$\alpha 4\beta 1$ Integrin/Ligand Interaction Inhibits $\alpha 5\beta 1$ -induced Stress Fibers and Focal Adhesions via Down-Regulation of RhoA and Induces Melanoma Cell Migration^V

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We have studied the function of the Hep III fibronectin domain in the cytoskeletal response initiated by $\alpha 5\beta 1$ integrin-mediated adhesion. Melanoma cells formed stress fibers and focal adhesions on the RGD-containing FNIII7–10 fragment. Coimmobilization of FNIII4–5, a fragment spanning Hep III and containing the $\alpha 4\beta 1$ ligand H2 with FNIII7–10, or addition of soluble FNIII4–5 to cells preattached to FNIII7–10, inhibited stress fibers and induced cytoplasmic protrusions. This effect involved $\alpha 4\beta 1$ since: 1) mutations in H2 reverted the inhibition; 2) other $\alpha 4\beta 1$ ligands (CS-1, VCAM-1), an anti- $\alpha 4$ mAb, or $\alpha 4$ expression in HeLa cells inhibited stress fibers. This activity was apparently cryptic in fibronectin or large fibronectin fragments, but exposed upon proteolytic degradation. Indeed purified peptic fragments containing H2, inhibited stress fibers when mixed with FNIII7–10 or fibronectin. RhoA activation with LPA or transfection with V14RhoA reverted the inhibitory effect and induced stress fibers on FNIII7–10+FNIII4–5. Furthermore, addition of $\alpha 4\beta 1$ ligands to FNIII7–10, down-regulated RhoA and activated p190RhoGAP, which localized to cytoplasmic protrusions. $\alpha 4\beta 1$ /ligand interaction induced cell migration, monitored by video microscopy and wound healing assays. These data indicate that $\alpha 4\beta 1$ provides an antagonistic signal to $\alpha 5\beta 1$ by interfering with the RhoA activation pathway and this leads to melanoma cell migration.

INTRODUCTION

Cellular interactions with the extracellular matrix (ECM) regulate cytoskeleton reorganization, cell migration, proliferation, survival and differentiation (Adams and Watt, 1993; Howe *et al.*, 1998). Cell adhesion to the ECM is mainly mediated by the integrin family of receptors and results in actin filament polymerization and assembly into stress fibers, and formation of focal adhesions (Sastry and Burridge, 2000; Geiger *et al.*, 2001). These multi-molecular complexes are active signaling centers which may assemble or disperse as cells attach and migrate, and therefore their precise regulation is crucial for normal cell behavior.

The cytoskeletal response following adhesion to fibronectin (Fn), a major component of the ECM, has been studied mainly in fibroblasts (reviewed in Sastry and Burridge, 2000; Geiger *et al.*, 2001). These reports have clearly shown that attachment to Fn fragments containing the central cell binding domain (RGD and PHSRN sequences) via $\alpha 5\beta 1$ integrin, induces cell spreading but is not sufficient for formation of stress fibers and focal adhesions (Woods *et al.*, 1986). This requires an additional signal provided by fragments containing the Hep II domain, a second cell-binding domain in Fn located in the carboxy-terminal region (Woods *et al.*, 1986). The main cellular receptor that interacts with Hep II is syndecan-4 (Woods *et al.*, 2000) and antibodies to syndecan-4 also provide a cooperative signal to $\alpha 5\beta 1$ integrin/RGD interaction that leads to formation of focal adhesions (Saoncella *et al.*, 1999). Using recombinant Fn fragments, Yoneda *et al.* (1995) and Bloom *et al.* (1999) further demonstrated that the Hep II domain ability to drive focal adhesion formation resides within the Fn III13 repeat, a region that we recently showed to bind syndecan-4 (Huang *et al.*, 2001). These intracellular effects are regulated by the Rho family of

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small GTPases (Bishop and Hall, 2000; Schmitz *et al.*, 2000; Ridley, 2001), which control the assembly of stress fibers and focal adhesions (RhoA), and the formation of lamellipodia (Rac) or filopodia (Cdc42). Control of actin filament assembly and disassembly is important for cell movement and this is also regulated by the coordinate contribution of RhoA, Rac and Cdc42 (Nobes and Hall, 1999).

We previously identified a third cell-binding region in Fn corresponding to the Hep III domain (repeats III4–III5, Moyano *et al.*, 1997, 1999). This domain contains the active sequences KLDAPT or H2 site, which is a ligand for activated $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins, and HBP/III5 which binds chondroitin-sulfate proteoglycans (CSPG). In this report we aimed to determine the biological role of the Hep III domain in the cytoskeletal response that follows adhesion to Fn and in particular, whether Hep III could provide a cooperative signal to the $\alpha 5\beta 1$ /RGD interaction leading to cytoskeleton reorganization. Since cell adhesion to Hep III involves binding to $\alpha 4\beta 1$ integrin and CSPG, we chose melanoma cells for these studies, which unlike most fibroblasts, express both $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins as well as CSPG. Using recombinant Fn fragments spanning the central cell binding domain (FNIII7–10) or the Hep III domain (FNIII4–5), as well as other $\alpha 4\beta 1$ ligands, we demonstrate that concomitant ligation of $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins results in antagonistic rather than cooperative signals that prevent formation of stress fibers and focal adhesions. We show that the inhibitory signal provided by $\alpha 4\beta 1$ engagement is due to sustained down-regulation of RhoA via p190RhoGAP activation, and this leads to stimulation of melanoma cell migration.

MATERIALS AND METHODS

Antibodies and Reagents

mAb anti-vinculin (clone hVIN-1) was purchased from Sigma (St. Louis, MO); anti-RhoA, from Santa Cruz Biotechnologies (La Jolla, CA); anti-p190RhoGAP, from Upstate Biotech (Lake Placid, NY); PY20 anti-phosphotyrosine was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA); anti- $\alpha v\beta 3$ integrin (LM609) and anti-Fn central cell binding domain (MAB1933), from Chemicon International, Inc., (Temecula, CA). P1D6 and P4C2, anti- $\alpha 5$ and $\alpha 4$ subunits respectively, have been previously described (Wayner *et al.*, 1989); IST-4, reactive with Fn repeat III5 (Carnemolla *et al.*, 1996) was obtained from Dr. Luciano Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy); anti-CS-1 mAb P1F11 was previously described (Garcia-Pardo *et al.*, 1992). Collagen type I, vitronectin, lysophosphatidic acid (LPA) and TRITC-labeled phalloidin were from Sigma. TPCK-Trypsin and pepsin were purchased from Merck (Darmstadt, Germany). FITC-Annexin-V was from Bender MedSystems (Vienna, Austria).

Fibronectin Fragments and VCAM-1

The 80 kDa Fn fragment (repeats III4–_{1/2}III11) was purified from tryptic digests of human plasma Fn (1:200 wt/wt, 90 min, 37°C) and further cleaved with pepsin (1:100 wt/wt, 1 h, 37°C) as reported (Sánchez-Aparicio *et al.*, 1994). Peptic fragments of 20/30 kDa, corresponding to the III4–5 region, were purified by FPLC using a Mono Q column (Amersham Biosciences Europe GmbH, Barcelona, Spain) as described (Sánchez-Aparicio *et al.*, 1994). These two fragments were separated from each other by SDS-PAGE and their N-terminal amino acid sequence determined using a 494 Protein Sequencer (Applied Biosystems, Foster City, CA).

The FNIII4–5 recombinant fragment, spanning the Hep III Fn domain (repeats III4–5), was prepared as previously described

(Moyano *et al.*, 1999). The FNIII7–10 fragment (repeats III7–10 including the RGD sequence) was kindly donated by Dr. Harold Erickson (Duke University Medical Center, Durham, NC) and purified as reported (Aukhil *et al.*, 1993). The recombinant H89 fragment comprising the Hep II domain (repeats III12–14), 89 residues of the V/IIICS region including the CS-1 but not the CS-5 site, and repeat III15, was obtained from Dr. Martin Humphries (University of Manchester, UK) and prepared as described (Mould *et al.*, 1994). Recombinant fragments FNIII13, (repeat III13) and FNIII7–15, containing repeats III7–15 and the entire IIICS/V region including CS-1 and CS-5, were obtained from Dr. Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA) and purified as reported (Bloom *et al.*, 1999), except that HiTrap™ Chelating HP columns (Amersham Biosciences) were used for the purification of FNIII7–15. Recombinant VCAM-1 was kindly provided by Dr. Roy Lobb (Biogen Inc., Cambridge, MA).

