N-terminal type I modules required for fibronectin binding to fibroblasts and to fibronectin's III₁ module

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Assembly of fibronectin fibrils occurs at the surface of substrate-attached cells and is mediated by the first to the fifth type I modules in the N-terminal 70 kDa portion of the molecule. The first type III module (III₁) of fibronectin, not present in the 70 kDa portion, contains a conformation-dependent binding site for the 70 kDa N-terminal region of fibronectin, suggesting that the III₁ module on cell-surface fibronectin may serve as a binding site for fibronectin's N-terminus on substrate-attached cells. To explore this possibility, we compared the ability of mutant recombinant 70 kDa proteins containing deletions of one or several of the first five type I modules to bind to fibroblasts and to III₁. Proteins containing the fourth and fifth type I modules (70KΔI₁₋₃) bound specifically to III₁ in solid-phase binding

assays; proteins lacking I₄ and I₅ did not bind. N-terminal molecules containing the fourth and fifth type I modules also bound to fibroblasts, suggesting that III₁-like binding sites are present on the cell surface. However, the high-affinity binding sites on fibroblasts for fibronectin or the 70 kDa protein displayed more complex determinants, inasmuch as 70 kDa deletion mutants lacking I₄ and I₅ also bound to the cell surface, and deletion mutants lacking I₁₋₃ and I₄₋₅ both competed only partially for binding of ¹²⁵I-labelled fibronectin or 70 kDa protein. These data indicate that the N-terminal part of fibronectin binds to III₁ via I₄ and I₅ and that interactions in addition to that of I₄ and I₅ with III₁ are important for cell-surface-mediated fibronectin polymerization.

INTRODUCTION

Multimeric fibronectin is a major constituent of extracellular matrices found throughout the body, where it mediates a variety of adhesive and migratory events associated with tissue repair, neovascularization and embryogenesis. The major functional form of fibronectin *in vivo* is the multimeric fibrillar form that is found in extracellular matrices [1–3]. *In vivo* studies have shown that inhibiting the interaction of mesodermal cells with fibronectin fibrils in amphibian embryos inhibits cell adhesion and migration and blocks gastrulation [4]. Recent studies with transgenic mice have shown that mice lacking fibronectin die in embryogenesis, emphasizing that fibronectin is essential for normal development [5].

Polymerization of fibronectin into the extracellular matrix is thought to be tightly regulated in order to insure that matrix is appropriately deposited. Altered deposition of matrix molecules, including fibronectin, has been associated with such pathological events as cancer, atherosclerosis and fibrosis [2,6–9]. Deposition of fibronectin in the extracellular matrix is a cell-mediated process that involves the binding of soluble fibronectin to specific sites on the surface of substrate-attached cells, termed matrix assembly sites. Although advances have been made in understanding how fibronectin is deposited in the extracellular matrix, many questions remain unresolved.

Most of the fibronectin molecule is comprised of a series of repeating modules, termed types I, II and III [10]. Binding of soluble fibronectin to the surface of adherent cells is mediated by the N-terminal 70 kDa portion of the molecule. This binding is

non-co-operative and to a single class of binding sites that can be up-regulated and down-regulated by addition or withdrawal of lysophosphatidic acid [11] or phorbol esters [12]. Binding of the 70 kDa fragment has been localized primarily to the 27 kDa N-terminal portion [13,14], which contains five type I modules. The presence of excess 70 kDa fragment blocks the binding of intact fibronectin to cells and thereby inhibits fibronectin matrix assembly [13,14]. Following the binding of fibronectin to the cell surface via the N-terminal 70 kDa region, interactions between fibronectin molecules leads to formation of fibronectin multimers. These multimers are insoluble in SDS and stabilized by disulphides [15]. Recently it has been shown that a dimeric form of the 70 kDa protein containing the 70 kDa N-terminal region of fibronectin directly linked to the C-terminal bridge region is assembled into disulphide-stabilized aggregates. Formation of SDS-insoluble complexes, however, required co-assembly with intact fibronectin [16].

The site on the cell surface that mediates binding of the N-terminal portion of fibronectin has not been identified. It has been suggested that the $\alpha_5\beta_1$ integrin receptor serves as the matrix assembly receptor on cells, since antibodies to $\alpha_5\beta_1$ block assembly of fibronectin into the matrix [17,18]. In addition, deposition of fibronectin into the matrix is modulated by the level of $\alpha_5\beta_1$ expression on cells [19–21]. However, direct binding of the 70 kDa N-terminal fragment of fibronectin to $\alpha_5\beta_1$ could not be demonstrated [3]. It has also been proposed that the III₁ module of fibronectin may serve as a binding site for the N-terminal region of fibronectin on the cell surface [22]. More recently, the integrin-binding III₁₀ module of fibronectin has

Abbreviations used: III₁, first type III module of fibronectin; DMEM, Dulbecco's modified Eagle medium; AcNPV, *Autographa californica* nuclear polyhedrosis virus; FBS, fetal-bovine serum; FITC, fluorescein isothiocyanate; 70KΔI₁₋₃ (etc.), 70 kDa deletion mutant lacking the first three type I modules; GST-III₁₋₂, glutathione S-transferase-III₁₋₂ fusion protein; LDL, low-density lipoprotein.

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been shown to contain a conformation-dependent binding site for the III₁ module [23]. Binding of fibronectin to the $\alpha_5\beta_1$ integrin may alter the conformation of III₁₀ and thus promote fibronectin–fibronectin interactions involving III₁ and the N-terminal region that lead to the self-assembly of fibronectin into the extracellular matrix [22–24].

