Further Localization of the Gelatin-binding Determinants within Fibronectin

ACTIVE FRAGMENTS DEVOID OF TYPE II HOMOLOGOUS REPEAT MODULES*

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Digestion of a 42-kDa gelatin-binding fragment (GBF) of fibronectin with pepsin followed by affinity chromatography on gelatin-Sepharose produces three fractions, a drop-through non-binding fraction, a retarded fraction that is dominated by a 13-kDa fragment whose NH₂ terminus is identical to that of 42-kDa GBF, and a binding fraction that contains a homogeneous fragment of apparent mass 21 kDa with an NH₂ terminus corresponding to Arg⁴⁸⁴. This 21-kDa GBF binds repeatedly to gelatin-Sepharose, eluting near 2.6 M in a urea gradient. It also binds in the fluid phase to a fluorescent-labeled collagen peptide with Kₐ = 10 μM and inhibits the binding of 42-kDa GBF to the same peptide with Kᵦ = 7.3 μM. Thus, major gelatin-binding determinants of fibronectin are located within a 21-kDa region that contains two type I homologous "finger" modules and is devoid of the type II "kringle-like" modules that were previously thought to be essential for this activity.

Fibronectin is a large glycoprotein that mediates the adhesion of numerous types of cells to various surfaces (1–3). It has two nearly identical polypeptide chains, each comprised of multiple domains that recognize other macromolecules such as collagen, heparin, and fibrin. The collagen binding domain is located near the NH₂ terminus within an approximately 40-kDa region that contains four type I and two type II homologous repeat units or modules (Fig. 1) (4, 5). The type I "finger" modules are also found in other domains of fibronectin, whereas the type II "kringle-like" modules are unique to the gelatin binding domain. It is thus logical to suspect that the type II modules are involved in the interaction with gelatin. Further evidence for this idea was provided by Owens and Baralle (6) based on experiments with fusion proteins expressed in Escherichia coli. This work also concluded that a short stretch of 14 amino acids linking the second type II with the adjacent type I is critical for gelatin binding activity.

We have worked extensively with a 42-kDa gelatin-binding fragment (GBF)¹ derived from chymotryptic and/or thermolysin digests of fibronectin (7–10). During the course of large scale preparation of this fragment, small quantities of an approximately 21-kDa gelatin-binding fragment were obtained as a by-product. Although there is evidence in the literature for the existence of gelatin-binding fragments smaller than 40 kDa, the binding properties and exact position of these fragments within the fibronectin molecule were not determined (11, 12). It thus appeared worthwhile to further purify and characterize the 21-kDa GBF and to attempt to generate it under controlled conditions. It is shown here that the 21-kDa fragment is comprised of two type I finger modules located in the COOH-terminal region of 42-kDa GBF, a region distinct from that which was concluded by Owens and Baralle (6) to be critical.

MATERIALS AND METHODS

Pepsin and thermolysin were obtained from Sigma. Fibronectin was purified by affinity chromatography on gelatin-Sepharose according to method B of Miekka et al. (13), starting with a side-fraction generated during large scale preparations of antihemophilic factor from human plasma. Fragments were generated by digestion of 1.6 g of purified fibronectin with thermolysin according to Zardi et al. (14). The 42- and 21-kDa gelatin-binding fragments were recovered by affinity chromatography on gelatin-Sepharose and further purified by exclusion chromatography on Bio-Gel P-60 (Bio-Rad). Small amounts of larger GBFs were removed from 42-kDa GBF by adsorption on heparin-Sepharose (10). A 21-kDa GBF was also obtained by digestion of 42-kDa GBF with pepsin as described in the text. The binding of these smaller fragments to gelatin is weaker than that of 42-kDa GBF, and the best yields are obtained with freshly prepared gelatin-Sepharose. Concentrations of 42-kDa GBF are based on an extinction coefficient of 7.3 × 10⁴ M⁻¹ cm⁻¹ (10). The corresponding value for 21-kDa GBF is 2.7 × 10⁴ M⁻¹ cm⁻¹, calculated by the method of Edelhoch (15) assuming 3 Trp, 7 Tyr, and 4 disulfide. Unless otherwise noted, all experiments were performed in 0.02 M Tris, 0.15 M NaCl, pH 7.4 (TBS).

Analytical affinity chromatography was performed as described by Isaacs et al. (10) using gelatin-Sepharose prepared according to Miekka et al. (13). SDS-PAGE was performed in the Pharmacia Phast system using 8–25% gradient gels followed by Coomassie Blue staining. NH₂-terminal sequences were determined on a Beckman 264A fluorometer as described previously (9). The collagen peptide used in the titration experiments was obtained by digestion of type I calf-skin collagen (Sigma) with CNBr (1% w/v, 0.1 M HCl, 4 h, 30 °C). After dialysis against 0.1 M NaHCO₃, pH 8, the mixture was labeled with fluorescein isothiocyanate as described previously (9) and applied to a column of immobilized 42-kDa GBF at 40 °C. A fluorescent peptide having an apparent mass of 70 kDa on SDS-PAGE was preferentially adsorbed under these conditions. It was eluted with 6 M urea, dialyzed into TBS, and stored at −70 °C for later titrations. Titration of dilute solutions of this peptide with fibronectin or gelatin-binding fragments produced a concentration-dependent increase in anisotropy, ΔA, which was fit to the following equation:

\[ \Delta A = \Delta A_{max}[GBF]/(K_a + [GBF]) \]  

where [GBF] = total concentration of fragment and Kₐ is the dissociation constant of the fragment.

