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Integrins and syndecan-4 make distinct, but critical, contributions to adhesion contact formation†

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Abstract

During cell adhesion to fibronectin there is a major reorganisation of the actin cytoskeleton and concomitant formation of adhesion complexes. Conflicting studies of adhesion receptors report that either integrin alone, or both integrin and syndecan-4 mediate the formation of vinculin-containing adhesions, and differences in these studies have been attributed to the density and conformational integrity of ligands used. We have endeavoured to resolve these issues by ELISA analysis of immobilised polypeptides, and found that ligands of both integrin $\alpha_5\beta_1$ and syndecan-4 are necessary for focal adhesion formation under conditions of equivalent density of folded ligand. We also demonstrate that integrin and syndecan-4 play quite distinct roles in adhesion contact maturation and are not interchangeable. These results help us to understand how cells respond efficiently to changes in matrix environment, which should prove useful for developing approaches to aid wound healing.

Introduction

Cells adhere to extracellular matrix proteins through a process that involves attachment to the matrix followed by spreading. These morphological changes are coupled to the sequential formation of adhesion contacts and signalling complexes. Integrins are essential for adhesion to the extracellular matrix, but in some cases are not sufficient for the complete adhesion response. For example, cells attach and spread on the central cell-binding domain of fibronectin *via* integrin $\alpha_5\beta_1$, but fail to form vinculin-containing focal adhesions unless co-stimulated with a second, heparin-binding, fragment of fibronectin.^{1,2} Similarly, migration of fibroblasts over the central cell-binding domain is compromised unless the heparin-binding fragment of fibronectin is also incorporated into the matrix.³ Antibody clustering and gene disruption experiments have identified the transmembrane proteoglycan, syndecan-4, as the major mediator of fibronectin-induced focal adhesion formation,⁴ and syndecan-4 has been linked to the activation of a number of signalling molecules that might influence adhesion contact formation.⁵ These include the direct activation of protein kinase C α in a phosphatidylinositol-4,5-bisphosphate-dependent manner,⁶ the activation of focal adhesion kinase (FAK), which is abolished in cells lacking syndecan-4 or a heparin-binding ligand,⁷ and the regulation of Rac.^{8,9}

In spite of these reports, the necessity of syndecan-4-engagement for focal adhesion formation has remained contentious.³ It is frequently debated whether the requirement for syndecan-4 is limited to circumstances of low integrin-ligand density¹⁰ and might depend

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on integrin activity,¹¹ although the second hypothesis does not preclude a role for syndecan-4 as an integrin regulator. At the other extreme, syndecan-4 rather than integrin has been reported to mediate cell spreading on ligands such as the cysteine-rich domain of ADAM-12,¹² although integrin activity was still necessary for focal adhesion formation. In the present study we resolve these issues through precise analysis of ligand density to demonstrate that syndecan-4 makes a necessary contribution to focal adhesion formation on fibronectin, and does so in cooperation with integrin $\alpha_5\beta_1$.

Results

Adhesion contact formation is a consequence of syndecan-4-signalling rather than integrin density

The initial aim was to determine whether the fibronectin fragments that are frequently used to dissect the role of syndecan-4 from integrin, act as suitable matrix ligands for adhesion studies. We compared the efficiency with which recombinant integrin ligands or plasma fibronectin, which acts as a ligand for both integrin $\alpha_5\beta_1$ and syndecan-4,^{13,14} coated glass or plastic surfaces by ELISA assays using an antibody against the central cell-binding domain of fibronectin. The 333 monoclonal antibody recognises a conformationally-sensitive epitope close to the integrin-binding RGD sequence and has been shown to block adhesion of fibroblasts to fibronectin.¹⁵ A recombinant fibronectin fragment (Fn6–10) that comprises type III repeats 6–10 and includes the $\alpha_5\beta_1$ -binding site, adhered poorly to clean glass coverslips, but could be coated more efficiently by derivatising the glass with a charged crosslinker, sulfo-MBS (Fig. 1A). Under these conditions, Fn6–10 coating was still limited to 26% of the efficiency of plasma fibronectin at the same concentration. Both fibronectin and the Fn6–10 fragment coated tissue culture plastic more efficiently than glass, regardless of pre-treatment with sulfo-MBS, and the difference between Fn6–10 and whole fibronectin was less pronounced (Fig. 1B). Recognition of both Fn6–10 and fibronectin by an adhesion-blocking antibody suggests that each of the immobilised proteins is in a suitable conformation to support cell adhesion. Significantly, shorter recombinant fragments, comprising repeats 9–10 and 8–10, coated surfaces very poorly, even at high concentrations (Fig. 1A + B). Consequently, the shorter fibronectin fragments would appear to be unsuitable ligands for cell spreading assays, despite including the entire integrin-binding motif. In order to achieve equivalent molar densities of Fn6–10 and fibronectin, proteins were coated at a range of concentrations, with the result that 100 $\mu\text{g ml}^{-1}$ Fn6–10 coated derivatised glass more efficiently than 5 $\mu\text{g ml}^{-1}$ fibronectin (Fig. 1C).