Several laboratories have demonstrated a role for III₁ in fibronectin matrix assembly [25,26]. Monoclonal antibodies against the ninth type I (I₉) and the first type III modules (III₁) inhibit matrix assembly, but do not block binding of the 70 kDa fragment, suggesting a role for these regions in some step subsequent to initial cell-surface binding [25]. A proteolytically derived fibronectin fragment containing a portion of III₁ was shown to bind to fibronectin and block matrix assembly [26]. In addition, a recombinant III₁ fragment was demonstrated to promote fibronectin–fibronectin self-association in solution and on the cell surface [24]. Conformationally altered III₁ [22] and a fusion protein containing III₁ and III₂ [27] have also been shown to bind to the 70 kDa N-terminus of fibronectin in solid-phase binding assays.

Experiments in which individual modules were deleted or mutated in a conserved tyrosine residue have localized the N-terminal binding site for substrate-attached cells to the first five type I modules (I_{1–5}) of a truncated 70 kDa protein [28]. Similarly, binding of recombinant fibronectin miniproteins to a fusion protein containing III₁ and III₂ requires the presence of module I₅ [27]. To localize further the site within the N-terminal part of fibronectin for the III₁ module, we examined the ability of 70 kDa deletion mutants to bind to conformationally altered III₁ in direct and competition binding assays. Results indicate that the binding site within the N-terminal region for III₁ is localized to I₄ and I₅. If the III₁ module serves as a binding site on the cell surface for the 70 kDa N-terminus of fibronectin, then the structural features of the N-terminal region necessary for binding to fibroblasts and to III₁ should be similar. 70 kDa deletion mutants containing I₄ and I₅ were also able to bind specifically to the surface of fibroblasts, indicating that III₁-like binding sites are present on the cell surface. However, other N-terminal type I modules apparently also participate in binding of 70 kDa protein to cell surfaces, because competition for fibronectin or 70 kDa protein binding to cells required all five type I modules.

MATERIALS AND METHODS

Cells and viruses

The insect cell line IPLB-SF-21, adapted to grow in the serum-free medium SF900-II, was obtained from Life Technologies (Gaithersburg, MD, U.S.A.). TJ6F normal human foreskin fibroblasts were established by Dr. Lynn Allen Hoffmann (University of Wisconsin, Madison, WI, U.S.A.). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the mutant AcNPV virus vΔ35K/lacZ [29] were generously given by Dr. Paul Friesen (University of Wisconsin). Baculogold DNA was purchased from PharMingen (San Diego, CA, U.S.A.).

Production of recombinant baculoviruses

The recombinant baculovirus Fn571/AcNPV has been previously described [30]. This virus directs the expression of a recombinant 70 kDa protein (70 kDa). 70 kDa deletion mutants (formerly called 'Gap proteins') were previously expressed in COS cells [28]. 70KΔI_{1–3} (Pro¹⁹-Ala¹⁵²); 70KΔI₃ (Asn¹⁰⁸-Ala¹⁵²);

70KΔI₄ (Glu¹⁵³-Arg¹⁹⁷); 70KΔI₅ (Asn¹⁹⁸-His²⁴²); 70KΔI_{4–5} (Glu¹⁵³-His²⁴²), and the 40 kDa protein (70KΔI_{1–5}, Pro¹⁹-His²⁴²). The recombinant 40 kDa protein contains tryptophan instead of valine at position 10. Trp¹⁰ is followed by four extra amino acids (Pro-Trp-Pro-Ser) not found in the wild-type sequence; the wild-type sequence resumes at Ala¹¹ [31]. Amino acids deleted from each mutant are shown in parentheses and are numbered from the N-terminal pyroglutamic acid in the processed protein [10]. Mutant DNAs were subcloned into the baculovirus expression vectors pAcYM1 [32] or pEV/35K (a gift from Dr. Paul Friesen). Recombinant viruses were generated according to established procedures [33,34]. Viral stocks were prepared using SF21 cells grown in serum-free SF900-II medium.

Recombinant mutant and wild-type 70 kDa proteins

Supernatant from infected cells was collected either 48 or 72 h post-infection. Recombinant proteins were purified from the supernatant on columns of gelatin–agarose essentially as described [30]. Protein concentrations were calculated from their absorbance at 280 nM. The absorbance 0.1% (= 1 g/l) of each protein was calculated from its amino acid composition using the computer program PHYSICHEM (IntelliGenetics, Mountain View, CA, U.S.A.). The absorbance 0.1% ranged from 1.95 to 2.0 for 70 kDa and 70 kDa deletion mutants.

SF21 cells do not produce any identifiable or detectable endogenous fibronectin. Thus recombinant mutant or wild-type 70 kDa proteins produced by these cells do not contain any contaminating insect cell fibronectin.

Recombinant III₁ and glutathione S-transferase–III_{1–2} fusion protein (GST–III_{1–2})

Recombinant III₁ was produced in bacteria and purified as described in [22]. To generate GST–III_{1–2}, PCR was used to amplify human fibronectin cDNA encoding the first and second type III modules of fibronectin. The primers used were: 5'-CCCGGATCCAGTGGTCCTGTCTGAAGTATT and 5'-CCCGAATTCCTATGTTGTTTGTGAAGTAGACAGG. PCR-amplified DNA was cloned into the bacterial expression vector pGEX-2T (Pharmacia) and sequenced [35] to confirm that no base changes had been introduced during amplification of the DNA. DNA was transfected into DH5α bacteria and the GST–III_{1–2} fusion protein purified from bacterial lysates on columns of glutathione–agarose as described for III₁ [22].

SDS/PAGE

SDS/PAGE was performed as described by Laemmli [36]. Proteins were mixed with sample buffer (final concentration 1% SDS/10% glycerol/0.02% Bromophenol Blue/50 mM Tris/HCl, pH 6.8/5% β-mercaptoethanol) before being applied to 9 or 14% slab gels. Proteins were revealed by staining with 0.05% Coomassie Blue. Molecular-mass standards were purchased from Life Technologies.

