of wound closure. For each data point, 10–30 nonoverlapping measurements were taken from multiple wells; mean and SDs were calculated from three independent experiments. Rates of cell proliferation were measured by immunohistochemical detection of 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) incorporation (Morgenbesser *et al.*, 1995). Cells were plated on FN-coated coverslips and wounded as described above. Fifteen minutes before the time point, the culture medium was replaced by medium containing 50 μM BrdU and 10% FBS. After 15-min culturing, cells were washed in PBS, fixed with cold methanol, washed with PBS, and treated with 1.5 M HCl in the tissue culture incubator for 40 min. Cells were then washed with PBS and stained with an anti-BrdU antibody (Sigma-Aldrich). For each cell line, microscopic fields (630 $\times$) from three coverslips were photographed. Percentage of cells with BrdU incorporation in each microscopic field was determined and mean and SDs were calculated.

The Transwell cell migration assay was performed as described in Liu *et al.* (1999) with the following exceptions. Transwell inserts were coated with 10 $\mu\text{g}/\text{ml}$ FN in serum-free F-12 for 2 h at 37°C. Media from the top chamber were replaced with 200 μl of cell suspension (1.5×10^5 cells/ml in F-12), and chambers were incubated for 4 h at 37°C.

Cell Surface Biotinylation and Immunoprecipitation

CHO- $\alpha 4$ cells were surface biotinylated by resuspending at 5×10^6 cells/ml in cell wash buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 1 mM CaCl_2 , and 5 mM MgCl_2) and incubating with EZ-Link NHS-LC-biotin (Pierce Chemical, Rockford, IL) for 60 min at room temperature. Cells were lysed for 15 min at 4°C in ice-cold extraction buffer (0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, and 0.02 mg/ml aprotinin in cell wash buffer). For coimmunoprecipitation studies, CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells were washed three times with PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.05% Tween 20, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 0.5 $\mu\text{g}/\text{ml}$ leupeptin) for 30 min at 4°C. The cell lysates were cleared with protein-G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to immunoprecipitation by using an anti- $\alpha 4$ antibody (5B10G; Upstate Biotechnology) and protein-G agarose beads. Immunoprecipitates were analyzed on Western blots for the presence of $\alpha 4$ /GFP, $\alpha 4$ Y991A/GFP,

and paxillin, by using an anti-GFP antibody (Molecular Probes, Eugene, OR) and an anti-paxillin antibody (Transduction Laboratories, Lexington, KY), respectively.

Fluorescence and Confocal Microscopy

Cells were plated on glass coverslips coated with 10 mg/ml FN and scratch-wounded. At 3 h or otherwise designated time points after scratch-wounding, the coverslips were collected. For GFP fluorescence, cells were washed three times in PBS, fixed for 15 min in 4% paraformaldehyde (Fluka Chemical, Ronkonkoma, NY) in PBS, and mounted. For immunofluorescence staining, cells were washed three times in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, permeabilized for 15 min in 0.5% NP-40 (Sigma-Aldrich) in PBS, and incubated with antibodies against paxillin (349 from Transduction Laboratories; and 165, a gift from Christopher Turner, SUNY Upstate Medical University, Syracuse, NY) at 37°C. After 30 min, coverslips were washed three times in PBS, incubated with a secondary antibody (BioSource International) at 37°C for 30 min, and washed three times in PBS. Both primary and secondary antibodies were diluted in 10% normal goat serum in PBS. Fluorescent images were obtained using an Axioskop 2 microscope (Carl Zeiss) in conjunction with a Coolsnap fx charge-coupled device camera (Photometrics, Tucson, AZ) controlled by IPLab-Spectrum software. Confocal images were obtained using the Oz confocal laser scanning microscope system (Noran, Middleton, WI), with Intervision software, version 6.5, on a Silicon Graphics O2 platform.

Online Supplemental Material

The online version of this article contains QuickTime movies that accompany Figures 1, 2, 5, and 7. The speed of the movies is 60 $\times$ faster than real time. Videos 1–5 accompany Figure 1, videos 6–8 accompany Figure 2, video 9 accompanies Figure 5, and video 10 accompanies Figure 7. Online supplemental material is available at www.molbiolcell.org

RESULTS

$\alpha 4 \beta 1$ Integrin Promotes Lamellipodia Protrusion When Ectopically Expressed in CHO Cells

It was previously shown that CHO cells express $\alpha 5 \beta 1$ but not $\alpha 4 \beta 1$ (Schreiner *et al.*, 1989; Kassner and Hemler, 1993). Using a Transwell assay, Martin Hemler and colleagues have shown that when $\alpha 4 \beta 1$ is ectopically expressed in CHO cells, this integrin enhances cell motility (Kassner *et al.*, 1995). To determine how $\alpha 4 \beta 1$ promotes cell motility, we used a scratch-wound assay and time-lapse microscopy to examine the migratory behaviors of a CHO cell line that stably expresses $\alpha 4$ (CHO- $\alpha 4$ cells) (Kassner *et al.*, 1995). In the scratch-wound assay, the CHO- $\alpha 4$ cells and the parental CHO cells were plated on FN-coated dishes; as the cells formed a confluent monolayer, a scratch-wound was made in the monolayer to induce cell migration into the wound. Two hours later (2-h time point), the cells at wound edges were imaged by time-lapse microscopy. As shown in Figure 1, A and B (Videos 1 and 2), CHO- $\alpha 4$ cells and the parental CHO cells displayed very different migratory behaviors. CHO cells at wound edges migrated as a mass. While the monolayer of CHO cells pushed toward the scratch-wound, individual cells protruded short-lived membrane extensions in random directions with little persistent polarity toward the wound. No prominent lamellipodia were observed (Figure 1A). In contrast, some CHO- $\alpha 4$ cells at the wound edge migrated into the wound as individual cells by forming

