A synthetic peptide from the heparin-binding domain III (repeats III4–5) of fibronectin promotes stress-fibre and focal-adhesion formation in melanoma cells

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Cell adhesion to fibronectin results in formation of actin stress fibres and focal adhesions. In fibroblasts, this response requires two co-operative signals provided by interactions of the RGD sequence with $\alpha 5 \beta 1$ integrin and the heparin-binding domain II (Hep II) domain with syndecan-4. Within Hep II, this activity was mapped to repeat III13 and to the peptide FN-C/H-V (WQPPRARITGY, repeat III14). We previously described that the synthetic heparin-binding peptide/III5 (HBP/III5) (WTPPRAQITGYRLTVGLTRR, repeat III5) binds heparin and mediates cell adhesion via chondroitin sulphate proteoglycans. We have now studied whether HBP/III5 co-operates with $\alpha 5 \beta 1$ and drives a full cytoskeletal response in melanoma cells. SKMEL-178 cells attached and spread on the RGD-containing FNIII7–FNIII10 (FNIII7–10) fragment, but did not form stress fibres or focal adhesions. Co-immobilization of HBP/III5 with FNIII7–10 or adding soluble HBP/III5 to cells prespread on FNIII7–10, effectively induced these structures. Cell transfection with dominant-negative N19RhoA, a member

of the small GTPase family, abolished the HBP/III5 effect. Both chondroitinase and heparitinase diminished focal adhesions, indicating that both types of proteoglycans bound HBP/III5 in melanoma cells. We have mapped the active sequence of HBP/III5 to YRLTVGLTRR, which is a novel sequence in fibronectin with focal-adhesion-promoting activity. The last two arginine (R) residues of this sequence are required for activity, since their replacement by alanine completely abrogated the HBP/III5 cytoskeletal effect. Moreover, this sequence is also active in the context of large fibronectin fragments. Our results establish that the Hep III region provides co-operative signals to $\alpha 5 \beta 1$ for the progression of the cytoskeletal response and that these include activation of RhoA.

Key words: cell-surface proteoglycans, cytoskeletal reorganization, fibronectin heparin-binding domain III (Hep III) domain, heparin-binding peptide, RhoA.

INTRODUCTION

Cell adhesion to fibronectin (FN), a major component of the extracellular matrix, leads to cell spreading and formation of stress fibres and focal adhesions [1,2]. Because this cytoskeleton reorganization is crucial for cell migration and delivery of intracellular signalling, it is important to determine the molecular interactions that regulate the initial post-adhesion events. Previous studies using proteolytic fragments of FN have established that the initial attachment and spreading is mediated by the interaction of the RGD (Arg-Gly-Asp)-containing cell-binding domain (repeats III9–10) with $\alpha 5 \beta 1$ integrin, but that further progression of the cytoskeletal response requires additional signals. These may be provided by the interaction of fragments containing the heparin-binding domain II (Hep II) domain (repeats III12–14) with cell-surface proteoglycans (PG), mainly syndecan-4 [3–5]. Further characterization of these interactions revealed that the synthetic peptide FN-C/H-V, corresponding to the sequence WQPPRARI in FN repeat III14 [6], was a potent inducer of stress fibres and focal adhesions in fibroblasts [7,8] and that endothelial cells [9] attached via RGD/ $\alpha 5 \beta 1$. Another report, however, found this sequence inactive when present in a recombinant fragment containing FN repeat III14, and the main focal-adhesion-promoting activity in that study was located to

repeat III13 [10], a ligand for syndecan-4 [11]. These apparent discrepancies suggest different signalling requirements dependent on the cell type and/or that the FN-C/H-V peptide may adopt a conformation which is similar to the ligand in repeat III13.

We previously reported that the WTPPRAQITGYRLTVGLTRR sequence in FN III5 repeat within the Hep III domain, binds heparin and mediates T cell adhesion via chondroitin surface proteoglycans (CSPG) when presented as the synthetic heparin-binding peptide/III5 (HBP/III5) [12]. Because this sequence is similar to FN-C/H-V, we tested in the present study whether HBP/III5 could also provide the additional signalling required for induction of stress fibres and focal adhesions. Using melanoma cells, which, unlike T-cells, form these cytoskeletal structures, we show that HBP/III5 efficiently promotes stress fibres and focal adhesions, and that both CSPG and heparan sulphate proteoglycan (HSPG) are involved in this response. Furthermore, signalling via HBP/III5 involves activation of the small GTPase RhoA.