Iodination of proteins

Mutant and wild-type 70 kDa proteins were iodinated using the chloramine-T method as previously described [13]. Labelled proteins were separated from unincorporated iodine by gel filtration on Pharmacia PD-10 columns. Iodinated proteins were dialysed against TBS at room temperature for 3 h. The specific activities of iodinated proteins were: 70KΔI_{1–3} (0.63 × 10¹¹ μCi/mol), 70KΔI₄ (1.71 × 10¹¹ μCi/mol),

70K Δ I₅ (1.86×10^{11} μ Ci/mol), 70K Δ I₄₋₅ (0.44×10^{11} μ Ci/mol) and 70 kDa (2.09×10^{11} μ Ci/mol).

Binding assays

Binding assays were performed essentially as described [13]. Briefly, human fibroblasts were seeded at 8×10^4 cells/well into 12-well cluster dishes in DMEM containing 10% FBS. Cells were allowed to grow to confluence for 3–5 days. Cells were washed three times with serum-free DMEM, then incubated with medium containing iodinated proteins. Equal molar amounts of 125 I-70 kDa protein and 125 I-deletion mutants were added to cells in DMEM containing 0.1% BSA. Cells were incubated at 37 °C for the times indicated in the Figure legends. Following this incubation period, cells were washed three times in PBS, pH 7.4, then solubilized in 0.1 M NaOH and radioactivity was measured in a γ -radiation counter. Non-specific binding was determined by incubating cells in the presence of excess unlabelled recombinant rat 70 kDa protein (15–20 μ g/ml; 0.2–0.3 μ M).

For competition experiments, 125 I-fibronectin or 125 I-70 kDa protein was incubated in the presence of various amounts of unlabelled 70 kDa or deletion mutants for 20 h at 37 °C. Bound proteins were processed as indicated above.

Solid-phase binding assay

Plates were coated with fibronectin or III₁ essentially as described [22]. III₁ was diluted to 2–10 μ g/ml in PBS and then incubated at 90 °C for 10 min. III₁ was then added to 24-well plates and incubated for an additional 60 min at 80 °C. Plates were allowed to cool at 37 °C for 30 min, after which time unbound protein was removed and sites that had not reacted were blocked with 1% BSA/PBS for 2 h at 37 °C. Plates were washed three times with PBS containing 0.1% BSA, then incubated with 1.4 pmol of 125 I-proteins at 37 °C for 90 min. Plates were washed as described above, bound proteins were solubilized with 1 M NaOH and radioactivity was measured in a γ -radiation counter. Non-specific binding was determined by incubating iodinated proteins in the presence of excess unlabelled 70 kDa protein (15–20 μ g/ml; 0.2–0.3 μ M). For competition experiments, 125 I-70K was incubated in the presence of various concentrations of unlabelled 70K Δ I₁₋₃ and 70K Δ I₄₋₅ and wild-type 70 kDa proteins.

For ELISA, various concentrations of III₁ were coated on to 96-well plates and processed as described above. Equal molar amounts (285 nM) of the 70 kDa protein or deletion mutants were added in PBS containing 0.2% BSA and incubated for 90 min at 37 °C. Plates were washed three times with 0.2% BSA/PBS, then incubated for 2 h at room temperature with a rabbit antiserum produced to recombinant 40 kDa protein (70K Δ I₁₋₅) at 1:1500. Plates were washed three times with TBS containing 0.05% Tween-20, then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) at 1:6000 for 2 h at room temperature. After three washes with TBS/0.05% Tween-20, plates were developed with 2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid) (Sigma) for 60 min at room temperature. Absorbance at 410 nm was read on a Bio-Kinetics EL312e Reader (Biotex Instruments, Winooski, VT, U.S.A.). The anti-(40 kDa protein) antibody was titred against the 70 kDa protein, the 40 kDa protein, and each of the deletion mutants using a direct ELISA. Half-maximal absorbance was obtained with an antibody dilution of 1:1500 for all of the proteins tested.

Indirect immunofluorescence

Human fibroblasts were seeded into 12-well cluster dishes on to 18 mm glass coverslips at 2.5×10^5 cells/well in DMEM con-

taining 10% fibronectin-depleted serum. Cells were seeded in the absence (control) or presence of 500 nM 70 kDa protein, 70K Δ I₁₋₃, 70K Δ I₄₋₅ or 70K Δ I₁₋₅. Cells were incubated for 25 h at 37 °C, and then incubated with a polyclonal antibody to human fibronectin at 1:1000 for 60 min at room temperature. After washing, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG for 30 min. Cells were washed, mounted, then examined using an Olympus microscope equipped with epi-fluorescence.

Direct immunofluorescence

70 kDa protein, 70K Δ I₁₋₃ and 70K Δ I₄₋₅ were conjugated to fluorescein isothiocyanate (FITC; Cappel, West Chester, PA, U.S.A.) or Texas Red sulphonyl chloride (Molecular Probes, Inc., Eugene, OR, U.S.A.) according to manufacturer's instructions. Unconjugated fluorochromes were removed using a PD10 gel-filtration column. Adherent fibroblasts were washed free of serum-containing medium and incubated in serum-free DMEM containing 0.4% Redu-SerII (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and 20 μ g/ml cycloheximide for 3 h. Cells were then trypsin-treated, washed three times with the above medium, and plated on to 18 mm-diameter round coverslips previously coated with 20 μ g/ml fibronectin. Cells were seeded in serum-free DMEM containing 0.4% Redu-SerII and 20 μ g/ml cycloheximide and incubated overnight at 37 °C. The next day the medium was supplemented with fluorescently labelled proteins (70 nM) and incubated for 2 h at 37 °C. Cells were fixed with paraformaldehyde, permeabilized with 0.5% Triton X-100 in Small's buffer [37], mounted in *p*-phenylenediamine in Small's buffer and examined under an Olympus microscope.