Figure 1. Time-lapse microscopy of CHO, CHO- $\alpha 4$, and CHO- $\alpha 4$ /GFP cells at wound edges. CHO cells on FN (row A), CHO- $\alpha 4$ cells on FN (row B), CHO- $\alpha 4$ /GFP cells on FN (row C), CHO- $\alpha 4$ cells on VCAM-1 (row D), and CHO- $\alpha 4$ cells on RGD (row E) were plated and wounded. Two hours after wounding, the cells were photographed every 2 min for 2 h. Four frames of each cell type at 0-, 40-, 80-, and 120-min time points are shown. Bar, 50 μ m.

fan-shaped broad lamellipodia with a persistent polarity toward the wound (Figure 1B). At the 4-h time point (2 h after starting to take the movies) 21% of all the cells at the wound edges exhibited this “fanning” behavior (Figure 3). We also performed the scratch-wound assay and photographed the cells at the 12- and 18-h time points. At these later time points, the majority of the CHO- $\alpha 4$ cells at wound edges had the fan shape, whereas none of the CHO cells at wound edges did (our unpublished data). We generated several stable cell lines expressing $\alpha 4$, which was tagged with GFP (CHO- $\alpha 4$ /GFP, see below for characterization of these cell lines), and found that all of the $\alpha 4$ /GFP-expressing cell lines also had the fanning phenotype (Figure 1C and Video 3) to a similar degree (Figure 3). Therefore, the fanning phenotype was not due to a cloning artifact.

$\alpha 4\beta 1$ Promotes Lamellipodia Protrusion Independent of $\alpha 5\beta 1$

The CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells also express $\alpha 5\beta 1$. Our flow cytometry analyses showed that the expression level of

$\alpha 5$ at the cell surface was slightly reduced in these cells (Figure 4A). Thus, the fanning behavior may be promoted directly by $\alpha 4\beta 1$, or indirectly due to decreased expression of $\alpha 5\beta 1$ at the cell surface. To distinguish between these possibilities and determine whether the fanning phenotype depends on $\alpha 4\beta 1$, we tested the CHO- $\alpha 4$ cells in the scratch-wound assay by using an $\alpha 4\beta 1$ -specific ligand, VCAM-1, or an $\alpha 5\beta 1$ -specific ligand, the RGD peptide, as the substrate. The cells fanned on VCAM-1 (Figure 1D and Video 4) but not on the RGD peptide (Figure 1E and Video 5). At the 4-h time point, CHO- $\alpha 4$ cells plated on RGD alone showed no evidence of fanning (Figure 3). However, 27% of the CHO- $\alpha 4$ cells on VCAM-1 at wound edges exhibited the fanning behavior. Because $\alpha 4\beta 1$ is the only receptor for VCAM-1 in CHO- $\alpha 4$ cells, this result indicates that the binding between $\alpha 4\beta 1$ and VCAM-1 is sufficient for the fanning phenotype, and that the RGD-recognizing integrins, including $\alpha 5\beta 1$, are not sufficient.

To test directly the roles of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ in the fanning phenotype, we took advantage of a CHO-derived cell line,

Figure 2. Time-lapse microscopy of CHO2, CHO2- $\alpha 5$ and CHO2- $\alpha 4$ cells at wound edges. CHO2 cells (row A), CHO2- $\alpha 5$ cells (row B), and CHO2- $\alpha 4$ cells (row C) were plated and wounded. Two hours after wounding, the cells were photographed every 2 min for 2 h. Three frames of each cell type at 0-, 40-, 80-, and 120-min time points are shown. Bar, 50 μ m.

named CHO2, which expressed a negligible level of $\alpha 5 \beta 1$ (Schreiner *et al.*, 1989). It has previously been shown that $\alpha 5 \beta 1$, when stably transfected into CHO2 cells, can rescue the ability of CHO2 cells to adhere to and migrate randomly on FN (Bauer *et al.*, 1992). When $\alpha 4 \beta 1$ is stably expressed in CHO2 cells, it can also rescue the cells for adhesion and migration (Wu *et al.*, 1995). We compared the $\alpha 5$ - and $\alpha 4$ -expressing CHO2 cell lines for their migratory behaviors at edges of scratch-wounds, by using FN as the substrate. The results are shown in Figure 2. CHO2 cells adhere poorly to FN. To examine their migratory behaviors at wound edges, the cells were plated and scratch-wounded, but the washes were omitted to allow the cells to remain on the dish. These cells (Figure 2A and Video 6) at wound

edges did not show any polarization, although the cells were able to protrude membrane extensions in a random manner. CHO2- $\alpha 5$ cells (Figure 2B and Video 7) adhered and migrated much in the same manner as the CHO cells. At the 4-h time point, CHO2 and CHO2- $\alpha 5$ both showed no evidence of fanning at wound edges (Figure 3). In contrast, some CHO2- $\alpha 4$ cells at wound edges (Figure 2C and Video 8) migrated by forming lamellipodia with persistent polarity toward the wound, although to a lesser extent compared with CHO- $\alpha 4$ cells. At the 4-h time point, 11% of CHO2- $\alpha 4$ cells at wound edges exhibited the fanning behavior (Figure 3). These results show that 1) $\alpha 5 \beta 1$ alone does not promote fanning on FN and $\alpha 4 \beta 1$ is required; and 2) $\alpha 4 \beta 1$ is sufficient to promote fanning on FN, but optimal fanning on FN also requires $\alpha 5 \beta 1$.

To test further whether $\alpha 4 \beta 1$ is required for the fanning phenotype, we performed an antibody perturbation experiment, by using an anti- $\alpha 4$ antibody that specifically disrupted binding between $\alpha 4 \beta 1$ and FN (Sechler *et al.*, 2000). When this antibody was added to scratch-wounded CHO2- $\alpha 4$ cells, considerably fewer cells exhibited the fanning activity than did the CHO2- $\alpha 4$ cells in the absence of the antibody (Table 1). This result indicates that the ligand-binding activity of $\alpha 4 \beta 1$ is required for promoting the fanning behavior.