EXPERIMENTAL

Fibronectin fragments and synthetic peptides

The FNIII7–FNIII10 (FNIII7–10) recombinant fragment (repeats III7–III10 in FN, contains the RGD sequence) was

Abbreviations used: CSIC, Consejo Superior de Investigaciones Científicas; CS(PG), chondroitin sulphate (proteoglycan); DMEM, Dulbecco's Modified Eagle's medium; FN, fibronectin; FNIII7–10, FNIII7–FNIII10; GFP, green fluorescent protein; HBP, heparin-binding peptide; Hep II, heparin-binding domain II; HS(PG), heparan sulphate (proteoglycan); KLH, keyhole-limpet haemocyanin; mAb, monoclonal antibody; TRITC, tetramethylrhodamine β -isothiocyanate.

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Table 1 Synthetic peptides used in the present study

Amino acid sequences are shown in the one-letter code. Mutated arginine (R)→alanine (A) residues are depicted in **bold**.

Peptide name	Peptide sequence
HBP/III5	WTPPRAQITGYRLTVGLTRR
HBP/III5.rv	RRTLGVTLRYGTIQARPPTW
HBP/III5-R1	WTPP AA QITGYRLTVGLTRR
HBP/III5-R2	WTPPRAQITGY ALT VGLTRR
HBP/III5-R3R4	WTPPRAQITGYRLTVGLT AA
HBP/III5-R1R2R3R4	WTPP AA QITGY ALT VGLT AA
N11	WTPPRAQITGY
C10	YRLTVGLTRR
C10-R2	Y ALT VGLTRR
C10-R3R4	YRLTVGLT AA
C10-R2R3R4	Y ALT VGLT AA
FN-C/H-V	WQPPRARITGY

prepared by transforming *Escherichia coli* DH5 α with FNIII7–10 cDNA in the pET vector, kindly donated by Dr Harold Erickson (Department of Cell Biology, Duke University Medical Center, Durham, NC, U.S.A.), and purified as described in [13]. The H0 recombinant fragment (FN repeats III12–III15, Hep II domain, lacks the IIICS region) was prepared as reported in [14]. The 80 kDa fragment (repeats III4–_{1/2}III11) was purified from tryptic digestions of plasma FN as previously described [15,16]. The synthetic peptides prepared and used in the present study are listed in Table 1. All peptides were synthesized on an automated multiple peptide synthesizer (AMS 422; ABIMED Analysen-Technik GmbH, Langenfeld, Germany) and conjugated to keyhole-limpet haemocyanin (KLH from *Megathura crenulata*; Calbiochem–Novabiochem Co., La Jolla, CA, U.S.A.) exactly as described in [12].

Antibodies, enzymes and other reagents

Monoclonal antibodies (mAbs) specific for vinculin and talin were purchased from Sigma (St. Louis, MO, U.S.A.); anti-paxillin mAb was from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.); mAbs P1D6 and P4C2, reactive with $\alpha 5$ and $\alpha 4$ integrins respectively, were previously described [17]; anti-($\alpha V\beta 3$ integrin) mAb (LM609) and polyclonal antibody anti-NG2 were purchased from Chemicon International Inc. (Temecula, CA, U.S.A.); anti-syndecan-4 mAb was from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Chondroitinase ABC, heparin, heparan sulphate and chondroitin sulphate were purchased from Sigma; heparitinase was from Seikagaku America (Falmouth, MA, U.S.A.).

Cells and immunofluorescence assays

The human cell lines SKMEL-178 (melanoma) and Jurkat (T lymphocytes) were obtained from Dr Francisco Real (Hospital del Mar, Barcelona, Spain) and Dr Margarita López-Trascasa (Hospital La Paz, Madrid, Spain) respectively. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; SKMEL-178) or RPMI (Jurkat), 10% fetal-bovine serum (Gibco, Paisley, Renfrewshire, Scotland, U.K.) and 40 μ g/ml gentamycin (Gibco). Prior to immunofluorescence assays, cells were serum-starved for 3 h, detached from culture flasks with 1 mM EDTA/PBS, pH 7.5, washed, and resuspended in attachment medium (DMEM/1% BSA/10 mM Hepes). Immunofluorescence assays were carried out on glass coverslips previously