RESULTS

Mutant 70 kDa proteins (Figure 1A) with deletions of one or several type I modules were expressed in insect cells using a baculovirus expression system (Figure 1A). After infection with recombinant viruses, conditioned media were isolated from SF21 cells, and 70 kDa and 70 kDa deletion mutants were purified by gelatin-agarose affinity chromatography and analysed by SDS/PAGE. The purified recombinant proteins are shown in Figure 1(B). Minor amounts of a 40 kDa protein were also present in some of the 70 kDa preparations. This represents the 40 kDa gelatin-binding domain of fibronectin [38], a frequent degradation product of the 70 kDa N-terminal fragment of fibronectin. Both the intact deletion mutants and the 40 kDa gelatin-binding domain cross-reacted with polyclonal antibodies to fibronectin in Western blots (results not shown).

Recent evidence has shown that the 70 kDa N-terminal region of fibronectin can bind to conformationally altered III₁ module of fibronectin [22] or to a fusion protein containing III₁ and III₂ [27]. The binding site for the 70 kDa N-terminus of fibronectin could be exposed in the isolated III₁ module by heat denaturation of intact fibronectin, by proteolytic cleavage of fibronectin from the cell surface or treatment with dithiothreitol [22]. The binding site within the 70 kDa region for III₁ has been localized to the 27 kDa N-terminus, which contains five type I modules [22]. To further localize the III₁ binding site within the first five type I modules we tested the ability of the 70 kDa deletion mutants to bind to III₁ in solid-phase binding assays. Figure 2 shows that a 70 kDa deletion mutant lacking the first three type I modules (70K Δ I₁₋₃) bound to heat denatured III₁ at 35–40% of the level of intact 70 kDa protein. In contrast, 70 kDa deletion mutants lacking both I₄ and I₅ (70K Δ I₄₋₅) bound at < 4% of the

Figure 1 (A) Diagram of recombinant wild-type and mutant 70 kDa proteins and (B) SDS/PAGE analysis of mutant and wild-type 70 kDa proteins

(A) Fibronectin (Fn) is comprised predominantly of type I modules (rectangles), type II modules (ovals), and type III modules (squares). Regions that are involved in matrix assembly are highlighted; the 70 kDa N-terminal region (70K), the first type III module (stippled box), and the tenth type III module (hatched box) containing the Arg-Gly-Asp sequence. Mutant proteins contained deletions of various type I modules (indicated by the inverted 'V'). The gelatin-binding region of fibronectin, comprised of type I modules I₆–I₉, and the type II modules were intact in all mutants. (B) Mutant and wild-type 70 kDa proteins were purified from the conditioned medium of SF21 cells on columns of gelatin–agarose as described in the Materials and methods section. Mutant proteins contained deletions of various type I modules as indicated by the Δ symbol. Proteins (4 μg) were analysed under reducing conditions on a 9% acrylamide gel, and were revealed with Coomassie Blue staining. Molecular-mass markers (dashes) were, from top to bottom: phosphorylase *a* (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa). A 40 kDa protein (arrowhead) was detected in some preparations. This protein represents the gelatin-binding region of fibronectin [38], a frequent degradation product of the 70 kDa N-terminus of fibronectin.

Figure 2 Binding of ¹²⁵I-labelled proteins to heat-denatured III₁

Various concentrations of heat-denatured III₁ were coated on to 24-well plates as described in the Materials and methods section. Equal molar amounts (3.8 nM) of iodinated 70 kDa protein (■), 70KΔ1-3 (○), and 70KΔ4-5 (●) were added to III₁-coated dishes for 90 min at 37 °C. Binding was performed in PBS with 0.2% BSA. After the 90 min incubation, wells were washed, solubilized in 1.0 M NaOH, then the radioactivity counted in a γ-radiation counter. Data represent the means for duplicate wells, and error bars show the range. Of the starting radioactivity added, 13.5% of 70 kDa protein, 5.8% of 70KΔ1-3, and 0.36% of 70KΔ4-5 bound specifically to III₁ coated at 10 μg/ml.

Figure 3 Inhibition of ¹²⁵I-70 kDa protein binding to heat-denatured III₁

¹²⁵I-70 kDa protein (5.8 nM) was added to dishes coated with 10 μg/ml III₁ in the presence of the indicated amounts of unlabelled 70 kDa protein (■), 70KΔ1-3 (○), and 70KΔ4-5 (●) and GST-III₁₋₂ (◆). After a 90 min incubation at 37 °C, wells were washed, solubilized in 1.0 M NaOH, then counted for radioactivity in a γ-radiation counter. Binding is expressed as a percentage of binding that occurred in the absence of competing proteins. Data represent the means for duplicate wells, and error bars show the range. This experiment was done twice, with similar results.

Figure 4 Binding of trypsin-treated proteins to heat-denatured III₁

Equal amounts of radioactively labelled iodinated 70 kDa protein, 70KΔI₁₋₃ and 70KΔI₄₋₅ were treated with 2 μg/ml *N*-tosyl-L-phenylalanylchloromethane ('TPCK')-treated trypsin for 3 min at room temperature. The reaction was stopped by addition of soybean trypsin inhibitor (10 μg/ml). Equal radioactivities of intact (A) or trypsin-treated (t) (B) proteins were added to wells coated with 10 μg/ml heat-denatured III₁ and incubated for 90 min at 37 °C. Binding was performed in the presence (+) or absence (-) of unlabelled 70 kDa protein (360 nM; 25 μg/ml). After washing with 0.2 % BSA/PBS, bound proteins were solubilized with sample buffer, and analysed on 14% polyacrylamide gels. Starting material (ST) shows proteins that were not incubated with III₁. The arrowhead shows the position of 40 kDa gelatin-binding fragment. Prestained molecular-mass markers (Life Technologies Inc.) are indicated by the dashes, and are (from top to bottom): myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