We conclude that $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ play different roles in the migration of CHO cells at wound edges, and $\alpha 4 \beta 1$ plays a unique role in promoting lamellipodia protrusion.

Figure 3. Percentage of fanning cells at wound edges. The last frame of each movie in Figures 1 and 2 were analyzed. Cells at the edge of the scratch-wound ($n = 30$ –50) were scored for fanning, and the percentage of these cells was calculated. This was done for each of the following cell lines: CHO, CHO- $\alpha 4$, CHO- $\alpha 4$ /GFP, CHO- $\alpha 4$ Y991A/GFP, CHO2, CHO2- $\alpha 5$, and CHO2- $\alpha 4$ on a FN substrate as well as for CHO- $\alpha 4$ on both VCAM-1 and RGD.

$\alpha 4$ Integrin Is Functional When Tagged with GFP

To study the mechanisms by which $\alpha 4 \beta 1$ promotes lamellipodia protrusion, we attached GFP to the cytoplasmic tail of the $\alpha 4$ subunit, producing the $\alpha 4$ /GFP fusion protein. Two independent cell lines stably expressing $\alpha 4$ /GFP were generated and analyzed. Cells from both cell lines migrated in the same manner as the CHO- $\alpha 4$ cells (Figure 1C and Figure

Figure 4. GFP-tagged $\alpha 4$ integrin is functionally normal. (A) Flow cytometry analysis. Unlabeled CHO cells served as negative controls (column 1). Cells were labeled with either anti-human $\alpha 4$ (column 2), anti-hamster $\beta 1$ (column 3), or anti-hamster $\alpha 5$ (column 4). Fluorescence intensity is shown in logarithmic scale. (B) $\alpha 4$ /GFP, $\alpha 4$, or GFP alone was each transfected into CHO cells. The CHO- $\alpha 4$ cells (a) and the parental CHO cells (c) were stained with an anti- $\alpha 4$ antibody by immunofluorescence. $\alpha 4$ /GFP (b) localized at the surface of transfected cell in the same pattern as $\alpha 4$ in the CHO- $\alpha 4$ cells (a). The parental CHO cells had only background staining (c). GFP alone localized diffusely in the nucleus and cytoplasm and did not reach the cell surface (d). Bar, 10 μ m. (C) Number of adherent cells per high-power field ($n = 7$) was determined for each of the triplicate wells for CHO, CHO- $\alpha 4$, and CHO- $\alpha 4$ /GFP cells adhering to FN, CS-1, or VCAM-1. The mean values and standard deviations for the triplicates were graphed. Note that the CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cell lines but not the CHO cells adhered to CS-1 and VCAM-1. (D) Cells were photographed at 0-, 12-, and 18-h time points. The percentage of wound closure was determined ($n = 20$ –30) from at least two (3 for all but $\alpha 4$ /GFP) independent experiments. The mean values and SDs were graphed. Note that CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells both had a much faster wound closure rate than that of CHO cells, with no significant difference between the wound closure rates of CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP.

Table 1. Percentage of cells fanning at the wound edge at the 2-h time point

Total number of cells was 340 and 654 for no antibody and with antibody, respectively.

No antibody ^b	7.65%
With antibody ^c	1.83%

^a In the absence of anti- $\alpha 4$ antibody.

^b In the presence of anti- $\alpha 4$ antibody.

3). To determine more closely whether the GFP tag interfered with the function of $\alpha 4\beta 1$, we used the CHO- $\alpha 4$ cell line as a control to characterize the GFP-tagged $\alpha 4$ for its surface expression, adhesive activity, and migration-promoting activity. Flow cytometry analysis showed that the surface expression levels of $\alpha 4$ /GFP in the transfected cell lines were similar to that of $\alpha 4$ in the control CHO- $\alpha 4$ line (Figure 4A). Both the CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cell lines also had surface expression levels of $\beta 1$ similar to CHO cells, yet they had slightly decreased levels of $\alpha 5$ compared with the parental CHO cells. We also compared the surface distribution of $\alpha 4$ with and without the GFP tag (Figure 4B). $\alpha 4$ /GFP and $\alpha 4$ were both expressed over the entire cell surface when the cells were plated sparsely, as assayed by

Table 2. Proliferation rates of CHO, CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells in wound assays

	% Cells with BrdU*
CHO	58 $\pm$ 10
CHO- $\alpha 4$	54 $\pm$ 8
CHO- $\alpha 4$ /GFP	55 $\pm$ 10

*Numbers represent the mean and standard deviation of the percentages of cells with BrdU incorporation (n = 523, n = 394, n = 288 cells for CHO, CHO- $\alpha 4$, and CHO- $\alpha 4$ /GFP, respectively). There are no statistically significant differences among the three cell lines ($p > 0.1$).

GFP fluorescence and anti- $\alpha 4$ immunofluorescence, respectively. $\alpha 4$ and $\alpha 4$ /GFP were also both detected within the cells, possibly in the ER and Golgi complexes. In control cells transfected with GFP cDNA alone, GFP was expressed in the cytoplasm and nucleus but not on the cell surface. These data showed that $\alpha 4$ /GFP was distributed normally at the cell surface.

Because $\alpha 5 \beta 1$ binds to the RGD region of FN (Pierschbacher and Ruoslahti, 1984), and $\alpha 4 \beta 1$ binds to the CS-1 region of FN, we predicted that the parental CHO cells, which express $\alpha 5 \beta 1$ but not $\alpha 4 \beta 1$, should adhere to full-length FN (containing both regions) but not to a fragment of FN that contains only the CS-1 region. In contrast, CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP should adhere to both. Because $\alpha 4 \beta 1$ also binds to VCAM-1, we predicted that, if the ($\alpha 4$ /GFP) $\beta 1$ protein was functional, the transfected cells would adhere to VCAM-1, whereas the parental CHO cells would not. Cell adhesion assays on these cells fulfilled our predictions (Figure 4C), demonstrating that ($\alpha 4$ /GFP) $\beta 1$ has normal adhesive activities.