Generation of FNIII4–5 Mutants

FNIII4–5 mutants were prepared by PCR using as template the FNIII4–5 cDNA cloned in the pQE-3/5 vector, a gift from Dr. Luciano Zardi. Primers used for each mutation were as follows: H2-E mutation: forward, 5'-GACTGCCAACAGACAACCAACT-GGAAGCTCCCACTAC-3'; reverse, 5'-GTTAGTGGGAGCTTC-CAGTTG-3'. H2-DL mutation: forward, 5'-CAAAGATCTTGCTC-CCACTAAC-3'; reverse, 5'-GAGCAAGATCTTTGGTTGTCTG-3'. HBP/III5-R1 mutation: forward, 5'-TACTGTCCTGGTGAGATG-GACTCCACCTGCGGCCAGATA-3'; reverse, 5'-TCTGGGCCG-CAGGTGGAGT-3'. HBP/III5-R2 mutation: forward, 5'-ACAG-GATACGCACTGACCGTG-3'; reverse, 5'-GCCTCTTCGGGTAA-GGCC CACGGTCAGGCGTATCCTGT-3'. HBP/III5-R3R4 mutation: forward, 5'-ACCGCAGCAGGCCAGCCAC-3'; reverse primer, 5'-GCCTGCTGCGGTAAGGCCAC-3'. For the double mutation HBP/III5-R1R2 we used HBP/III5-R2 as template and the primers used to generate HBP/III5-R1. The DNA sequence of all mutants cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI) was verified in an automated ABI-Prism 377 sequencer (Applied Biosystems-Prism-Perkin Elmer-Cetus, Foster City, CA), subcloned at the *Mfe* I and *Hind* III sites in the pQE3 vector and transfected into DH5 α *E. coli*. Mutated fragments were induced and purified as described for the parental FNIII4–5 fragment (Moyano *et al.*, 1999).

Other Recombinant Proteins and Constructs

$\alpha 4$ subunit cDNA (obtained from Dr. Martin Hemler, Dana-Farber Cancer Institute, Boston, MA) was subcloned into the pCI-neo vector (Promega) at the original restriction sites *Xba*I/*Sal*I and used to transform *E. coli* JM109. Plasmidic DNA was obtained by performing minipreps of positive colonies with the Plasmix kit (Talent Technologies, Torino, Italy).

Recombinant C3 transferase (C3) in the pGEX-2T vector (a gift from Dr. Alan Hall, University College, London, UK) was expressed in *E. coli* DH5 α and purified as described (Dillon and Feig, 1995), except that bacteria were lysed in 50 mM Tris pH7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT with 1 mM PMSF, 2.5 μ g/ml leupeptin, and 10 μ g/ml aprotinin (C3 buffer). Supernatants containing glutathione-S-transferase (GST)-C3 were incubated overnight at 4°C with glutathione-agarose beads, centrifuged, and cleaved with 5 U/ml thrombin (Sigma). Thrombin was removed by incubating with 10 μ l of p-aminobenzamidine-agarose bead suspension for 30 min at 4°C. Beads were removed by centrifugation and C3 was dialyzed into C3 buffer without the inhibitors. Purity was checked by SDS-PAGE.

Green fluorescent protein (GFP) fused to RhoA cDNAs encoding for V14RhoA (active mutant) or N19RhoA (dominant negative) cloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA) in *E. coli* DH5 α , were a gift from Dr. Francisco Sanchez-Madrid. Plasmidic DNA from *E. coli* was isolated by performing minipreps.

Cells and Cell Cultures

The human melanoma cell lines SKMEL-178 and A375 were obtained from Dr. Francisco Real (Hospital del Mar, Barcelona, Spain). The human epithelial cell line HeLa was obtained from Dr. Angel Corbí (Centro de Investigaciones Biológicas, Madrid, Spain). All cells were maintained in DME medium, 10% FBS (Life Technologies, Paisley, Scotland, UK), 40 $\mu\text{g}/\text{ml}$ gentamicin. Before all assays (except when indicated) cells were serum-starved for 3 h, detached from culture flasks with 1 mM EDTA, PBS pH 7.5, washed with PBS, and resuspended in attachment medium (DME, 1% BSA, 10 mM HEPES).

Immunofluorescence Assays

Glass coverslips were coated with Fn, fragments, VCAM-1 or antibodies diluted in 40 μl PBS for 2 h at 37°C and placed inside 24-well plates. Wells were washed and blocked with 1% BSA/PBS for 30 min. Serum-starved 2×10^4 cells in attachment medium were added to each well and incubated for 1 h at 37°C. Attached cells were fixed with 3.5% formaldehyde/PBS, permeabilized with cold 0.5% Triton X-100/PBS for 3 min and blocked with 1% BSA/PBS for 30 min. F-actin was stained with 25 $\mu\text{g}/\text{ml}$ TRITC-phalloidin in PBS, 1% BSA, 10 mM NaN_3 for 15 min. Vinculin was detected with specific mAbs. Samples were visualized on an epifluorescence Axioplan microscope (Zeiss, Germany) and photographed with a CCD camera (Photometrics Inc., Tucson, AZ). Viability of cells in these assays was confirmed by DAPI staining and flow cytometry using propidium iodide and FITC-Annexin-V.

Cell Transfection

Subconfluent cells in fresh complete medium were transiently transfected using FuGENE™ 6 (Roche Biochemicals, Mannheim, Germany) following the manufacturer's instructions. Briefly, 2 μg of cDNA were mixed with 3 μl of FuGENE 6, diluted to a final volume of 100 μl with DME, incubated for 30 min at room temperature, and added to the cells. After 24 h transfected cells were used in immunofluorescence assays as described. Transfection efficiency was ~60% for SKMEL-178 cells and 20% for HeLa cells, assessed by flow cytometry.

Wound Healing Assays and Video-Microscopy

Confluent cells on 12-well plates were wounded by scraping a yellow-tip across the cell monolayer. After washing with DME, complete medium containing either 2.1 μM FNIII7–10 alone or mixed with 4.9 μM FNIII4–5, FNIII4–5-DL, or H89 or with 1.48 μM VCAM-1 was added to the wells. Wound closing was checked at different times during 24 h. Photographs were taken on an inverted phase-contrast microscope equipped with a CCD camera (Nikon Diaphot/DXM-1200, Tokyo, Japan). For video microscopy, 2×10^4 cells were added to glass coverslips previously coated with the various substrata and placed inside a 24-well plate. Individual wells were analyzed on a microscope equipped with a thermally-controlled plate set at 37°C. Photograms were taken every 30 s >3 h, using a CCD camera with the Act-1 acquisition software (Nikon). Digital videos were composed using the VideoMach software. Quantitation of wound and cell spread areas was performed using the IP Lab Spectrum program (Signal Analytics Co., Vienna, VA). Single cell tracking measurement was performed using the MetaMorph program (Universal Imaging Co., Downingtown, PA).

Regulation and Analysis of RhoA Activity

For C3 inactivation of RhoA, subconfluent cells were incubated overnight in DME, 0.5% FBS, 50 $\mu\text{g}/\text{ml}$ C3 in 6-well plates. For LPA activation, cells with or without previous treatment with C3, were detached from plates with 1 mM EDTA/PBS, washed and incubated with 1 μM LPA in attachment medium for 60 min at room

temperature on a rotary shaker. Fresh C3 was added again during this incubation to cells previously treated with this inhibitor.