coated for 2 h at 37 °C with 0.41 μ g (0.71 μ M) or 0.06 μ g (0.1 μ M) of FNIII7–10 in 40 μ l of PBS, with or without 11 μ l (at 135 μ g/ml) of appropriate peptides. After washing and blocking with 1% BSA/PBS, serum-starved 2×10^4 SKMEL-178 cells in attachment medium were added to each well and incubated for 1 h at 37 °C. In some experiments cells were incubated with chondroitinase (1 unit/ml) or heparitinase (2 munit/ml) or both for 45 min at room temperature prior to their addition to substrate-coated wells. Attached cells were fixed with 3.5% formaldehyde/PBS, permeabilized with cold 0.5% Triton X-100/PBS and blocked with 1% BSA/PBS for 30 min. F-actin was stained with tetramethylrhodamine β -isothiocyanate (TRITC)–phalloidin, and focal-adhesion components were detected with specific mAbs (1 h, 37 °C) followed by rabbit FITC-labelled anti-mouse IgG Ab (Dako A/S, Glostrup, Denmark). For inhibition with chondroitin sulfate, heparan sulphate and heparin, the 80 kDa fragment, FNIII7–10 or the mixture FNIII7–10 + HBP/III5 were incubated in solution with 100 μ g/ml of either of these reagents for 1 h at 37 °C prior to their immobilization on glass coverslips, and the assay was continued as explained above in the presence of these reagents. Samples were visualized under an epifluorescence Axioplan microscope (Zeiss, Oberkochen, Germany) and photographed with a CCD camera (Photometrics Inc., Tucson, AZ, U.S.A.). At least 100 cells were scored in each assay.

Cell-adhesion assays

Flat-bottomed eight-well strips with an *N*-oxysuccinimide amine binding surface (Costar, Cambridge, MA) were coated for 1–2 h with increasing concentrations of FNIII7–10 fragment diluted in 0.1 M sodium borate, pH 8.5. SKMEL-178 cells (20×10^3) with or without previous incubation with P1D6, LM609 or P4C2 mAbs or with enzymes [45 min at room temperature (23 °C)] were added to the wells and adhesion was allowed to occur for 1 h at 37 °C. Attached cells were fixed with 1.25% formaldehyde and stained with 0.1% Toluidine Blue in PBS. A_{620} was determined on a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland), and quantification of cell attachment was done using calibration curves as described in [12,16]. The absorbance was found to be practically a linear function of the number of cells attached (1 A unit = 80×10^3 cells).

Cell transfection

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, U.S.A.) in *E. coli* DH5 α , were a gift from Dr Francisco Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Plasmidic DNA from *E. coli* was isolated by performing minipreps. Subconfluent cells in fresh complete medium were transiently transfected with these cDNAs or with the empty vector using FuGENE™ 6 (Roche Biochemicals, Mannheim, Germany), following the manufacturer's instructions. After 24 h, transfected cells were used in immunofluorescence assays.

Statistical analyses

Significance of the difference between means was determined by the Student's *t* test for non-paired samples using the GraphPad Instat program (GraphPad Software, San Diego, CA, U.S.A.). Two-tailed statistical significances were determined. A *P* value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The WTPPRAQITGYRLTVGLTRR sequence (HBP/III5) promotes stress fibres and focal adhesions in melanoma cells

Initial characterization of SKMEL-178 cell adhesion to the FNIII7–10 fragment revealed that these cells attached to FNIII7–10 in a dose-dependent manner and that adhesion was completely blocked by the anti- $\alpha 5$ integrin mAb P1D6 but not by LM603 (anti- $\alpha V\beta 3$) or P4C2 (anti- $\alpha 4$) mAbs (Figure 1), indicating that $\alpha 5\beta 1$ was the receptor for FNIII7–10 in SKMEL-178 cells. To characterize the cytoskeletal response following attachment to this fragment, SKMEL-178 cells were added to glass coverslips coated with FNIII7–10 at 0.1 μM or 0.71 μM respectively. As shown in Figure 2(A) (left panels), melanoma cells attached to both concentrations of FNIII7–10, but the resulting morphological patterns were very different: at 0.1 μM , more than 80% of cells showed limited spreading with extension of lamellipodia and absence of stress fibres and focal adhesions (Figures 2A and 2C); at the higher concentration of 0.71 μM , most cells displayed an elongated morphology, and approx. 70% of them formed stress fibres (Figure 2A) and weak focal adhesions containing vinculin, little paxillin and no talin (Figure 2B).