levels of wild-type 70 kDa protein, suggesting that the III₁ binding site is contained within the I₄ and I₅ modules. The 70 kDa fragment has also been shown to bind to intact fibronectin after treatment with dithiothreitol [22]. Dithiothreitol treatment results in a 2.5-fold enhancement of 70 kDa binding, presumably by causing a conformational change in III₁ [22]. In results not shown, we examined binding of the 70 kDa protein and 70 kDa deletion mutants to untreated and dithiothreitol-treated fibronectin. As with the 70 kDa fragment, binding of the 70KΔI₁₋₃ protein to dithiothreitol-treated fibronectin was enhanced 2.5-fold in comparison with untreated fibronectin, whereas binding of 70KΔI₄₋₅ mutant was low and similar to both types of fibronectin.

To confirm that 70KΔI₁₋₃ contains a binding site for III₁, competition binding experiments were performed. Figure 3 shows that 70KΔI₁₋₃ competed almost as well as 70 kDa protein for ¹²⁵I-70 kDa binding. The 70 kDa protein had an IC₅₀ of approx. 15 nM, whereas 70KΔI₁₋₃ had an IC₅₀ of approx. 45 nM. In contrast, 70KΔI₄₋₅ had an IC₅₀ of > 500 nM. The 40 kDa gelatin-binding domain of fibronectin does not compete for ¹²⁵I-70 kDa binding in this assay [22]. In addition, the GST-III₁₋₂ fusion protein was a poor competitor of ¹²⁵I-70 kDa binding, presumably because the III₁ module in this construct is in the 'closed' conformation, and cannot compete for binding to heat-denatured 'open' III₁ coated on the dish.

To localize further the III₁ binding site in 70KΔI₁₋₃ to the N-terminal type I modules, ¹²⁵I-70 kDa, ¹²⁵I-70KΔI₁₋₃ and ¹²⁵I-70KΔI₄₋₅ were trypsin-treated to separate the N-terminal type I modules from the gelatin-binding region. Cleavage of the 70 kDa protein, 70KΔI₁₋₃ and 70KΔI₄₋₅ yielded a 40 kDa gelatin-binding fragment, and N-terminal fragments that migrated with apparent molecular masses of approx. 27 kDa (I₁₋₅), 15 kDa (I₄₋₅) and

Figure 5 Dose-response of binding to heat-denatured III₁ as assayed by ELISA

The indicated concentrations of heat-denatured III₁ were coated on to 24-well plates as described in the Materials and methods section. Control wells were coated with 10 μg/ml BSA. Equal molar concentrations (285 nM) of unlabelled 70 kDa protein (■), 70KΔI₁₋₃ (○), 70KΔI₄ (□), 70KΔI₅ (◆) and 70KΔI₄₋₅ (●), and 70KΔI₁₋₅ (the 40 kDa protein; ◇) were added to III₁-coated dishes for 90 min at 37 °C. Binding was performed in PBS with 0.2 % BSA. After the 90 min incubation, wells were washed, and incubated with polyclonal anti-40K antibody at 1:1500 for 2 h at room temperature. Wells were washed with TBS containing 0.05 % Tween-20, then incubated for 2 h at room temperature with horseradish peroxidase-coupled goat anti-rabbit IgG at 1:6000. After washing with TBS/0.05 % Tween-20, wells were incubated with substrate, and the absorbance at 410 nm read in an ELISA plate reader. Absorbance of wells coated with BSA were subtracted from absorbance in wells coated with heat-denatured III₁. This background absorbance ranged from 0.06 (wells incubated with the 70 kDa protein) to 0.003 (wells incubated with 70KΔI₁₋₅). Data represent the means for duplicate wells, and error bars show the range.

20 kDa (I₁₋₃). Intact and trypsin-treated proteins were incubated with plates coated with heat-denatured III₁. Bound proteins were solubilized with sample buffer and revealed by SDS/PAGE. Both the 27 kDa (I₁₋₅) and 15 kDa (I₄₋₅) N-terminal fragments bound specifically to III₁ (Figure 4B). In contrast, even though large amounts of the 40 kDa fragment are present in each of the trypsin-treated preparations, the 40 kDa fragment failed to bind to III₁. These data demonstrate that binding of 70 kDa protein to heat-denatured III₁ is mediated by the fourth and fifth type I modules.

To determine whether I₄ and I₅ are both involved in binding to III₁, deletion mutants lacking I₄ or I₅ were tested in solid-phase binding assays using an ELISA assay. Figure 5 shows that 70KΔI₁₋₃ bound at 75 % of the levels of intact 70 kDa protein. 70KΔI₄ and 70KΔI₅ bound at 65 and 50 % of the levels of intact 70 kDa protein. Deletion of both I₄ and I₅ resulted in minimal binding to III₁ (< 0.2 absorbance units). The small amount of 70KΔI₁₋₅ binding probably represents non-specific binding. In direct binding assays using iodinated mutant proteins, 70 kDa proteins lacking either I₄ or I₅ showed greatly reduced binding to III₁ in comparison with the 70 kDa protein or to 70KΔI₁₋₃, binding at levels similar to that seen with 70KΔI₄₋₅ (results not shown). These data indicate that both I₄ and I₅ are required for binding of the N-terminus to heat-denatured III₁.

