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Collagen-binding recombinant fibronectin fragments containing type II domains

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Abstract

Each of the two type II domains and four larger fragments, containing one or two type II domains of fibronectin, have been expressed in *Escherichia coli*. A special vector, containing a fragment encoding the cleavage site for Factor X_a, Ile-Glu-Gly-Arg, inserted immediately before the protein fragment of interest, was used. After treatment of the purified fusion proteins with reduced/oxidized glutathione, the correctly folded fibronectin fragments were released by proteolytic digestion with Factor X_a. The largest fragment, consisting of two type II and two type I domains, was the only fragment able to bind to immobilized gelatin.

Key words: Fibronectin; Type II domains; Gelatin-binding; Refolding; Factor X_a

1. Introduction

Fibronectin (FN) is a large multidomain/multifunctional glycoprotein found in blood plasma and in the extracellular matrix (reviewed in [1]). Its two, almost identical chains are built from three types of repeating units, the type I, type II and type III domains [2,3]. The type II domains, of which there are two, consist of approx. 60 amino acid residues and contain four cysteinyl residues connected in a 1–3, 2–4 disulfide bridge pattern [4]. Type II domains have been identified in other proteins, such as blood clotting factor XII [5], bovine seminal fluid proteins PDC-109 and BSP A3 [6,7], insulin-like growth factor II receptor/mannose 6- phosphate receptor [8,9], mannose receptor [10], 72 kDa and 92 kDa type IV collagenases [11,12]. Five of these seven proteins (two have not been tested) can bind to denatured collagen (gelatin), and the type II domains are believed to play an important role in this binding. The two type II units of FN are unique to the 42 kDa gelatin-binding fragment (GBF) of FN, which also contains four type I domains in the sequence: I_6-II_1-II_2-I_7-I_8-I_9 [4] (numbers refer to the actual domain number, starting from the amino terminus of FN). Subfragments of the 42 kDa GBF (I_6-II_1, II_2-I_7 and I_8-I_9) have been isolated [13,14] in order to further localize the gelatin- binding site. Of these were II_2-I_7 and I_8-I_9 strongly gelatin-binding and I_6-II_1 was weakly gelatin-binding. Thus, separate gelatin-binding sites may be located on the type I and type II domains of the 42 kDa GBF. Other type II containing fragments of FN have been produced by gene expression in *E. coli*. These are II_1, II_1-II_2 and II_1-II_2-I_7-I_8-I_9, expressed as β-galactosidase fusion proteins [15], and they all bind immobilized gelatin. In contrast, one of the same fragments, II_1-II_2, expressed by others [16], also as a β-galactosidase fusion protein, did not bind gelatin, unless I_7 or the 14 amino terminal residues of I_7 was added to the carboxy-terminus of the fragment.

In the mentioned, more or less conflicting reports, the type II_1 or type II_2 domains were either bound to each other, to a type I domain or to a leader protein. In the present work, we have isolated recombinant type II domains of FN that is free of type I domains and other fragments, and tested their ability to bind immobilized gelatin. Our results show, that only the largest fragment, I_6-II_1-II_2-I_7, was able to bind to immobilized gelatin. None of the smaller fragments, consisting of one or two type II domains, bound to gelatin. The disulfide bridge patterns in the two small fragments, II_1 and II_2a (II_2 with a carboxy-terminal extension) were determined and found to be correct.

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Abbreviations: FN, fibronectin; GBF, gelatin-binding fragment; HPLC, high-performance liquid chromatography; FX, plasma factor X; PMSF, phenylmethyl sulfonyl flouride; NTA, nitrilo-triacetic acid

2. Materials and methods

2.1. Materials

Restriction enzymes and chymotrypsin were purchased from Boeringer (Mannheim, Germany). A Qiagen kit for plasmid preparation and sequencing kit (ver. 2.0) used for dideoxy sequencing of cloned frag-

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ments, were from USB (Cleveland, OH, USA). *E. coli* N4830–1 cells were purchased from Pharmacia (Sweden). Ni^{2+}- NTA-Sepharose was synthesized as in [17]. Gelatin was coupled to Sepharose 6B as decribed [18]. FX was isolated from bovine plasma and activated essentially as described in [19] or purchased from Denzyme, Denmark. Trypsin was purchased from Worthington (Freehold, NJ, USA). Gelatin from swine skin (type I) was from Sigma (Sigma product no: G-2500).

