Comparison of the fibrin-binding activities in the N- and C-termini of fibronectin

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Fibronectin (Fn) binds to fibrin in clots by covalent and non-covalent interactions. The N- and C-termini of Fn each contain one non-covalent fibrin-binding site, which are composed of type 1 (F1) structural repeats. We have previously localized the N-terminal site to the fourth and fifth F1 repeats (⁴F1-⁵F1). In the current studies, using proteolytic and recombinant proteins representing both the N- and C-terminal fibrin-binding regions, we localized and characterized the C-terminal fibrin-binding site, compared the relative fibrin-binding activities of both sites and determined the contribution of each site to the fibrin-binding activity of intact Fn. By fibrin-affinity chromatography, a protein composed of the ¹⁰F1 repeat through to the C-terminus of Fn (¹⁰F1-COOH), expressed in COS-1 cells, and ¹⁰F1-¹²F1, produced in *Saccharomyces cerevisiae*, displayed fibrin-binding activity. However, since ¹⁰F1 and ¹⁰F1-¹¹F1 were not active, the presence of ¹²F1 is required for fibrin binding. A proteolytic fragment of 14.4 kDa, beginning 14 residues N-terminal to ¹⁰F1, was isolated from the fibrin-affinity matrix. Radio-iodinated

14.4 kDa fibrin-binding peptide/protein (FBP) demonstrated a dose-dependent and saturable binding to fibrin-coated wells that was both competitively inhibited and reversed by unlabelled 14.4 kDa FBP. Comparison of the fibrin-binding affinities of proteolytic FBPs from the N-terminus (25.9 kDa FBP), the C-terminus (14.4 kDa) and intact Fn by ELISA yielded estimated *K_d* values of 216, 18 and 2.1 nM, respectively. The higher fibrin-binding affinity of the N-terminus was substantiated by the ability of both a recombinant ⁴F1-⁵F1 and a monoclonal antibody (mAb) to this site to maximally inhibit biotinylated Fn binding to fibrin by 80 %, and by blocking the 90 % inhibitory activity of a polyclonal anti-Fn, by absorption with the 25.9 kDa FBP. We propose that whereas the N-terminal site appears to contribute to most of the binding activity of native Fn to fibrin, the specific binding of the C-terminal site may strengthen this interaction.

Key words: dissociation constant, ELISA, fibrin clot, thrombus, wound healing.

INTRODUCTION

Fibronectin (Fn), a glycoprotein that exists in a soluble form in body fluids and in an insoluble form in the extracellular matrix, plays a major role in many physiological processes, such as embryogenesis, wound healing, haemostasis and thrombosis, and extracellular matrix assembly [1–3]. Fn is secreted as a dimer of two nearly identical polypeptide chains of approximately 220–250 kDa that are linked together by two disulphide bonds near the C-terminus of the protein. Similar to many proteins of the extracellular matrix, Fn is a mosaic protein composed of three types of repeating modular consensus amino acid sequences, referred to as F1, F2 and F3 [3–9]. In Fn, the modules are organized into proteolytically resistant functional domains that contain binding sites for extracellular matrix proteins, cell-surface receptors, circulating blood proteins, glycosaminoglycans, proteoglycans and certain bacteria. These specific binding interactions of Fn confer its varied physiological functions.

The twelve F1 modules of Fn are encoded by a single exon (except ¹²F1), consist of approximately 45 amino acids, and share between 22 and 55 % sequence identity [1–3,5–7]. They occur in the N-terminal (¹F1–⁵F1), the collagen-binding (⁶F1-⁷F2-⁸F2-⁹F1–¹⁰F1) and the C-terminal (¹⁰F1–¹²F1) domains. Single F1 modules have also been identified in tissue-type plasminogen activator (t-PA) and in the blood coagulation Factor XII [6,9–11]. F1 modules contain four highly conserved cysteine residues, which form two disulphide bonds in a 1–3, 2–4

arrangement, thus creating a ‘finger’-like structural configuration (as shown in Figure 2, see below); the ¹²F1 has an extra pair of cysteines and has been reported to form a 1–4, 2–6, and 3–5 disulphide-bonding pattern [12,13]. NMR studies of F1 modules have revealed similar secondary structures composed of five β -strands that comprise a consensus fold characterized by a short double-stranded anti-parallel β -sheet near the N-terminus that folds over a triple-stranded anti-parallel β -sheet [6,10,11,14]. The majority of the conserved residues contribute to the hydrophobic core of the protein or are located in the turns between the β -strands. The highly conserved residues that are of structural significance are buried in the hydrophobic core, whereas surface regions of the modules, which are variable and predominantly hydrophilic, are exposed and probably confer varied ligand-binding interactions.

Proteolytic cleavage of Fn followed by specific ligand affinity chromatography and amino acid sequencing of bound fragments have enabled the localization of the various ligand-binding activities of Fn [1–3]. Using this approach, two fibrin-binding sites have been identified, one in the N-terminus and one in the C-terminus [15–23]. The N-terminal fibrin-binding site/domain contains the first five tandem F1 repeat modules (¹F1–⁵F1) and binds to fibrin by both non-covalent and covalent interactions. Using recombinantly expressed proteins, we and others have localized the non-covalent interaction in the N-terminus to the ⁴F1-⁵F1 module pair [11,23,24]. The covalent interaction involves a transglutaminase (Factor XIIIa)-catalysed

Abbreviations used: Fn, fibronectin; FBP, fibrin-binding peptide/protein; FBB, fibrin-binding buffer; mAb, monoclonal antibody; t-PA, tissue-type plasminogen activator; TFA, trifluoroacetic acid.

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cross-linking between a lysine residue in the C-terminus of the α -chain of fibrin and Gln³ and Gln¹⁶ in Fn [25–27]. It has been proposed that binding of Fn to fibrin involves a two-step process in which the non-covalent interaction precedes the covalent cross-linking [24].

The secondary and tertiary structure, thermodynamic properties and fibrin-binding activity of the N-terminal non-covalent fibrin-binding site have been well-characterized [11,23,24,28,29]. Although F1 modules exist as independent folded structures, a unique interaction between the N-terminal ⁴F1 and ⁵F1 modules was confirmed by NMR and thermodynamic studies [11,28] and, coincidentally, fibrin-binding activity was not exhibited by the individual modules but required the module pair [24]. The localization of the C-terminal fibrin-binding site to a minimal molecular structure and a comparative analysis of the biochemical characteristics of the fibrin-binding activity of the N- and C-terminal sites have remained largely undefined. As the role of the Fn–fibrin-binding interaction is critical in fibrin matrix assembly, tissue remodelling and blood clotting [1–3,7,25,30–34], the current studies were designed to compare the biochemical nature of the both the non-covalent C- and N-terminal fibrin-binding sites and determine the contribution of each site to the fibrin-binding activity of the entire Fn molecule.

The C-terminal fibrin-binding site is found in a 31 kDa tryptic fragment [19], a thermolysin-generated fragment of 19 kDa [24,28] and in various other proteolytic fragments [16,17,20]; all fragments included the ¹⁰F1–¹³F1 modules. In the current study, we report fibrin-binding activity in a subtilisin-generated fragment of 14.4 kDa that commences at Thr²¹⁵⁸, and in a protein encompassing the ¹⁰F1–¹³F1 modules, cloned and expressed in *Saccharomyces cerevisiae* cells. These data further confine the fibrin-binding activity to the beginning of ¹⁰F1 and extending at least 21 residues into ¹³F1. This is the smallest amino acid sequence from the C-terminus of Fn, known to date, that demonstrates fibrin-binding affinity. Direct binding assays, competitive inhibition and displacement experiments employing ¹²⁵I-labelled 14.4 kDa fibrin-binding protein (FBP) demonstrate that the binding of the isolated Fn C-terminal site to fibrin is specific, saturable and reversible. However, since both an antibody specific to the Fn N-terminus and the N-terminal binding site itself (⁴F1–⁵F1) inhibited the binding of Fn to fibrin by nearly 80%, it appears that the C-terminal site contributes comparatively less than the N-terminal site to the binding of Fn to fibrin. Accordingly, the 14.4 kDa FBP demonstrated a 12-times lower estimated binding affinity (K_d) than the 25.9 kDa FBP.

EXPERIMENTAL

Materials

Human plasma Fn was purchased from the New York Blood Center and further purified by affinity chromatography on lysine–Sepharose and gelatin–Sepharose, as described [35]. CNBr-activated Sepharose, Protein A–Sepharose, bovine fibrinogen, human thrombin and aprotinin were from Sigma (St. Louis, MO, U.S.A.), lysine–Sepharose, heparin–Sepharose and gelatin–Sepharose were from Pharmacia Biotech Inc. (Piscataway, NY, U.S.A.). ¹²⁵I-Na was purchased from Dupont-NEN Research Products (Boston, MA, U.S.A.).