To determine whether the HBP/III5 synthetic peptide (see Table 1) could provide a co-operative signal to $\alpha 5\beta 1$ -integrin-mediated cytoskeletal reorganization, similar experiments were performed on glass coverslips coated with a mixture of FNIII7–10 and HBP/III5 peptide (135 $\mu\text{g}/\text{ml}$) coupled to KLH. Co-immobilization of HBP/III5 with FNIII7–10 dramatically induced cell spreading and formation of stress fibres on 86% of cells plated on 0.1 μM FNIII7–10 (Figures 2A and 2C). HBP/III5 also clearly enhanced spreading and the number of stress fibres at the higher concentration of 0.71 μM FNIII7–10 (Figure 2A).

Figure 1 Melanoma-cell adhesion to the FNIII7–10 fragment is mediated by $\alpha 5\beta 1$ integrin

Cells (20×10^3), with or without (control) previous incubation with mAbs P1D6, LM609 or P4C2 (2 μg each), were added to wells coated with the indicated concentrations of FNIII7–10. After 1 h at 37 $^{\circ}\text{C}$, attached cells were fixed, stained with 0.1% Toluidine Blue and quantified by measuring the A_{620} as described in the Experimental section. Average values from three different experiments are shown.

Figure 2 HBP/III5 peptide promotes stress fibres and focal adhesions

(A) SKMEL-178 cells (2×10^4) were added to glass coverslips previously coated with FNIII7–10 (0.1 or 0.7 μM respectively) alone or mixed with 135 $\mu\text{g}/\text{ml}$ of HBP/III5 or HBP/III5.rv peptides coupled with KLH. After 1 h at 37 $^{\circ}\text{C}$, actin filaments were visualized with TRITC/phalloidin. Cells were also added to coverslips coated with HBP/III5-KLH or HBP/III5.rv-KLH alone. The bar represents 10 μm . (B) Focal adhesions on cells plated on 0.7 μM FNIII7–10 + HBP-KLH were detected by staining with specific monoclonal antibodies to vinculin, paxillin and talin. The bar represents 10 μm . (C) Quantification of the effect of co-immobilization of peptides (135 $\mu\text{g}/\text{ml}$) with FNIII7–10 (0.1 μM) or addition of soluble (sol) peptides to cells prespread on FNIII7–10 for 1 h. The effect of immobilized peptides alone is also indicated. See Table 1 for the sequence of each peptide. SF, stress fibres; FA, focal adhesions. Values represent the average for at least three different experiments. *** $P \leq 0.001$.

Additionally, HBP/III5 induced formation of focal adhesions on cells plated on both concentrations of FNIII7–10 (shown in Figure 2B for 0.71 μM). These structures were more developed than those observed on 0.71 μM FNIII7–10 alone, since they now contained paxillin and talin besides vinculin (Figure 2B).

The primary structure of HBP/III5 was important for the activity, since reversing the entire sequence (HBP/III5.rv, Table 1) rendered the peptide inactive (Figures 2A and 2C). Cell adhesion to HBP/III5–KLH alone produced very limited spreading and no stress fibres or focal adhesions (Figures 2A and 2C). Cells attached to the control HBP/III5.rv (or to KLH; results not shown) remained round, with no appreciable spreading (Figure 2A).

The HBP/III5 peptide was also equally active (80% of cells with stress fibres) when co-immobilized with FNIII7–10 as a free peptide rather than coupled to KLH (results not shown). Moreover, soluble HBP/III5 (135 µg/ml) efficiently induced stress fibres and focal adhesions on cells pre-spread on FNIII7–10 for 60 min, whereas the control peptide HBP/III5.rv had no effect (Figure 2C). However, cells attached to HBP/III5–KLH did not form stress fibres or focal adhesions upon addition of FNIII7–10 (0.1 µM) in soluble form, indicating that immobilization of this fragment was required for the observed cytoskeleton reorganization (results not shown).

