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The collagen-binding site of type-II units of bovine seminal fluid protein PDC-109 and fibronectin

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A single type-II domain has been isolated by limited proteolysis of the collagen-binding bovine seminal fluid protein, PDC-109. The 45-residue fragment corresponding to the second type-II domain of the parent molecule was found to have retained affinity for immobilized collagen, indicating that this minidomain carries critical regions of the collagen-binding site. Studies on various fragments of fibronectin have also implicated the two type-II units of this molecule in collagen-binding. In the present work we have found that type-II domains of human fibronectin, expressed in *Escherichia coli* as β-galactosidase fusion proteins, bind specifically to immobilized collagen.

The majority of proteins in body fluids, components of the extracellular matrix and receptor proteins, are mosaics assembled from a variety of module-types (Patthy, 1985; 1987; 1988). Kringle-modules are typical building blocks of mosaic proteins: the non-protease parts of prothrombin; plasminogen; urokinase- and tissue-type plasminogen activators; factor XII; apolipoprotein a; and hepatocyte growth factor, were found to contain kringles (Magnusson et al., 1975; Sottrup-Jensen et al., 1978; Pennica et al., 1983; Gunzler et al., 1982; McMullen and Fujikawa, 1985; McLean et al., 1987; Nakamura et al., 1989). It is now well established that kringles correspond to autonomous structural/functional units that mediate protein-protein interactions regulating the activity of these proteases (Trexler and Patthy, 1983; Patthy, 1985; Van Zonneveld et al., 1986; Cleary et al., 1989).

We have shown that the type-II domains of fibronectin are also related to protease kringles and suggested that these domains may also function as protein-binding sites (Patthy et al., 1984). The proposed homology of the three-dimensional structures of type-II and kringle domains is also supported by molecular modeling based on the coordinates of kringle domains (Holland et al., 1987). The homologous type-II units appear to be as versatile as protease kringles. They have been found in the seminal fluid proteins PDC-109 and BSP-A3 (Esch et al., 1983; Seidah et al., 1987); in blood coagulation factor XII (McMullen and Fujikawa, 1985); in insulin-like growth factor II receptor/cation-independent mannose-6-phosphate receptor (Morgan et al., 1987; Lobel et al., 1987); and in 72-kDa and 92-kDa type-IV collagenases (Collier et al., 1988; Wilhelm et al., 1989). The wide-spread occurrence of this module type and its presence in proteins with diverse functions makes this domain an interesting object for structure/function studies. In the present work we have chosen the type-II domains of PDC-109 and fibronectin to study their possible involvement in protein-protein interactions.

Fibronectin is a large glycoprotein found in plasma, in the extracellular matrix of tissues and on the surface of various cell types. The protein consists of several structurally homologous domains belonging to types I, II and III. Distinct regions of this multidomain protein serve as binding sites for a variety of macromolecules such as collagen, fibrin, actin, heparin, DNA etc. The two type-II units of fibronectin are found in the collagen-binding region of the molecule and have long been suspected to be involved in collagen binding (for a recent review on fibronectin see Ruoslahti, 1988). The collagen-binding site of fibronectin, however, could be only roughly delineated by limited proteolysis of the molecule; the smallest collagen-binding fragments were found to contain, beside the two type-II homologous units, a variable number of type-I domains (Skorstengaard et al., 1984). A finer mapping of the collagen-binding site has been performed recently by the analysis of the gelatin-Sepharose affinity of a series of fibronectin fragments expressed in *E. coli* as β-galactosidase fusion proteins (Owens and Baralle, 1986). The data obtained in these experiments suggested that type-II domains may be insufficient for collagen binding.

Recent studies have revealed that two major constituents of bovine seminal fluid, proteins PDC-109 and BSP-A3, contain two type-II domains, homologous to those of fibronectin; both proteins, PDC-109 and BSP-A3, containing 109 and 115 amino acid residues, respectively, are essentially made up of two type-II domains (Esch et al., 1983; Seidah et al., 1987). The observation that PDC-109 and BSP-A3 are also bound to gelatin-Sepharose (Manjunath et al., 1987) has therefore revived the suspicion that type-II units may be capable of collagen binding. This suspicion gained additional support by the recent finding that a 72-kDa and a 92-kDa type-IV collagenase, which display marked affinity for denatured

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Abbreviations. PDC-109, bovine seminal fluid protein PDC-109; BSP-A3, bovine seminal fluid protein BSP-A3; IPTG, isopropyl-β-D-thiogalactopyranoside; β-gal 1278 and β-gal 12, fusion proteins from phages M13mp18 1278 and M13mp18 12, respectively.