Isolation of a 14.4 kDa proteolytic fragment of Fn that binds to fibrin and localization within the C-terminus of Fn

Isolation of a 14 kDa fibrin-binding fragment by affinity chromatography

Fibrin–Sepharose was prepared from fibrinogen coupled to Sepharose beads exactly as described previously [23]. Purified

human plasma Fn was incubated with subtilisin Carlsberg type VIII (Sigma) at an enzyme/substrate ratio of 1:100 (w/w) for 1 h at 37 °C [35]. Briefly, the digested Fn was subjected to gelatin–Sepharose chromatography and the effluent unbound fraction was combined with fibrin–Sepharose (80 mg/15 ml of beads) equilibrated in fibrin binding buffer (FBB: 0.05 M Tris/HCl/0.1 M NaCl, pH, 7.4) in a batchwise manner. Following incubation overnight at 4 °C, the affinity matrix was poured into a column and washed with the equilibration buffer. Upon warming the column to 22 °C, a 25.9 kDa fragment from the Fn N-terminus was eluted with FBB. The biochemical characterization of the interaction of the 25.9 kDa FBP with fibrin has been described in a previous publication [23]. Following washes with FBB, a proteolytic fragment of 14.4 kDa was eluted from the fibrin-affinity matrix with 50 mM Tris/HCl/0.5 M NaCl/6 M urea buffer, pH 7.4, as originally described for the elution of intact Fn [36]. Alternatively, the 14.4 kDa FBP was eluted with 50% ethylene glycol, pH 11.0, for better preservation of biological activity, as described in the Results section. The 14.4 kDa FBP was further purified to approximately 99% homogeneity by heparin-affinity chromatography (Pharmacia) to remove the 25.9 kDa N-terminal FBP (which remained bound to the fibrin–Sepharose after the temperature change to 22 °C and subsequent washing) that partially co-eluted with the 14.4 kDa FBP with the urea or ethylene glycol buffer. The 25.9 kDa FBP binds to heparin–Sepharose because it contains the ‘intermediate’ N-terminal heparin-binding site of Fn, as described [37]. The 14.4 kDa FBP was suspended in 0.1 M acetic acid and subjected to molecular-sieve chromatography using, separately, Sephadex G-100 and G-75, which had previously been calibrated with standard-molecular-mass markers. By SDS/PAGE analysis of both the void volume and individual fractions, it was shown that the 14.4 kDa fragment eluted as a single sharp peak in fractions corresponding to the elution volume of cytochrome *c*, as a marker (12.3 kDa). The molecular mass of 14.4 kDa was estimated using SDS/PAGE and was derived from its migration distance (R_F) on a 5–20% gradient of acrylamide monomer under non-reducing conditions. The molecular mass was estimated by comparison with a calibration curve of migration distances of standard proteins (Pharmacia).

Amino acid sequence analysis of the 14.4 kDa FBP

The 14.4 kDa FBP of Fn was subjected to SDS/PAGE and electrotransferred to a PVDF membrane (Immobilon; Millipore Corp., Bedford, MA, U.S.A.), as described previously [35]. The protein band was subjected to N-terminal amino acid sequence analysis using a 477A protein sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.), as described [35]. Following N-terminal sequence determination and based on the estimated mass of the 14.4 kDa FBP by SDS/PAGE and the molecular mass of the composite amino acids, the potential C-terminal cleavage site was predicted by using the software GPMA/W (Lighthouse Data, Odense SV, Denmark), together with the probable preferred amino acid specificity of subtilisin cleavage, according to the formula: $P_3-P_2-P_1-\downarrow-P_1'-P_2'-P_3'$, where $\downarrow$ marks the cleavage site for subtilisin [P_1 = neutral or acidic amino acid (broad specificity)]. Enhanced cleavage was seen when (i) P_1' = Gly or (ii) P_2 = bulky/hydrophobic amino acid [38].

Cloning and expression of recombinant Fn type-1 modules

Transient expression of type-1 modules in COS-1 cells

Two recombinant rat Fn cDNAs encoding the N- and C-terminal fibrin-binding domains were constructed in the vector

pcDNAI (Invitrogen, Carlsbad, CA, U.S.A.) for expression in COS-1 cells. pcDNAI-⁴F1-⁵F1 spanned two type-1 repeats from the N-terminal domain. *Xba*I sites were engineered at the 5' and 3' ends of the cDNA encoding these repeats using a *Dde*I site at position 551 and a *Bst*YI site at 844. The latter *Xba*I site introduced a termination codon following ⁵F1. This fragment was then inserted behind the Fn signal sequence (cDNA residues 1–150 from the vector pLPS [39]) between *Bam*HI and *Xba*I sites of the vector pcDNAI. The plasmid pcDNAI-¹⁰F1-COOH encodes the C-terminal fibrin-binding domain plus the flanking sequence extending from the tenth type-1 module to the end of the polypeptide chain. Starting with the plasmid pLJ-V0-SS [39], a *Bam*HI site was engineered at a *Stu*I site at position 6860, immediately 5' of ¹⁰F1. V0-SS lacks a 20 amino acid segment including the pair of cysteines that form the interchain disulphide bonds in Fn. A *Bam*HI-*Sal*I fragment (positions 6860–7607) containing the entire 3' end of the coding region, including some 3'-untranslated sequences, was excised and ligated behind the Fn signal sequence into the vector pcDNAI. Newly engineered sites were confirmed by DNA sequencing using Sequenase (US Biochemical, Cleveland, OH, U.S.A.). COS-1 cells were transfected with recombinant Fn plasmids using DEAE-dextran essentially as described in [40]. Post-transfection (24 h), cells were metabolically labelled with [³⁵S]methionine (25 µCi/ml; Dupont-NEN). After 24 h, conditioned media was collected and the supernatants were tested for their ability to bind to fibrin-Sepharose. The expression of the recombinant proteins in the COS-cell supernatants was confirmed by immunoprecipitation. Culture supernatants (0.15 ml) were incubated with 20 µl of a polyclonal rabbit anti-Fn antiserum [39] and 40 µl of Protein A-Sepharose beads by rotation overnight at 4 °C. The beads were separated by centrifugation, washed twice with Tris-buffered saline, resuspended in Laemmli sample buffer and subjected to SDS/PAGE, under non-reducing conditions, employing a 5–20 % gradient of acrylamide monomer. The presence of [³⁵S]methionine-labelled proteins was determined by autoradiography using X-OMAT AR5 imaging film (Kodak Scientific, Rochester, NY, U.S.A.).

Expression of recombinant type-1 modules of the C-terminus of Fn in *S. cerevisiae*

The ⁴F1-⁵F1 [11], ¹⁰F1-¹¹F1, ¹⁰F1-¹²F1 and ¹⁰F1-COOH polypeptides were cloned and expressed, using the yeast α -factor secretion pathway, as described in [7,11,23,41]. The following primers were used (note, the amino acids for the 5' primers correspond to the first codon). ⁴F1-⁵F1: A, 5'-GCT GAG AAG TGT TTT GAT CAT-3' (Ala¹⁵²); and B, 5'-T AAT GGA TCC TTA AGA GGT GTG CCT CTC ACA CTT C-3' (Ser²⁴⁴). ¹⁰F1: A, 5'-GAT GAC TCG TGC TTT GAC CC-3' (Asp²¹⁷²); and B, 5'-T AAT GGA TCC TTA AGA TGA ATC ACA TCT GAA GAT AC-3' (Ser²²¹⁷). ¹⁰F1-¹¹F1: A, 5'-GAT GAC TCG TGC TTT GAC CC-3' (Asp²¹⁷²); and B, 5'-T AAT GGA TCC TTA CGT TGC CTC ATG AGG GTC-3' (Thr²²⁶³). ¹⁰F1-¹²F1: A, 5'-GAT GAC TCG TGC TTT GAC CC-3' (Asp²¹⁷²); and B, 5'-T AAT GGA TCC TTA AGT GTT TGT TCT CTG ATG GTA-3' (Thr²³³²). ¹⁰F1-COOH: A, 5'-GAT GAC TCG TGC TTT GAC CC-3' (Asp²¹⁷²); and B, 5'-T AAT GGA TCC TTA CTC TCG GGA ATC TTC TCT GTC-3' (Glu²³⁵⁵). The construction of the vectors and the expression and purification of the ¹⁰F1-¹¹F1, ¹⁰F1-¹²F1 and ¹⁰F1-COOH polypeptides were described previously [11,23,29,41]. Briefly, the PCR product was first ligated into the pMB50 plasmid, which contains the yeast α -factor leader sequence, to enable secretion of the cloned proteins. Subsequently, the entire α -factor-Fn construct was ligated into

the non-inducible pMA91 plasmid and vector, containing the Leu gene for positive selection. Finally, the pMA91-type-1 modules were transformed into the leucine(–) yeast strain *S. cerevisiae* and positive transformants selected for their growth on leucine(–) medium. At each point, the inserted sequence was confirmed by oligonucleotide sequencing (Core Facility, NYU Medical School, New York, NY, U.S.A.). The secreted expressed protein only consisted of the Fn construct since the leader sequence was cleaved from the recombinant peptides by the endogenous KEX2 protease (which recognizes a Lys–Arg cleavage site during secretion of the protein) and because of the presence of a stop codon immediately C-terminal to the Fn construct [29]. Following culture overnight [in 0.17 % yeast nitrogen base (without amino acids or ammonium sulphate)/0.5 % ammonium sulphate/2 % glucose (YNB medium)], at least 20 cultures from different clones were tested for maximal production of the recombinant proteins in culture supernatants, by SDS/PAGE. Upon selection of an adequate clone that yielded optimal quantities of recombinant protein, preparative cultures of the yeast cells were grown for 2–3 days, centrifuged to remove the yeast cells, and the proteins adsorbed on to C₁₈ silica beads (3 g/l) for 2 h. The beads were washed with 20 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) and the proteins that remained bound were eluted with 60 % acetonitrile/0.1 % TFA. Following SDS/PAGE, the recombinant proteins were electroblotted on to PVDF membranes (described above); they were all shown to have the correct N-terminal amino acid sequence corresponding to the appropriate oligonucleotide codons. ¹⁰F1-¹¹F1 was further purified by reversed-phase HPLC and its purity determined by matrix-assisted laser desorption ionization–time of flight MS (NYU Medical School Core Facility).