For comparison, we also tested the effect of the previously described FN-C/H-V peptide [6–8] (Table 1) in our melanoma cell system. As shown in Figure 2(C), immobilized FN-C/H-V–KLH peptide or soluble FN-C/H-V (135 µg/ml) significantly induced stress fibres and focal adhesions, but was 20% less efficient than HBP/III5. Furthermore, FN-C/H-V was inactive when co-immobilized with FNIII7–10 as a free peptide, in contrast with HBP/III5 (results not shown). While these differences suggest that HBP/III5 may be a better ligand for melanoma cell surface PG (see below), it should also be considered that HBP/III5 is a 20-residue peptide, while FN-C/H-V contains only 11 residues, and this may affect binding of the free peptide to the glass surface.

In a previous study the major activity of FN-C/H-V was mapped to the sequence PRARI [7], which is homologous to PRAQI, present in HBP/III5 (see Table 1). To determine whether PRAQI was the active sequence in HBP/III5 or there was a different site responsible for the cytoskeletal effect of this peptide, we prepared two smaller peptides, N11 and C10, corresponding to the N- and C-terminal halves respectively of HBP/III5 (see Table 1). As shown in Figure 2(C), C10 fully retained the ability to induce stress fibres and focal adhesions, while N11, containing PRAQI, did not. These results are in agreement with our previous findings showing that the C10 peptide retained partial heparin-binding activity, while the N11 peptide did not bind to heparin [12]. The lack of activity of the PRAQI sequence (HBP/III5) compared with the previously described sequence PRARI (FN-C/H-V) suggests that the presence of two basic residues may be important for receptor recognition. To determine the role of the arginine residues in the activity of HBP/III5, we replaced each arginine with an alanine residue in both the HBP/III5 and C10 peptides (Table 1) and tested the effect of the mutated peptides on cell adhesion and cytoskeleton reorganization. As shown in Figure 3(A), mutation of the first arginine residue in HBP/III5 (HBP/III5-R1) did not affect the adhesion-mediating properties of this peptide, in agreement with the lack of cytoskeletal activity of the N11 sequence (Figure 2C). Mutation of the second arginine residue (HBP/III5-R2) or the equivalent arginine residue in C10 (C10-R2) decreased their ability to mediate adhesion by 25% (Figure 3A), suggesting a minor involvement of this residue in the activity of the peptides. In contrast, when the last two arginine residues were replaced by alanine (HBP/III5-R3R4 and C10-R3R4; Table 1), the resulting peptides no longer mediated cell adhesion (Figure 3A). Likewise, peptides HBP/III5-R1, HBP/III5-R2 and C10-R2 promoted stress fibres and focal adhesions as effectively as the parent

Figure 3 Effect of point mutations on the biological activity of HBP/III5 and C-10 peptides

(A) Melanoma-cell adhesion to the parental peptide HBP/III5 and various mutated peptides (see Table 1). Cells (20×10^3) were added to wells coated with the indicated peptides at $2.5 \mu\text{g}/\text{cm}^2$. After 1 h, attached cells were fixed, stained with 0.1% Toluidine Blue, and quantified by measuring the A_{620} as explained above. Values represent the average for two independent experiments. (B) Parental or mutated peptides (135 µg/ml) were co-immobilized with FNIII7–10 (0.1 µM) and the number of cells showing stress fibres (SFs) and focal adhesions (FAs) was quantified using an epifluorescence microscope. Values are the average for three independent experiments. *** $P \leq 0.001$.

peptides (Figure 3B). However, mutation of the two C-terminal arginine residues (HBP/III5-R3R4 and C10-R3R4 peptides) completely inhibited their ability to promote stress fibres and focal adhesions (Figure 3B). As expected, mutation of all four arginine residues in HBP/III5 or the three arginine residues in C10 abolished the activity of both peptides (results not shown). These findings clearly demonstrate that the stress-fibre/focal-adhesion-promoting function of the HBP/III5 peptide resides in the C-terminal sequence YRLTVGLTRR and that the last two arginine residues are required for its activity.

CS and HS proteoglycans are melanoma-cell receptors for the HBP/III5 sequence

We previously showed that the cellular receptor for the HBP/III5 sequence in Jurkat T cells is a CSPG [12]. To determine the nature of the receptor involved in the cytoskeleton effect observed in melanoma cells, SKMEL-178 cells were incubated with chondroitinase ABC or heparitinase prior to their addition to FNIII7–10 or FNIII7–10+HBP/III5. As shown in Table 2, these enzymes had no effect on the morphological pattern observed on cell adhesion to FNIII7–10. However, on mixed substrata, both chondroitinase and heparitinase diminished the proportions of cells with stress fibres and focal adhesions to 49 and 51% respectively (Table 2). Interestingly, the effect of the individual enzymes was additive, since treatment with both enzymes simultaneously completely reverted the focal-adhesion-

Table 2 Effect of enzymes on stress-fibre/focal-adhesion formation

Cells were treated with either or both enzymes and added to coverslips coated with FNIII7–10 or FNIII7–10 + HBP/III5. Values represent the mean for three different experiments.