As discussed above, we have shown that CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells both display a fanning behavior at edges of scratch-wounds. We also compared their rates of wound closure and found that both CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells closed the scratch-wounds much faster than the CHO cells, with the migration rates of CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells not significantly different from each other (Figure 4D). In parallel with the wound assay, cell proliferation rates were measured for these cell lines under the same plating and wounding conditions as the cells in the wound assays. Our data showed that CHO, CHO- $\alpha 4$, and CHO- $\alpha 4$ /GFP cells proliferated at the same rate (Table 2). Thus, the faster wound-closure rates of CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells were not due to a faster proliferation rate but due to a faster migration rate of these cells.

In summary, we show that ($\alpha 4$ /GFP) $\beta 1$ has the same localization, adhesive activities, and migration-promoting activities as untagged $\alpha 4 \beta 1$. We conclude that ($\alpha 4$ /GFP) $\beta 1$ is functionally normal.

$\alpha 4 \beta 1$ Forms Transient Puncta at Leading Edge of Cells That Begin to Protrude Lamellipodia in Response to Scratch-Wounding

Using a fluorescence microscope attached to a time-lapse imaging system, we examined the surface dynamics of ($\alpha 4$ /GFP) $\beta 1$ on the CHO- $\alpha 4$ /GFP cells that displayed fanning

activity (Figure 5). At low magnification, ($\alpha 4$ GFP) $\beta 1$ was seen over the entire surface of the cells. The $\alpha 4$ /GFP fluorescence was particularly strong in membrane ruffles at the leading edge (arrowheads in Figure 5, a–c). At higher magnification, transient $\alpha 4$ /GFP-positive puncta were found in cells that had just begun to fan into the scratch-wounds. The $\alpha 4$ /GFP-positive puncta were seen in some areas at the leading edge where the ruffles flattened out and the membrane extended into smooth edged but small lamellipodia (arrows in Figure 5, e and k). The puncta were located right along the leading edge of these small lamellipodia. As the lamellipodia continued to extend and a new cell front formed, the puncta stayed at their original positions (arrow in Figure 5f). The extended membrane then began to ruffle again, whereas the $\alpha 4$ /GFP puncta gradually disappeared (Figure 5 g, h, and l). As the new cell front was ruffling, $\alpha 4$ /GFP fluorescence was again seen in the ruffles (arrowheads in Figure 5, j and l). This sequence of events was repeated continuously as the cell formed a broad lamellipodia and moved forward toward the wound (Video 9). This pattern of surface dynamics may be related to the unique function of $\alpha 4 \beta 1$ in promoting broad lamellipodia protrusion.

Disrupting $\alpha 4$ /Paxillin Binding Allows a Faster Response of Cells to Scratch-Wounding That Correlates with Formation of $\alpha 4$ -Positive Puncta at the Leading Edge

Paxillin is a signaling adaptor protein (Turner, 2000), which binds to the cytoplasmic tail of $\alpha 4$ (Liu *et al.*, 1999). A point mutation at the $\alpha 4$ tail, Y991A, disrupts this binding (Liu *et al.*, 1999). It has been shown that this mutation reduces random cell motility (Liu *et al.*, 1999). We reasoned that, if this mutation affects the $\alpha 4 \beta 1$ -dependent fanning phenotype as well as the formation of the $\alpha 4$ -positive puncta at the leading edge, we would be able to establish a mechanistic relationship between fanning and puncta formation. Therefore, we generated and analyzed two independent CHO cell lines that stably express $\alpha 4$ cDNA carrying this mutation; the mutant $\alpha 4$ was tagged with GFP (the cell lines are referred to as CHO- $\alpha 4$ Y991A/GFP). To confirm that the Y991A mutation disrupted the paxillin-binding in the CHO- $\alpha 4$ Y991A/GFP cells, we performed a coimmunoprecipitation experiment. We showed that the amount of paxillin that coimmunoprecipitated with the mutant $\alpha 4$ was much less than that coimmunoprecipitated with the wild-type $\alpha 4$ (Figure 6B). We also showed that both $\alpha 4$ /GFP and $\alpha 4$ Y991A/GFP remained intact when expressed in CHO cells (Figure 6A). To confirm that the Y991A mutation reduces random motility as reported by Ginsberg and colleagues (Liu *et al.*, 1999), we compared the abilities of the CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells to migrate in the Transwell assay. We found that the motility of CHO- $\alpha 4$ Y991A/GFP cells on FN was reduced by ~55% (Figure 6C).

We then compared the CHO- $\alpha 4$ Y991A/GFP and CHO- $\alpha 4$ /GFP cells by using the scratch-wound assay. To our surprise, all CHO- $\alpha 4$ Y991A/GFP cell lines, when plated on FN and tested in the scratch-wound assay, had a faster wound closure rate than CHO- $\alpha 4$ /GFP cells (Figure 6D). The faster wound closure rate was not due to faster cell proliferation, because CHO- $\alpha 4$ Y991A/GFP and CHO- $\alpha 4$ /

Figure 5. Time-lapse microscopy of $\alpha 4$ /GFP on the surface of CHO- $\alpha 4$ /GFP cells migrating into a scratch-wound. CHO- $\alpha 4$ /GFP cells were plated and wounded. After 3 h, the cells with fanning activity were photographed under a fluorescence microscope at 2-min intervals for 26 min. Three frames at low magnification (a–c) and nine frames at high magnification (d–l) are shown representing this time period. Asterisks are fixed reference marks to highlight the movement of the cell. Note that $\alpha 4$ /GFP fluorescence was very strong in ruffles (arrowheads). As the ruffles flattened out, $\alpha 4$ /GFP fluorescence was seen as puncta along the leading edge of small lamellipodia protrusions (e and k, arrows). As the leading edge continued to extend forward, the puncta of fluorescence stayed at a fixed position behind of the new cell front (f, arrow), and they disappeared when the cell front formed new ruffles (j and l, arrowheads). Bars, 10 μ m.