Preparation of antiserum to recombinant ¹⁰F1-¹¹F1 expressed in *S. cerevisiae* (R620)

The purified ¹⁰F1-¹¹F1 was used to immunize 12-week-old female New Zealand white rabbits (Cocalico Biologicals, Reamstown, PA, U.S.A.). The rabbits were immunized with an initial injection of 250 µg of ¹⁰F1-¹¹F1 (1 mg/ml in PBS) emulsified with an equal volume of Ribi adjuvant (Sigma) and boosted every 3 weeks with 100 µg of the immunogen without adjuvant. Antibody titre was optimal after approximately 3 months. The antiserum was purified by affinity chromatography on recombinant streptococcal Protein-G-Sepharose (Gamma G-agarose; Genex Corp., Gaithersburg, MD, U.S.A.) equilibrated in PBS. The IgG fraction was eluted from the affinity matrix with 0.5 M acetic acid, pH 3.0, immediately neutralized, and finally dialysed against PBS. The antiserum was shown to immunoreact with intact Fn and ¹⁰F1-¹¹F1, by Western blot analysis, and with the 14.4 kDa FBP by ELISA.

Fibrin-binding assays

Fibrin-Sepharose chromatography of recombinant proteins

Proteins expressed by COS cells. Each of the COS-cell culture supernatants containing the [³⁵S]methionine-labelled expressed proteins (1.5 ml) was combined with 0.4 ml of fibrin-Sepharose and incubated overnight at 4 °C with end-over rotation. The fibrin-Sepharose beads were centrifuged at 4 °C, washed extensively with ice-cold FBB (10 times with 0.5 ml each) and warmed to 22 °C; the FBPs were eluted with FBB. Following this, the remaining bound proteins were eluted with 0.05 M Tris/HCl/0.5 M NaCl/6 M urea, pH 7.6, until radioactivity was no longer detected. An aliquot (50 µl) of each fraction was counted in an LS 6000 Scintillation Counter (Beckman Instru-

ments, Fullerton, CA, U.S.A.) to identify protein-containing fractions and the corresponding peak fractions were pooled, and analysed by SDS/PAGE followed by autoradiography, as described above. The autoradiograms were exposed for 2 weeks, scanned with a pdI desktop scanner, and bands quantified using the software Quantity One, version 2.4, pdI (Huntington Station, NY, U.S.A.).

Proteins expressed by *S. cerevisiae*. Since ample yields of $^{10}\text{F1}^{11}\text{F1}$ (1–3 mg/l) were present in the yeast culture supernatants, this protein was purified to homogeneity, as described above, solubilized in FBB and subjected to fibrin–Sephacryl chromatography. Conversely, only minute quantities of $^{10}\text{F1}^{12}\text{F1}$ and $^{10}\text{F1}^{13}\text{F1}$ were secreted by the transfected yeast clones. The yeast culture supernatants (50 ml) were concentrated by absorption on to silica C_{18} beads and eluted with 60% acetonitrile/0.1% TFA. The proteins were equilibrated in FBB and subsequently chromatographed on fibrin–Sephacryl. The affinity matrix was washed extensively with FBB at 4 °C, warmed to 23 °C, washed, and eluted with the 6.0 M urea buffer (as above). The column fractions were subjected to SDS/PAGE and confirmation that the FBBs in the bound fraction were the $^{10}\text{F1}^{12}\text{F1}$ and $^{10}\text{F1}^{13}\text{F1}$ recombinant proteins was achieved by electroblotting and amino acid sequence analysis, as described above.

Solid-phase fibrin-binding assays

The purified 14.4 kDa FBP from the C-terminus of Fn, the purified 14.4 kDa FBP was iodinated and employed in the following assays: (i) direct binding to fibrin-coated microtitre plates; (ii) competitive inhibition with unlabelled 14.4 kDa FBP for binding to fibrin; and (iii) reversibility of ^{125}I -labelled 14.4 kDa FBP binding to fibrin by unlabelled 14.4 kDa FBP. The 14.4 kDa FBP (100 μg in FBB) was radiolabelled with 2.0 mCi of ^{125}I -Na in the presence of Iodobeads (Pierce Chemical Co., Rockford, IL, U.S.A.) as described previously [42] (specific activity obtained, 5.0 $\mu\text{Ci}/\mu\text{g}$; 90% trichloroacetic acid precipitable counts).

For direct binding of the ^{125}I -labelled 14.4 kDa FBP to fibrin-coated ELISA plates; fibrin-coated (200 ng) polystyrene detachable microtitre wells (Immulon 2 Removawells), prepared as previously described [11,23], were blocked with 1% BSA and incubated with increasing concentrations of the 14.4 kDa FBP (50–1000 ng/0.1 ml of FBB; 35–690 nM) for 1 h at room temperature. The concentration of fibrin on the wells was measured using the Quantigold Protein Assay (Diversified Biotech, Boston, MA, U.S.A.) adapted for microtitre plates. Following removal of the unbound proteins, the plates were washed extensively with FBB containing 0.05% Tween 20, and bound ^{125}I -labelled 14.4 kDa FBP (^{125}I -14.4 kDa FBP) was determined by counting the radioactivity bound to each detachable well in an LKB-Wallac 1272 CitiGamma Counter (Wallac, Finland). Non-specific binding was calculated by determining the radioactivity bound to BSA-coated wells.

To examine competitive inhibition of ^{125}I -labelled 14.4 kDa FBP fragment binding to fibrin by unlabelled 14.4 kDa FBP fragment, the ^{125}I -14.4 kDa FBP [400 ng/0.1 ml of FBB (300 nM)] was combined with increasing concentrations of unlabelled 14.4 kDa peptide (140–6500 ng/0.1 ml of FBB [100–4800 nM]) and immediately added to the fibrin-coated wells (200 ng) for 1 h at 22 °C. Bound ^{125}I -14.4 kDa FBP was determined as described above. As a negative control, unlabelled egg lysozyme [120–6000 ng/0.1 ml of FBB (100–5000 nM)] was competed with the 14.4 kDa FBP for fibrin binding. Non-specific binding was calculated as above by determining the radioactivity

attached to BSA-coated wells. The results are expressed as the percentage of ^{125}I -14.4 kDa FBP binding to fibrin in the presence of unlabelled proteins compared with the value of ^{125}I -14.4 kDa FBP binding alone (100%).

To test the reversibility of ^{125}I -labelled 14.4 kDa FBP binding to fibrin, fibrin-coated (200 ng) microtitre wells were incubated with a constant concentration of ^{125}I -14.4 kDa [400 ng/0.1 ml of FBB (300 nM)], for 1 h at 22 °C. Following removal of the unbound radiolabelled protein and extensive washes with FBB containing 0.1% Tween 20, the bound ^{125}I -14.4 kDa FBP was reversed/displaced by incubation with increasing concentrations of unlabelled 14.4 kDa FBP [1260–12600 ng/0.1 ml of FBB (900–9000 nM)] for 1 h. The quantity of ^{125}I -14.4 kDa FBP that remained bound to each well was counted as described above. Results are expressed as the percentage of control wells (100%) that were incubated with buffer instead of the unlabelled 14.4 kDa FBP.