Enzymes. Trypsin (EC 3.4.21.4); calf intestinal alkaline phosphatase (EC 3.1.3.1); DNA polymerase I (EC 2.7.7.7); restriction endonucleases (EC 3.1.21.4); T4 DNA ligase (EC 6.5.1.1); deoxyribonuclease I (EC 3.1.21.1); ribonuclease A (EC 3.1.27.5).

collagen, differ from other collagenases in containing type-II units (Collier et al., 1988; Wilhelm et al., 1989).

In the present work we isolated type-II domains of PDC-109 and fibronectin and studied their collagen affinity. Here we show that a 45-residue fragment of PDC-109, corresponding to a single type-II domain of the molecule, displays specific affinity for immobilized collagen. Type-II domains of human fibronectin, expressed in *E. coli* as β-galactosidase fusion proteins were also found to have an affinity for collagen.

EXPERIMENTAL PROCEDURES

Materials

Citraconic anhydride (Merck); tosylphenylalanylchloromethane trypsin (Worthington); gelatin, type I (Sigma); phenylethylamine (Fluka); ethidium bromide, tetracyclin HCl, ampicillin, deoxyribonuclease I, ribonuclease A (Boehringer); low-melting-temperature agarose (Sigma) were commercial preparations. [γ-^{32}P]ATP and [α-^{32}P]dATP (280–800 μCi/mmol) were obtained from IZINTA (Budapest).

Restriction endonucleases were from New England Biolabs (Beverly, MA), Amersham International plc (England), Pharmacia (Uppsala) or Reanal (Budapest). T4 DNA ligase, *E. coli* DNA polymerase I *E. coli* polymerase I (Klenow large fragment) were from New England Biolabs (Beverly, MA); alkaline phosphatase of calf intestinal mucosa was obtained from Pharmacia; isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were purchased from Boehringer. The M13 sequencing kit used for dideoxy sequencing of cloned DNA fragments was obtained from Amersham International plc.

Gelatin-Sepharose 4B was prepared by coupling gelatin to CNBr-activated Sepharose 4B (Pharmacia) according to the instruction of the manufacturer.

Human fibronectin and its 42-kDa and 29-kDa gelatin-binding fragments were isolated according to described methods (Engvall and Ruoslahti, 1977; Gold et al. 1979; Ruoslahi et al., 1979; Hahn and Yamada, 1979; Sekiguchi et al., 1981).

Isolation of PDC-109

Fresh ejaculates from adult bulls were centrifuged to remove spermatozoa and seminal plasma, and stored frozen at −20 °C until use. Bovine seminal plasma (10 ml) was loaded onto a gelatin-Sepharose 4B column (50 ml) equilibrated with 0.1 M ammonium bicarbonate buffer, pH 8.0, and the non-bound proteins were eluted with two bed volumes of the same buffer. PDC-109 was eluted with buffer containing 4 M urea and the fractions containing PDC-109 were pooled, subjected to gel filtration on Sephadex G-25 equilibrated with 0.1 M ammonium bicarbonate, pH 8.0 to remove urea and the protein was lyophilized. Amino acid analysis and sequence analysis of the protein verified its identity with PDC-109.

Isolation and characterization of the second type-II unit of PDC-109

Lyophilized PDC-109 (100 mg) was dissolved in 13 ml 0.25 M sodium borate buffer at pH 9.0 and was citraconylated with 250 μl of citraconic anhydride at 25 °C; the pH of the reaction mixture was maintained at 9.0 by the addition of 4 M NaOH. The reaction mixture was subjected to gel filtration on a Sephadex G-25 column (2 cm internal diameter × 50 cm) equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, and the citraconylated protein was lyophilized. The protein was dissolved in 10 ml 0.1 M ammonium bicarbonate, pH 8.0, and digested with 2 mg of trypsin for 15 min at 25 °C. The reaction was stopped by the addition of acetic acid (10%, final concentration) and the solution was incubated for 24 h at 37 °C to decitraconylate the protein.

