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The role of the ninth and tenth type III domains of human fibronectin in cell adhesion

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Abstract

Fibronectins (FN) contain sites, in addition to the cell recognition site RGD in the tenth type III domain (FIII10), that are required for adhesive activity. The role of FIII10 and the adjacent FIII9 was analysed in functional cell adhesion assays recombinant FIII domains in which the domain boundaries were strictly conserved. FIII9 had no adhesive activity. FIII10, and FIII9 plus FIII10 had less activity than FN, whereas the activity of FIII9–10 was similar to FN. We conclude that FIII9 acts synergistically with FIII10 in cell adhesion, and that this synergy is dependent upon the structural integrity of the FIII9–10 pair of domains.

Key words: Fibronectin; FIII domain; RGD; Cell adhesion; Cell spreading assay; Protein expression

1. Introduction

Fibronectins (FN) are a group of large glycoproteins that mediate the adhesion of many cell types to the extracellular matrix (ECM), and have a critical role in many biological processes such as development, differentiation, wound healing, tumorigenesis and metastasis [1]. The adhesive function of FN is facilitated by multiple binding sites for cells and for components of the ECM, such as collagen, fibrin and heparin. The binding sites are located in distinct structural domains on the FN molecule, called type I, type II and type III domains [2], (Fig. 1). FN type III (FIII) domains are common structural motifs that have been identified in many eukaryotic and prokaryotic proteins with diverse functions [3].

Many cell types bind to the central cell binding domain (CCBD) which spans the eighth, ninth and tenth type III domains (FIII8, FIII9 and FIII10) via the integrin family of cell surface receptors [4]. The minimal sequence in the CCBD for cell recognition and binding has been mapped to RGD [5], at the N-terminus of FIII10. However RGD does not mimic the full adhesive capacity of FN, or of a 37 kDa proteolytic fragment of FN containing the CCBD, in cell adhesion assays [6], suggesting that this region contains sites, in addition to RGD, that are required for cell adhesion. The presence of additional sites in the CCBD that are required for the interaction of FN with both $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ integrins, has been confirmed in functional, cell binding and cell adhesion studies of recombinant fragments of FN containing deletions in this region, and by mapping the epitopes of monoclonal antibodies to the CCBD that disrupt adhesive function [6–9].

Structural studies of FIII domains [10–12] have led to a greater understanding of the mechanisms involved in molecular recognition and cell adhesion. The studies also indicate that it is important to understand how the functional sites required for cell adhesion interact in the context of the structural domains in which they reside.

We have previously shown that the intact FIII10 domain has only slightly more adhesive activity than a short peptide containing RGD [10]. In the present study we investigated the interaction of FIII9 and FIII10 structural domains in the adhesion of fibroblasts which bind to FN via $\alpha 5\beta 1$. We assessed the adhesive activity of FIII9 and FIII10, expressed singly or as a pair, as fusion proteins of glutathione *S*-transferase (GST), in which the FIII domain boundaries had been strictly maintained.

2. Materials and methods

2.1 Expression of recombinant FIII–GST fusion proteins

cDNAs encoding the FIII9 (residues 1,325–1,415) and FIII10 (residues 1,416–1,509) domains, and the FIII9–10 pair of domains, were amplified from a human FN cDNA fragment, pFH154 [13], by the use of oligonucleotide primers in polymerase chain reactions: FIII9, 5′ GGGTCTTGATTCCCCAACTGG, 3′ TTATTATGTTGATTGT-

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Abbreviations: FN, fibronectins; ECM, extracellular matrix; CCBD, central cell binding domain; BHK cells, baby hamster kidney cells; kDa, kilodalton; FCS, fetal calf serum; MEM, minimal essential medium; GST, glutathione *S*-transferase.

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TGGCCAATCA; FIII10 5′, GGTTTCTGATGTTCCGAGGGA, 3′ TTATTATGTTCGGTAATTAATGGAAA.