GFP cells had the same proliferation rates as assayed under the same plating and wounding conditions as in the wound assays (Table 3). The same results were obtained from two independent cell lines, indicating that the faster wound closure rate was not due to a cloning artifact. This result suggests that the scratch-wound assay and the Transwell assay measure different types of motile activities.

We suspected that the faster wound closure rate of the mutant cells was likely due to enhanced fanning activity of the CHO- $\alpha 4$ Y991A/GFP cells at wound edges. To test this idea, we compared the migratory behaviors of CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells at wound edges by time-lapse microscopy and found that the CHO- $\alpha 4$ Y991A/GFP cells indeed displayed a much higher degree of fanning at wound edges (Figure 7A and Video 10). At the 4-h time point (2 h after starting the time-lapse movie), the percentage of the CHO- $\alpha 4$ Y991A/GFP cells that exhibited fanning activity exceeded that of CHO- $\alpha 4$ and CHO- $\alpha 4$ /GFP cells by at least 45% (Figure 3). To evaluate the fanning activities more closely, the CHO- $\alpha 4$ Y991A/GFP and CHO- $\alpha 4$ /GFP

cells at wound edges ($n = \sim 300$) were photographed at 0.5-, 1-, 2-, and 3-h time points and scored for the percentage of cells that displayed the fanning behavior (Figure 7B). We found that at the 0.5-h time point, the CHO- $\alpha 4$ /GFP cells at wound edges had little fanning activity ($< 5\%$ fanning cells), whereas at this time point, $\sim 30\%$ fanning CHO- $\alpha 4$ Y991A/GFP cells was seen at wound edges (Figure 7B). There was a steady increase of the percentage of fanning cells for both cell types over time. At the 3-h time point, while the percentage of fanning CHO- $\alpha 4$ /GFP cells remained low (18.6%), that of fanning CHO- $\alpha 4$ Y991A/GFP cells reached 53.8%. This result showed that the CHO- $\alpha 4$ Y991A/GFP cells responded to scratch-wounding faster and fanned earlier than the CHO- $\alpha 4$ /GFP cells.

To relate the formation of $\alpha 4$ -positive puncta at the leading edge to the fanning activity, we examined a large number of CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells at wound edges and scored the percentage of cells with the $\alpha 4$ -positive puncta ($n = \sim 110$) (Figure 8K). The $\alpha 4$ -positive puncta were found at the leading edge of both cell types

Figure 6. Characterization of CHO- $\alpha 4$ Y991A/GFP cells. (A) Tagged GFP was not cleaved from the $\alpha 4$ /GFP fusion protein when the protein is expressed in CHO cells. CHO- $\alpha 4$ cells were surface biotinylated, lysed, and immunoprecipitated with an anti- $\alpha 4$ antibody, by which $\alpha 4$ integrin was detected as cleavage fragments of 70 and 80 kDa. CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells were lysed and subjected to immunoblot analysis by using an anti-GFP antibody, which revealed a 100-kDa cleavage fragment of $\alpha 4$ /GFP fusion protein (the 70-kDa cleavage fragment of $\alpha 4$ plus 30 kDa of GFP), showing that the tagged GFP was not cleaved from the fusion protein. (B) Y991A mutation reduced binding between $\alpha 4$ and paxillin. CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells were lysed, immunoprecipitated with an anti- $\alpha 4$ antibody, and analyzed by immunoblotting by using anti-paxillin or anti-GFP antibody. Total cell lysate for each cell type was analyzed alongside. Note that the amount of paxillin that coimmunoprecipitated with $\alpha 4$ Y991A/GFP was significantly less than that with the wild-type $\alpha 4$ /GFP. (C) Transwell cell migration assay with the membrane coated on both sides with FN. The number of cells migrated per field ($n = 3$) was determined for each of the triplicate wells for CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells. Data were means and standard derivations of triplicate experiments ($p < 0.001$). Note that the Y991A mutation reduced random cell motility. (D) Cells were photographed at 0-, 6-, and 12-h time points. The percentage of wound closure was determined ($n = 10$), and the mean values and SDs were graphed ($p < 0.01$). Note that the Y991A mutation enhanced the wound closure rate.

(Figure 8, E and F), and the cells that displayed the $\alpha 4$ -positive puncta were largely those that had just begun to fan and migrate into the scratch-wounds, which were frequently found among CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells at the 3-h (Figure 8C) and 0.5-h (Figure 8B) time points, respectively. In these cells, the puncta were again located along the leading edge of small, newly formed lamellipodia protrusions (arrows in Figure 8, E and F). However, the cells that had already formed broad lamellipodia and migrated into the wounds did not display the $\alpha 4$ -positive puncta at the leading edge (Figure 8D). Therefore, while the CHO- $\alpha 4$ Y991A/GFP cells had fanning activity at earlier time points after scratch-wounding than the CHO- $\alpha 4$ /GFP cells, the onset of puncta formation was also earlier in these cells. To compare the fanning activity and puncta formation more closely, we scored CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP

Table 3. Proliferation rates of CHO- $\alpha 4$ /GFP and CHO- $\alpha 4$ Y991A/GFP cells in wound assay

The numbers represent the mean and standard deviation of the percentages of cells with BrdU incorporation ($n \approx 500$). There are no statistically significant differences between the two cell lines ($p > 0.1$).