The trypsin-treated, decitraconylated protein was subjected to gel filtration on a Sephadex G-50 column (fine resin type, 3 cm internal diameter × 64 cm) equilibrated with 10% acetic acid and elution of the PDC-109 fragments was monitored spectrophotometrically and by SDS/PAGE on 16% polyacrylamide gels. The fractions containing the 10-kDa and 5-kDa fragments (Fig. 1) were pooled and lyophilized. The fragments were dissolved in 0.1 M ammonium bicarbonate, 4 M urea, pH 8.0, desalted on Sephadex G-25 equilibrated with 0.1 M ammonium bicarbonate and lyophilized. For structural characterization of the tryptic fragments of PDC-109, the alkylated peptides were sequenced using standard procedures.

The 10-kDa and 5-kDa fragments of PDC-109 (25 mg each) were dissolved in 10 ml 0.1 M Tris/HCl buffer, pH 8.0 containing 5 mM EDTA, 0.1 M dithioerythritol and 6 M guanidinium chloride and incubated for 30 min at 25 °C. The reduced/denatured protein was alkylated with iodoacetic acid used in 1.1-fold molar excess with respect to the sulfhydryl groups. The alkylated proteins were digested with trypsin and the peptides were isolated by HPLC (LKB) on Mono-S HR 5/5 or Rep RPC HR 5/5 columns using procedures recommended by Pharmacia.

Peptides and proteins were hydrolysed in 6 M HCl at 110 °C for 24 h. The composition of the hydrolysates was analysed using a Biotronik LC 2000 analyzer. Peptides and proteins were sequenced according to described procedures (Gray, 1967; Tarr, 1977); the phenylthiohydantoin and dansyl derivatives of amino acids were identified by HPLC according to recommended protocols (Pharmacia) using a Mino RPC S 5/20 column.

Recombinant plasmids, phage and bacteria

Plasmid pFH134, encoding the collagen binding region of human fibronectin (Kornblihtt et al., 1983 and 1985; Owens et al., 1986) was obtained from Professor F. Baralle (University of Oxford). The *E. coli* expression plasmid pmed23, which contains the strong p2 promoter of the *rrnB* ribosomal gene and the ribosome-binding site and operator region of the *lacZ* gene (Lukachovich et al., 1987) were obtained from Dr P. Venetianer (Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged).

E. coli JM 109 (*recA1, endA1, gyrA96, thi 1, hsdR17, supE44, relA1, Δ(lac-proAB), [CF',traD36, proAB$^+$, lacI^{q}ZΔM15*]) was used as the host for M13 and plasmid vectors (Messing, 1983; Boyer and Rouland-Dussoix, 1969). M13mp18 DNA (Yanisch-Perron et al., 1985) from the *E. coli*-specific filamentous phage M13 was used as the sequencing vector.

Propagation of M13 virus stocks, isolation of single-stranded phage DNA and transformation of competent cells were carried out as described by Messing (Messing, 1983). Replicative-form phage DNA and plasmid DNA were isolated from cell pellets by an alkaline lysis procedure (Birnboim and Doly, 1979). DNA fragments obtained by cleavage with restriction enzymes were separated by electrophoresis in 1–

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3% low-melting-temperature agarose gels and were recovered from the gels by the procedure of Wieslander (1979) or by electroelution with Extraphor (Pharmacia).

Construction of plasmid-expressing domains of the collagen-binding site of human fibronectin

Standard procedures were used for restriction, digestion and plasmid construction (Maniatis et al., 1982). Plasmid pFH134 was digested with *Hin*dIII and *Bam*HI and the insert was isolated and digested with *Pvu*II and *Hae*III. The fragment was ligated into the *Sma*I site of M13mp18 and was sequenced using the dideoxy-chain-termination method (Sanger et al., 1977). Replicative forms of the resulting recombinant phage (M13mp18 1278) were digested with *Nco*I and *Sph*I to remove the seventh and eighth type-I units and the fragment was blunt ended and religated. *E. coli* JM 109 cells were transformed with the ligate and the recombinant phage (M13mp18 12) was isolated and sequenced.

Replicative forms of M13mp18 1278 and M13mp18 12 were digested with *Eco*RI and *Hin*dIII. The isolated inserts were ligated into the *Eco*RI/*Hin*dIII site of the pmed 23-plasmid vector yielding clones β-gal 1278 and β-gal 12 from phages M13mp18 1278 and M13mp18 12, respectively. The β-galactosidase part of the recombinant gene was truncated by *Pvu*II and *Sac*I digestion of the plasmid DNA gave clones Δβ-gal 1278 and Δβ-gal 12.