Amplified cDNAs were cloned into the *SmaI* site of pGEX2T [14], and the sequence verified by using the Sequenase kit (Pharmacia) and 3′ pGEX2T oligonucleotide sequencing primer TTTCACCGTCATCACCGA. Recombinant FIII–GST fusion proteins were expressed and purified as described by Smith et al. [14]. The purity and M_r of the fusion proteins was confirmed by 15% SDS-PAGE (Fig. 2) and C8 reverse-phase HPLC. For the inhibition assays, the domains were purified further by cleavage of the fusion protein with 1–5 U human plasma thrombin (Sigma) per mg fusion protein as described [14]. The thrombin was inactivated by incubation with 1 mM phenylmethylsulfonylfluoride, and the FIII domains concentrated and equilibrated with PBS by centrifugation through a Centricon 3 (Amicon). Cleaved domains were analysed by SDS-PAGE, as above, and the molecular weights of the proteins confirmed by injecting a solution of the protein into the electrospray system of a VG BIO Q quadrupole mass spectrometer. The concentration of the proteins was determined by measuring the absorbance at 280 nm, and the extinction coefficient for each protein was calculated on the basis of the number of tyrosines and tryptophans in the amino acid sequence.

2.2. Cell spreading assays

Baby hamster kidney (BHK) cells were maintained in MEM and 10% FCS at 37°C. Cell spreading assays were carried out according to methods described previously by others [15], with some modifications. In each experiment, the surface of replicate wells of 96 well plates (Nunclon) was coated with doubling dilutions of 200 μg/ml of the FIII–GST fusion proteins, FN (human plasma; Sigma) or GST, in PBS, or PBS, for 16 h at 4°C. Uncoated plastic was blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 20°C. 10^4 BHK cells were inoculated into each well in MEM, and incubated for 1 h at 37°C. Adherent cells were washed gently with PBS and fixed with 4% formaldehyde; 4% gluteraldehyde in PBS. Cells were viewed by the use of phase contrast microscopy. In each experiment, at least 200 cells in four fields were counted in each duplicate well, and scored for either rounded or spread morphology. Cells that appeared elongated and non-refractile, with the nucleus and nucleoli clearly visible, were scored as spread; refractile cells in which the nucleus was not visible were scored as round. The number of spread cells was expressed as a percentage of the total number of cells counted.

For the inhibition of spreading assays, wells of 96-well plates were coated with 5 μg/ml FN, and blocked as described above. 25 μl cleaved FIII domain in PBS, or PBS, were added to each well. 10^4 BHK cells in 75 μl MEM were inoculated into each well and incubated for 1 h at 37°C. Adherent cells were fixed and scored as described for the cell spreading assays.

3. Results

3.1. Expression of FIII–GST fusion proteins

FIII9, FIII10, and FIII9–10 were expressed as fusion proteins of GST. Fig. 2 shows SDS-PAGE analysis of the FIII–GST fusion proteins after purification by affinity adsorption onto glutathione-Sepharose beads. FIII10–GST and FIII9–10–GST were highly expressed and the expressed protein was highly soluble in the bacterial lysate, whereas expression of FIII9–GST was low, and the protein was only partially soluble. The fusion proteins and the cleaved FIII domains migrated as single bands on SDS-PAGE, and eluted in a single peak from a C8, reverse-phase column by HPLC (data not shown).

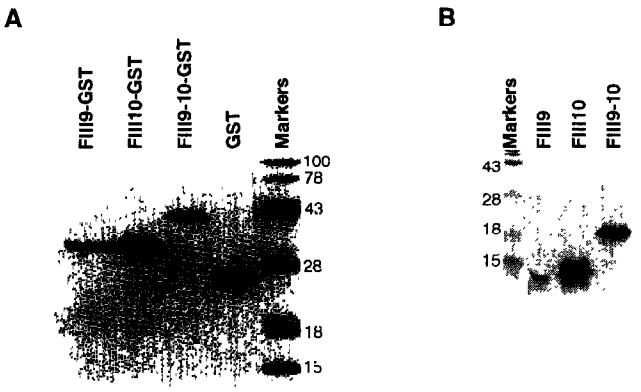

Fig. 2. 15% SDS-PAGE of purified GST-FIII fusion proteins FIII9, 36 kDa; FIII10, 36 kDa; FIII9–10, 46 kDa and GST, 26 kDa (A) and the cleaved domains FIII9, 9 kDa; FIII10, 9.5 kDa and FIII9–10, 18.5 kDa (B) stained with Coomassie blue. The molecular weight markers and their relative mass are shown.