The Rho binding domain of Rhotekin C21 fused to GST and cloned into the pGEX-3 $\times$ vector (Pharmacia) was a gift of Dr. John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Protein was expressed in *E. coli* DH5 α and purified as described (Sander *et al.*, 1999). For pull-down assays, serum-starved cells were added to 6-well plates ($7 \times 10^5/\text{well}$) previously coated with recombinant fragments. A total of 8×10^6 cells was used for each condition. At different times attached cells were lysed in 500 μl of cold 50 mM Tris pH7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM NaVO_4 , 1 mM NaF, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 2.5 $\mu\text{g}/\text{ml}$ leupeptin (RIPA buffer) and lysates clarified by centrifugation. 15 μl of the supernatant were used to measure total RhoA and the remaining volume was mixed with 50 μl of C21-GST and 60 μl of glutathione-agarose beads for 60 min at 4°C. Beads were washed and bound protein was eluted by boiling in Laemmli buffer. Samples were separated on 15% SDS-PAGE and transferred to nitrocellulose membranes (BIO-RAD, Hercules, CA). Total and bound RhoA were detected with an anti-RhoA mAb, followed by HRP-conjugated antimouse IgG antibody (Dako), and visualized with the ECL SuperSignal West Pico kit (Pierce, Rockford, IL). Protein bands were quantified on a densitometer (Molecular Dynamics, Sunnyvale, CA) using the Quantity-One™ program (BIO-RAD).

Analysis of p190RhoGAP Phosphorylation

Cells were incubated in 6-well plates and lysed in RIPA buffer. 15 μl of the supernatant were used for analysis of total protein and the remaining 485 μl were incubated with protein G-agarose beads (Pierce) coated with 4 μg of anti-p190RhoGAP mAb or mock beads without antibody, at 4°C overnight. Beads were washed with RIPA buffer, and bound proteins extracted by boiling in Laemmli buffer and separated on 7.5% SDS-PAGE followed by western blotting. Phosphorylated proteins were detected using PY20 mAb followed by an HRP-labeled secondary antibody, and total p190RhoGAP with a specific mAb. Protein bands were visualized and quantitated as explained above.

Statistical Analyses

Significance of the difference between means was determined by the Student's *t* test for nonpaired samples using the GraphPad InStat program (GraphPad Software, San Diego, CA). A P-value of ≤ 0.05 was considered significant.

Online Supplemental Material

SKMEL-178 cells were added to glass coverslips coated with FNIII7–10 (video 1), FNIII7–10+FNIII4–5 (video 2), FNIII7–10+FNIII4–5-DL (video 3), FNIII7–10+H89 (video 4), or FNIII7–10+VCAM-1 (video 5). Images were recorded every 30 s for 3 h and videos were compiled with the VideoMach software. In videos 1 and 3, cells displayed slow membrane activity and spreading, but remained stationary. However, in the presence of FNIII4–5, H89 or VCAM-1 (videos 2, 4, 5) cells showed fast membrane activity, no spreading, and substantial random motility. See also Figure 9.

RESULTS

Melanoma Cells Form Stress Fibers and Focal Adhesions Upon Attachment to the FNIII7–10 Fragment

The SKMEL-178 and A375 melanoma cell lines used in this study expressed several integrins including the two main Fn receptors $\alpha 5 \beta 1$ and $\alpha 4 \beta 1$, and $\alpha \text{V} \beta 3$, as determined by flow cytometry (our unpublished results). To study the cytoskel-

Figure 1. (A) Cytoskeletal organization following adhesion of melanoma cells to the FNIII7-10 fragment (2.1 μ M) alone or mixed with 4.9 μ M FNIII4-5 (+FNIII4-5). 2×10^4 SKMEL-178 cells were added to coverslips previously coated with the indicated fragments for 1 h at 37°C. In a different experiment, soluble FNIII4-5 (4.9 μ M; +sol FNIII4-5) was added to cells previously attached to FNIII7-10 for 10 min and incubation continued for 1 h. After fixing and permeabilization, actin was visualized with TRITC-phalloidin and vinculin with specific antibodies. The morphological pattern of the few cells attached to FNIII4-5 alone is also shown. (B) Cells plated on FNIII7-10 or FNIII7-10+FNIII4-5 for 1 h were detached, incubated with propidium iodide (PI; 50 μ g/ml) and 6 μ l FITC-Annexin-V for 10 min in the dark, and analyzed by flow cytometry. Values represent the % of apoptotic cells before (control) and after the two different conditions of the assay. Bar, 10 μ m.

eton reorganization upon melanoma cell adhesion to the central cell-binding domain of Fn, serum-starved subconfluent SKMEL-178 cells were placed on glass coverslips previously coated with the FNIII7-10 fragment (2.1 μ M, 40 μ g/ml). Nearly 80% of cells plated on FNIII7-10 had a characteristic elongated morphology and formed fine stress fibers and some focal adhesions visualized by vinculin staining (Fig. 1A). $\alpha 5\beta 1$ integrin (but not $\alpha V\beta 3$ or $\alpha 4\beta 1$) localized to focal adhesions (our unpublished results), in agreement with its role as receptor for the FNIII7-10 fragment (Moyano

et al., 2003). This cytoskeletal pattern was observed for the range of FNIII7-10 concentrations tested (0.71 μ M - 2.88 μ M), and did not change significantly by expanding the time of the assay up to six h.

Cell Interaction with the FNIII4-5 Fragment Disrupts the Cytoskeleton Reorganisation Induced by Attachment to FNIII7-10

To determine whether the Hep III domain of Fn could amplify the cytoskeletal response induced by $\alpha 5\beta 1$ -mediated adhesion, as previously shown for the Hep II/syndecan-4 interaction, glass coverslips were simultaneously coated with FNIII7-10 and FNIII4-5 fragments at 2.1 and 4.9 (135 μ g/ml) μ M respectively. This molar ratio was found to be optimal after initial dose response experiments using several concentrations of FNIII4-5. As shown in Figure 1A, addition of FNIII4-5 produced a dramatic morphological change compared with cells plated on FNIII7-10 alone, with ~75% of cells lacking stress fibers and extending protrusions where actin patches accumulated. Additionally, vinculin had a diffused pattern on these cells (Figure 1A). This altered cytoskeletal pattern was not due to a diminished FNIII7-10 coating concentration caused by the presence of FNIII4-5 on the same surface, as confirmed by ELISA using specific mAbs reactive with either fragment (our unpublished results). Neither it was due to induction of apoptosis by the FNIII4-5 fragment since cell viability was almost identical for cells plated on FNIII7-10 or the mixed substrate (Figure 1B). Cells incubated in suspension with soluble FNIII4-5 (4.9 μ M) for 40 min, did not form stress fibers or focal adhesions when added to FNIII7-10-coated coverslips (our unpublished results). Furthermore, addition of soluble FNIII4-5 fragment to cells preattached to FNIII7-10 also inhibited stress fiber formation by 68% (Figure 1A). This effect was clearly evident if FNIII4-5 was added at early times of up to 10 min after cells had been plated on FNIII7-10; at later times, there was a progressive loss of the ability of the soluble fragment to alter the cytoskeleton organization induced by FNIII7-10 (not shown). Very few cells (15%) attached to FNIII4-5 when coated alone and they remained round after 1 h (Figure 1A).