The replicative form of M13mp18 12 was digested with *Eco*RI and *Hin*dIII, the isolated insert was cleaved with *Hin*fI and the *Eco*RI/*Hin*fI fragment encoding the first type-II domain was ligated into pmed 23 digested with *Eco*RI/*Hin*dIII. The resulting plasmid, pmed23 1 was truncated with *Pvu*II and *Sac*I to give Δβ-gal 1.

Bacterial expression of fusion proteins

E. coli cells carrying recombinant pmed 23 plamsids were grown to stationary phase in 2TY medium (5 ml) containing 0.1 mg/ml ampicillin at 37 °C. The culture was diluted 20-fold with fresh 2TY medium containing ampicillin (0.1 mg/ml) and IPTG (1 mM) and incubation was continued at 37 °C for 2 h. The cells were harvested by centrifugation and the pellet was washed with 10 ml ice-cold 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, buffer and centrifuged. The pellet was suspended in 10 ml of the same buffer and the cells were disrupted with an MSE ultrasonicator. The insoluble material (containing the cloned protein in inclusion bodies) was collected by centrifugation, dissolved in 1 ml 0.1 M Tris/HCl, 6 M guanidinium chloride buffer, pH 8.0 containing 0.1 M dithiothreitol and was incubated at 25 °C for 1 h with constant stirring. This step served to solubilize misfolded proteins and to cleave any mismatched disulphide bonds that may have formed in the oxidizing microenvironemt of the inclusion bodies (Schoenmaker et al., 1985). Any insoluble cellular debris was removed by centrifugation and the supernatant was subjected to gel filtration on a Sephadex G-25 column (1.4 cm internal diameter × 20 cm) equilibrated with 0.1 M Tris/HCl buffer, pH 8.0 containing 3 M urea, 5 mM EDTA, 1 mM oxidized and 10 mM reduced glutathione. The solution was incubated for 24 h at 25 °C, then subjected to gel filtration on a Sephadex G-25 column (1.4 cm internal diameter × 20 cm) equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, and applied to a gelatin-Sepharose 4B column (HR10/30) equilibrated with the same buffer. The bound protein was eluted with buffer containing 4 M urea, desalted and the assay on gelatin-Sepharose 4B column was repeated.

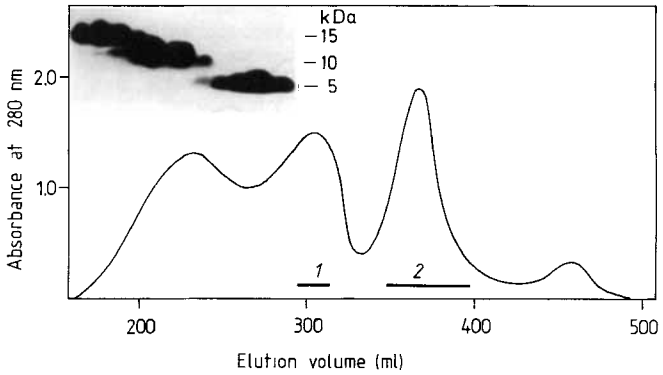

Fig. 1. *Gel filtration of a tryptic digest of PDC-109.* Tryptic hydrolysate of PDC-109 (0.1 g) was chromatographed on a Sephadex G-50 column (fine particle size, 3 cm internal diameter × 64 cm) equilibrated with 10% acetic acid and 18-ml fractions were collected. The inset shows the SDS/PAGE pattern of the elution profile using 16% polyacrylamide gel; fractions were pooled as indicated by the solid bars. The first peak contained uncleaved PDC-109 (15 kDa); pool 1 contained disulphide bonded peptides, residues 1–31, 34–57 and 58–64 corresponding to the A domain of PDC-109 (10 kDa); pool 2 contained residues 65–109 corresponding to the intact B domain of PDC-109 (5 kDa)

Other procedures

Analytical affinity-chromatography experiments on gelatin-Sepharose 4B columns (HR10/30) were performed using HPLC. In the affinity-chromatography experiments the samples exhausted less than 20% of the total column capacity.