Treatment	Proportion of cells with stress fibres or focal adhesions (%)	
	FNIII7–10	FNIII7–10 + HBP/III5
None	17.3 ± 2.2	75.2 ± 6.9
Chondroitinase ABC	20.9 ± 7.1	49.3 ± 2.8
Heparitinase	28.4 ± 4.7	50.9 ± 4.4
Chondroitinase ABC + heparitinase	21.5 ± 3.4	23.2 ± 2.5

Table 3 Effect of enzymes on cell adhesion

Cells treated with individual or combined enzymes were added to wells coated with the different peptides or the recombinant fragment H0 (Hep II domain). Values represent the average for three different experiments. Abbreviation: ND, not determined.

Treatment	Cells ... Substrate ...	Cell adhesion (%)				
		Jurkat			SKMEL-178	
		HBP/III5	C10	H0	HBP/III5	C10
None		100	100	100	100	100
Chondroitinase ABC		0	0	90.5 ± 3.31	70.0 ± 7.1	54.0 ± 8.4
Heparitinase		96.0 ± 2.1	ND	5.5 ± 6.36	65.0 ± 3.5	54.5 ± 10.7
Chondroitin ABC + heparitinase		ND	ND	ND	17.5 ± 3.5	18.5 ± 8.5

promoting activity of HBP/III5 (Table 2). To confirm that the partial inhibition obtained with either enzyme alone was not due to poor enzymic activity, in parallel experiments we tested the effect of chondroitinase and heparitinase on the adhesion of Jurkat cells to HBP/III5, C10 and the H0 fragment. We have previously shown that Jurkat cell adhesion to HBP/III5 is completely dependent on CSPG but not HSPG and, by contrast, adhesion to the H0 fragment is mediated by HSPG [12]. In agreement with these previous results, Table 3 shows that chondroitinase ABC, at the same concentration used for the SKMEL-178 assays, completely inhibited Jurkat-cell adhesion to the HBP/III5 and C10 peptides, indicating that the enzyme was fully active. Similarly, preincubation of Jurkat cells with heparitinase completely blocked their adhesion to the H0 fragment, again confirming the activity of this enzyme (Table 3). These results suggested that, in contrast with our previous findings on Jurkat cells, recognition of the HBP/III5 sequence by melanoma cells involves both CSPG and HSPG. To confirm this, we performed adhesion assays to HBP/III5 and C10 peptides after preincubation of SKMEL-178 cells with either or both enzymes. As shown in Table 3, either chondroitinase or heparitinase when used alone, partially inhibited cell adhesion to both peptides, whereas the combination of both enzymes produced almost complete inhibition, in agreement with the results observed on cytoskeleton reorganization. Identical results were obtained for inhibition of adhesion to the HBP/III5-R1, HBP/III5-R2 and C-10-R2 peptides (results not shown). Altogether these data indicate that the sequence HBP/III5 interacts with both CSPG and HSPG at the melanoma-cell surface or, alternatively, with a unique PG that carries both CS and HS glycosaminoglycan chains, and that both chains appear to contribute to peptide

Figure 4 RhoA is involved in the cytoskeletal effect of HBP/III5 peptide

(A) SKMEL-178 cells were transiently transfected with GFP–V14RhoA (panel a), GFP–N19RhoA (panel b), or GFP-empty vector (panels c and d) and added to coverslips coated with mixtures of FNIII7–10 (0.1 μM) + HBP/III5 (135 μg/ml) (panels a–c) or FNIII7–10 alone (panel d). Actin was visualized with TRITC/phalloidin. Only transfected cells, identified by fluorescence, are shown. The bar represents 10 μm. (B) Quantification of the effect of transfecting cells with V14RhoA or N19RhoA on formation of stress fibres (SF) and focal adhesions (FA). 'Mock' denotes transfection with the empty vector. The coating substrate (FNIII7–10 or FNIII7–10 + HBP/III5) is also indicated.

recognition. It is likely that the type of PG that recognizes HBP/III5 is cell-dependent, as already shown for the FN-C/H-V peptide, which interacts with HSPG in fibroblasts, but with both HS and CS PG in monocytic cells [18].