$\alpha 4\beta 1$ Integrin Interaction with the H2 Site in FNIII4-5 is Responsible for the Inhibition of Stress Fibers on Cells Attached to FNIII7-10

FNIII4-5 contains two cell-binding sites namely H2 and HBP/III5, which bind $\alpha 4\beta 1$ integrin or CSPG respectively (Moyano *et al.*, 1997, 1999). To determine which of these sites was responsible for the observed inhibition of stress fibers, we performed directed mutations on both active sequences (Figure 2A) and tested their effect on cytoskeletal organization. SKMEL-178 cells were added to glass coverslips coated with either FNIII7-10 alone or mixed with FNIII4-5 mutants (all at 135 μ g/ml). Figure 2B shows that the FNIII4-5-DL mutation efficiently reverted (78% of cells with stress fibers) the inhibitory effect produced by parental FNIII4-5. In contrast, the FNIII4-5-E mutant behaved as FNIII4-5 (not shown). Fragments carrying mutations on either or both of the first two R residues in HBP/III5 behave as parental FNIII4-5, with 80% of cells lacking stress fibers and extending protrusions (Figure 2B and not shown for the R1R2 mutant). Substitution of the last two R residues of HBP/III5

Figure 2. (A) Specific mutations (bold type) performed on the H2 and HBP/III5 sequences of the FNIII4-5 fragment. (B) A mutation on the $\alpha 4\beta 1$ integrin binding site in FNIII4-5 reverts the inhibitory effect of this fragment. Cells were incubated on glass coverslips previously coated with FNIII7-10 (2.1 μ M) combined with either of the indicated FNIII4-5 mutants (all at 4.9 μ M) for 1 h, 37°C. Cells were fixed, permeabilized, and stained for actin. Each experiment was performed at least three times. Values represent the % of cells with stress fibers. Bar, 10 μ m.

by A residues (FNIII4-5-R3R4 mutant, Figure 2A) had a partial effect and ~56% of cells formed stress fibers (Figure 2B). These results showed that although some positively charged residues in HBP/III5 may play a certain role, altering the $\alpha 4\beta 1$ binding site in FNIII4-5 abolished its ability to inhibit the $\alpha 5\beta 1$ /FNIII7-10-induced cytoskeletal response, thus implicating $\alpha 4\beta 1$ integrin in this signaling.

To determine whether $\alpha 4\beta 1$ inhibitory signals were specific for $\alpha 5\beta 1$ or could also affect other integrins, we coimmobilized FNIII4-5 with 2.1 μ M collagen or vitronectin which are ligands for $\alpha 2\beta 1$ and $\alpha V\beta 3$ integrins respectively. As shown in Figure 3, melanoma cells spread and formed stress fibers and focal adhesions on collagen (74.2% of cells) and vitronectin (85.1% of cells) when coated alone. Concomitant ligation of $\alpha 4\beta 1$ by FNIII4-5 inhibited the cytoskeleton organization induced via $\alpha 2\beta 1$ (10% of cells formed stress fibers) but did not affect the response mediated by $\alpha V\beta 3$ ligation. This indicates that $\alpha 4\beta 1$ cross-talk signaling is not exclusive for $\alpha 5\beta 1$ but is specific for certain integrins.

Other $\alpha 4\beta 1$ Integrin Ligands and an Anti- $\alpha 4$ mAb also Inhibit the FNIII7-10-Induced Stress Fiber Formation

To further establish that the observed cytoskeletal alterations were due to $\alpha 4\beta 1$ engagement, we tested the effect of other ligands of this integrin such as the H89 Fn fragment which contains the CS-1 site (Mould *et al.*, 1994), and the endothelial protein VCAM-1 (Elices *et al.*, 1990). Co-immobilization of FNIII7-10 with either 4.9 μ M H89 or 1.48 μ M VCAM-1 resulted in a morphological pattern resembling that induced by FNIII4-5, with cells extending lamellipodia where actin accumulated (69.3% of cells on H89; 68% of cells on VCAM-1) (Figure 4A). Lower concentrations of H89 or VCAM-1 (tested up to 0.7 μ M, not shown) also inhibited stress fibers, probably due to their higher affinity for $\alpha 4\beta 1$ compared with FNIII4-5. In contrast, addition of 4.9 μ M FNIII13, a recombinant Fn fragment which does not bind $\alpha 4\beta 1$, had no effect (Figure 4A). We also tested whether the anti- $\alpha 4$ subunit P4C2 mAb would mimic the effect of $\alpha 4\beta 1$ ligands. As shown in Figure 4B, 74% of cells plated on mixtures of FNIII7-10 and P4C2 lacked stress fibers and formed cytoplasmic protrusions. The same concentration of a control mAb (LM609, anti- $\alpha V\beta 3$) had no effect on the original cytoskeleton pattern (87% of cells with stress fibers) (Figure 4B). Moreover, cell incubation with soluble P4C2 for 40 min before their addition to FNIII7-10-coated coverslips also prevented formation of stress fibers on 72.1% of cells (Figure 4B), further confirming that P4C2 was mimicking the effect of $\alpha 4\beta 1$ ligands. Incubation with soluble LM609 had no effect (Figure 4B). Neither antibody induced a particular morphological pattern when coated alone (Figure 4B).

Inhibition of Stress Fibers on FNIII7-10+FNIII4-5 Is also Observed on A375 Melanoma Cells and $\alpha 4$ Integrin Subunit-Transfected HeLa Cells

To determine whether the cytoskeletal effect of FNIII4-5/ $\alpha 4\beta 1$ interaction was also observed on other cells, we carried out similar experiments using A375 melanoma cells, which as mentioned above express $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins, and HeLa cells, which do not express $\alpha 4\beta 1$ and were transiently transfected with the $\alpha 4$ subunit. Approximately 73% of A375 cells plated on FNIII7-10 spread and formed mainly peripheral stress fibers (Figure 5A). These fibers were completely lost upon adhesion to mixed substrata of FNIII7-10+FNIII4-5 and 78.7% of cells became smaller and extended protrusions (Figure 5A). As observed for SKMEL-178 cells, addition of the FNIII4-5-DL mutant to FNIII7-10 produced no effect (Figure 5A). Likewise, parental HeLa cells (72.3%) attached and formed stress fibers on FNIII7-10 and this pattern was not altered when cells were plated on mixtures of FNIII7-10+FNIII4-5 or FNIII7-10+FNIII4-5-DL (69.1% of cells; Figure 5B). A similar morphological pattern was obtained when $\alpha 4$ subunit-transfected HeLa cells were added to FNIII7-10 alone (72% of cells; Figure 5B). However, when added to mixtures of FNIII7-10+FNIII4-5, 69.1% of $\alpha 4$ -HeLa cells did not spread and did not form stress fibers as previously observed for melanoma cells. As observed for parental cells, 71% of $\alpha 4$ -HeLa cells formed stress fibers on mixed substrata of FNIII7-10+FNIII4-5-DL

Figure 3. Interaction of FNIII4–5 with $\alpha 4\beta 1$ inhibits the cytoskeletal response induced via $\alpha 2\beta 1$ but not via $\alpha V\beta 3$. 2×10^4 SKMEL-178 cells were added to glass coverslips coated with $2.1 \mu\text{M}$ collagen (A) or vitronectin (B) alone or mixed with $4.9 \mu\text{M}$ FNIII4–5 fragment. After 1 h, actin was visualized with TRITC-phalloidin and vinculin with specific antibodies. Experiments were done at least three times. Values represent the % of cells with stress fibers on each condition. Bar, $10 \mu\text{m}$.

(Figure 5B). Moreover, coinmobilization of the H89 fragment or VCAM-1 with FNIII7–10 also altered the morphological pattern of $\alpha 4$ -HeLa cells resulting in inhibition of stress fibers in 68.8% of transfected cells (Figure 5B). Therefore, the presence of an engaged $\alpha 4\beta 1$ integrin clearly disturbs formation of stress fibers after $\alpha 5\beta 1$ -mediated adhesion in several cell types.