HPLC of the second type-II unit of PDC-109 on Alkyl-Superose HR 5/5 (Pharmacia) columns was performed in 10 mM Tris/HCl buffer, pH 8.0 containing 0.1 M NaCl, 0.2 mM EDTA.

SDS/PAGE was performed according to a described procedure (Laemmli, 1970), proteins were stained with Coomassie brillant blue and the molecular masses of the proteins were estimated using a Pharmacia low molecular mass calibration kit.

RESULTS AND DISCUSSION

The second type-II domain of PDC-109 has a collagen-binding site

The second type-II domain of PDC-109 was isolated by limited tryptic hydrolysis of the protein. Citraconylation was used to block the lysyl residues of PDC-109 and to limit tryptic cleavage to the arginine residues. Cleavage at Arg64, present in the region separating the first (A) and second (B) type-II domains of PDC-109 (domain A, residues 24–61; domain B, residues 69–109) was expected to yield two fragments containing single type-II domains. Sequence analysis of the 10-kDa and 5-kDa fragments obtained by tryptic hydrolysis of PDC-109 (Fig. 1) revealed that the A domain was cleavaged at Arg31, Arg33 and Arg57 (10-kDa fragment), whereas the peptide bond of the single arginyl residue (Arg104) of the B domain was not cleaved by trypsin, thus yielding a 5-kDa fragment (residues 65–109) containing the intact B domain (Fig. 2). The probable explanation for the lack of tryptic

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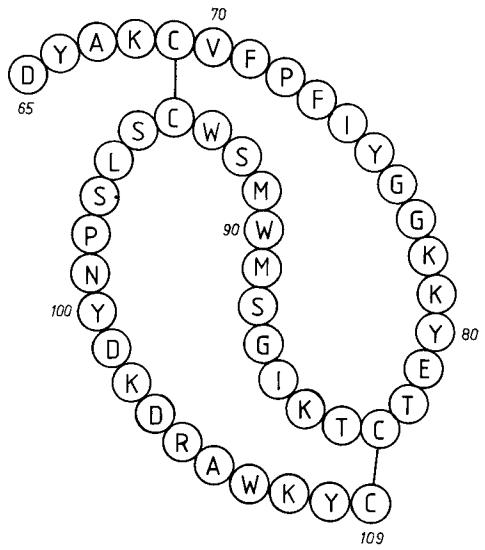

Fig. 2. *Structure of the 5-kDa tryptic fragment of PDC-109 containing the second (B) type-II domain.* The numbering refers to the sequence of PDC-109 (Esch et al., 1983)

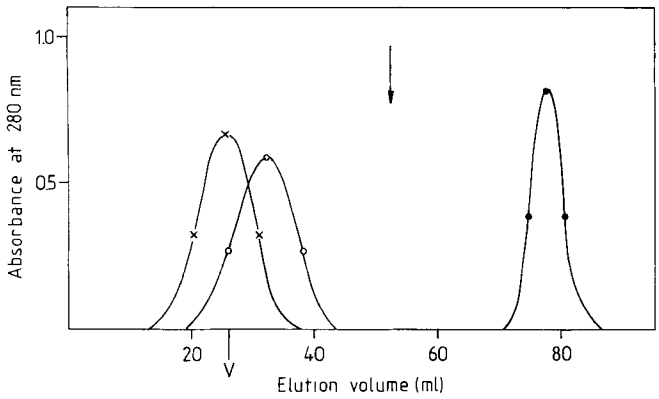

Fig. 3. *Affinity chromatography of PDC-109/B by HPLC using a gelatin-Sepharose 4B column.* Chromatography was performed using an HR 10/30 column of gelatin-Sepharose 4B, equilibrated with 10 mM Tris/HCl buffer, pH 8.0 containing 1 M NaCl. Samples are: PDC-109/B (●), PDC-109/B in the presence of 50 mM phenylethylamine (○), reduced-denatured PDC-109/B (×). The arrow marks the start of elution of PDC-109/B with buffer containing 4 M urea. V indicates the breakthrough volume of the column