	% Cells with BrdU	
	6 h	12 h
CHO- $\alpha 4$ /GFP	39 ± 7	52 ± 7
CHO- $\alpha 4$ Y991A/GFP	38 ± 6	43 ± 10

cells at wound edges for the percentage of cells with fanning activity ($n \approx 300$) at the 0.5- and 3-h time points after scratch-wounding, by using phase micrographs at a lower magnification, which provided a better view of cell morphology at wound edges (Figure 8, G–J). The cells with fanning activity were placed in one of two categories: 1) initiating fanning or 2) having formed broad lamellipodia and migrated into the scratch-wounds. We found that the degree to which cells had initiated fanning at the wound edge correlated with the percentage of cells exhibiting $\alpha 4$ -positive puncta. CHO- $\alpha 4$ /GFP cells at the 0.5-h time point (Figure 8G) showed little fanning activity at the wound edges and little evidence of puncta formation (Figure 8K). However, by the 3-h time point, when some cells began to fan and move into the wound (Figure 8I), the appearance of puncta increased correspondingly (Figure 8K). On the other hand, CHO- $\alpha 4$ Y991A/GFP cells had considerable number of cells initiating fanning at the 0.5-h time point (Figure 8H), and at this time point a corresponding percentage of the cells had the $\alpha 4$ -positive puncta (Figure 8K). By the 3-h time point (Figure 8J) when the majority of the CHO- $\alpha 4$ Y991A/GFP cells have formed broad lamellipodia and migrated into the scratch-wounds, the percentage of the cells with $\alpha 4$ -positive puncta had drastically decreased (Figure 8K). These results show that there is a close correlation between the formation of $\alpha 4$ -positive puncta and the initial protrusion of broad lamellipodia into the scratch-wound.

$\alpha 4 \beta 1$ Colocalizes with Paxillin Partially in Leading Edge Ruffles But Is Not Localized in Focal Adhesions and Focal Complexes

To understand how the paxillin-binding regulates the lamellipodia-promoting activity of $\alpha 4 \beta 1$, we performed immunofluorescence studies to determine whether $\alpha 4 \beta 1$ and paxillin colocalize at the leading edge. It has been shown by others that in the CHO cells that do not express $\alpha 4 \beta 1$, paxillin is localized in focal adhesions, focal complexes, and ruffles (Nakamura *et al.*, 2000). In migrating CHO cells, paxillin is recruited into newly formed focal complexes near the leading edge (Laukaitis *et al.*, 2001). We found that paxillin was also localized in these areas in CHO- $\alpha 4$ /GFP cells. ($\alpha 4$ /GFP) $\beta 1$ partially colocalized with paxillin in ruffles (Figure 9J, arrow). At the leading edge of cells migrating into scratch-wounds, ($\alpha 4$ /GFP) $\beta 1$ was localized in puncta as seen in the time-lapse studies (Figure 9F, arrows), but these $\alpha 4$ -positive puncta clearly did not colocalize with the paxillin-positive focal complexes, which are also seen at the lead-

Figure 7. Y991A mutation enhances cell fanning and migration into the scratch-wound. (A) CHO- α 4/GFP and CHO- α 4Y991A/GFP cells were plated on FN and wounded. Two hours after wounding, the cells were photographed every 2 min for 2 h. Four frames of both cell types at 0-, 40-, 80-, and 120-min time points are shown. Bar, 50 μ m. (B) CHO- α 4/GFP and CHO- α 4Y991A/GFP cells were plated on FN-coated coverslips and wounded. Percentages of cells at the wound edge ($n = \sim 300$) with fanning activity were scored at 0.5-, 1-, 2-, and 3-h time points.

ing edge. (α 4/GFP) β 1 was also absent from most of the paxillin-positive focal adhesions (Figure 9, C and F). A very small number of paxillin-positive focal adhesions overlapped with some α 4-positive spots (Figure 9F, arrowhead), but given that the majority of focal adhesions had no α 4 staining, we conclude that α 4 β 1 is not localized in focal adhesions. We also examined the CHO- α 4Y991A/GFP cells by paxillin staining and GFP fluorescence and found that the localization patterns of α 4 and paxillin were not altered by the Y991A mutation (our unpublished data). Paxillin and the mutant α 4 β 1 were both found in ruffles, although our biochemical data clearly showed that the binding between α 4 β 1 and paxillin was drastically reduced by the Y991A mutation (Figure 6B).

DISCUSSION

Cell migration is an integrated process involving multiple steps, including membrane protrusion, formation of stable attachments near the leading edge of the protrusion, forward locomotion of the cell body, release of adhesions, and cell rear retraction (Lauffenburger and Horwitz, 1996). The assembly and disassembly of focal adhesions play critical roles in the formation and release of stable attachments of the cell to its substratum (Webb *et al.*, 2002). The assembly of focal adhesions involves sequential recruitment of adhesion components, including integrins such as α 5 β 1, into nascent focal complexes at the leading edge of membrane protrusions (Laukaitis *et al.*, 2001). The integrins in focal complexes and focal adhesions not only provide anchors for the cells to generate motile force (Smilenov *et al.*, 1999) but also their

adhesive activities, when modulated, can regulate migration speed (DiMilla *et al.*, 1993; Cox *et al.*, 2001). Furthermore, when the cell forms membrane protrusions, these protrusions are stabilized by focal complexes that mediate stable cell-substratum adhesion. This relatively stable adhesion allows persistent membrane protrusions but is not required for the initial formation of the protrusions (Bailly *et al.*, 1998).