2.2. Plasmids

Plasmid pFH134, containing the cDNA insert of the entire 42 kDa GBF of FN [20], was a kind gift from K. Vibe-Pedersen (Aarhus, Denmark). The *E. coli* expression plasmid pLcIIMLCH6 was constructed from the vector pLcII [19,21] as described in [22].

2.3. Construction of plasmids expressing the fibronectin fragments

DNA fragments encoding six type II containing fragments of FN (I_6-II_1-II_2-I_7, II_1, II_2, II_2a (II_2 with a six residue carboxy-terminal extension peptide), II_1-II_2 and II_2-I_7, see Fig. 1) were amplified from pFH134 by PCR, using the primer pairs shown in Fig. 2. The amplified DNA fragments were purified by electrophoresis on 1% agarose, cleaved by *Bam*HI and *Hin*dIII and cloned into the *Bam*HI/*Hin*dIII sites of pLcIIMLCH6. CII encodes the 31 amino terminal residues of the λcII protein, MLC encodes the 118 amino terminal residues of human myosin light chain and H6 codes for six histidines, providing an afinity purification handle for a single-step purification on Ni^{2+}- NTA-Sepharose [17].

2.4. Bacterial expression

E. coli QY13 cells [19] or N4830–1 cells were transformed with the pLcIIMLCH6FXFIB expression vector, containing the inserted fibronectin fragment (FIB) and the factor X_a recognition site, Ile-Gly-Glu-Arg, (FX) introduced by the amino-terminal oligonucleotides used in the PCR reaction (see Fig. 2). The cells were grown at 30°C until $A_{600} = 0.8$ and expression of the fusion protein was induced by heat shock at 42°C (15 min) [19]. Following expression at 37°C for 4–5 h, cells were harvested by centrifugation at 4°C and taken up in equal volumes of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 and detergent buffer [19]. Inclusion bodies were then isolated essentially as described [19]. After the final centrifugation the inclusion bodies were resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 and mixed with the same volume of neutralized phenol and then sonicated. Two volumes of ethanol was added to the phenol extract and a crude protein fraction was obtained after centrifugation.

2.5. Purification of the fusion protein

The pellet containing the crude protein was dissolved in 6 M guanidinium chloride, 50 mM Tris-HCl, pH 8.0 containing 100 mM dithiothreitol and incubated overnight at 20°C. Insoluble cellular debris was removed by centrifugation and the solution was gel-filtered on a Sephadex G-25 column, equilibrated with 8 M urea, 1 M NaCl, 0.2 mM ascorbic acid, 50 mM Tris-HCl, pH 8.0 to remove the dithiothreitol. The fractions containing the protein were pooled and applied to a Ni^{2+}-NTA-Sepharose column, equilibrated with the same buffer. Following extensive wash with the same buffer and with 6 M guanidinium chloride, 50 mM Tris-HCl, pH 8.0, the bound fusion protein was eluted with 8 M urea, 1 M NaCl, 0.2 mM ascorbic acid, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0.