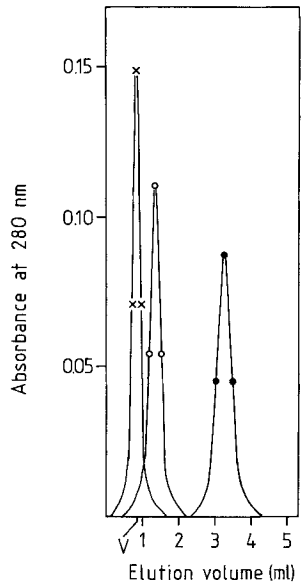

Fig. 4. *Affintiy chromatography of PDC-109/B on Alkyl-Superose.* Chromatography was performed using an HR 5/5 column of alkyl-Superose equilibrated with 10 mM Tris/HCl buffer, pH 8.0, containing 0.2 mM EDTA and 0.1 M NaCl. Samples are: PDC-109/B (●), PDC-109/B in the presence of 50 mM phenylethylamine (○), reduced/denatured PDC-109/B (×). V indicates the breakthrough volume of the column

hydrolysis of this arginyl bond is that Arg104 is preceded by acidic groups Asp101 and Asp103. Moreover, in citraconylated PDC-109, Lys102 also carries an acidic moiety. It has frequently been observed that such an acidic enrivonment hinders tryptic hydrolysis of susceptible bonds (Trexler et al., 1982).

The isolated second type-II unit of PDC-109 (PDC-109/B) was found to have an affinity for gelatin-Sepharose 4B (Fig. 3), the binding being specific for immobilized collagen as shown by the fact that the protein was not bound to or retarded on unsubstituted Sepharose 4B. The specificity of the interaction is also supported by the observation that PDC-109/B was not bound to gelatin-Sepharose 4B when applied to a column equilibrated with buffer containing 0.5% collagen (data not shown).

The affinity of PDC-109/B for gelatin-Sepharose 4B was found to increase with increasing solvent ionic strength, suggesting that hydrophobic interactions play a major role in binding. The presence of a hydrophobic binding pocket was also indicated by the observation that the protein has an affinity for the neopentyl groups of alkyl-Superose (Fig. 4). The specificity of the above interactions is underlined by the observation that they require the native conformation of the molecule, reduced/denatured PDC-109/B displayed no affinity for either of the two gels mentioned above (Figs 3 and 4).

We have found that low concentrations of phenylethylamine were able to elute the protein from gelatin-Sepharose 4B and Alkyl-Superose columns (Figs 3 and 4), suggesting that a binding pocket for this hydrophobic ligand may form a subsite of the collagen-binding site.

Type-II domains of fibronectin carry critical regions of the collagen-binding site

Different domains of the collagen-binding site of human fibronectin (Fig. 5) have been expressed in *E. coli* as β-galactosidase fusion proteins. In all cases studied, the fusion proteins accounted for more than 40% of the total bacterial proteins synthesized in the presence of 1 mM IPTG (Fig. 6). The expressed proteins were found to form inclusion bodies, facilitating their isolation in pure form. To solubilize the fusion proteins the inclusion bodies had to be reduced and denatured in buffer containing 6 M guanidine/HCl and 0.1 M dithiothreitol.

To determine the optimal conditions for the refolding of reduced-denatured fibronectin fragments, we first studied the refolding of the 42-kDa and 29-kDa gelatin-binding fragments of human fibronectin. Both fragments lost their affinity for gelatin-Sepharose 4B when their disulphide-bonds were cleaved in 0.1 M Tris/HCl buffer, pH 8.0 containing 6 M guanidine/HCl and 0.1 M dithiothreitol. Upon incubation in 0.1 M Tris/HCl buffer, pH 8.0 containing 5 mM EDTA, 1 mM oxidized and 10 mM reduced glutathione, these

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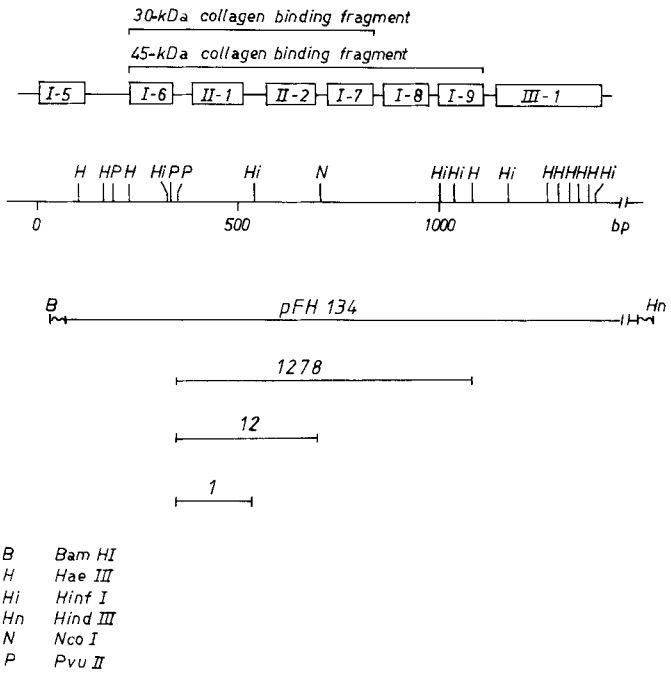