Functional Role of the $\alpha 4\beta 1$ Ligands H2 and CS-1 in a Physiological Context

To establish the role of the III4–5 region in the context of intact Fn, we first analyzed the cytoskeletal response of melanoma cells upon attachment to Fn. Figure 6A shows that 80.2% of these cells formed prominent actin stress fibers

Figure 4. Inhibition of stress fibers by other $\alpha 4 \beta 1$ integrin ligands and anti- $\alpha 4$ mAbs. (A) Cells were incubated on glass coverslips previously coated with 2.1 μM FNIII7-10 alone or mixed with either 4.9 μM H89 fragment, 1.48 μM VCAM-1, or 4.9 μM FNIII13. After 1 h, cells were fixed, permeabilized, and stained for actin. (B) Cells were added to FNIII7-10 coimmobilized with 1 $\mu\text{g}/\text{ml}$ P4C2 (anti- $\alpha 4$; +P4C2) or LM609 (anti- $\alpha V \beta 3$; +LM609) mAbs. In a different experiment, cells were incubated in suspension for 40 min with soluble P4C2 (+sol P4C2) or LM609 (+sol LM609) (5 $\mu\text{g}/\text{ml}$ each) and added to glass coverslips coated with 2.1 μM FNIII7-10. Finally, cells were also added to coverslips coated with mAbs alone (lower panels). Actin was visualized in all cases with TRITC-phalloidin. Each experiment was done three times. Bar, 10 μm .

Figure 5. Effect of FNIII4-5, H89 and VCAM-1 in other cells. (A) The FNIII4-5 fragment inhibits stress fiber formation on A375 melanoma cells. 2×10^4 A375 cells were added to glass coverslips coated with FNIII7-10 alone or mixed with FNIII4-5 or the mutant FNIII4-5-DL. Cells were permeabilized after 1 h and stained with TRITC-phalloidin. (B) Expression of $\alpha 4$ subunit inhibits spreading and stress fibers on HeLa cells plated on mixed substrata of FNIII7-10 plus FNIII4-5, H89 or VCAM-1. Parental HeLa cells or transfected with $\alpha 4$ integrin subunit (HeLa- $\alpha 4$) were added to coverslips coated with FNIII7-10 alone, or mixed with $\alpha 4 \beta 1$ integrin ligands. After 1 h, attached cells were stained with TRITC-phalloidin (top and center panels) or P4C2 mAb + FITC-labeled secondary antibodies (lower panels). Two different experiments were performed with identical results. Bar, 10 μ m.

terminating in focal adhesions which contained vinculin (Figure 6A), paxillin and talin (not shown). This suggested two independent possibilities: 1) that the inhibitory activity of the III4-5 (and possibly CS-1) region was cryptic in Fn; 2) that the molar ratio for $\alpha 5 \beta 1 / \alpha 4 \beta 1$ binding sites required to achieve the observed morphological change was not optimal in plasma Fn. To address these possibilities, we first studied the actin organization upon cell attachment to proteolytic digests of Fn and whether fragments similar to FNIII4-5 were produced by the degradation process. Trypsin digestion of Fn rendered several fragments including a major 80 kDa product (Figure 6B) which we previously showed to have the N-terminal sequence SDTVPS (beginning of repeat III4) and contain the RGD sequence (Sánchez-Aparicio *et al.*, 1994). The molar ratio of $\alpha 4 \beta 1 / \alpha 5 \beta 1$ binding sites (H2/RGD) in the 80 kDa fragment was therefore 1:1. Melanoma cells attached to an 80 kDa-enriched fraction (devoid of CS-1 containing fragments; Figure 6B) displayed a similar morphological pattern to that observed on Fn, with >80% of cells forming stress fibers and focal adhesions (Figure 6A). However, cells attached to a peptic digest of 80 kDa (a mixture of H2- and RGD-containing fragments) (Figure 6B) acquired a rounded morphology and lacked stress fibers and focal adhesions (Figure 6A). Western blot analysis of 80 kDa

proteolytic products using mAb IST-4, which specifically recognizes Fn III5 repeat (Carnemolla *et al.*, 1996), revealed two positive fragments of 20 and 30 kDa respectively with similar molecular size as the recombinant FNIII4-5 fragment used in this study (Figure 6B). Further characterization of the 20/30 kDa fraction after purification by FPLC and analytical separation by SDS-PAGE, rendered the N-terminal sequences ESKPLT (corresponding to the end of repeat III4, residue 985 in Fn) for the 20 kDa, and SDTVPS (also the beginning of the 80 kDa parental fragment at residue 904) for the 30 kDa fragment.

To determine whether the 20/30 kDa proteolytic fragments could reproduce the cytoskeletal effects of FNIII4-5, we coimmobilized the purified 20/30 kDa fraction (95 μ g/ml, Figure 6B) with FNIII7-10 and studied the cytoskeleton organization after 1 h. As shown in Figure 6C, the 20/30 kDa mixture efficiently inhibited (63.5% of cells) the stress fibers and focal adhesions induced by FNIII7-10. mAb IST-4 did not abolish the 20/30 kDa (or the FNIII4-5) effect (not shown) indicating that the epitope recognized by this mAb was outside the H2 sequence. We next studied whether the 20/30 kDa fragments affected the cytoskeleton organization induced by adhesion to Fn. As shown in Figure 6C, coimmobilization of either 20/30 kDa (95 μ g/ml) or FNIII4-5 (4.9

Figure 6. Functional role of $\alpha 4 \beta 1$ ligands in the context of Fn or large Fn fragments. (A) 2×10^4 SKMEL-178 cells were added to glass coverslips coated with Fn ($0.13 \mu\text{M}$), an 80 kDa-enriched tryptic fraction of Fn ($30 \mu\text{g/ml}$; panel B) or a peptic digest of the 80 kDa fragment ($70 \mu\text{g/ml}$; panel B). After 1h, actin and vinculin were visualized as explained. (B) Analysis of the 80 kDa tryptic fraction and peptic digests of 80 kDa by 12.5% SDS-PAGE and western blotting with mAb IST-4, specific for repeat III5. Purified 20/30 kDa peptic fragments and their comparison with FNIII4-5 are also shown. (C) Cells were added to coverslips coated with mixtures of purified 20/30 kDa fragments and FNIII7-10 or Fn. The effect of coimmobilizing Fn with FNIII4-5 ($4.9 \mu\text{M}$) was also studied. (D) Cytoskeletal organization of melanoma cells attached to the FNIII7-15 recombinant fragment ($2.1 \mu\text{M}$). The expression of the CS-1 epitope in FNIII7-15, H89 and Fn was determined by ELISA using the P1F11 mAb. ** $p \leq 0.01$. Bar, $10 \mu\text{m}$

μM) with Fn ($0.13 \mu\text{M}$) altered the morphological pattern observed on Fn and 74.6% of cells extended protrusions and lacked stress fibers and focal adhesions. At higher concentrations of Fn ($0.3 \mu\text{M}$) there was a progressive reversion of the inhibitory effect (not shown).

We also performed similar studies using the recombinant fragment FNIII7-15 which, like the 80 kDa fragment, contained binding sites for $\alpha 5 \beta 1$ (RGD) and $\alpha 4 \beta 1$ (CS-1) integrins at a 1:1 M ratio. FNIII7-15 also contains CS-5 but its affinity for $\alpha 4 \beta 1$ is very low compared with CS-1 (Mould *et al.*, 1991), thus its effect within the fragment is probably very minor. 66.8% of melanoma cells plated on FNIII7-15 spread and formed stress fibers and focal adhesions (Figure 6D), resembling the pattern obtained on the 80 kDa fragment. To further determine whether the CS-1 site was exposed on FNIII7-15 (or in Fn), we performed ELISA assays using the anti-CS-1 mAb P1F11 and equimolar concentrations of Fn, H89 and FNIII7-15. As shown in Figure 6D, CS-1 was significantly more exposed on the H89 than on the FNIII7-15 fragment, whose reactivity was similar to that of intact Fn. Altogether these results suggested that in the context of Fn or large Fn fragments, the $\alpha 4 \beta 1$ ligands H2 and CS-1 appear

to be at least partially cryptic and proteolytic cleavage uncovers their biological activity.