2.6. Refolding of the fusion protein

The purified fusion protein was reduced with 25 mM dithiotreitol and gel-filtered on a Sephadex G-25 column, equilibrated with 8 M urea, 1 M NaCl, 2 mM dithiotreitol, 50 mM Tris-HCl, pH 8.0. Ten mM thiopyridyl-glutathione (a blocking agent, reacting with free thiols) was added to the pooled fractions containing the protein, and the mixture was incubated at 4°C overnight with constant stirring. The blocked fusion protein was gel-filtered on a Sephadex G-25 column in 8 M urea, 1 M NaCl, 50 mM Tris-HCl, pH 8.0. The pooled fractions containing the protein were reapplied to the Ni^{2+}-NTA-Sepharose column (in the same buffer) to initiate the refolding on the column. First, 5 ml of 8 M urea, 1 M NaCl, 5 mM ascorbic acid, 50 mM Tris-HCl, pH 8.0 was applied, then extensive wash with 1 M NaCl, 50 mM Tris-HCl, pH 8.7 and incubation overnight in this buffer. The bound fusion protein was eluted with 1 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.7 and subjected to disulfide exchange (16 h, 20°C) by addition of reduced/oxidized glutathione at final concentrations of 5 mM/0.5 mM.

2.7. Cleavage with factor X_a

The refolded fusion protein was cleaved with factor X_a (16 h, 20 °C, 1:100; w/w) either directly in the elution buffer from the Ni^{2+}-NTA-Sepharose column, or in 1 M NaCl, 50 mM Tris-HCl, pH 8.0, following gel filtration on Sephadex G-25.

2.8. Affinity chromatography on gelatin-Sepharose

The collagen affinity of the individual type II containing FN fragments was assayed by affinity chromatography on a gelatin-Sepharose column (1.6 × 11 cm), equilibrated with 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5. Bound protein was eluted with 5 M urea, 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5 (Fig. 3A).

2.9. Determination of disulfide bridges

The two small fragments containing domains II_1 and II_2a, respectively, were purified by reverse-phase HPLC (Pharmacia LKB HPLC system), on a Vydac C_4 column for II_1 and on a Nucleosil C_{18} for II_2a. SDS-PAGE (according to [23]) or amino terminal protein sequencing (using an Applied Biosystems 477A/120A sequencer) was performed to identify the peaks containing II_1 and II_2a, respectively. These were dried down, dissolved in 0.1 M ammonium bicarbonate and digested, first with chymotrypsin (3 h, 37°C, 1:100; w/w) and then with trypsin (3 h, 37°C, 1:200; w/w). Chymotrypsin was inhibeted by PMSF and trypsin by soybean trypsin inhibitor. Peptides were separated by HPLC, using a Nucleosil C_{18} column. Aliquots from each peak were hydrolyzed (6 M HCl, 16 h, 110°C) and the amino acid composition was determined by a modification of the procedure described in [24]. Prior to hydrolyzation, performic acid oxidation was performed [25] to allow identification of cystines as cysteic acid. The bridge-containing peptides were sequenced, further treated with performic acid and rechromatographed by HPLC (Nucleosil C_{18}) to verify the shift from one to two peaks. These peaks were analyzed by amino acid analysis and sequencing.

3. Results and discussion

Six fragments of fibronectin, containing type II domains, have been expressed in *E. coli* as cIIMLCH6FXFIB fusion products, isolated from inclusion bodies, denatured, reduced, purified and refolded/reoxidized. Sequence specific cleavage with FX_a liberated the fibronectin fragments. The six fibronectin fragments are: I_6-II_1-II_2-I_7, II_1, II_2, II_2a, II_1-II_2 and II_2-I_7 (II_2a is II_2 with six extra residues (AAHEEI) in the carboxy terminus) and their position in the 42 kDa GBF of FN are

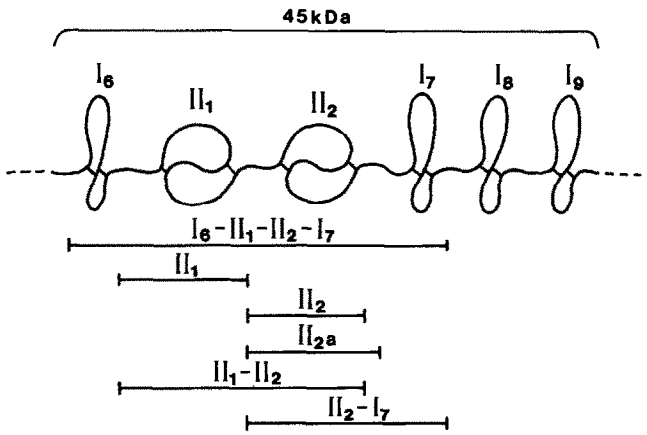

Fig. 1. Location of the six recombinant type II containing fragments, I_6-II_1-II_2-I_7, II_1, II_2, II_2a (II_2a is II_2 with a carboxy-terminal extension peptide, AAHEEI), II_1-II_2 and II_2-I_7, in the gelatin-binding 42 kDa fragment of fibronectin.