Fig. 5. *cDNA fragments of human fibronectin expressed in* E. coli. The figure shows a part of the fibronectin structure, where blocks marked I-5, I-6, I-7, I-8, I-9, II-1, II-2 and III-1 represent type-I, type-II and type-III domains. The position and size of the cDNA fragments expressed and the positions of the restriction sites used in the construction of expression plasmids are also shown

fibronectin fragments showed recovery of gelatin-Sepharose affinity, in 24 h a significant portion (30% of the 29-kDa fragment and 20% of the 42-kDa fragment) of the proteins regained their affinity for immobilized collagen.

In contrast, reduced-denatured fusion proteins β-gal 1278 and Δβ-gal 1278 (essentially corresponding to the 42-kDa gelatin-binding fragment of human fibronectin but differing in the size of the β-gal region) did not recover gelatin-Sepharose affinity under the same conditions. Upon removal of the denaturing agents, a large proportion of the proteins (50–60%) precipitated even when refolded at low (0.1 mg/ml) protein concentrations. Since 1–3 M urea is known to facilitate refolding of proteins solubilized from inclusion bodies (Marston, 1986; Tsuji et al., 1987; Baldwin and Schulz, 1989) we have incubated the reduced/denatured fusion proteins in refolding buffers containing 1–3 M urea. We have found that in the presence of 3 M urea, a significant portion of Δβ-gal 1278 (15–20%) regained its gelatin-Sepharose affinity in 24 h (Fig. 6); essentially no active protein was recovered, however, in the case of β-gal 1278.

Refolding of β-gal fusion proteins isolated from inclusion bodies was therefore performed in 0.1 M Tris/HCl buffer, pH 8.0 containing 3 M urea, 5 mM EDTA, 1 mM oxidized and 10 mM reduced glutathione for 24 h at 25 °C. Under these conditions, fusion proteins Δβ-gal 1 and Δβ-gal 12 were found to regain affinity for gelatin-Sepharose (30–35% and 40–45% of the total, respectively, Fig. 6).

It should be noted, however, that no detectable activity was recovered from fusion proteins β-gal 1 and β-gal 12 which contain untruncated β-galactosidase regions. This observation suggests that the large hydrophobic β-galactosidase fragment interfers with proper refolding of the fibronectin-derived moiety of the fusion proteins.