Exogenous Activation of RhoA by LPA or Transfection with V14 RhoA Reverts the Stress Fiber-Inhibitory effect Caused by $\alpha 4 \beta 1$ Integrin Ligand

It is well established that formation of actin stress fibers in several cell types involves activation of RhoA (Bishop and Hall, 2000; Ridley, 2001). To determine whether $\alpha 4 \beta 1$ engagement was interfering with the function of RhoA in melanoma cells, we first carried out exogenous activation using the RhoA-specific activator LPA (Ridley and Hall, 1992). As shown in Figure 7A, activation of RhoA resulted in enhanced spreading and stress fiber formation for cells attached to FNIII7-10 as expected. Interestingly, treatment with LPA had a dramatic effect on cells attached to FNIII7-10+FNIII4-5, as cells spread and formed stress fibers similar to those observed on FNIII7-10. Quantitation of these results (Figure 7B) revealed that activation of RhoA by LPA reverted the phenotype observed on untreated cells (26% of cells with stress fibers and 69% with cytoplasmic protru-

Figure 7. Active RhoA reverts the effect of FNIII4-5. (A) Cells, with or without previous incubation with 50 μ g/ml C3 were treated with 1 μ M LPA for 1 h and added to coverslips previously coated with FNIII7-10 alone or mixed with FNIII4-5. After 1 h, actin was visualized with TRITC-phalloidin. (B) Quantitation of the different morphologies was performed by counting a minimum of 100 cells/condition by two independent observers. Values represent the mean of three different experiments. SF, stress fibers. Bar, 10 μ m. (C) SKMEL-178 cells were transiently transfected with GFP-V14RhoA (active mutant), N19RhoA (dominant negative), or empty vector (mock), and added to coverslips coated with FNIII7-10 alone or mixed with FNIII4-5. Fluorescence images of GFP (green) and actin filaments (red) in the same cell are shown. Bar, 10 μ m.

sions) resulting in 69% of cells with stress fibers and 33% without fibers. Preincubation of cells with the RhoA inhibitor C3, abolished the effect of LPA on both substrata (Figure 7A, B).

These results indicated that exogenous activation of RhoA overcomes the effect induced by α 4 β 1 ligation. To further confirm the involvement of RhoA in this process, we transiently transfected SKMEL-178 cells with the constitutively active GFP-V14Rho form and studied their cytoskeletal response after attachment to FNIII7-10 or FNIII7-10+FNIII4-5 fragments. As shown in Figure 7C, 74% of GFP-V14Rho-transfected cells showed enhanced spreading and actin stress fibers on FNIII7-10 similar to the pattern obtained after activation with LPA. Likewise, 72% of GFP-V14Rho-transfected cells spread and formed stress fibers on the mixed substrate FNIII7-10+FNIII4-5, clearly indicating that constitutive activation of RhoA was sufficient to overcome the cytoskeletal effects induced by α 4 β 1 integrin engagement (Figure 7C). As expected, transfection with the dominant negative mutant GFP-N19Rho resulted in reduced spreading and lack of stress fibers on both substrata. Transfection with the empty vector (mock-GFP) had no effect (Figure 7C). Altogether these results indicate that the stress fiber inhibitory activity induced by the α 4 β 1 ligand FNIII4-5 appears to involve the RhoA activation pathway.

α 4 β 1 Integrin Ligation Prevents RhoA Activation Due to Increased p190RhoGAP Phosphorylation

To determine whether RhoA was inactivated as a result of α 4 β 1/ligand interaction, we performed affinity binding

assays using the C21-GST fusion protein which only binds GTP-Rho (Sander *et al.*, 1999; Ren *et al.*, 1999). Lysates from SKMEL-178 cells that had been serum starved for 3 h and plated on FNIII7-10 alone or mixed with either FNIII4-5, H89, P4C2 mAb or P1D6 mAb for 50 min, were analyzed for the presence of active RhoA. As shown in Figures 8A and 8C, there was a basal RhoA activity (time 0), which increased after the 50 min of adhesion to FNIII7-10. In contrast, active RhoA levels at this time, for cells attached to mixtures of FNIII7-10 and α 4 β 1 integrin ligands or P4C2 were similar to basal levels (Figure 8AC) and significantly lower than those induced by FNIII7-10 (Figure 8C). Coimmobilization of FNIII7-10 with the anti- α 5 subunit mAb P1D6 did not reduce the active RhoA levels induced by FNIII7-10 (Figure 8AC), thus confirming the specificity of the α 4 β 1-mediated effect. Adhesion to H89 alone did not modify the basal activity of RhoA (Figure 8AC) in agreement with the lack of cells forming stress fibers on this substrate (not shown).

Down-regulation of RhoA has been attributed to activation/phosphorylation of p190RhoGAP by c-Src (Arthur *et al.*, 2000). To establish whether the observed lack of activation of RhoA in the presence of α 4 β 1 ligands followed this pathway, we measured the levels of p190RhoGAP tyrosine phosphorylation upon adhesion to FNIII7-10 or to the above mentioned mixed substrata. As shown in Figures 8B and 8C, these levels were significantly elevated in the presence of FNIII7-10 mixed with α 4 β 1 ligands or P4C2 but not when FNIII7-10 was used alone or mixed with P1D6. Adhesion to the H89 fragment

Figure 8. Regulation of RhoA and p190RhoGAP by $\alpha 4\beta 1$ interaction with its ligands. (A) Cells (7×10^5 /well) were added to FNIII7-10 alone or mixed with the indicated fragments or mAbs. Cells were also added to H89-coated wells. After 50 min, attached cells were lysed, lysates incubated with C21-GST and bound and total RhoA analyzed by western blotting using an anti-RhoA mAb. A representative experiment out of four performed is shown. (B) p190RhoGAP was immunoprecipitated from cell lysates after 50 min of adhesion to FNIII7-10 or the indicated mixed substrata with a specific mAb, and phosphorylation measured in western blots using PY20 mAb. Mock, protein G-agarose beads without antibody; BL, basal levels at time 0. Total p190RhoGAP was also analyzed by Western blotting (WB: p190). A representative experiment out of three performed is shown. (C) Quantitation (arbitrary units) of the relative amount of active RhoA and p190RhoGAP on cells attached to the various substrata. Average values from three independent experiments are shown. pp190, phosphorylated p190RhoGAP. $**p \leq 0.01$; $***p \leq 0.001$. (D) Time course of RhoA and p190RhoGAP regulation upon cell attachment to mixtures of FNIII7-10 and FNIII4-5. Values represent the mean of two experiments. (E) Localization of p190RhoGAP on cells attached to FNIII7-10 or FNIII7-10+FNIII4-5. Cells were fixed after 1 h and stained with anti-p190RhoGAP. Notice the colocalization of p190RhoGAP and actin at the protrusions. Bar, 10 μ m.

alone also induced p190RhoGAP phosphorylation (Figure 8BC), in agreement with the low RhoA active levels observed on this substrate (Figure 8AC).

To determine whether there was a time course correlation between the observed RhoA inactivation and p190RhoGAP phosphorylation, we performed kinetic analysis on cells attached to mixtures of FNIII7-10 and FNIII4-5. As shown in Figure 8D, there was an early enhanced RhoA activity after 10 min followed by a decrease to basal levels over the 50 min

of the assay. In parallel to this inactivation, there was a progressive increase of p190RhoGAP phosphorylation (Figure 8D). These results suggested that $\alpha 4\beta 1$ ligation prevents RhoA activation and stress fiber formation by inducing p190RhoGAP phosphorylation. In support of an important role for p190RhoGAP in $\alpha 4\beta 1$ signaling, p190RhoGAP colocalized with actin in the cytoplasmic protrusions of cells plated on FNIII7-10+FNIII4-5, while it remained diffused on cells attached to FNIII7-10 (Figure 8E).