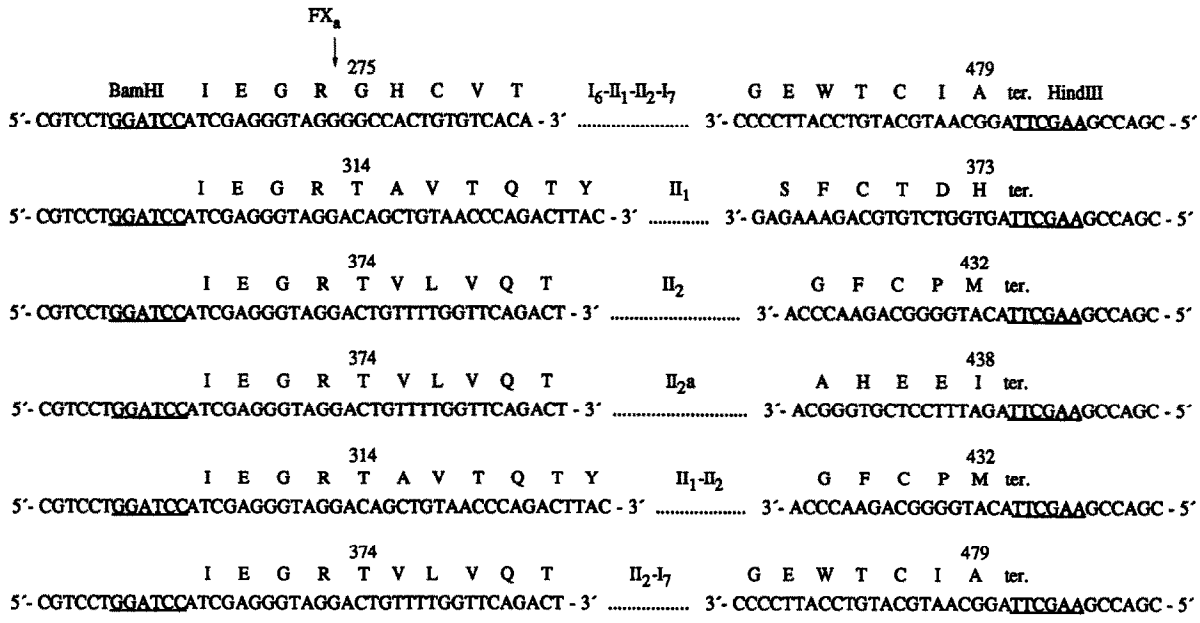

Fig. 2. Primer pairs used for PCR amplification of DNA fragments encoding the six fibronectin fragments shown in Fig. 1. The corresponding amino acid sequence is shown above each primer. The carboxy-terminal amino acid sequences (the right part) are translated from the strands complementary to the primers. Numbers refer to the protein sequence [2]. Restriction sites are underlined. The arrow indicate the FX_a cleavage site.

shown in Fig. 1. DNA sequencing of the part of pFH134 encoding II_2, showed CGA (coding for arginine, in position 380 of the protein sequence) instead of CAA (coding for glutamine) as reported by Kornblihtt et al. [20]. This brings the human FN sequence [20] in accordance with the bovine FN sequence [2] in this position, resulting in identical type II_2 domains in the two species, indicating that this observed sequence difference is likely to represent an original sequencing error rather than mutation of the cDNA clone.