Inhibition of Fn binding to fibrin-coated ELISA plates

Purified human plasma Fn was biotinylated exactly as described with complete preservation of the fibrin-binding activity [23]. The binding of biotin-labelled plasma Fn to fibrin-coated microtitre wells was competitively inhibited by: (i) a polyclonal rabbit antiserum (Calbiochem, La Jolla, CA, U.S.A.) that immunoreacted with both the N-terminal (25.9 kDa FBP) and the C-terminal fibrin-binding sites (14.4 kDa FBP) by Western-blot analysis; (ii) a mouse monoclonal antibody (mAb) (kindly provided by A. Garcia-Pardo, CSIC, Madrid, Spain) that immunoreacted with the 25.9 kDa proteolytic fragment; (iii) the purified N-terminal fibrin-binding site (recombinant $^4\text{F1}^{5}\text{F1}$) [11,23]; and (iv) the polyclonal anti-Fn antiserum (Calbiochem) absorbed with the purified 25.9 kDa N-terminal Fn proteolytic fragment overnight at 4 °C at a ratio of 250 μg of purified 25.9 kDa FBP per 100 μl of antiserum. The fibrin-coated wells (200 ng) were reacted with a constant concentration of biotinylated Fn [150 ng/0.1 ml (3.4 nM)] that had been pre-incubated overnight at 4 °C with increasing concentrations (0.001–50 $\mu\text{g}/\text{ml}$ FBB) of each of the inhibitors described above. Bound biotin-labelled Fn was quantified by incubation with alkaline-phosphatase-labelled streptavidin (Amersham Life Science Inc., Arlington Heights, IL, U.S.A.), as previously described [23].

Comparative binding of Fn and its constituent N-terminal and C-terminal fibrin-binding sites to fibrin by ELISA

To compare the fibrin-binding activity of the Fn molecule and its constituent N- and C-terminal fibrin-binding sites and to estimate fibrin-binding affinities, the direct binding of intact Fn, the C-terminal 14.4 kDa FBP, the N-terminal 25.9 kDa FBP and the $^4\text{F1}^{5}\text{F1}$ N-terminal site to fibrin-coated wells was assessed. Increasing concentrations of Fn (10–500 ng/0.1 ml [0.23–11.35 nM]), the 14.4 kDa FBP (10–500 ng/0.1 ml [7.1–350 nM]), the 25.9 kDa FBP (10–300 ng/ml [4–116 nM]) and the $^4\text{F1}^{5}\text{F1}$ (37–226 ng/0.1 ml [35–215 nM]) were incubated for 1 h at 22 °C with fibrin-coated microtitre wells (200 ng). Fibrin binding was determined by incubating the microtitre plates with either rabbit R620 antiserum (4.0 $\mu\text{g}/\text{ml}$ in Tris-buffered saline containing 0.1% BSA and 0.05% Tween 20) to detect intact Fn and the 14.4 kDa FBP, or (mouse) mAb N288 (Chemicon, Temecula, CA, U.S.A.; 5.0 $\mu\text{g}/\text{ml}$ in the same buffer) to detect the 25.9 kDa FBP and the $^4\text{F1}^{5}\text{F1}$ polypeptide. We have previously shown that mAb N288 immunoreacts with an epitope in $^4\text{F1}^{5}\text{F1}$ (contained within the 25.9 kDa FBP fragment) by Western-blot analysis [23]. The wells were then incubated with alkaline-

phosphatase-labelled anti-rabbit or anti-mouse IgGs, the colour developed, as described above [23], and specific binding to fibrin quantified by subtracting values obtained in control wells coated with BSA.

Analysis of the binding data

Non-linear regression analysis, and estimation of dissociation constants and IC_{50} values were calculated using the software GraphPad Prism, version 1.0 (GraphPad Software, San Diego, CA, U.S.A.). The binding-affinity constants were estimated following non-linear regression analysis of the saturation curves obtained and were calculated based on the concentrations of Fn, the proteolytic FBP or the recombinant modules required to reach 50% saturation of binding to fibrin coated on the wells.

RESULTS

Isolation of a fibrin-binding fragment from the C-terminus of Fn

Proteolytic digestion of Fn generates fragments that retain biological activity and can subsequently be isolated by specific ligand affinity chromatography [1–3,15–24,35,37,42–44]. Fn was digested with subtilisin, subjected to gelatin-affinity chromatography to remove fragments containing the gelatin-binding domain, and the unbound effluent fraction from gelatin-Sepharose [43] was incubated with fibrin-Sepharose at 4 °C [23]. As illustrated in Figure 1, a broad peak containing a 25.9 kDa fragment (from the N-terminal fibrin-binding domain of Fn) was eluted by warming the affinity matrix to 22 °C (Figure 1, inset, lane 1). Following elution with 0.5 M NaCl/6 M urea, a sharp protein peak was eluted that contained a 14.4 kDa FBP (Figure 1, inset, lane 2); this fraction represented approximately 3–5% of the total protein digest applied to the column. Approximately

10% of the 25.9 kDa fragment was retained by the fibrin-affinity matrix after warming and was released with the urea buffer [23]. Similarly, < 10% of the 14.4 kDa FBP co-eluted with the 25.9 kDa FBP following warming of the fibrin-Sepharose. We have previously reported this heterogeneous fibrin-binding phenomenon [23]. In separate experiments, 2.0 and 4.0 M urea in FBB only partially eluted the 14.4 kDa FBP with values of 32% and 50%, respectively, of the total amount eluted with 6.0 M urea (maximum yield). Thus 6.0 M urea, which produced the highest yield of 14.4 kDa, was chosen as the elution buffer for the 14.4 kDa FBP. After elution of the 14.4 kDa FBP with 6.0 M urea from fibrin-Sepharose and dialysis against FBB, only 50% of the protein retained binding activity to a subsequent fibrin-Sepharose matrix. In an effort to retain optimal fibrin-binding activity, a variety of reagents were tested for elution of the 14.4 kDa from fibrin-Sepharose. We found that the 14.4 kDa FBP could also be released from the fibrin-affinity matrix with 50% ethylene glycol, pH 11.0 (pH 8.0 did not allow elution the 14.4 kDa FBP), permitting retention of 90% of the protein on a second fibrin-Sepharose column; 1.0 M KBr only preserved 7–10% of the fibrin-binding activity. In contrast, high salt concentrations (1 M NaCl) or low pH (4.0) did not release the binding of the 14.4 kDa FBP from the fibrin-affinity matrix. The disruption of the interaction of the 14.4 kDa fragment with fibrin by these reagents suggests that the protein requires denaturation for its elution and/or may bind through hydrophobic interactions and hydrogen bonding.

Localization of the 14.4 kDa fibrin-binding fragment within Fn

The 14.4 kDa FBP was subjected to N-terminal amino acid sequence analysis for localization within the Fn molecule. The sequence TVGNSVNE was obtained from the excised band (containing 30 pmol of 14.4 kDa FBP), indicating that the

Figure 1 Purification of the 14.4 kDa proteolytic fragment of Fn by fibrin-affinity chromatography

Purified human plasma Fn was digested with subtilisin Carlsberg VIII and subjected to gelatin-Sepharose chromatography as described. The effluent unbound fraction was incubated overnight at 4 °C with fibrin-Sepharose equilibrated in FBB, and unbound proteins eluted at 4 °C. The N-terminal 25.9 kDa FBP was predominantly released from affinity matrix following warming to 22 °C. The C-terminal 14.4 kDa FBP eluted with 6.0 M urea in 0.05 M Tris/HCl/0.5 M NaCl, pH 7.4. Inset: gradient SDS/PAGE (5–20% acrylamide monomer). Lane 1, 25.9 kDa FBP from Fn N-terminus eluted by warming the fibrin-Sepharose matrix from 4 °C to 22 °C; lane 2, 14.4 kDa FBP from Fn C-terminus eluted by the 6 M urea buffer. All samples were electrophoresed under reducing conditions using a final concentration of 0.1 M dithiothreitol.

Figure 2 Diagram of the type-1 structure of the FBPs from Fn

The finger-like configuration is formed by the conserved cysteine residues within each module. Type-1 modules are composed of conserved consensus repeats that fold to form a characteristic tertiary structure composed of a predominantly hydrophobic backbone core [6,10,11,14]. **(A)** 25.9 kDa FBP from the N-terminus of Fn. The subtilisin-generated FBP, indicated by broken lines, encompasses the $^1\text{F1}$ – $^5\text{F1}$ extending from Ser¹⁷ to Gln²⁴⁶. Fibrin-binding activity has been confined to the $^4\text{F1}$ – $^5\text{F1}$ module pair [11,23,24]. **(B)** 14.4 kDa FBP from the C-terminus of Fn. The subtilisin-generated FBP, indicated by broken lines, commences at amino acid residue Thr²¹⁵⁸, 14 residues before the beginning of the $^{10}\text{F1}$, and extends at least 21 amino acid residues into the $^{12}\text{F1}$ at Leu²²⁸². The diagram depicts the primary amino acid sequence of the 14.4 kDa FBP from the N-terminal subtilisin cleavage site and including the entire $^{12}\text{F1}$. The fibrin-binding site, indicated by a continuous line, commences at the beginning of the $^{10}\text{F1}$ and extends at least 21 residues into the $^{12}\text{F1}$ (amino acids are shown to the end of the $^{12}\text{F1}$ as x-x-x). Putative subtilisin-cleavage sites within the $^{12}\text{F1}$ are either C-terminal to Leu²¹ (residue 2282 of Fn) or N-terminal to Gly³¹ (residue 2292 of Fn). A second proposed cleavage site occurs either immediately C-terminal to the last Pro of the $^{12}\text{F1}$ at Gly²³⁰⁶ or Gly²³¹³.