Having established conditions of equivalent integrin-ligand density, we tested the ability of primary fibroblasts to spread and form adhesion contacts on the two polypeptides. At 10 $\mu\text{g ml}^{-1}$, both Fn6–10 and fibronectin supported spreading equally ($92 \pm 2\%$ and $89 \pm 3\%$ cells spread respectively). The major difference between ligands was established by scoring cells for the ability to form focal adhesions. The majority of cells plated onto fibronectin formed vinculin-containing adhesions, while only a small minority of cells formed focal adhesions on Fn6–10, even at high ligand density (Fig. 1D). As reported previously,² focal adhesion formation could be restored to cells spread on Fn6–10 by stimulation of syndecan-4 with a soluble fibronectin fragment (H/0) that encompasses the type III repeats 12–15 and includes an extended heparin-binding interface.¹⁶ The ability of H/0 to restore focal adhesion formation was independent of the density of immobilised integrin ligand, demonstrating that the activities of the two receptors are not interchangeable (Fig. 1D). Further reduction of fibronectin density did not result in spread cells failing to form focal adhesions, but instead passed below the threshold necessary to support adhesion with the result that cells plated onto fibronectin coated at 1 $\mu\text{g ml}^{-1}$ were unable to spread (Fig. 1E).

Syndecan-4 works cooperatively with integrins to support adhesion

The absolute requirement of a syndecan-4 ligand for focal adhesion formation raises the possibility that syndecan-4 acts as the primary adhesion receptor, and can support spreading and focal adhesion formation that is independent of an integrin ligand. However, fibroblasts plated onto H/O-coated coverslips attached very weakly and failed to spread or assemble adhesion contacts, even in the presence of soluble integrin-ligand (Fig. 2). These observations indicate that firstly, syndecan-4 is incapable of supporting cell spreading, and secondly, the integrin, rather than the syndecan-4, ligand must be immobilised to supply the mechanical tension necessary for cell adhesion. Thus it would appear that integrin and syndecan-4 cooperate, but fulfil distinct roles, in cell adhesion by contributing to spreading and adhesion contact maturation, respectively.

We tested the hypothesis that receptors influence different aspects of adhesion by disrupting signals downstream of syndecan-4, in an attempt to mimic the behaviour of cells spread on Fn6–10. Although syndecan-4 has been linked with a number of signalling cascades,⁵ the best characterised involves the direct activation of PKC α through association of paired syndecan-4 cytodomains with the catalytic domain of PKC α , in a phosphatidylinositol-4,5-bisphosphate-dependent manner.^{6,17} We compared the effects of inhibition of PKC α on cell spreading and adhesion formation by treating mouse embryonic fibroblasts with the pharmacological inhibitor, bisindolylmaleimide I (BIM-1). Inhibition of PKC had no effect on cell spreading ($89 \pm 2\%$ cells spread) (Fig. 3A), but limited recruitment of vinculin in response to H/O to levels similar to MEFs spread on Fn6–10 ($11 \pm 1\%$ and $9 \pm 3\%$ of cells respectively) (Fig. 3B). In summary, we show that engagement of syndecan-4 is responsible for signals that drive the development of focal adhesions, but are quite separate from the mechanical influences of integrins that mediate cell spreading.