RhoA is involved in the cytoskeleton organization induced by HBP/III5-PG interaction

Although induction of stress fibres and focal adhesions in several cell types generally involves activation of the small GTPase RhoA [19,20], it was important to determine whether the novel signalling pathway provided by interaction of HBP/III5 with melanoma-cell-surface PG involved RhoA. We therefore tran-

Figure 5 The HBP/III5 sequence is active in the context of a tryptic FN fragment

(A) SKMEL-178 cells (2×10^4) were added to glass coverslips previously coated with $0.1 \mu\text{M}$ 80 kDa fragment. After 1 h at 37°C , actin filaments were visualized with TRITC/phalloidin and focal adhesions with specific mAbs to vinculin, paxillin and talin. The bar represents $10 \mu\text{m}$. (B) The 80 kDa or FNIII7–10 fragments ($0.1 \mu\text{M}$) were incubated with $100 \mu\text{g/ml}$ of CS, HS, heparin or the mixture CS + HS for 1 h and used to coat glass coverslips. Cells were added and after 1 h, stress fibres (SF) and focal adhesions (FA) were quantified as described above. Values represent the mean for three different experiments.

siently transfected SKMEL-178 cells with the constitutively active V14RhoA or the dominant-negative N19RhoA forms, and studied their cytoskeleton reorganization upon attachment to FNIII7–10 + HBP/III5 or FNIII7–10 alone. On mixed substrata, the presence of the active GFP-V14RhoA did not significantly affect the cytoskeletal structures induced by HBP/III5, and 68 % of transfected cells displayed stress fibres (Figures 4A and 4B) and focal adhesions (results not shown), suggesting that maximal activation of RhoA was already achieved by the HBP/III5–PG interaction. By contrast, transfection with the dominant-negative GFP-N19RhoA resulted in reduced spreading and complete absence of stress fibres on 71 % of transfected cells (Figures 4A and 4B), a pattern that resembled that shown by mock-transfected cells on FNIII7–10 (Figure 4A). In agreement with this, transfection with GFP-V14RhoA induced spreading and stress-fibre formation on cells attached to FNIII7–10 alone (not shown). Transfection with the empty vector did not alter the stress-fibre

pattern observed on cells attached to FNIII7–10 + HBP/III5 (Figure 4A). These results indicate that an active RhoA is required for induction of stress fibres and focal adhesions by HBP/III5 and that constitutively active RhoA can substitute for the effect of HBP/III5.

The YRLTVGLTRR sequence is active in the context of an 80 kDa FN tryptic fragment

Our results therefore establish that PG interaction with the YRLTVGLTRR sequence contained in the HBP/III5 and C10 peptides provides a novel co-operative signal to $\alpha 5 \beta 1$ -mediated cytoskeleton reorganization. The role of this sequence in the context of the entire FN molecule may be difficult to evaluate because of the presence of the Hep II domain, which is known to effectively induce stress fibres and focal adhesions on fibroblasts attached via $\alpha 5 \beta 1$ [3]. We therefore studied whether the YRLTVGLTRR sequence was active in the context of large FN fragments devoid of the Hep II domain and which are usually present in many normal and pathological situations [21]. To this purpose, we analysed the melanoma cytoskeleton response upon adhesion to an 80 kDa tryptic fragment corresponding to FN repeats III4– $1/2$ –III11 and thus containing the YRLTVGLTRR sequence (located in repeat III5) as well as the entire FNIII7–10 fragment [16]. As shown in Figure 5(A), adhesion of SKMEL-178 cells to the 80 kDa fragment ($0.1 \mu\text{M}$) resulted in cell spreading and formation of stress fibres and focal adhesions, which contained vinculin, paxillin and talin. This cytoskeletal response resembled that obtained when HBP/III5 was co-immobilized (or added in solution) with FNIII7–10 (see Figure 2A) and was very different from the response induced by FNIII7–10 alone (Figure 2A). These results suggested that the YRLTVGLTRR sequence (the only sequence in repeats III4–6 reported to bind heparin and PG [12]) was active when present in large FN fragments such as the 80 kDa and promoted stress-fibre and focal-adhesion formation. Indeed, we have previously shown that the 80 kDa fragment can be purified by heparin–Sepharose affinity chromatography, thus confirming that the heparin/PG-binding site is exposed in the fragment [15]. To prove further that the cytoskeleton organization induced by the 80 kDa fragment was mediated by interaction with cell-surface PG, this fragment was incubated in solution with CS, HS, heparin, or the mixture CS + HS, prior to its use as adhesion substrate. Treatment with these reagents partially inhibited cell spreading, stress fibres and focal adhesions, whereas preincubation with the mixture CS + HS completely abolished these structures (Figure 5B), resulting in a pattern very similar to that induced by the FNIII7–10 fragment (see Figure 2A). As expected, preincubation of FNIII7–10 with HS, CS or heparin did not affect the melanoma cytoskeletal response to this fragment (Figure 5B). These results confirmed that the HBP/III5 sequence was functional in the context of a large 80 kDa FN fragment and that disruption of its interaction with cell surface PG clearly impaired the cytoskeletal response induced by the 80 kDa fragment. Our results also establish that the requirements for this response may be different depending on the cell type. In this regard, fibroblasts have been shown to spread upon attachment to 85 or 105 kDa FN fragments, very similar to the 80 kDa used in the present study, but did not form the stress fibres or focal adhesions [3,7] that we observe on melanoma cells.