fragment commences at residue 2158 from the N-terminus of Fn, 14 residues before the beginning of the $^{10}\text{F1}$ (Figure 2B). Based on the molecular mass of the 14.4 kDa FBP determined by migration on SDS/PAGE and the molecular mass of the composite amino acids, we have determined, employing the software GPMA/W, that this fragment extends at least 21 residues (Leu residue at position 2282 from the N-terminus of Fn) into the $^{12}\text{F1}$, as designated in Figure 2(B) by an arrow. According to a report denoting probable cleavage-site specificities for subtilisin (see [38]), we propose that the cleavage site may be C-terminal to Leu²¹ (2282 of Fn, flanked by Tyr and Gly) or N-terminal to Gly³¹ (2292 in Fn, flanked by Phe and Gly). According to the reported intrachain disulphide-bonding pattern [12,13], either internal cleavage would permit the entire $^{12}\text{F1}$ to remain attached, as illustrated in Figure 2. Because of the size of the 14.4 kDa FBP and the fact that it migrated identically on SDS/PAGE whether under non-reducing or reducing conditions (results not shown), a cleavage C-terminal to the $^{12}\text{F1}$ must have occurred. Possible cleavage sites close to the end of the $^{12}\text{F1}$ are N-terminal to Gly²³⁰⁶ (immediately C-terminal to the last Pro of the $^{12}\text{F1}$) or Gly²³¹³ (eight residues past the last Pro). Accordingly, the region C-terminal to the compact type-1 structure of the $^{12}\text{F1}$ represents an elongated polypeptide stretch, highly vulnerable to proteolytic cleavage. Upon disruption of the 3 intrachain disulphide bonds, by reduction, depending on whether the cleavage within the $^{12}\text{F1}$ occurs at Leu²¹ or Gly³¹ (as described above), a cleavage fragment of between 23 and 14 amino acids, respectively, must have been liberated. Since by

SDS/PAGE we did not observe a faster migrating 14.4 kDa species that would have been commensurate with the detachment of this fragment from the $^{12}\text{F1}$, we presume that the higher frictional ratio, caused by releasing the compact structure following reduction, caused the 14.4 kDa FBP to migrate more slowly, thereby compensating in migration distance for the loss of the fragment.

Fibrin-affinity chromatography of recombinant Fn proteins expressed by COS cells

To determine whether the recombinant proteins from the N- and C-termini of Fn demonstrated similar fibrin-binding characteristics as the fibrin-binding fragments obtained by proteolytic digestion, the labelled supernatants from each of the COS cell transfectants were incubated with fibrin–Sephacrose and the proteins eluted exactly as described above. The eluted fractions were analysed by SDS/PAGE followed by autoradiography and densitometric quantification. As illustrated in Figures 3(A) and 3(B), since both the recombinant $^4\text{F1}$ – $^5\text{F1}$ from the N-terminus and $^{10}\text{F1}$ – $^{12}\text{F1}$ from the C-terminus were not detected in the unbound fraction, these proteins were completely biologically active in fibrin binding. Figure 3(A) shows that 80% of the recombinant $^4\text{F1}$ – $^5\text{F1}$ was released by warming the fibrin–Sephacrose beads to 22 °C, leaving only 12% more tightly retained, which was subsequently eluted with 6.0 M urea in FBB. These results are identical to our previously reported findings using the $^4\text{F1}$ – $^5\text{F1}$ expressed in yeast [23]. In contrast, as shown

Figure 3 Densitometric scanning of autoradiogram of fractions of metabolically labelled COS-cell supernatants containing the $^4\text{F1.5F1}$ and $^{10}\text{F1-COOH}$ recombinant proteins chromatographed on fibrin-Sepharose

Fibrin-Sepharose chromatography of the (A) $^4\text{F1.5F1}$ recombinant protein from Fn N-terminus and of the (B) $^{10}\text{F1-COOH}$ from Fn C-terminus. Cell-culture supernatants of [^{35}S]methionine-labelled COS cells transiently transfected with the $^4\text{F1.5F1}$ and $^{10}\text{F1-COOH}$ were incubated with fibrin-affinity matrices at 4 °C as described. The recombinant proteins bound to fibrin-Sepharose were released stepwise by FBB following warming of the affinity matrices to 22 °C and by 6.0 M urea in 0.05 M Tris/HCl/0.5 M NaCl, pH 7.4. The pooled effluent unbound fraction and the two bound fractions were analysed by SDS/PAGE followed by autoradiography as described in the Experimental section. The bands of the autoradiograms corresponding to the $^4\text{F1.5F1}$ and $^{10}\text{F1-COOH}$ were scanned with a pdl desktop scanner and their quantities in the different fractions determined by image analysis as described. Neither the $^4\text{F1.5F1}$ nor the $^{10}\text{F1-COOH}$ expressed proteins were found in the unbound fraction and, thus, the expressed polypeptides were completely active in binding to fibrin. RT, room temperature.

in Figure 3(B), 65 % of recombinant $^{10}\text{F1-COOH}$ was eluted with 6.0 M urea buffer and 35 % was released by the temperature shift. Thus nearly identical to the results obtained using the proteolytic fragments containing the N-terminal (25.9 kDa) and C-terminal (14.4 kDa) fibrin-binding sites, the $^{10}\text{F1-COOH}$, representing the C-terminal fibrin-binding site, required more stringent conditions for elution than the N-terminal site, represented by the $^4\text{F1.5F1}$, suggesting that the C-terminal site may have a higher relative affinity for fibrin. The only notable difference between the recombinant and proteolytic fragments is that more of the recombinant $^{10}\text{F1-COOH}$ was eluted by temperature change (Figure 3B), whereas the 14.4 kDa FBP appeared to bind more tightly, since more of this protein required 6.0 M urea for elution (Figure 1, inset).

Fibrin-affinity chromatography of recombinant type-1 modules from the C-terminus of Fn expressed in *S. cerevisiae*

Since ample quantities of properly folded and biologically active $^4\text{F1.5F1}$ were produced using *S. cerevisiae* [11,23], the yeast system was again employed for the cloning and expression of the $^{10}\text{F1.11F1}$, $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ recombinant proteins,

Figure 4 Fibrin-Sepharose chromatography of culture supernatants of *S. cerevisiae* expressing the recombinant $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ proteins

Yeast culture supernatants (50 ml) were concentrated by absorption on to silica C_{18} beads, eluted with 60 % acetonitrile/0.1 % TFA, equilibrated in FBB and incubated overnight at 4 °C with fibrin-Sepharose. Unbound protein was washed from the column at 4 °C, the affinity matrix warmed to 22 °C, and bound proteins eluted with 6.0 M urea in 0.05 M Tris/HCl/0.5 M NaCl, pH 7.4. The unbound and bound fractions were pooled separately, dialysed against deionized water, lyophilized, electrophoresed on gradient SDS/polyacrylamide gels (5–20 % acrylamide monomer), transferred to PVDF membranes and stained with 0.1 % Coomassie Brilliant Blue R250 in 50 % methanol. (A) Recombinant $^{10}\text{F1-12F1}$. (B) Recombinant $^{10}\text{F1-COOH}$. Lanes 1, proteins in the yeast cell-culture supernatants after concentration by absorption on C_{18} beads (represents 1/12 of the total protein content of 1.5 mg); lanes 2, unbound fraction (represents 1/6 of the total pooled fraction); lanes 3, fraction bound to fibrin-Sepharose (represents the total bound fraction). The bands corresponding to the expected molecular masses of the $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ recombinant proteins were excised from the membranes and amino acid sequence analysis performed. The open arrows indicate the electrophoretic migration of the confirmed $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ recombinant proteins.

representing the C-terminal fibrin-binding site of Fn. The $^{10}\text{F1.11F1}$ was expressed in similar quantities to the $^4\text{F1.5F1}$, and following HPLC purification it migrated as one band by SDS/PAGE. The approximate yield of pure protein was 1–3 mg/l of culture supernatant. By MS, the $^{10}\text{F1.11F1}$ resolved into one peak with a molecular mass of 10373 Da. The $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ proteins represented < 1 % of the total yeast proteins and therefore they could not be purified by HPLC. Whereas both HPLC purified and non-purified $^{10}\text{F1.11F1}$ did not bind to fibrin-Sepharose, the $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ demonstrated fibrin-binding activity and were eluted with the 6.0 M urea buffer. Confirmation that the $^{10}\text{F1-12F1}$ and $^{10}\text{F1-COOH}$ recombinant proteins bound to fibrin-Sepharose was achieved by electroblotting the bound fraction followed by excising the band that corresponded to the molecular masses of the $^{10}\text{F1-12F1}$ (18.1 kDa; Figure 4A), $^{10}\text{F1-COOH}$ (25 kDa; Figure 4B) and amino acid sequencing. The correct recombinant proteins are demonstrated by an arrow, as shown in Figures 4(A) and 4(B) of the Coomassie Brilliant Blue-stained PVDF membrane (prior to band excision). The dark band shown at approximately 50 kDa in Figure 4(A) was also excised and sequenced. The sequence