In this article, we provided evidence for a role of α 4 β 1 integrin in the formation of membrane protrusions that is independent of focal complexes and focal adhesions. We show that α 4 β 1 promotes broad lamellipodia protrusion when ectopically expressed in CHO cells that do not express this integrin endogenously, whereas α 5 β 1 does not have this effect. This protrusion-promoting activity of α 4 β 1 is consistent with an observation that the protrusive activity of T lymphocytes on FN can be inhibited by an anti- α 4 antibody but not an anti- α 5 antibody (Szabo *et al.*, 1995). In migrating cells, while α 5 β 1 is recruited into focal complexes (Laukaitis *et al.*, 2001), we show that α 4 β 1 forms transient puncta at the leading edge, which do not colocalize with focal complexes and focal adhesions. It is likely that the α 4-positive puncta contribute to the lamellipodia protrusion activity of the cells, because the Y991A mutation in the cytoplasmic tail of α 4 results in an earlier onset of α 4-positive puncta formation as well as earlier initiation of lamellipodia protrusion in response to scratch-wounding. We found that the α 4-positive puncta formed along the leading edge of small protrusions before they developed into broad lamellipodia, supporting a role of these puncta in the initiation stage of lamellipodia formation. The dynamic nature of the α 4-positive puncta

Figure 8. $\alpha 4$ -Positive puncta are present in cells that begin to protrude broad lamellipodia. CHO- $\alpha 4$ /GFP (A, C, E, G, and I) and CHO- $\alpha 4Y991A$ /GFP (B, D, F, H, and J) cells were plated on FN-coated coverslips and scratch-wounded. At 0.5- (A, B, F, G, and H) and 3-h (C, D, E, I, and J) time points, cells were fixed and analyzed by fluorescence (A–F) and phase microscopy (G–J). E and F were higher magnification of C and B, respectively. The histogram (K) shows the percentage of cells ($n = \sim 110$) with the $\alpha 4$ -positive puncta, and the percentages of cells ($n = \sim 300$) that begin to fan and of those that have migrated into the scratch-wounds. Note that at the 0.5-h time point, CHO- $\alpha 4$ /GFP cells (A and G) showed little fanning activity, whereas CHO- $\alpha 4Y991A$ /GFP cells (B and H) had already initiated fanning. By the 3-h time point, although CHO- $\alpha 4$ /GFP cells (C and I) had just begun to fan, many CHO- $\alpha 4Y991A$ /GFP cells (D and J) had already formed broad lamellipodia and migrated into the scratch-wounds. The percentage of cells with the presence of $\alpha 4$ -positive puncta closely correlated with the percentage of cells initiating fanning (K), in which the puncta were located along the leading edge of small lamellipodia protrusions (E and F, arrows). Asterisks (*) in C and B and arrowheads in E and F indicate locations of $\alpha 4$ -positive puncta. Bar, 10 μm .

strongly suggests that the puncta result from transient clustering of the $\alpha 4 \beta 1$ molecules.

While $\alpha 4 \beta 1$ is sufficient and required for CHO cells to protrude broad lamellipodia in response to scratch-wounding, under the same conditions CHO cells expressing endogenous or exogenous $\alpha 5 \beta 1$ only randomly protrude short-lived membrane extensions. This result indicates that $\alpha 5 \beta 1$ is not sufficient to promote broad lamellipodia protrusion, but optimal lamellipodia protrusion of CHO- $\alpha 4$ cells requires $\alpha 5 \beta 1$. This result is consistent with $\alpha 5 \beta 1$ playing a role in stabilizing broad lamellipodia after they are formed. We propose that transient clustering of $\alpha 4 \beta 1$ molecules at the leading edge mediates

strong but transient adhesion of the leading edge membrane to the ECM substrate, whereas the focal complexes evolve into focal adhesions that mediate stable adhesion. Both transient and stable adhesions are required for optimal protrusive activity, but the transient clustering of $\alpha 4 \beta 1$ may be the primary adhesive event that initiates broad lamellipodia protrusion in response to scratch-wounding.

Lamellipodia are broad membrane extensions of cells comprised of a planar meshwork of actin filaments (Small *et al.*, 1999). The formation of lamellipodia involves proteins that regulate actin dynamics, such as vasodilator-stimulated phosphoprotein (Reinhard *et al.*, 1992), Wiskott-Aldrich syn-

Figure 9. $\alpha 4\beta 1$ is localized in ruffles but not in paxillin-positive focal complexes/focal adhesions. CHO- $\alpha 4$ /GFP cells were wounded and allowed to heal for 3 h, after which the cells were fixed, stained with an anti-paxillin antibody, and analyzed by fluorescence (A–F) or confocal (G–J) microscopy. Paxillin (A, D, and G, single exposures) was localized in focal adhesions and ruffles. $\alpha 4$ /GFP was localized in ruffles and puncta at the leading edge (B, E, and H, single exposures). Paxillin and $\alpha 4$ /GFP were colocalized in ruffles, giving yellow color in double exposures (pointed by arrowheads in C, I, and J). An edge view of ruffles show that paxillin and $\alpha 4$ /GFP colocalize partially in the ruffles (J, oblique edge view of I). However, $\alpha 4$ /GFP did not localize with paxillin-positive focal complexes/focal adhesions (C and F, double exposures), and paxillin did not localize in $\alpha 4$ -positive puncta (pointed by arrows in F). The area marked between two asterisks (*) in J shows artifact colors of reflection from glass coverslips beneath the cell. Bar, 5 μ m.