Discussion

Through careful manipulation of matrix ligands we have been able to resolve the issues surrounding the role of syndecan-4 in focal adhesion formation, and demonstrate that the integrin and syndecan-4 receptors play distinct roles in adhesion. The major practical consideration in these types of experiments, and the reason that controversy has persisted for so long, is that, in order to isolate syndecan-4 engagement, it is essential to eliminate potential syndecan-4 ligands from the experiment. Syndecan-4 can be engaged *via* its extracellular polysaccharide chains by a large number of ligands,¹⁸ including matrix molecules such as fibronectin,¹³ growth factors⁹ and cell adhesion molecules.¹⁹ Analyses of integrin function using short, RGD-containing, peptides have frequently been conducted in serum-containing media and indeed given stronger biochemical responses to integrin ligands in the presence of serum that is itself indicative of cooperation with additional receptors. The diversity of syndecan-4 ligands means that, even in the absence of serum, *de novo* synthesis of ligands by the cells themselves permits limited signalling by syndecan-4 and must be blocked. $25 \mu\text{g ml}^{-1}$ cycloheximide has been reported to be sufficient to block protein synthesis in fibroblasts²⁰ and was sufficient to establish syndecan-4-dependence in our experiments. This point was illustrated by the ability of fibroblasts spread on Fn6–10, without cycloheximide treatment, to form focal adhesions regardless of H/O stimulation (data not shown). Similarly, plating cells at high density compromised the difference in focal adhesion area on different ligands, with cells forming more focal adhesions than individual cells on Fn6–10, but fewer than cells on fibronectin (data not shown).

Inevitably, experiments of this nature are artificial and cells are never confronted with an isolated matrix fragment *in vivo*. However, such approaches enable us to distinguish the contributions of different receptors, and provide clues as to how cells respond to matrix cues in whole organisms. One of the major differences between cell spreading assays and *in vivo*

matrix interactions is the time scale over which cell responses are observed. *In vitro*, cells respond to matrix stimuli to form adhesion contacts within 30 minutes, whereas the wound healing defects that are manifested in whole animals following disruption of syndecan-4 are recorded over 2–7 days.²¹ The fact that syndecan-4-null mice are viable and fertile²¹ clearly rules out any suggestion that adhesion is abrogated in the absence of syndecan-4, but rather that cooperation with the integrin allows efficient maturation of focal adhesions in response to exposed matrix. The ability of cells to balance the strength of adhesive contacts at an intermediate level is crucial to rapid cell migration and consequently wound healing,²² and the dose-dependent effect of syndecan-4 expression is demonstrated by syndecan-4 +/- heterozygous mice, which exhibit the same delay in wound closure as syndecan-4 null mice.²¹ Embryogenesis requires a different level of cell proliferation and migration to the maintenance of an adult animal, so it comes as no surprise that different adhesive regulators affect the two processes. Variation in the signals underlying development and physiological responses to injury will benefit the development of pharmacological reagents that target a specific physiological problem. By separating the roles of integrin and syndecan-4 into spreading and cytoskeletal reorganisation, we move a step closer to modulating the efficiency with which cells respond to wounding *in vivo*.

Experimental

Reagents

Mouse monoclonal antibody against vinculin (hVIN-1) (Sigma, Poole, UK), FITC-conjugated anti-mouse IgG (Stratech Scientific, Luton, UK), HRP-conjugated anti-rat IgG (Dako UK Ltd, Ely, UK), and TRITC-conjugated phalloidin (Invitrogen Ltd, Paisley, UK) were all used according to manufacturers' instructions. The rat monoclonal antibody (333)15 was diluted to 1 $\mu\text{g ml}^{-1}$ for ELISA assays. Recombinant fibronectin polypeptides encompassing type III repeats 6–10, 8–10, 9–10 and 12–15 (H/0) were expressed as recombinant polypeptides as described previously,²³ and human plasma fibronectin was purchased from Sigma (Poole, UK).

Ligand coating

13 mm diameter glass coverslips or tissue culture-treated plastic (Corning BV) were derivatised for 30 minutes with 1 mM sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Perbio Science UK Ltd) and washed three times with Dulbecco's PBS containing calcium and magnesium (BioWhittaker UK, Ltd). Surfaces were coated for 2 hours at room temperature with 0.5–10 $\mu\text{g ml}^{-1}$ plasma fibronectin (Sigma, Poole, UK) or 10–100 $\mu\text{g ml}^{-1}$ recombinant fibronectin polypeptides in PBS containing calcium and magnesium, and washed three times with Dulbecco's PBS lacking divalent cations (PBS-) (BioWhittaker UK, Ltd).