Interaction of $\alpha 4\beta 1$ with FNIII4–5 or with Other Ligands Stimulates Melanoma Cell Migration

To determine whether the observed $\alpha 4\beta 1$ -mediated signaling resulted in cell migration, we first monitored SKMEL-178 cell movement on FNIII7–10, FNIII7–10+FNIII4–5, or FNIII7–10+FNIII4–5-DL substrata using a CCD camera and the VideoMach software. Video 1 (see supplemental material) shows that cells seeded on FNIII7–10 (2.1 μM) attached, spread and displayed certain membrane activity but remained at their original position without any detectable migration over the 150 min monitored. In contrast, when $\alpha 4\beta 1$ was simultaneously engaged to FNIII4–5 (4.9 μM), cells became very motile, extended protrusions and displayed significant random migration (Video 2). Cells plated on the control substrate FNIII7–10+FNIII4–5-DL behave very similar to those plated on FNIII7–10 (Video 3). These results are also shown in Figure 9A, which displays four representative photograms taken at different times during the assay. As can be observed with the help of black and white arrows (Figure 9A) and by single cell tracking measurements (Figure 9B), melanoma cells did not move from their initial position (black arrows) when plated on FNIII7–10 or FNIII7–10+FNIII4–5-DL. Cells however clearly migrated on FNIII7–10+FNIII4–5 to new positions, indicated by white arrows (Figure 9AB).

To determine whether the H89 fragment or VCAM-1 also stimulated cell migration when coimmobilized with FNIII7–10, we monitored cells added to these substrata over the same period of time. As shown in Figures 9C and 9D and videos 4 and 5 (see supplemental material), cells became very motile in the presence of either H89 (4.9 μM) or VCAM-1 (0.75 μM) and extended cytoplasmic protrusions. Cell movement on FNIII7–10+H89 was more limited than in the presence of VCAM-1 or FNIII4–5 but it was clearly evident when compared with cells plated on FNIII7–10 alone or mixed with FNIII4–5-DL (see corresponding videos). The concentration of VCAM-1 had to be adjusted in these experiments to obtain maximal movement, in agreement with the reported short range of VCAM-1 concentrations that support cell migration (Mould *et al.*, 1994). These results were further confirmed by cell surface area measurements, which showed a significant decrease overtime for cells plated on mixed substrata of FNIII7–10 and FNIII4–5, H89 or VCAM-1 (Figure 9E). These areas however showed a moderate increase with a maximum at 60 min, for cells plated on FNIII7–10 alone or mixed with FNIII4–5-DL (Figure 9E).

We also performed wound-healing assays using the various $\alpha 4\beta 1$ ligands. Nearly confluent SKMEL-178 cells were monitored for their ability to fill a small wound in the presence of FNIII7–10, FNIII7–10+FNIII4–5, or FNIII7–10+FNIII4–5-DL. The response to wounding in all cases was slow and after 3–6 h no appreciable migration could be detected. However, after 24 h there were clear differences in cell migration depending on the ligand used. In the presence on FNIII7–10 or FNIII7–10+FNIII4–5-DL, cells moved very similarly to control cells in the absence of Fn fragments and 33–40% of the area of the wound remained open when compared with the wound at time 0 (Figure 10A, 10C). In contrast cells cultured in the presence of FNIII7–10+FNIII4–5 clearly migrated from both edges and filled nearly 93% of the wound surface after 24 h (Figure 10A,

10C). This was not due to increased cell proliferation in the presence of FNIII4–5 fragment since quantitation of cells by trypan blue at the end of the assay revealed almost identical numbers on all three substrata: $438 \times 10^3/\text{ml}$ (FNIII7–10), $440 \times 10^3/\text{ml}$ (FNIII7–10+FNIII4–5) and $443 \times 10^3/\text{ml}$ (FNIII7–10+FNIII4–5-DL), and no significant growth with respect to the number of starting cells ($440 \times 10^3/\text{ml}$).

A similar wound-healing response was obtained in the presence of FNIII7–10 plus H89 fragment or VCAM-1. As shown in Figures 10B and 10C, 95 and 92% of the wound respectively had closed after 24 h in the presence of H89 or VCAM-1. As in the case of FNIII4–5, these ligands did not induce increased cell proliferation compared with FNIII7–10 alone (not shown). The results from video microscopy and wound healing assays therefore showed that $\alpha 4\beta 1$ signaling upon interaction with its ligands leads to melanoma cell migration.

DISCUSSION

In this report we have studied the role of the Hep III domain of Fn on cytoskeleton organization, in an effort to clarify the physiological function of this region. We show that melanoma cells, unlike previous findings on fibroblasts (Woods *et al.*, 1986; Bloom *et al.*, 1999), formed stress fibers and focal adhesions upon attachment to the FNIII7–10 fragment via $\alpha 5\beta 1$ integrin, although the response was not as developed as on Fn. Addition of FNIII4–5 (a fragment representing the Hep III domain), either immobilized or in soluble form, to FNIII7–10 inhibited these cytoskeletal structures and induced formation of cytoplasmic protrusions. Thus, although FNIII4–5 was only weakly adhesive (this study and Moyano *et al.*, 1999) it effectively induced intracellular signals to cells attached via $\alpha 5\beta 1$. A similar behavior was previously described for a FNIII13 fragment which was also poorly adhesive but induced stress fibers and focal adhesions on fibroblasts attached via $\alpha 5\beta 1$ (Bloom *et al.*, 1999). In our present study, we provide conclusive evidence for a primary role of $\alpha 4\beta 1$ integrin in the cytoskeleton response induced by FNIII4–5. First, point mutation experiments clearly showed that disruption of the native H2 sequence KLDAPT ($\alpha 4$ ligand) rendered the FNIII4–5 fragment inactive. The D/E mutation had no effect, in agreement with the fact that the KLEAPT peptide inhibited cell adhesion to FNIII4–5 while the KDLAPT peptide did not (Moyano *et al.*, 1997). Similarly, a D/E substitution in the GRGDS peptide was inactive, while the peptide GRDGS did not inhibit cell adhesion (Mould *et al.*, 1991). Second, other $\alpha 4\beta 1$ ligands such as the H89 fragment, containing the CS-1 site or VCAM-1 as well as an anti- $\alpha 4$ mAb, produced the same cytoskeletal response when mixed with FNIII7–10 as FNIII4–5, while the non-integrin binding fragment FNIII13 had no effect. Third, transfection of HeLa cells with $\alpha 4$ subunit clearly impaired their ability to form stress fibers in the presence, but not in the absence, of FNIII4–5.

Our results differ from a previous study (Huhtala *et al.*, 1995) in which rabbit synovial fibroblasts, which unlike most fibroblasts express $\alpha 4\beta 1$ integrin, formed weak focal adhesions when attached to mixtures of a 120 kDa Fn fragment ($\alpha 5\beta 1$ ligand) and the CS-1 synthetic peptide ($\alpha 4\beta 1$ ligand). This apparent discrepancy may be explained by the different cell type used in both studies and/or by the use of a peptide

Figure 9. Time-lapse analysis of melanoma cell movement. SKMEL-178 cells were added to coverslips coated with FNIII7-10 alone or mixed with either FNIII4-5 or FNIII4-5-DL (A) or with H89 or VCAM-1 (C), placed on a thermally-controlled plate and photographed every 30 s for 3 h. Four representative times are shown. Black arrows indicate the initial position of selected cells and white lines the movement and final position of those cells after 24 h. Single cell tracking analyses for all substrata are also shown (B, D). See online material for the corresponding videos. Bar, 100 μm . (E) Cell surface areas were quantified using the IP Lab Spectrum program. At least six different determinations were done for each panel. A representative experiment out of three performed is shown. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 10. Stimulation of melanoma cell migration (wound-healing) by $\alpha 4\beta 1$ /ligand interaction. Confluent SKMEL-178 cells in 12-well plates were wounded and incubated with complete medium (control) or medium containing 2.1 μ M FNIII7-10 alone or mixed with 4.9 μ M FNIII4-5 or FNIII4-5-DL (A), or with 4.9 μ M H89 or 1.48 μ M VCAM-1 (B). Wound closure was monitored >24 h and photographed. Bar, 200 μ m. (C) The wound area (μ m²) that remained open for each condition after 24 h was calculated as in Figure 9. Values are expressed as % of the area of the original wound (time = 0) and represent the average of two different experiments with duplicate determinations. *** $p \leq 0.001$.

vs. a fragment (our study) as substrata, which certainly affects the concentration/density and conformation of the active sites, probably modifying the cellular response.