Co-operative interactions between integrins and PG have been extensively studied in fibroblasts attached to FN, where it is clear that $\alpha 5 \beta 1$ and syndecan-4 co-operate in the progression of the cytoskeletal response [3–5]. These studies have shown that syndecan-4 signalling depends on RhoA activation [5], in agree-

ment with our present findings on HBP/III5 signalling. It is also clear that syndecan-4 is present in focal adhesions [22] and that the FN-C/H-V peptide causes syndecan-4 to relocate to these complexes [4]. The SKMEL-178 cells used in the present study did not express syndecan-4 (results not shown) and therefore another HSPG must recognize HBP/III5 (and peptide FN-C/H-V). Previous studies have shown that the main CSPG in melanoma cells is NG2 [23], which co-operates with $\alpha 4 \beta 1$ integrin to promote spreading and focal adhesions, but is not found in these complexes [24]. In agreement with this, SKMEL-178 cells expressed NG2, but we were unable to detect it in focal adhesions (results not shown).

Our present results constitute a novel example of collaboration between integrins and PG that leads to a full cytoskeletal response upon attachment to FN. As mentioned above, previous studies on melanoma cells have focused on the co-operation between $\alpha 4 \beta 1$ integrin and CSPG [24,25]. We now show, in the present study, that CSPG (and HSPG) can also signal in a co-operative manner with $\alpha 5 \beta 1$ integrin in melanoma cells. We have identified the sequence in HBP/III5 that triggers this signalling as YRLTVGLTRR, which corresponds to the C-terminal portion of this peptide. It is noteworthy that the N-terminal sequence of HBP/III5 (WTPPRAQITGY), which is homologous with the previously described FN-C/H-V peptide (WQPPRARITGY), was clearly inactive in our system and YRLTVGLTRR is therefore a novel sequence in FN with the ability to induce stress fibres and focal adhesions. As we show, on the basis of point-mutation experiments, the last two arginine residues of this sequence are required for the biological effect. This may explain why the FN-C/H-V peptide was also active in the present study, since it contains two arginine residues (PRARI) which are absent in the N-terminus of HBP/III5 (PRAQI). Our results also suggest that the two arginine residues must be in the proper conformation, since altering the primary sequence of HBP/III5 (as in HBP/III5.rv peptide) abolished the cytoskeletal effect. The novel YRLTVGLTRR sequence described here is located in repeat III5 within the Hep III domain. Thus our results establish that at least two regions in FN (the previously described Hep II domain and the Hep III domain reported here) can modulate the cytoskeletal response initiated by $\alpha 5 \beta 1$ integrin engagement and induce intracellular signalling. We show that the YRLTVGLTRR sequence is active in the context of large FN fragments and possibly also in the context of the entire FN molecule. The contribution of either or both heparin-binding domains to the regulation of cytoskeleton organization will probably depend on the presence of appropriate receptors in a given cell type and environment.

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