3.2. BHK cells adopt a more spread morphology on FIII9–10–GST than on FIII10–GST

The FIII–GST fusion proteins were tested for their capacity to support spreading of BHK cells on plastic coated with 100 μg/ml fusion proteins (Fig. 3). BHK cells attached poorly to FIII9–GST, and the cells remained rounded (Fig. 3A). Cells adhered to FIII10–GST more efficiently, although many cells remained rounded and appeared more refractile. Some cells exhibited a spread morphology, but to a lesser extent than cells plated onto FN (compare Fig. 3B with 3E). The morphology of cells plated onto FIII9–GST + FIII10–GST (Fig. 3C) was similar to that of cells on FIII10–GST. Most cells plated onto plastic coated with FIII9–10–GST were well spread, and very few cells remained rounded (Fig. 3D), and were morphologically comparable to cells plated onto FN (compare Fig. 3C with 3E). Very few cells attached to GST (Fig. 3F).

3.3. FIII9–10–GST mimics the adhesive activity of FN

The graph in Fig. 4A shows the quantitative data for

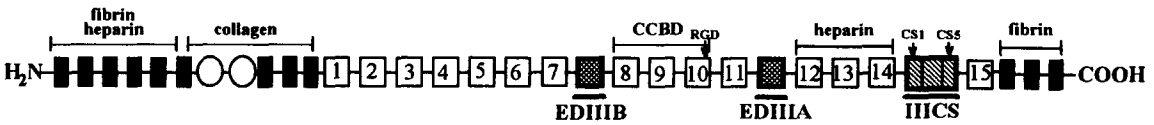

Fig. 1. Diagram of the FN subunit illustrating the organisation of the type I (black boxes), type II (circles) and type III (white boxes, numbered 1–15) domains and the alternatively spliced domains EDIIIA, EDIIIB and IIICS. Fibrin, heparin and collagen binding sites, the central cell binding domain (CCBD) and cell binding sites CS1 and CS5 are also shown.