Enzyme-linked immunosorbent assays (ELISA)

Ligand-coated surfaces were blocked for 30 minutes with 5% BSA in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN_3) before incubating for 1 hour in mAb 333 diluted in blocking solution. Surfaces were rinsed with PBS-, 0.1% Tween-20 before incubating for 30 minutes in anti-rat HRP diluted in 0.1% BSA in PBS-, and rinsing thoroughly with PBS-, 0.1% Tween-20. The ELISA was developed by addition of 2 mM ABTS, 2.5 mM H_2O_2 , 0.1 M NaOAc, 50 mM NaH_2PO_4 , pH 5.0, stopped by addition of 1% SDS, and absorbance readings at 405 nm were measured using a multiscan plate reader.

Immunofluorescence

Primary human foreskin fibroblasts, passage number 8–25, or immortalised mouse embryonic fibroblasts were passaged one to two days before each experiment to ensure an

active proliferative state. 50% confluent cells were treated with 25 $\mu\text{g ml}^{-1}$ cycloheximide (Sigma, Poole, UK) for 2 hours prior to detachment to prevent *de novo* matrix synthesis, and inhibited with bisindolylmaleimide I (BIM-I; 200 nM) for 20 minutes as appropriate. Ligand-coated coverslips were blocked with 10 mg ml^{-1} heat-denatured BSA for 30 minutes at room temperature and rinsed with PBS-. Detached cells were resuspended in DME-25 mM HEPES, 25 $\mu\text{g ml}^{-1}$ cycloheximide (supplemented with 200 nM BIM-I as appropriate), plated at a density of 1.25×10^4 cells per coverslip and allowed to spread at 37 °C for 2 hours. Spread cells were stimulated with 10 $\mu\text{g ml}^{-1}$ H/O for 30–60 minutes, and then fixed with 4% (w/v) paraformaldehyde, permeabilised with 0.5% (w/v) Triton X-100 diluted in PBS-, and blocked with 3% (w/v) BSA in PBS-. Fixed cells were stained for vinculin and actin, mounted in Prolong® Antifade (Molecular Probes, Invitrogen Ltd, Paisley, UK) and photographed on a DeltavisionRT microscope using a 60x NA 1.42 PlanApo objective and Photometrics CH350 camera. Images were compiled and analyzed using ImageJ software.

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Fig. 1.

Receptor cooperation rather than ligand density determines focal adhesion formation. (A–C) Fibronectin polypeptides ($10 \mu\text{g ml}^{-1}$ unless stated) were coated onto surfaces, and the density of folded ligand was measured by ELISA using a conformation-specific antibody. (A) Polypeptides coated onto glass either directly or following treatment with sulfo-MBS. (B) Polypeptides coated onto plastic either directly or following treatment with sulfo-MBS. (C) Polypeptides coated onto sulfo-MBS-derivatised glass at a range of concentrations. (D + E) Human fibroblasts were spread for two hours on polypeptide-coated, derivatised glass before staining for vinculin and scoring 100 spread cells for the ability to form adhesions that exceeded 2% of cell area (* $p = 0.009$, ** $p = 0.003$ in comparison with $5 \mu\text{g ml}^{-1}$ fibronectin). Error bars represent the standard error of four experiments, results were obtained on three separate occasions.

Fig. 2.

Focal adhesion formation depends upon both an immobilised integrin ligand and a syndecan-4 ligand. Human fibroblasts were spread for 2 hours on immobilised ligands (**bold**) in the presence of soluble ligand (brackets) before fixing and staining for vinculin and actin. Images are representative of 400 cells photographed on four separate occasions. Bar = 20 μm .

Fig. 3.

Integrin and syndecan-4 make divergent contributions to adhesion. (A) MEFs were spread for 10–120 minutes on Fn6–10 or plasma fibronectin either with or without 200 nM BIM-I, before measuring the average area of 50 cells. (B) MEFs were spread for 2 hours on fibronectin either with or without 200 nM BIM-I, before fixing and staining for vinculin and actin to determine focal adhesion formation. Error bars represent the standard error of four experiments, images are representative of 100 cells and results were obtained on three separate occasions, bar = 20 μm .