(A)

(B)

Figure 6 (A) Binding of fluorescently labelled proteins to fibroblasts and (B) binding of ^{125}I -labelled $70\text{K}\Delta\text{I}_{1-3}$ to fibroblasts

(A) Cycloheximide-treated fibroblasts were double stained with FITC-labelled 70 kDa protein and Texas-Red-labelled $70\text{K}\Delta\text{I}_{1-3}$ (a–c) or with FITC-labelled $70\text{K}\Delta\text{I}_{4-5}$ and Texas-Red-labelled $70\text{K}\Delta\text{I}_{1-3}$ (d–f) for 2 h at 37 °C as described in the Materials and methods section. The cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and Texas-Red-labelled $70\text{K}\Delta\text{I}_{1-3}$ (a, d), FITC-labelled 70 kDa protein (b), and FITC-labelled $70\text{K}\Delta\text{I}_{4-5}$ (e) revealed by fluorescence microscopy. The corresponding interference reflection microscopy images (c, f) are also shown. Mutant and non-mutant 70 kDa proteins co-localized at regions of focal contact are shown by the arrowheads. The bar represents 10 μm . (B) ^{125}I - $70\text{K}\Delta\text{I}_{1-3}$ (5.2 nM) was added to confluent wells of fibroblasts in the presence of the indicated amounts of unlabelled 70 kDa protein (■), $70\text{K}\Delta\text{I}_{1-3}$ (○) and $70\text{K}\Delta\text{I}_{4-5}$ (◇). After a 4 h incubation at 37 °C, cell layers were washed, solubilized in 0.1 M NaOH, then radioactivity was counted in a γ -radiation counter. Data represent the means of values for duplicate wells. Binding is expressed as a percentage of binding that occurred in the absence of competing proteins.

If III_1 serves as a binding site for the 70 kDa N-terminus of fibronectin on the cell surface, then 70 kDa deletion mutants containing I_4 and I_5 should bind to the cell surface and should compete for binding of intact 70 kDa protein. Previous studies

have shown that the 70 kDa N-terminus of fibronectin mediates the binding of fibronectin to the surface of substrate-attached cells [13]. Most of this binding has been localized to the first five type I modules in the 27 kDa N-terminus [13,14,28]. To determine

Figure 7 Inhibition of ^{125}I -fibronectin and ^{125}I -70 kDa protein binding to fibroblasts

Equal molar amounts (0.32 nM) of ^{125}I -fibronectin (**A**) and ^{125}I -70 kDa (**B**) were added to confluent wells of fibroblasts in the presence of the indicated amounts of unlabelled 70 kDa protein (■), 70K Δ_{1-3} (○), 70K Δ_{1-5} (□), 70K Δ_{4-5} (◇), 70K Δ_{1-3} + 70K Δ_{4-5} (◻), 70K Δ_{1-5} (◆), 70K Δ_{4-5} (●), and 70K Δ_{1-3} + 70K Δ_{4-5} (◼). After a 20 h incubation at 37 °C, cell layers were washed, solubilized in 0.1 M NaOH, then counted for radioactivity in a γ -radiation counter. Data represent the means of values for duplicate wells, and error bars show the range. Binding is expressed as a percentage of binding that occurred in the absence of competing proteins. This experiment was done four times, with similar results. Similar results were obtained when 90 min binding experiments were performed.

whether III₁-like binding sites are present on the cell surface, fluorescently labelled mutant and wild-type 70 kDa proteins were incubated with fibroblasts. Mutant 70 kDa proteins containing I₄ and I₅, but lacking I₁₋₃ (70K Δ_{1-3}) bound to the cell surface (Figure 6A, panels a and d). 70K Δ_{1-3} binding sites (panel a) co-localized with 70 kDa binding sites (panel b) at focal adhesions as shown by interference reflection microscopy in panel c. These data indicate that binding sites that recognize I₄ and I₅ exist on the cell surface. However, binding does not appear to be an exclusive property of 70 kDa molecules containing I₄ and I₅. Mutant 70 kDa proteins lacking I₄ and I₅ also bound to fibroblasts at regions of focal adhesions (panel e). Texas Red-labelled 70K Δ_{1-5} did not bind to fibroblasts and could not be detected in focal adhesions (results not shown). In direct binding assays with iodinated mutant proteins, 70K Δ_{1-3} (Figure 6B) and 70K Δ_{4-5} (results not shown) bound specifically to the cell surface. Binding of ^{125}I -70K Δ_{1-3} competed with either unlabelled 70K Δ_{1-3} or intact 70 kDa protein, but not by 70K Δ_{1-5} (Figure 6B). None of the mutant 70 kDa proteins

Figure 8 Inhibition of ^{125}I -fibronectin binding to fibroblasts: effects of combining 70K Δ_{1-3} and 70K Δ_{4-5}

^{125}I -fibronectin (1.5 nM) was added to confluent wells of fibroblasts in the presence of the indicated amounts of unlabelled 70 kDa protein (■), 70K Δ_{1-3} (○), 70K Δ_{4-5} (●), and 70K Δ_{1-3} + 70K Δ_{4-5} (◻) and 70K Δ_{1-5} (◆). After a 20 h incubation at 37 °C, cell layers were washed, solubilized in 0.1 M NaOH, then counted for radioactivity in a γ -radiation counter. Data represent the means of values for duplicate wells, and error bars show the range. Binding is expressed as a percentage of binding that occurred in the absence of competing proteins.

tested bound at levels comparable with intact 70 kDa protein; binding ranged from 10 to 30% of the levels of the wild-type protein.

In competition binding assays, the ability of various deletion mutants to inhibit binding of ^{125}I -fibronectin and ^{125}I -70 kDa to the cell surface was assessed. As shown in Figure 7(A), unlabelled 70 kDa protein was the best competitor of ^{125}I -fibronectin binding in a 20 h assay, with half-maximal inhibition of binding (IC_{50}) occurring at approx. 30 nM. In contrast, all of the deletion mutants tested had IC_{50} values of > 300 nM. Similar results for 70K Δ_{1-3} and 70K Δ_{4-5} were found with ^{125}I -70 kDa protein binding to fibroblasts (Figure 7B).