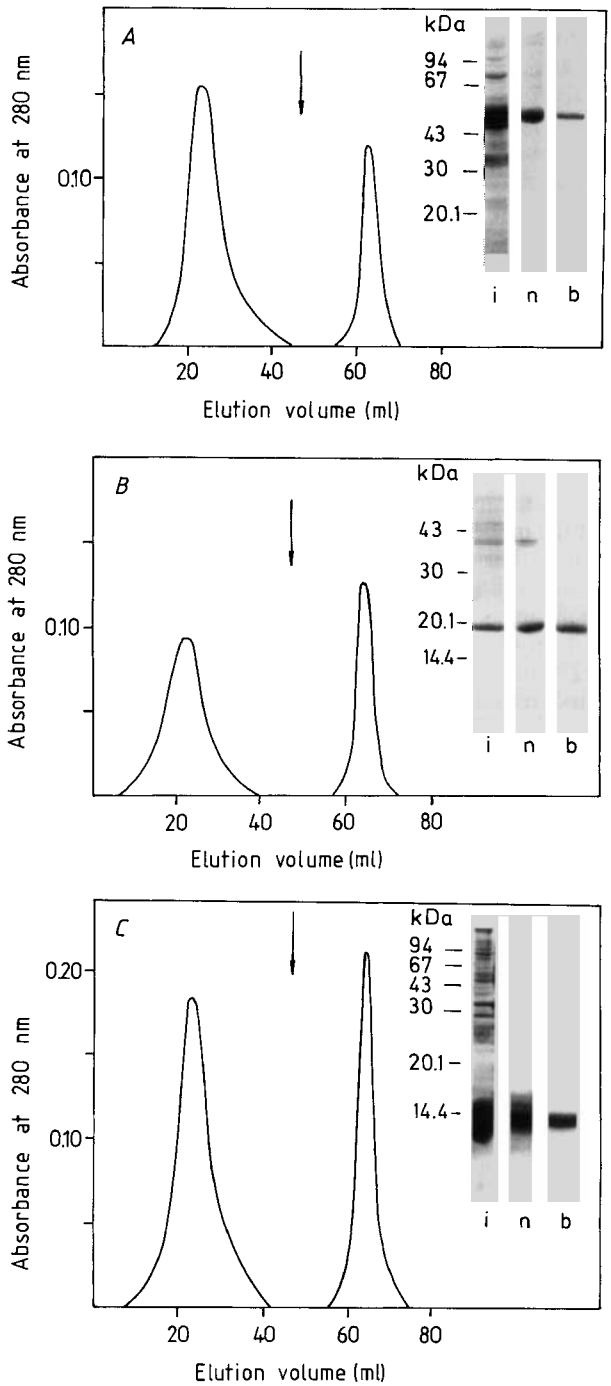

Fig. 6. *Affintiy chromatography of fusion proteins Δβ-gal 1278, Δβ-gal 12 and Δβ-gal 1 on gelatin-Sepharose 4B.* Fusion proteins Δβ-gal 1278 (A), Δβ-gal 12 (B) and Δβ-gal 1 (C) expressed in *E. coli* were isolated from inclusion bodies and refolded as described in Experimental Procedures. The proteins were applied to a gelatin-Sepharose 4B column (HR 10/30) equilibrated with 0.1 M ammonium bicarbonate buffer, pH 8.0 and the bound proteins were eluted with buffer containing 4 M urea. The arrow marks the start of elution with buffer containing urea. The insets show the SDS/PAGE patterns of the proteins of IPTG-induced bacteria (i), fusion proteins not bound (n) and bound (b) to gelatin-Sepharose 4B. The numbers indicate the positions of the molecular mass marker proteins

Binding of Δβ-gal 1 and Δβ-gal 12 fusion proteins to gelatin-Sepharose 4B was shown to be specific for immobilized collagen since no adsorption of expressed proteins was ob-

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served when the samples were loaded onto unsubstituted Sepharose 4B. The specificity of the gelatin-Sepharose affinity of the fusion products was also checked by reapplying the bound proteins to gelatin-Sepharose. In these experiments complete rebinding of the bound proteins was observed.

The observation that fragments containing only type-II domains of fibronectin ($\Delta\beta$-gal 12, $\Delta\beta$-gal 1) are capable of binding denatured collagen is at variance with previous suggestions that type-II domains alone may be insufficient to specify collagen-binding by fibronectin (Owens and Baralle, 1986). The latter conclusion was based on experiments where fibronectin fragments were expressed in *E. coli* as cro/β-galactosidase fusion proteins. In these experiments fusion proteins were recovered from inclusion bodies using urea. Following extensive dialysis only a small fraction (1–5%) of the fusion proteins were found to be functionally active even in the case of proteins that carry the entire collagen-binding site of fibronectin, raising the possibility that lack of activity may reflect the inability of the protein to acquire its native conformation, rather than the absence of structures of a potential collagen-binding site. In the present work we introduced two major changes that increased the yields of active protein, truncation of the β-galactosidase portion (reducing its size from 279 to 34 residues with *Pvu*II/*Sac*I digestion of the vector) and refolding in the presence of urea appeared to be essential for efficient reactivation of fibronectin domains.

CONCLUSION

In the present work we have shown that single type-II domains of fibronectin and PDC-109 carry binding sites for collagen. The availability of small, functionally active domains of the type-II module family may greatly facilitate further studies on the molecular mechanism of their interaction with ligands and may contribute to the understanding of the biological activity of various proteins containing homologous type-II modules. Studies to determine the three-dimensional structure of the second type-II domain of PDC-109 and to localize its binding site by NMR spectroscopy are in progress.

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