Figure 5 Characterization of the fibrin-binding activity of the ^{125}I -labelled 14.4 kDa proteolytic FBP from the C-terminus of Fn by solid-phase assays

(A) Direct binding of the ^{125}I -14.4 kDa FBP to fibrin-coated ELISA microtitre wells. Increasing concentrations of ^{125}I -14.4 kDa FBP (50–1000 ng/0.1 ml of FBB [35–690 nM]) were incubated with fibrin-coated (200 ng) ELISA plates and bound 14.4 kDa FBP on individual Removawells determined in a gamma counter. Results represent the means (± 2 S.D.) of two independent triplicate experiments. (B) Competitive inhibition of ^{125}I -labelled 14.4 kDa FBP binding to fibrin. The fibrin-coated microtitre wells were incubated with 300 nM iodinated 14.4 kDa fragment (400 ng/0.1 ml of FBB) premixed with increasing concentrations of unlabelled 14.4 kDa FBP (140–6500 nM) as described. Egg lysozyme was used as a negative control. Bound ^{125}I -14.4 kDa was determined as above. Results are expressed as percentage of the fibrin-binding activity determined in the absence of inhibitor. Data represent the means (± 2 S.D.) of quadruplicate experiments. (C) Reversibility of ^{125}I -labelled 14.4 kDa FBP binding to fibrin. The fibrin-coated wells were incubated with a constant concentration of ^{125}I -14.4 kDa FBP (400 ng/0.1 ml of FBP [300 nM]). Following removal of unbound radiolabelled protein, the bound ^{125}I -14.4 kDa FBP was displaced/reversed by incubation with increasing concentrations of unlabelled 14.4 kDa FBP (900–9000 nM) for 1 h. The quantity of labelled FBP that remained bound to the wells was determined as above. Results are expressed as percentage of control wells (100%) that were incubated with buffer instead of the unlabelled 14.4 kDa FBP. Data represent the means (± 2 S.D.) of duplicate experiments.

corresponded to the γ -chain of Fn, indicating that the fibrin had leached from the column in the 6.0 M urea eluent. Both of the $^{10}\text{F1}$ - $^{12}\text{F1}$ and $^{10}\text{F1}$ -COOH recombinant proteins were also observed in the fibrin-bound fractions following immunoblotting with the R620 antiserum (produced to purified recombinant $^{10}\text{F1}$ - $^{11}\text{F1}$ [results not shown]).

Characterization of the fibrin-binding activity of the 14.4 kDa fragment

To gain insight into the biochemical nature of the interaction of the C-terminus of Fn with fibrin, the 14.4 kDa FBP was radiolabelled and employed in solid-phase binding assays.

Direct binding

As demonstrated in Figure 5(A), the ^{125}I -14.4 kDa FBP bound to fibrin in a dose-dependent manner that reached saturation with 700 ng/0.1 ml (500 nM) reacting with 200 ng of fibrin coated on the microtitre well. Non-linear regression analysis indicated that the data fitted to a rectangular hyperbola, thereby defining a one-site binding interaction; the estimated K_d was 202 nM. Therefore, the 14.4 kDa proteolytic FBP specifically binds to fibrin. In addition, the derivatization of its constituent tyrosine residues with iodine did not disrupt fibrin-binding activity and the inherent structure of the 14.4 kDa FBP was apparently retained.

Competition by unlabelled 14.4 kDa FBP

Competitive inhibition of ^{125}I -14.4 kDa FBP binding to fibrin by increasing concentrations of unlabelled 14.4 kDa FBP was performed at a concentration of ^{125}I -14.4 kDa FBP that achieved 75% saturation (400 ng/0.1 ml [300 nM] per 200 ng of fibrin), as derived from the saturation curve obtained above. Figure 5(B) shows that the unlabelled 14.4 kDa FBP competitively inhibited the binding of ^{125}I -14.4 kDa FBP in a dose-dependent manner. In contrast, the control protein, egg lysozyme, did not inhibit ^{125}I -14.4 kDa FBP from binding to fibrin. The data fit a one-site competition curve and further substantiated the specificity of the interaction of the 14.4 kDa FBP with fibrin. Half-maximal inhibition (IC_{50}) of the ^{125}I -14.4 kDa FBP was obtained with 880 ng/0.1 ml (630 nM) of unlabelled 14.4 kDa FBP. Thus, a 2.1-fold molar excess of unlabelled 14.4 kDa FBP was required to inhibit 50% of the radiolabelled 14.4 kDa FBP from binding to fibrin. A maximum inhibition of 80% of binding was achieved at a concentration of 6.4 μg /0.1 ml (4500 nM) of unlabelled 14.4 kDa FBP (15-fold molar excess of unlabelled FBP).

Reversibility of binding of ^{125}I -14.4 kDa FBP

After a constant concentration of ^{125}I -14.4 kDa FBP (400 ng/0.1 ml [300 nM]) was incubated with the fibrin-coated wells (200 ng), bound ^{125}I -14.4 kDa FBP was reversibly displaced by incubation with increasing concentrations of unlabelled 14.4 kDa FBP. As shown in Figure 5(C), 42% of the bound ^{125}I -14.4 kDa FBP was displaced by a concentration of 14 μg /0.1 ml (10000 nM) of unlabelled 14.4 kDa FBP (33-fold molar excess). Egg lysozyme, used as a negative control, was unable to disrupt the binding of the labelled peptide to fibrin. These results further support the specificity of the interaction of the C-terminal fibrin-binding site/14.4 kDa FBP with fibrin.

Inhibition of Fn binding to fibrin

Whereas two fibrin-binding sites have been localized in Fn, it is not known whether both sites are active in the intact molecule and the extent to which each fibrin-binding site contributes to the fibrin-binding capacity of Fn. To clarify these points, the following reagents were used to inhibit the binding of biotin-labelled Fn to fibrin-coated ELISA plates: (i) an mAb to the 25.9 kDa N-terminal FBP; (ii) the $^4\text{F1}$ - $^5\text{F1}$ recombinant protein

Figure 6 Inhibition of Fn binding to fibrin-coated microtitre wells by antibodies and the $^4\text{F1.}^5\text{F1}$ using ELISA

Fibrin-coated (200 ng) microtitre wells were incubated with biotinylated Fn (150 ng/0.1 ml of FBB [3.4 nM]) preincubated with increasing concentrations (0.001–50 $\mu\text{g/ml}$ FBB) of: (i) anti-Fn polyclonal rabbit antiserum that immunoreacts with both the N-terminal 25.9 kDa and C-terminal 14.4 kDa FBPs by Western blot (■); (ii) a mouse mAb that immunoreacts with the 25.9 kDa FBP (○); (iii) purified recombinant $^4\text{F1.}^5\text{F1}$ expressed in yeast (●); and (iv) anti-Fn polyclonal anti-Fn antiserum [same as in (i)] that was absorbed with purified 25.9 kDa FBP (▲). Bound Fn was detected by incubation with alkaline-phosphatase-labelled streptavidin as described. Results are expressed as percentage binding compared with control wells incubated with the same concentration of biotinylated Fn without preincubation with any inhibitor. Data represent the means (± 2 S.D.) of quadruplicate experiments.

(N-terminal fibrin-binding site); and (iii) the polyclonal antibody to Fn (immunoreacts with both the 14.4 and 25.9 kDa FBPs and $^4\text{F1.}^5\text{F1}$ by ELISA and immunoblot analysis) to inhibit both fibrin-binding sites simultaneously. We had previously demonstrated that biotinylated Fn possessed equal fibrin-binding activity to unlabelled Fn [23]. A constant concentration of Fn (150 ng/0.1 ml [3.4 nM]) that achieved 75% saturation of binding to fibrin [23] was preincubated with increasing concentrations of the two different antibodies or the $^4\text{F1.}^5\text{F1}$ prior to the addition to the fibrin plates. As shown in Figure 6, 90% inhibition of the fibrin-binding activity of Fn was obtained by the polyclonal anti-Fn antiserum, and both the mAb and the $^4\text{F1.}^5\text{F1}$ showed a maximum of 80% inhibition of binding. A dose-response inhibition of binding was obtained, indicating the specificity of the inhibition by the specific antibodies and the $^4\text{F1.}^5\text{F1}$. Following absorption of the polyclonal antiserum to Fn with a 10-fold molar excess of purified 25.9 kDa FBP, the antiserum lost its ability to inhibit the binding of biotinylated Fn to fibrin (except at 10 $\mu\text{g/ml}$, when 13% inhibition was detected, similar to background). We were unable to obtain enough $^{10}\text{F1}$ – $^{12}\text{F1}$ or $^{10}\text{F1}$ –COOH to raise antibodies to an active recombinant protein to test for its ability to block the C-terminal fibrin-binding site directly.