Affinity chromatography on gelatin-Sepharose showed that only the largest fragment, I_6-II_1-II_2-I_7, bound to gelatin (Fig. 3A). When bound protein was diluted and reapplied to the gelatin-Sepharose column, complete rebinding was observed (Fig. 3B), confirming the specificity of the interaction. None of the smaller fragments bound to the gelatin-Sepharose column. This is in disagreement with the results of Bányai et al. [15], who reported that type II domain containing fragments, II_1 and II_1-II_2, of FN, expressed as b-galactosidase fusion proteins, bind immobilized gelatin. Our findings also disagree with those of Ingham et al. [13,14] and Owens and Baralle [16], who report that the fragment, II_2-I_7, by itself [14], or as a β-galactosidase fusion protein [16], can bind gelatin-Sepharose. The cause for the discrepancy is unresolved, but may relate to different types or sources of

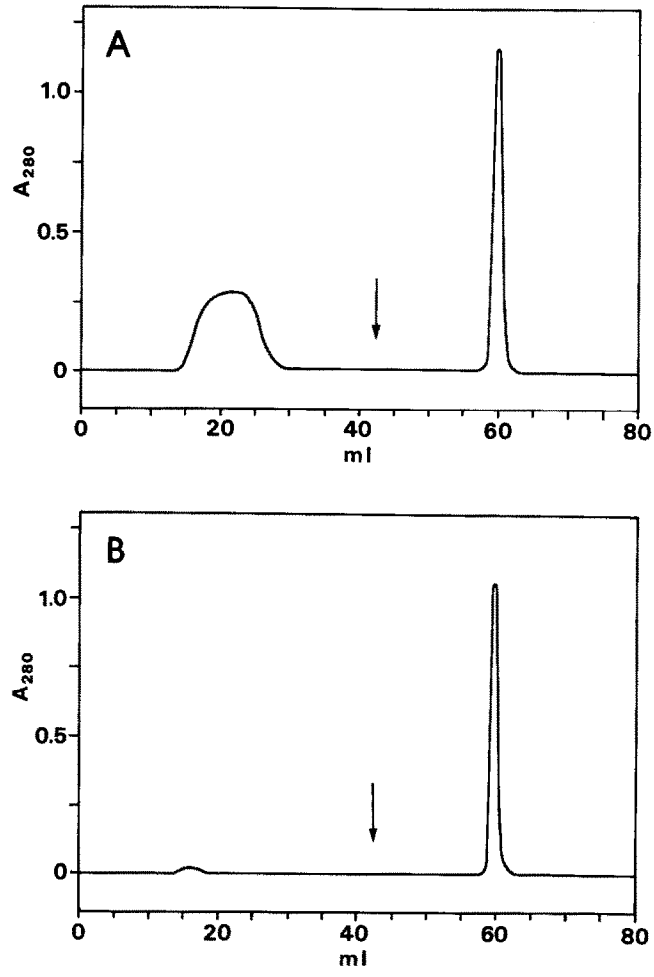

Fig. 3. (A) Affinity chromatography of the fibronectin fragment I_6-II_1-II_2-I_7 on a gelatin-Sepharose column. (B) Rechromatography of diluted, bound protein from Fig. 3A. Arrows indicate the start of elution with buffer containing 5 M urea.

gelatin. Also it could be questioned if our fragments are correctly folded. To test correct folding in two of the smallest fragments, II_1 and II_2a, the disulfide bridge patterns were determined, and in both cases found to be correct. One may also speculate if a correct disulfide-bridge pattern is enough to ensure correct folding. We believe it is, and therefore the discrepancy between our results and those of Bányai et al. [15] may rely on unspecific binding of their fragments to the gelatin-Sepharose or on the gelatin source as mentioned.

In conclusion, our results do not support the conclusion of others [15], that one type II domain of fibronectin is capable of binding to gelatin on its own, similar to those of PDC-109 and BSP A3 [6,7]. In contrast, our data suggest that the four-domain cluster I_6-II_1-II_2-I_7 is the smallest type II containing fragment of fibronectin that harbours an authentic binding site for gelatin.

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