To determine if simultaneous addition of more than one deletion mutant results in competition similar to that seen with wild-type 70 kDa protein, 70K Δ_{1-3} and 70K Δ_{4-5} were added together in competition binding assays. Figure 8 indicates that addition of both 70K Δ_{1-3} and 70K Δ_{4-5} did not result in greater inhibition than that observed with 70K Δ_{1-3} alone. The competition curves would be dominated by the 70K Δ_{1-3} inhibition curve if 70K Δ_{4-5} binding is of lower avidity than 70K Δ_{1-3} binding.

To examine whether 70 kDa deletion mutants can also inhibit endogenous fibronectin matrix assembly, fibroblasts were seeded in the absence and presence of 500 nM intact 70 kDa protein or various deletion mutants in medium containing fibronectin-depleted serum. Fibronectin fibrils polymerized by cells were revealed by indirect immunofluorescence. As shown in Figure 9, control fibroblasts (Figures 9A and 9B) elaborated an extensive fibronectin matrix. Incubation of cells with either the 70 kDa protein (Figure 9C) or 70K Δ_{1-3} (Figure 9D) resulted in a reduction in the amount of fibrils present. Fibronectin staining in these cells appeared in the form of short stitches or thin fibrils. Incubation of cells with 70K Δ_{4-5} (Figure 9E) also resulted in a decrease in the amount of fibronectin fibrils, but to a lesser extent than that observed with either 70 kDa protein or 70K Δ_{1-3} . In contrast, the presence of 70K Δ_{1-5} (Figure 9F) had only a slight inhibitory effect on fibronectin fibril formation. These data are consistent with the results of the competition binding assays

Figure 9 Inhibition of endogenous fibronectin matrix assembly

Fibroblasts were seeded at 2.5×10^5 cell/well in 12-well cluster dishes in DMEM containing 10% fibronectin-depleted FBS in the absence (**A, B**) or presence of 500 nM 70 kDa protein (**C**), 70K Δ I₁₋₃ (**D**), 70K Δ I₄₋₅ (**E**), or 70K Δ I₁₋₅ (**F**). Cells were incubated for 25 h at 37 °C, and then processed for immunofluorescence as indicated in the Materials and methods section. These selected micrographs are representative of multiple fields. Cells were photographed at 250 $\times$ magnification using the same exposure times.

using 500 nM concentrations of 70 kDa constructs and 125 I-labelled fibronectin as shown in Figure 7.

DISCUSSION

The present data indicate that polymerization of fibronectin into the extracellular matrix involves at least two cell-surface binding sites which interact with multiple sites within the 70 kDa N-terminus of fibronectin. Despite the efforts of numerous investigators to characterize the binding site on the cell surface for soluble fibronectin, identification has been elusive. Recent data have suggested the possibility that a conformationally altered III₁ module on cell-surface fibronectin may serve as an N-terminal binding site on the cell surface [22], and that integrin-mediated binding of fibronectin's III₁₀ module to III₁ may cause

a conformational change in III₁ [23]. In the present study we have used 70 kDa proteins lacking one or several type I modules to determine the role of individual type I modules in binding to fibroblasts and to fibronectin's III₁ module.

The 70 kDa N-terminal region of fibronectin interacts with the surface of substrate-attached fibroblasts [13,14], *Staphylococcus aureus* [39], fibrin [40,41] and III₁ [22]. Previous studies using 70 kDa deletion mutants expressed in COS cells showed that all five type I modules were important for binding to fibroblasts and *S. aureus*, but that binding to fibrin appeared to be a general property of type I modules [28]. In the present study we show that binding of the 70 kDa N-terminus to fibronectin's III₁ module is localized predominantly to modules I₄ and I₅. These data indicate that intermodule interactions between type I modules are necessary for the function of the N-terminal part of

fibronectin. Little or no III₁ binding was detected in mutants lacking both I₄ and I₅. 70KΔ₁₋₃ was approx. 3-fold less effective at inhibiting ¹²⁵I-70kDa binding to heat-denatured III₁ than the intact 70 kDa protein. It is not unusual for smaller fragments of proteins to show alterations in activity when compared with the intact protein or to larger fragments. For example, the 27 kDa N-terminal fragment of fibronectin is 3–5-fold less effective in blocking ¹²⁵I-70 kDa binding to fibroblasts than is the 70 kDa fragment, even though the 40 kDa fragment does not directly participate in binding [13,14]. Similarly, small peptides containing the Arg-Gly-Asp sequence in the tenth type III module of fibronectin are less effective in promoting cell adhesion than larger peptides or proteolytic fragments of fibronectin containing this sequence [42]. It is unlikely that the 40 kDa gelatin-binding region directly contributes to III₁ binding, since ¹²⁵I-labelled 40 kDa fragment could not be demonstrated to bind to heat denatured III₁ (Figure 4). In addition, Hocking et al. [22] showed that the 27 kDa and 70 kDa N-terminal fragments competed equally well for ¹²⁵I-70 kDa binding to heat-denatured III₁. Taken together, these data demonstrate that all or most of the binding activity to III₁ is localized to I₄ and I₅.

The structures of single and tandem fibronectin type I modules have been analysed by NMR [43,44]. The seventh type I module consists of two antiparallel β-sheets that are stacked on top of one another and which enclose a hydrophobic core containing conserved tyrosine and tryptophan residues and two disulphide bonds [43]. Structural analysis of two tandem type I modules, I₄ and I₅, indicates that each module contains a compact structure with the same consensus fold as I₇ [44]. Calorimetry studies also indicate that fibronectin type I modules are independently folded units [45]. Recently, tandem I₄ and I₅ modules produced in yeast [46] and bacteria [47,48] were shown to retain the ability to bind to sequences on *S. aureus* [47] and to fibrin [46,48]. Thus, even though structural studies indicate that type I modules fold independently, these modules can clearly interact to form functional domains critical for binding to III₁, *S. aureus* and fibroblasts [28,47; the present paper].