Comparative binding of Fn, the 14.4 kDa FBP and the recombinant $^4\text{F1.}^5\text{F1}$ to fibrin

Since the studies using the antibodies specific for the N- and C-terminal fibrin-binding sites suggested that the C-terminus may contribute less than the N-terminal site to the fibrin-binding activity of Fn, the direct binding interaction of each site with fibrin was compared with that of the native Fn molecule. Since we had previously shown that the $^4\text{F1.}^5\text{F1}$ (composed of 93

amino acids) contained all of the N-terminal fibrin-binding activity in Fn, both the recombinant $^4\text{F1.}^5\text{F1}$ and the 25.9 kDa FBP were employed in these experiments. Only the 14.4 kDa FBP was used to represent the Fn C-terminal site because only low yields of recombinant C-terminal polypeptides were obtained with both the yeast and COS-cell expression systems. Separately, increasing concentrations of Fn, the 14.4 kDa FBP, the 25.9 kDa FBP and the $^4\text{F1.}^5\text{F1}$ were incubated with fibrin-coated microtitre wells. Binding of Fn and 14.4 kDa FBP was detected with purified R620 IgG and binding of the 25.9 kDa FBP and $^4\text{F1.}^5\text{F1}$ was detected with mAb (N288). Direct binding for all four proteins was dose-dependent and reached saturation at approximately 4.5, 350, 75 and 200 nM for Fn, 14.4 kDa FBP, 25.9 kDa FBP and $^4\text{F1.}^5\text{F1}$, respectively. Non-linear regression analysis of the specific binding data indicated that, for each protein, the data fitted a rectangular hyperbola; the mathematical equation was consistent with the presence of one binding site. Therefore, although Fn possesses two fibrin-binding sites, it displays the characteristics of a molecule composed of a single binding site (presumably due to the predominant affinity of the N-terminus). Based on the concentration of protein needed to achieve half-maximal saturation of binding, the following K_d values for fibrin were estimated: 2.1 nM for Fn (Figure 7A), 216 nM for the 14.4 kDa FBP (Figure 7B; similar K_d to the ^{125}I -14.4 kDa FBP shown in Figure 5A), 18 nM for the 25.9 kDa FBP (Figure 7C) and 60 nM for the $^4\text{F1.}^5\text{F1}$ (Figure 7D).

The native Fn molecule exhibits a high binding affinity for fibrin (compared with t-PA at 1 μM [45,46]). Our data show that the 14.4 kDa FBP exhibits 100-fold lower affinity for fibrin than intact Fn (14.4 kDa FBP, $K_d = 216$ nM versus Fn, $K_d = 2.1$ nM). Whereas the $^4\text{F1.}^5\text{F1}$ harbours the fibrin-binding activity in the N-terminus (out of the $^1\text{F1}$ – $^5\text{F1}$, no other single module or module pair demonstrated fibrin-binding activity [23,24]), the 25.9 kDa FBP (which contains the $^1\text{F1}$ – $^5\text{F1}$, see Figure 2A) possessed at least a three-times (3.3) greater estimated binding affinity to fibrin than the $^4\text{F1.}^5\text{F1}$ (25.9 kDa FBP, $K_d = 18$ nM versus $^4\text{F1.}^5\text{F1}$, $K_d = 60$ nM). These results agree with our previous studies that showed that the relative affinity of the 25.9 kDa FBP for fibrin was four times higher than that of the $^4\text{F1.}^5\text{F1}$ (data based on IC_{50} of each protein needed to inhibit the binding of biotinylated Fn to fibrin [23]). The N-terminal fibrin-binding site displays a 12-fold higher binding affinity for fibrin than the C-terminal site (25.9 kDa FBP, $K_d = 18$ nM versus 14.4 kDa, $K_d = 216$ nM). However, since the K_d of Fn binding to fibrin is 8.6 times greater than that of the N-terminal site (Fn, $K_d = 2.1$ nM versus 25.9 kDa FBP, $K_d = 18$ nM), the C-terminal site may increase the affinity of Fn for fibrin by this degree. Alternatively, the greater binding affinity of Fn for fibrin may be due to the dimeric structure and thus bivalency of the intact Fn molecule.

DISCUSSION

Employing both proteolytic fragments derived from Fn and recombinantly produced type-1 modules, the tertiary solution structure, thermodynamic properties and fibrin-binding characteristics of the N-terminal fibrin-binding domain, precisely localized to the $^4\text{F1.}^5\text{F1}$ (93 amino acids), has been well studied [11,23,24]. Whereas thermodynamic studies have revealed that the N- and C-terminal fibrin-binding domains (referred to as Fib-1, Fib-2) share similar thermal stability and fluorescence spectra [24,28] and all the reported C-terminal proteolytic fragments of various sizes contain the $^{10}\text{F1}$ – $^{12}\text{F1}$, the interaction of the C-terminal fibrin-binding site with fibrin has not been well

Figure 7 Comparative binding of Fn and its constituent N- and C-terminal fibrin-binding sites to fibrin by ELISA

(A) Binding of Fn to fibrin. Increasing concentrations of Fn (10–500 ng/0.1 ml of FBB [0.23–11.35 nM]) were incubated with fibrin-coated (200 ng) microtitre wells. Bound Fn was detected by incubation with R620 antiserum (raised against purified recombinant $^{10}\text{F1}$, $^{11}\text{F1}$ produced in yeast; R620 immunoreacts with the 14.4 kDa FBP by Western blot) followed by alkaline-phosphatase-labelled anti-rabbit IgGs as described. (B) Binding of 14.4 kDa FBP to fibrin. Increasing concentrations of 14.4 kDa FBP (10–500 ng/0.1 ml of FBB [7.1–350 nM]) were incubated with fibrin-coated microtitre wells and binding detected with R620 polyclonal antiserum as above. (C) Binding of the 25.9 kDa FBP to fibrin. Increasing concentrations of 25.9 kDa FBP (10–300 ng/0.1 ml of FBB [4–116 nM]) were incubated with fibrin-coated wells and bound FBP detected using a mAb to the N-terminus of Fn (N288). (D) Binding of $^4\text{F1}$, $^5\text{F1}$ recombinant type-1 module pair expressed in *S. cerevisiae* to fibrin. Increasing concentrations of $^4\text{F1}$, $^5\text{F1}$ (37–226 ng/0.1 ml of FBB [35–215 nM]) were incubated with fibrin-coated wells and binding quantified using mAb N288 as previously described [21]. In all cases data represent the means (± 2 SD.) of triplicate experiments.

studied. The studies described herein were designed to elucidate the structure/function relationship of the C-terminal fibrin-binding site by both further localizing this site within Fn and by demonstrating the specificity of the binding interaction of this site with fibrin. Moreover, to gain insight into the interaction of the Fn molecule with fibrin, we determined the contribution of each site to the binding of intact Fn to fibrin.

Following electroblotting and amino acid sequence analysis, the 14.4 kDa FBP, from the C-terminus of Fn, was shown to commence at Thr²¹⁵⁸, 14 residues before the beginning of the $^{10}\text{F1}$, and to extend at least 21 (Leu²¹) to 30 (Gly³⁰) residues into the $^{12}\text{F1}$, depending on the cleavage site. In either case, the entire $^{12}\text{F1}$ would remain attached in the 14.4 kDa FBP, due to the proposed disulphide-bonding pattern illustrated in Figure 2 [12,13]. It is notable that the internal cleavage in the 14.4 kDa FBP did not apparently affect the fibrin-binding function of the fragment, perhaps due to the maintenance of the tertiary structure and intramodule interactions that result from the intrachain disulphide-bonding pattern of the $^{12}\text{F1}$. A 19 kDa thermolysin-generated fibrin-binding fragment was shown to commence at Ile²¹⁶⁷, five residues before the $^{10}\text{F1}$, and to end before Cys²³³⁶, which forms the interchain disulphide bridge, joining the two Fn monomers [24,28]. This fragment includes and extends at

least 30 residues past the $^{12}\text{F1}$. Thus the 14.4 kDa FBP that we have obtained by subtilisin digestion is the smallest proteolytic fragment, to date, that displays fibrin-binding activity. Attempts to further localize the C-terminal fibrin-binding site were made by cloning and expressing the constituent overlapping type-1 modules of this region of Fn. We have previously shown that the $^{10}\text{F1}$ single module did not inhibit biotinylated Fn from binding to fibrin [23] and, in the current studies, it was shown that the pure and properly folded $^{10}\text{F1}$, $^{11}\text{F1}$ module pair, produced using the yeast expression system, did not bind to fibrin–Sepharose or to fibrin-coated ELISA plates. In contrast, the $^{10}\text{F1}$ –COOH and $^{10}\text{F1}$ – $^{12}\text{F1}$ recombinant proteins in the yeast cell-culture supernatants bound to fibrin–Sepharose. Our data suggest that no less than the first 21–30 amino acids of the $^{12}\text{F1}$ may be required to promote the fibrin-binding function of the 14.4 kDa FBP. The internally cleaved, but intact, $^{12}\text{F1}$ may function to stabilize the $^{10}\text{F1}$, $^{11}\text{F1}$ for interaction with fibrin or contain primary structural residues directly involved in binding to fibrin. Expression of the $^{11}\text{F1}$, $^{12}\text{F1}$ and $^{12}\text{F1}$ and assessment of their fibrin-binding activity are necessary to further localize this site.