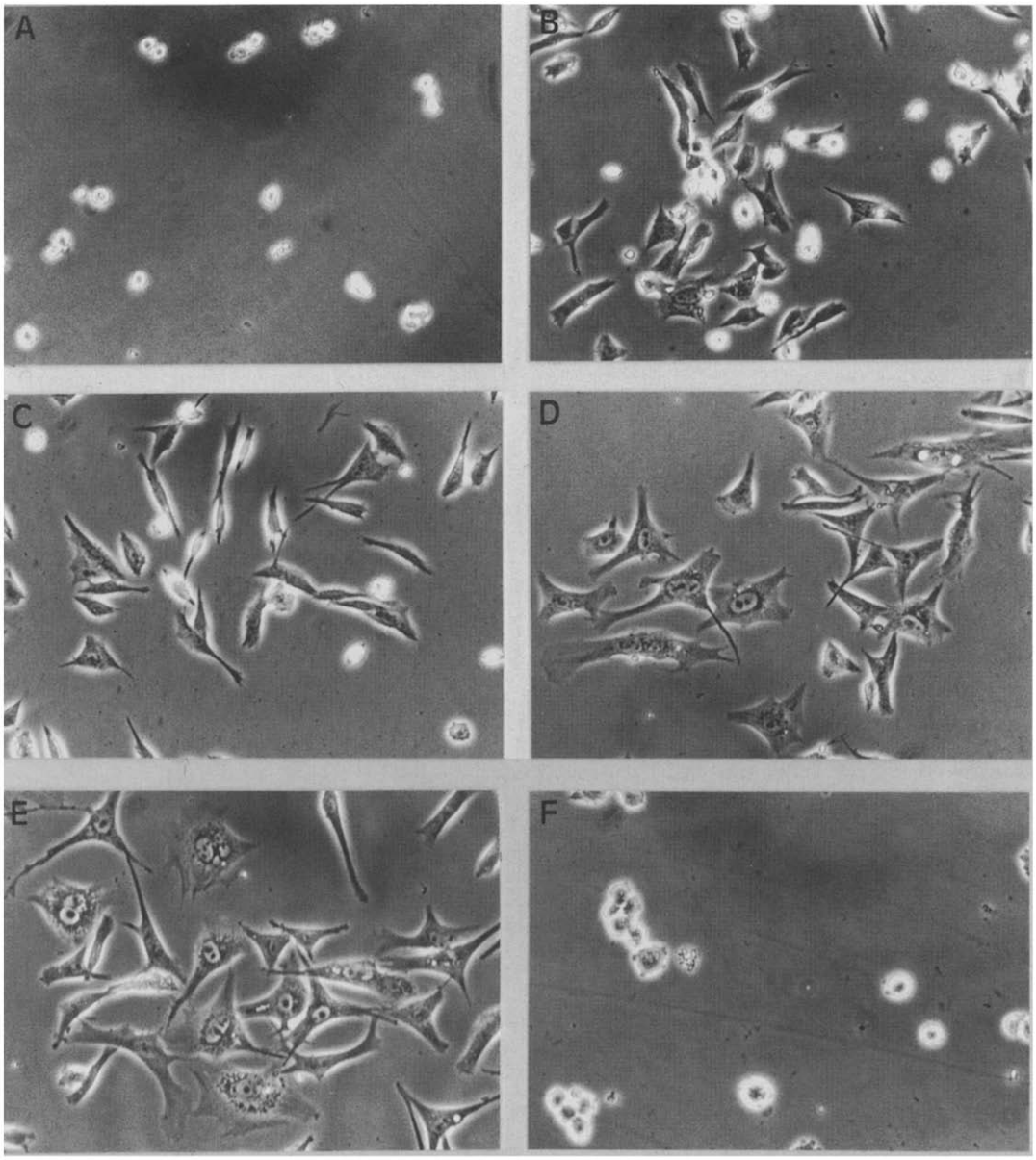

Fig. 3. Phase contrast microscopy of BHK cells cultured for 1 hour on plastic coated with 100 μg/ml FIII9–GST (A); FIII10–GST (B); FIII9–GST + FIII10–GST (C); FIII9–10–GST (D); FN (E) and GST (F). × 660 magnification.

BHK cell spreading assays. Cells attached very poorly to plastic coated with up to 200 μg/ml either FIII9–GST or GST, or with 1% BSA, and these substrates supported minimal cell spreading (< 3%). 40% of cells spread on plastic coated with 100 μg/ml FIII10–GST (~ 0.8 μM with respect to FIII10), whereas 86% of cells spread on plastic coated with 100 μg/ml FIII9–10–GST (~ 1 μM with respect to FIII9–10). The maximal spreading activity observed on FIII9–10–GST was similar to FN, on which 90% of cells exhibited a spread morphology. Half- maximal spreading occurred at coating concentrations of ~ 10 μg/ml of FIII9–10–GST (~ 0.1 μM with respect to FIII9–10), and ~ 3 μg/ml FN (~ 0.01 μM). FN was thus 10-fold more active than FIII9–10 at low concentrations, athough FIII9–10 achieved a similar spreading activity to FN at a coating concentration of 100 μg/ml.

3.4. *FIII9–10 inhibits spreading of BHK cells on FN more efficiently than FIII10*

The inhibitory effect of FIII9, FIII10 and FIII9–10 on cell spreading was studied by culturing BHK cells on plastic coated with 5 μg/ml FN in the presence of the purified domains. These results are shown in Fig. 4B. FIII9–10 inhibited spreading of 69% cells, at a concentration of 50 μM, whereas 31% cells were inhibited by FIII10. FIII9 did not inhibit the spreading of BHK cells on FN.