In the present study, the high-affinity binding site for fibroblasts could not be localized to I₄ and I₅, consistent with previous data using 70 kDa deletion mutants produced in COS cells [28]. The inability to localize a binding site to one module in a multimodule array has also been described in other systems. Lipoproteins bind to the N-terminal region of the low-density lipoprotein (LDL) receptor, which contains seven cysteine-rich repeats. However, binding of the LDL receptor to LDL could not be localized to one cysteine-rich repeat, but was found to require repeats 3–7 [49]. Our data demonstrate that 70 kDa deletion mutants lacking I₁₋₃ or I₄₋₅ bind specifically to the surface of fibroblasts at regions of focal adhesions. This is consistent with data showing that 70 kDa binding sites co-localize with α₅β₁ integrins in focal adhesions [50]. Further, the ability of 70KΔI₁₋₃ and 70KΔI₄₋₅ to partially inhibit ¹²⁵I-fibronectin binding and endogenous fibronectin matrix assembly suggests that these mutant proteins bind to sites on the cell surface that are involved in polymerization of fibronectin into the extracellular matrix.

These data provide the first evidence that more than one 70 kDa binding site may exist on the cell surface. The existence of two cell-surface sites that interact with different regions within type I modules 1–5 would explain the inability to localize the high-affinity fibroblast binding activity to specific type I modules (the present paper; [28]). Evidence that multiple binding sites may be present within the first five type I modules is also shown in Figure 7, where 70KΔI₁₋₃ and 70KΔI₄₋₅ were both able to compete partially for ¹²⁵I-fibronectin and 70 kDa binding to the cell surface. In addition, deletion mutants lacking two or more

Figure 10 Proposed model of fibronectin matrix assembly

The 70 kDa N-terminus of fibronectin interacts with an unidentified cell-surface protein (site 1, A), using modules I₁₋₃ or I₄₋₅. Following this step, bound fibronectin is transferred from site 1 to site 2 on a growing fibronectin fibril. This interaction involves binding of I₄₋₅ to conformation-dependent site in the III₁ module on another cell-surface fibronectin molecule. It has been proposed that binding of fibronectin's III₁₀ module to the α₅β₁ integrin may alter the conformation of III₁, leading to exposure of a binding site for the N-terminus of fibronectin [23].

modules competed more poorly for ¹²⁵I-fibronectin binding than mutants lacking single modules (Figure 7A). This may reflect the presence of multiple sites within the first five type I modules that can interact with the cell surface. If the second cell-surface binding site contains subsites for both I₁₋₃ and I₄₋₅ then simultaneous addition of 70KΔI₁₋₃ and 70KΔI₄₋₅ in competition binding assays with ¹²⁵I-fibronectin would not be expected to result in competition greater than that of 70KΔI₁₋₃ alone (Figure 8).

Our data suggest that one 70 kDa-cell surface interaction may involve binding of I₄₋₅ to the III₁ module on cell surface fibronectin (site 2, Figure 10). An additional interaction appears to involve the interaction of I₁₋₃ or I₄₋₅ with an unidentified cell-surface protein (site 1, Figure 10). The striking effect of 70KΔI₁₋₃ on elaboration of a fibrillar fibronectin matrix suggests that strong binding of I₄₋₅ to III₁ may occur after modules I₁₋₃ bind to this as-yet unidentified cell-surface receptor. These data are consistent with data showing that antibodies to III₁ block fibronectin binding to the cell surface in long-term assays, but do not block binding of the 70 kDa protein to the cell surface [25] or to heat-denatured III₁ [23]. In addition, these findings are consistent with the suggestion that the interaction of 70 kDa protein with III₁ occurs subsequent to the binding of the 70 kDa N-terminus to the cell surface [23,25].

We and others have proposed models for matrix assembly in which soluble fibronectin is assembled into disulfide stabilized aggregates in a stepwise fashion [23,26,27,51,52]. Figure 10 depicts a proposed model in which two distinct cell-surface sites exist which can interact with the N-terminus of fibronectin. The 70 kDa N-terminus of fibronectin interacts with an unidentified cell-surface protein (site 1, Figure 10A), using modules I₁₋₃ or I₄₋₅. Antibodies to III₁ would not be expected to interfere with 70 kDa binding to site 1. Following this step, bound fibronectin is transferred from site 1 to site 2 on a growing fibronectin fibril. This interaction involves binding of I₄₋₅ to a conformation-

dependent site in the III₁ module on another cell-surface fibronectin molecule. Antibodies to III₁ [25], the cell-binding domain of fibronectin [53], or the $\alpha 5 \beta 1$ integrin [17,18], as well as addition of excess III₁₀ [23] would interfere with binding to site 2, but not to site 1, and account for the ability of these reagents to inhibit fibronectin polymerization but not 70 kDa binding to cell surfaces [18,23,25].

Recombinant tandem repeats of I₄ and I₅ contain a very interesting intermodule interaction in which a tryptophan in I₄ interacts with the peptide backbone in I₅ [44]. It is possible that binding of fibronectin via I₁₋₅ or I₁₋₃ (site 1) provides an initial low-affinity interaction with the cell surface that precedes the higher-affinity interaction which occurs between III₁ and I₄₋₅ (site 2). One can imagine a mechanism in which conformationally altered III₁ displaces I₅ from its interaction with the tryptophan in I₄, thus allowing a stable protein-protein interaction between III₁ and I₄.

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