Fibrin binding is a function of type-1 repeats in the N- and C-termini of Fn and also in a single type-1 module in the N-terminus of t-PA [10]. Given the degree of structural similarity

between the different Fn modules studied to date, which are superimposable [6,10,11,14], one can predict that the tertiary structures of other F1 modules, such as those within the C-terminus of Fn, will likewise be similar.

The relative orientation of successive F1 modules in this region may depend, however, on the length of the linker between the modules and on other intermodule interactions. Intermodule interaction stabilizes the interaction between the ⁴F1 and ⁵F1 [11,28] and is also integral to the fibrin-binding function of the ⁴F1.⁵F1. The individual ⁴F1 and ⁵F1 modules do not bind to fibrin, indicating that the interaction between the two fingers induces the proper conformation necessary for fibrin binding. Whether a single type-I module, as in the N-terminal finger of t-PA [10,45,46], or a module pair (or triplet) of the constitutive C-terminal fibrin-binding site, is required for fibrin binding remains to be determined. Three-dimensional structural analysis of the individual modules and of the C-terminal putative fibrin-binding unit should reveal whether intermodule interactions, as occur in the N-terminal site, may be required for fibrin binding. Since the fluorescence spectra of the 19 kDa thermolysin fibrin-binding proteolytic fragment from the C-terminus of Fn is dominated by one or more exposed tryptophans [28], it is possible that one of these tryptophans may be involved in the type of fibrin binding that we have observed for the C-terminus during fibrin-affinity chromatography, which requires disruption of hydrophobic interactions by urea and ethylene glycol.

Since the C-terminal fibrin-binding site required denaturing buffers for its elution and the N-terminal site was mainly released by temperature change, we originally proposed that the C-terminal site binds to fibrin with a relatively higher affinity than the N-terminal site. Interestingly, N- and C-terminal fibrin-binding fragments generated by thermolysin digestion showed similar binding affinities to fibrin-Sepharose when they were both applied and eluted at 4 °C [24]. However, on fibrin-Sepharose chromatography at 22 °C and 37 °C, less of both the N- and C-terminal fragments bound and a higher concentration of urea was required for elution of the C-terminal site than the N-terminal site at these higher temperatures [24]. In classic experiments, the binding of native Fn to immobilized fibrin was shown to be temperature-sensitive and to decrease as the temperature is increased to physiological values [36,37,47]. In these experiments, plasma Fn was bound to fibrin-Sepharose at 4 °C (67 % of total Fn applied), partially eluted at 22 °C (28 %) and completely eluted with 4.0 M urea (35 %) [36]. Therefore, the proteolytic fragments recapitulate the heterogeneous characteristic fibrin-binding activity portrayed by the intact Fn molecule except that most of the Fn applied to the fibrin-affinity matrix demonstrated an interaction with fibrin analogous to the C-terminal fibrin-binding site requiring urea for elution. The temperature-dependent heterogeneous binding/elution characteristics of the recombinant or proteolytic fragments of Fn may be an artifact specific to affinity chromatography or alternatively may reflect different states of equilibrium of binding at these different temperatures. The estimated K_d values for fibrin binding that we have obtained for the N- and C-terminal sites by ELISA contrast with the relative affinities suggested by their binding characteristics to fibrin-Sepharose. Similar discordant results were reported previously for the interaction of Fn and its proteolytic fragments with both fibrin and IgG linked to affinity matrices compared with ELISAs [23,42]. Whereas affinity matrices have proven useful for the isolation and characterization of sites on proteins that interact with specific ligands [1–3,15–24,35,42–44], these matrices may be limited in their usefulness for predicting relative binding affinities/characteristics. Although the results obtained by affinity chromatography appeared not to

vary among individual experiments, the high concentration of protein and random orientation of the protein chemically coupled to the matrix may contribute to the observed artifactual binding. Moreover, the elevated density of immobilized ligand has been reported to directly affect binding activities, with an increase in non-specific hydrophobic interactions [48], perhaps related, in part, to the elution characteristics observed for the 14.4 kDa FBP from fibrin-Sepharose.

On the other hand, our experiments using solid-phase binding assays clearly demonstrated that the N-terminal fibrin-binding site contributes to most of the fibrin-binding activity of intact Fn, as shown by the following results. (i) An mAb to the N-terminal site and the ⁴F1.⁵F1 both inhibited the binding of Fn to fibrin by 80 %. Since the same degree of inhibition was achieved by the ⁴F1.⁵F1 (the N-terminal fibrin-binding site of 10.5 kDa) as by the antibody to this site, blocking Fn from binding to fibrin was not due to steric hindrance by the antibody. Furthermore, we have previously shown that a different mAb to an epitope within the ⁴F1.⁵F1 achieved the same 80 % inhibition [23]. (ii) A polyclonal antibody to Fn, which inhibited biotinylated Fn binding to fibrin by 90 %, failed to inhibit Fn from binding to fibrin following absorption with the 25.9 kDa FBP. (iii) Using an ELISA in which Fn, the 25.9 kDa FBP and the 14.4 kDa FBP were directly bound to fibrin-coated wells, the estimated binding-affinity dissociation constants obtained were consistent with the role for the N-terminal site in providing most of the fibrin-binding function of Fn (2.1 nM for Fn, 18 nM for the 25.9 kDa FBP and 216 nM for the 14.4 kDa FBP). These data agree with a direct binding solid-phase assay using radiolabelled 14.4 kDa FBP, yielding an estimated K_d of 202 nM. Taken together, it appears that the C-terminal site may contribute less than 10 % of the binding of intact Fn to fibrin. Consistent with these results, a similar relative fibrin-binding affinity of Fn and its separate composite fibrin-binding sites was demonstrated in a recent study [49]. These studies used a competitive ELISA to test the plasma transglutaminase-catalysed covalent fibrin-binding ability of Fn and two recombinant peptides of Fn, one containing the N-terminus through to ⁵F1 (31 kDa) (similar to the region of the 25.9 kDa FBP, except the 25.9 kDa FBP does not contain the covalent transglutaminase site, Gln³, since it commences 17 residues in from the N-terminal of Fn; it does contain the non-covalent ⁴F1.⁵F1) and one of the N-terminus through to ²F1 (12 kDa) (containing the transglutaminase cross-linking site, Gln³, but not the non-covalent N-terminal fibrin-binding site, ⁴F1.⁵F1). It was demonstrated that Fn possessed at least 10-fold higher fibrin-binding activity than the 31 kDa protein and 100-fold higher activity than the 12 kDa protein [50]. The 10-fold higher binding of intact Fn compared with the 31 kDa N-terminal polypeptide to fibrin implicates a role for the C-terminal site in the binding of intact Fn to fibrin of approximately 10 % of the total fibrin-binding affinity of Fn. Moreover, in general, even in the presence of covalent cross-linking, the non-covalent interactions of Fn contribute greatly to fibrin binding. Interestingly, in another study, it was found that plasma Fn composed of one subunit containing an alternatively spliced variable region (V+) and one subunit lacking this spliced region (V0) was incorporated into an *in vitro* fibrin clot at a faster rate than cellular Fn, composed of two V+ subunits [50]. It was proposed that the absence of the V regions in one subunit of plasma Fn may affect interactions with fibrin, differentially exposing the C-terminal fibrin-binding site.

Based on the relative binding affinities obtained for both sites compared with native Fn, we hypothesize that the N-terminal site would bind first due to its higher rate/affinity of fibrin-binding, followed by the C-terminal site, which may then

strengthen the fibrin-binding interaction of Fn. Furthermore, the binding of the N-terminal site to fibrin may cause a requisite conformational change that subsequently exposes the C-terminal site for binding. In addition, *in vivo*, the C-terminus may have a distinctly different interaction, since the binding of Fn to other matrix constituents (e.g. collagen) may expose these sites differently than on binding to fibrin alone (as coated on the ELISA plate). Alternatively, one cannot exclude the possibility that the C-terminal fibrin-binding site is an artifact of enzymic digestion and does not have physiological function. The temporal order of binding of both the N- and C-terminal non-covalent fibrin-binding sites in Fn, as well as of the covalent transglutaminase-catalysed cross-linking of Fn to fibrin *in vivo*, warrants further studies. Determination of the molecular structure that dictates fibrin binding in the C-terminus of Fn, comparative quantification of binding-affinity constants of pure proteolytic or recombinant proteins from the N- and C-termini of Fn, for example by biospecific interaction analysis in real time, as well as assessment of the role for the N- and C-terminal fibrin-binding sites, in *in vitro* clot-binding assays in the presence and absence of transglutaminase, should provide answers to these questions. Most importantly, future studies of this nature should elucidate the role of the interaction of Fn with fibrin in haemostasis and thrombosis and in wound repair.

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