drome protein (Rohatgi *et al.*, 1999; Winter *et al.*, 1999), and the Arp2/3 complex (Machesky and Gould, 1999), and their upstream regulators, including Rac (Hall, 1998) and its effector p21-activated kinase (Bagrodia and Cerione, 1999). Broad lamellipodia protrusion does not necessarily require cell-substratum contacts. When stimulated by soluble chemoattractants, some cell types can protrude broad lamellipodia rapidly in the absence of any contact with the substratum (Bailly *et al.*, 1998). But lamellipodia protrusion stimulated by mechanical cues does require cell-substratum contact (Pelham and Wang, 1997). We speculate that scratch-wounding somehow generated a mechanical cue at the wound edge, which induced clustering of $\alpha 4\beta 1$ molecules at the leading edge of the cells. The clustering event not only provides transient contact of the leading edge to the substratum but also may activate a signaling cascade leading to

actin cytoskeletal reorganization and the formation of broad lamellipodia. The cytoplasmic domain of $\alpha 4$ can be phosphorylated (Han *et al.*, 2001) and may play an active role in these signaling events. Therefore, we propose that while the integrins in focal complexes/focal adhesions mediate cell-substratum adhesion to stabilize protrusions, $\alpha 4\beta 1$ may act at the leading edge as a mediator for sensing migratory cues to initiate polarized protrusions. Besides $\alpha 4\beta 1$, other integrins may play a similar role at the leading edge. For example, activated $\alpha V\beta 3$ can be recruited to the leading edge of cells when stimulated with fibrinogen or basic fibroblast growth factor, and this recruitment is required for directional motility (Kiosses *et al.*, 2001). It is conceivable that integrin clustering and activation may play a similar role at the leading edge in regulating polarized membrane protrusions.

We show that the lamellipodia-promoting activity of $\alpha 4 \beta 1$ is negatively regulated by paxillin. This negative regulation is likely to occur in the ruffles at the leading edge where paxillin and $\alpha 4 \beta 1$ colocalize. We propose that the binding between paxillin and the $\alpha 4$ tail prevents $\alpha 4 \beta 1$ in the ruffles from clustering at the leading edge. This negative regulation may be relieved by an inside-out signaling pathway, which is activated in response to scratch-wounding. We found that, after scratch-wounding, the cells expressing $\alpha 4 Y 991 A$ began to fan sooner than the cells expressing wild-type $\alpha 4$, suggesting that ($\alpha 4 Y 991 A$) $\beta 1$ may be constitutively active in its lamellipodia-promoting activity due to reduced paxillin binding.

Interestingly, the Y991A mutation reduces random motility of the cells in Transwell assays while enhancing lamellipodia protrusion in response to scratch-wounding. Therefore, lamellipodia protrusion and random motility are distinct types of motile activities, which are differentially regulated by interactions between $\alpha 4 \beta 1$ and paxillin. Paxillin is a multidomain adaptor protein that is recruited into focal complexes and focal adhesions, where it provides docking sites for cytoskeletal and signaling proteins (Turner, 2000). Furthermore, paxillin has been shown to play essential roles in cell motility, possibly by recruiting signaling components into focal complexes via the PKL-PIX-PAK complex (Turner *et al.*, 1999) and by regulating the turnover of focal adhesions via a FAK-mediated pathway when paxillin and FAK interact in focal adhesions (Hagel *et al.*, 2002). Ginsberg and colleagues show that disrupting the binding between paxillin and $\alpha 4 \beta 1$ affects the kinetics of FAK phosphorylation. They propose that the $\alpha 4$ tail changes the kinetics of FAK phosphorylation through $\alpha 4$ /paxillin interactions and promotes random cell motility by allowing a more efficient activation of FAK signaling that is primarily mediated by the integrins in focal adhesions, including $\alpha 5 \beta 1$ (Liu *et al.*, 1999). Thus, in addition to the cooperative role of $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ in promoting lamellipodia protrusion as discussed above, these two integrins may also play a synergistic role in regulating random cell motility, where $\alpha 4 \beta 1$ somehow amplifies the $\alpha 5 \beta 1$ -mediated signaling events although not localized in focal adhesions. In fact, $\alpha 4 \beta 1$ can rescue the ability of CHO2 cells, which lack $\alpha 5 \beta 1$, to adhere and migrate on FN (Wu *et al.*, 1995).

Alternatively, paxillin may play a more active role. It has been reported that overexpression of a paxillin LD4 domain deletion mutant, which disrupted binding of paxillin to PKL and localization of PKL to focal adhesions, caused an increase in cell protrusiveness and random motility but an inhibition of the cells to migrate into scratch-wounds (West *et al.*, 2001). Therefore, paxillin/PKL binding and paxillin/ $\alpha 4 \beta 1$ binding seem to have opposite effects on cell motility, suggesting that paxillin may play an active role in achieving a balance between random and polarized lamellipodia protrusive activities.

In summary, we show that $\alpha 4 \beta 1$ can mediate specific events at the leading edge of migrating cells to initiate the formation of broad lamellipodia and that this lamellipodia-promoting activity of $\alpha 4 \beta 1$ is independent of focal complexes/focal adhesions. In addition, we demonstrated that lamellipodia protrusion and random motility can be differentially regulated by interactions between $\alpha 4 \beta 1$ and paxillin. These findings provide new insight into how cell

migration can be regulated by integrin-mediated cell-ECM interactions.

ACKNOWLEDGMENTS

We thank Martin Hemler, Michael Dipersio, and Cary Wu for the CHO- $\alpha 4$, CHO2- $\alpha 5$, and CHO2- $\alpha 4$ cell lines; Rudy Juliano for the PB1 and 7E2 antibodies; Christopher Turner for the anti-paxillin antibody; and Richard Hynes and Roy Lobb for the CS-1 and VCAM-1 peptides. We also thank Gina Hamlin (Johns Hopkins School of Medicine Center for Analytical Cytology), Doug Murphy and Michael Delannoy (Johns Hopkins School of Medicine Microscope Facility) Jennifer Sengbusch, Stephen Liu, and James Hildreth for technical assistance. We are grateful to Susan Craig and Richard Hynes for helpful discussions regarding this project, and to Susan Craig, Peter Devreotes, Katherine Wilson, Monn Monn Myat, and Michael Dipersio for helpful suggestions and critical comments on this article. This work was supported by a grant from the American Cancer Society (RPG-98-229-01-DDC).

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