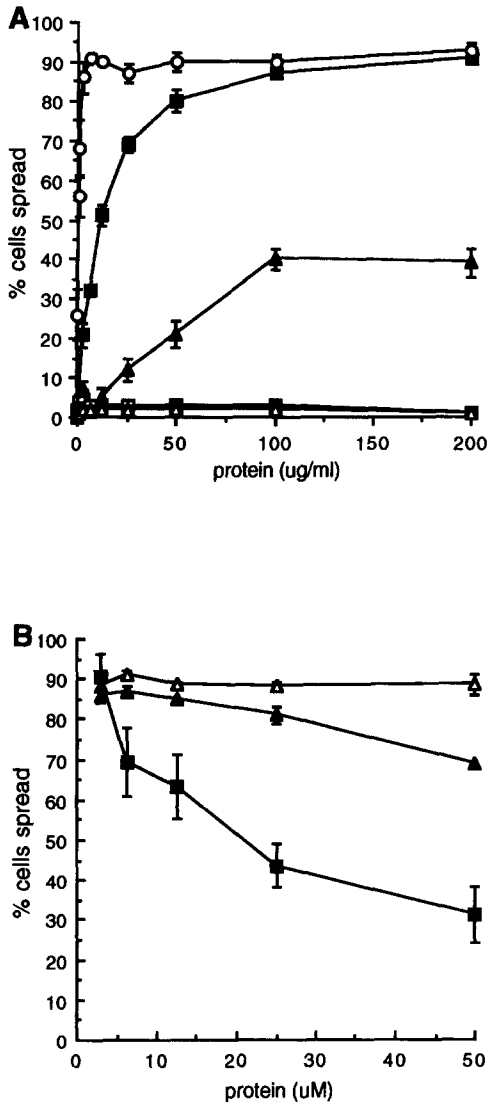

Fig. 4. (A) Spreading of BHK cells on FIII–GST fusion proteins cultured for 1 h on plastic coated with FIII9–GST (△), FIII10–GST (▲), FIII9–10–GST (■), FN (○) or GST (□). (B) Inhibition of spreading of BHK cells cultured on plastic coated with 5 μg/ml FN in the presence of FIII9 (△), FIII10 (▲) or FIII9–10 (■). The data represent the mean number of spread cells in four fields of duplicate wells from at least four independent experiments ± S.E.M.

4. Discussion

In this study we have shown that (1) the ninth and tenth FIII domains, expressed as a pair with conserved domain boundaries, mimic the adhesive activity of the FN molecule, (2) FIII9 and FIII10 act synergistically in mediating the adhesion of fibroblasts, (3) this synergy is dependent upon the structural contiguity of FIII9 and FIII10, and (4) FIII9 alone does not mediate the adhesion of BHK cells.

The requirement of sites for cell adhesion in the CCBD, in addition to RGD, has been established by the use of deletion mutants, and monoclonal antibodies that disrupt adhesive function. A site that is strongly synergistic with RGD for fibroblast adhesion has been mapped to a region at the N-terminus of FIII9. A second, weaker synergistic site has been mapped to the C-terminus of FIII8 [6–8,]. Additional binding sites required for binding of FN to the platelet-specific integrin αIIbβ3 have also been identified within a region spanning FIII9 and the N-terminal region of FIII10 [9].

Our results from both the cell spreading and inhibition assays confirm that FIII10 has some adhesive activity that is less than that of FN. However, Aukhil et al. [16] reported that FIII10 does not support the adhesion of fibroblasts, although different expression systems and coating concentrations were used in the two studies. FIII7, FIII8, and FIII9 domains were required in addition to FIII10 to mimic the full cell adhesive activity shown by the native FN molecule in a study in which recombinant FN proteins were generated by expression of restriction fragments of FN cDNA [17]. The authors reported that a protein fragment containing FIII9–10 has only 0.5% of the activity of the FN molecule as assessed by the relative ED_{50}, whereas in our study, FIII9–10 exhibited more than 10% of the activity of FN. The results we present here suggest that the full adhesive activity of FN can be achieved by the FIII9–10 pair of domains, although higher concentrations of FIII9–10 than FN were required. The domain boundaries were strictly retained in the FIII proteins we describe in this study, which may explain the differences between our findings and previous reports.

FIII domains, like many other protein domains, have probably arisen as a result of exon shuffling and duplication. Structural integrity of these domains may therefore be necessary for the full activity of their functional sites. Structural studies of FIII10 have revealed that the RGD motif exists in a mobile loop between two of the seven β strands that comprise the FIII domain [12]. Our results suggest that the activity of the functional site, RGD, in FIII10 is dependent upon a site(s) in FIII9 and on the structural integrity of the FIII9 and FIII10 pair of domains. The elucidation of the structure of the FIII9–10 pair should thus provide important information about how the domains interact to achieve their adhesive function, and the availability of recombinant FIII9–10 domains will permit further structure–function studies.

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