Several reports have documented the existence of cross-talk between integrins, which frequently leads to antagonistic signaling (Huhtala *et al.*, 1995; Porter and Hogg, 1997; Retta *et al.*, 1998). Our present results are the first to show that induction of stress fibers and focal adhesions upon $\alpha 5\beta 1$ engagement is inhibited by concomitant ligation of $\alpha 4\beta 1$, thus establishing that $\alpha 4\beta 1$ may be an important regulator of $\alpha 5\beta 1$ -mediated cytoskeletal responses. Our results also show that $\alpha 4\beta 1$ cross-talk is not restricted to $\alpha 5\beta 1$ since it can also affect $\alpha 2\beta 1$ (but not $\alpha V\beta 3$)-induced cytoskeletal response. The antagonistic roles of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins in melanoma cytoskeleton reorganization contrast with the well established cooperative effect of syndecan-4 and $\alpha 5\beta 1$ integrin that promotes formation of stress fibers and focal adhesions in fibroblasts (Woods *et al.*, 2000; Saoncella *et al.*, 1999; Bloom *et al.*, 1999). Coordinate functions for $\alpha 4\beta 1$ integrin and the melanoma cell CSPG that lead to cell spreading and focal contact formation have also been clearly demonstrated (Iida *et al.*, 1995). These previous findings support the conclusion that simultaneous engagement of an integrin and a proteoglycan results in a cooperative response. Since melanoma cells also express CSPG, our current results suggest that signaling via $\alpha 4\beta 1$ dominates oversignaling via CSPG. We believe that inhibition of $\alpha 5\beta 1$ function was not previously observed because most cytoskeleton organization studies have been done in fibroblasts, which generally do not express $\alpha 4\beta 1$ integrin, and thus only the

cooperative effect of syndecan-4/Hep II interaction was functional.

Our results indicate that the activity of the H2 and CS-1 sites appears to be cryptic in the context of native Fn, since melanoma cells formed stress fibers and focal adhesions when attached to Fn. This conclusion is supported by the fact that large Fn fragments such as 80 kDa and FNIII7-15, which contain equimolar ratios of $\alpha 5\beta 1$ and $\alpha 4\beta 1$ binding sites, also induced stress fibers. Moreover, our results also show that the inhibitory activity mediated by $\alpha 4\beta 1$ was clearly exposed upon proteolytic degradation of Fn, an important physiological process essential during development, cell migration and invasion, wound healing, and in pathological situations (Basbaum and Werb, 1996; Davis *et al.*, 2000), that we reproduced here using trypsin and pepsin digestions as a model system. Indeed, cells attached to peptic digests of the 80 kDa fragment lacked stress fibers and we were able to purify fragments of 20/30 kDa from these digests which contained the H2 sequence and produced similar cytoskeletal effects as the recombinant FNIII4-5 used throughout our study. Therefore, our present results clearly establish that fragments containing $\alpha 4\beta 1$ integrin ligands are released upon proteolytic degradation of Fn and may modify the cytoskeletal response in physiological situations. The fact that the 20/30 kDa fragments also affect the morphological pattern induced by adhesion to Fn, further extends the physiological role of proteolytic fragments. Although we have focused this study on the functional role of the H2 sequence in repeat III5 and thus have purified fragments containing this region, a similar situation is likely to occur

for CS-1-containing fragments, which we previously showed to be readily produced upon trypsin digestion of Fn (Garcia-Pardo *et al.*, 1986). These fragments would be equivalent to the recombinant H89 fragment used here, which produced inhibition of stress fibers and focal adhesions. Interestingly, we found that the CS-1 epitope was partially buried in Fn and in FNIII7–15, in agreement with a previous study showing that CS-1 was cryptic in Fn and exposed upon proteolytic degradation, mainly by acidic proteases such as cathepsins and pepsin (Ugarova *et al.*, 1996).

We have aimed to identify some of the biochemical signals triggered by $\alpha 4\beta 1$ integrin engagement, which account for the inhibition of stress fibers and focal adhesions in melanoma cells. Previous reports on fibroblasts have shown that during the early phase of attachment to Fn (via $\alpha 5\beta 1$ integrin), RhoA is rapidly and transiently inhibited and this is followed by an activation phase which peaks at 60–90 min and induces formation of stress fibers and focal adhesions (Ren *et al.*, 1999). The initial suppression of RhoA requires FAK (Ren *et al.*, 2000) and is apparently due to c-Src phosphorylation and activation of p190RhoGAP leading to the formation of membrane protrusions, which allow fibroblast spreading and migration (Arthur *et al.*, 2000; Arthur and Burridge, 2001).

In the present study we show that activation of RhoA or transfection with constitutively active V14Rho, restored formation of stress fibers in cells attached to FNIII7–10+FNIII4–5 fragments. Furthermore, our results establish a different modulation of RhoA by $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins in melanoma cells. Thus, ligation of $\alpha 5\beta 1$ resulted in sustained activation of RhoA while concomitant engagement of $\alpha 4\beta 1$, down-regulated RhoA after an early activation phase. Interestingly, this correlated with an increased p190RhoGAP phosphorylation, indicating that $\alpha 4\beta 1$ ligation prevents activation of RhoA by activating p190RhoGAP. At difference with the previously described pathway for the early RhoA inactivation in fibroblasts (Ren *et al.*, 1999), $\alpha 4\beta 1$ mediated signaling apparently lacks the stimulus that results in RhoA activation at a later time, and RhoA activity remains low. The involvement of p190RhoGAP in $\alpha 4\beta 1$ signaling is further supported by its localization at cytoplasmic protrusions on FNIII7–10+FNIII4–5 but not on FNIII7–10. This is in agreement with a role for p190RhoGAP in protrusion formation as previously shown for the melanoma cell line LOX following $\alpha 6\beta 1$ engagement (Nakahara *et al.*, 1998) and for dominant negative p190-fibroblasts (Arthur and Burridge, 2001).

Although a role for $\alpha 4\beta 1$ in cell motility has already been established (Holzmann *et al.*, 1998), we describe here a novel mechanism by which cross-talk between $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins results in cell migration. We also report for the first time that the Hep III domain of Fn induces melanoma cell migration as effectively as the “classical” CS-1 and VCAM-1 $\alpha 4\beta 1$ ligands. This involves down-regulation of RhoA whose levels appear to be critical for melanoma cell motility, as previously found for fibroblasts and some leukocytes (Nobes and Hall, 1999; Schmitz *et al.*, 2000; Alblas *et al.*, 2001; Worthylake *et al.*, 2001).

The finding that Fn contains positive and negative regulators of RhoA may be particularly important for melanoma cells in which the presence of an unligated or engaged $\alpha 4\beta 1$ integrin may determine whether the cytoskeletal response

leads to firm adhesion or to cell migration. $\alpha 4\beta 1$ /VCAM interaction has already been shown to regulate the metastatic capacity of melanoma cells (Holzmann *et al.*, 1998). Moreover, high expression of $\alpha 4\beta 1$ was shown to inhibit invasiveness of murine B16 cell lines and induction of homotypic aggregation, which resulted in retention of the cells at the primary injection site (Quian *et al.*, 1994). The regulation of $\alpha 4\beta 1$ expression and function may therefore be critical for the progression of melanoma tumors. A previous report has shown that the adaptor protein paxillin binds to the $\alpha 4$ subunit cytoplasmic domain and this results in inhibition of stress fibers and focal adhesions and enhanced cell migration (Liu *et al.*, 1999). Since this is similar to the pattern observed in the present report, it is possible that paxillin and the $\alpha 4$ subunit also associate in melanoma cells leading to the migratory phenotype characteristic of this cell type. While this possibility remains to be clarified, our present results provide new data on the role of $\alpha 4\beta 1$ integrin in regulating melanoma cell cytoskeleton organization and migration. They also establish novel functions for $\alpha 4\beta 1$ integrin ligands in the Hep III domain and the IIICS region that although latent in the native Fn molecule, become exposed during physiological processes.

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