*The abbreviations used are: GBF, gelatin-binding fragment; TBS, Tris-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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cation constant \( K_d \). The quantity \( \Delta G_{\text{max}} \) was treated as a fitting parameter because the amount of fragment added is seldom sufficient to give complete saturation.

Titration with 42-kDa GBF in the presence of fixed amount of 21-kDa GBF produced apparent inhibition from which an inhibition constant, \( K_i \), was calculated as follows:

\[
K_i = \frac{[I]}{(K_d/[I]) - 1)} \tag{2}
\]

where \( K_d \) is the apparent dissociation constant for 42-kDa GBF in the presence of 21-kDa GBF at concentration \([I]\), and \( K_i \) is the dissociation constant in the absence of 21-kDa GBF. This equation is analogous to that used in enzyme kinetics to describe the effect of a competitive inhibitor on the \( K_m \) of an enzyme for its substrate. In the present case, \( K_i \) provides an estimate of the dissociation constant for the binding of 21-kDa GBF to the fluorescent gelatin peptide.

RESULTS

Fig. 2A illustrates the time course of digestion of 42-kDa GBF with pepsin at pH 2.5 and 35 °C as analyzed by SDS-PAGE. Within 5 min, the parent fragment is converted into smaller ones, two of which appear to resist further proteolysis. The largest of these has an apparent size of 24 kDa prior to reduction and 21 kDa after reduction. The smaller has an apparent mass of 13 kDa before and after reduction. The appearance of these fragments in SDS-PAGE and their failure to decompose after reduction of disulfides suggests that a major pepsin cleavage site is located between disulfide-bonded modules (Fig. 1). Application of a 10-min pepsin digest of 42-kDa GBF to a column of gelatin-Sepharose results in the production of three fractions, as shown in Fig. 3A. The first is a non-binding fraction containing numerous fragments of varying size (Fig. 2B, lanes 5). The second is a retarded fraction containing primarily the 13-kDa fragment (Fig. 2B, lanes 6). The third is a binding fraction that is essentially homogeneous, being comprised of the 21-kDa fragment, hereafter referred to as 21-kDa GBF (Fig. 2B, lanes 7). NH2-terminal sequence analysis of this material produced a single sequence beginning at Arg4, between the second and third type I modules of the parent fragment (Table I, Fig. 1, arrow 1). The appearance of a single sequence for the nonreduced fragment indicates that the apparent decrease in mass from 24 to 21 kDa upon reduction is not due to the presence of a small disulfide-associated peptide. The 13-kDa fragment has an NH2-terminal sequence identical to that of the parent 42-kDa GBF (Table I). Also shown in Fig. 2B (lanes 8) is the 21-kDa GBF obtained as a byproduct while preparing 42-kDa GBF from a thermolysin digest of 42-kDa GBF and the pools obtained after affinity fractionation on gelatin-Sepharose as in Fig. 3A. Lanes 1 and 9, molecular weight markers; lane 2, blank; lanes 3, 42-kDa GBF; lanes 4, pepsin digest; lanes 5, non-binding pool 1 from Fig. 3A; lanes 6, weak binding pool 2 from Fig. 3A; lanes 7, binding pool 3 from Fig. 3A; lanes 8, 21-kDa GBF from thermolysin digest.

![Fig. 1. Schematic illustration of the modular structure of the 42-kDa gelatin-binding fragment (42-kDa GBF) of human fibronectin showing four type I and two type II modules (5, 24). Single-letter codes are given within the circles, except for cysteines which are filled in to accentuate disulfide bonds. Shaded circles represent deletions in the alignment of type I modules. Arrows indicate cleavage sites for pepsin. Carbohydrates are represented by filled squares. The dashed line reflects uncertainty in the exact length of this fragment, which terminates at Trp99 in the chymotrypsin and plasmin-generated versions (6). The brackets in the center of the molecule designate the region that was deemed critical for gelatin binding by Owens and Baralle (6). In contrast to the published cDNA-derived sequence of human fibronectin (24), we consistently find an arginine at position 380, in agreement with the bovine (5) and rat (25) species.](image-url)
Amino-terminal sequences of fragments derived from the 42-kDa gelatin-binding fragment of fibronectin

| Fragment  | Cycle 1 | Cycle 2 | Cycle 3 | Cycle 4 | Cycle 5 | Cycle 6 |
|-----------|---------|---------|---------|---------|---------|---------|
| 42 kDa*   | Ala (1.0) | Val (0.93) | Tyr (0.74) | Gln (0.53) | Pro (0.35) |         |
|           | Val (0.5) | Tyr (0.25) | Gln (0.18) | Pro (0.10) | Gln (0.15) |         |
| 21 kDa (pepsin) | Arg (1.0) | Asp (1.6) | Gln (0.89) | Cys (ND) | Ile (1.6) |         |
| 21 kDa (thermolysin) | Leu (0.5) | Arg (0.31) | Asp (0.50) | Gln (0.36) | Cys (ND) |         |
| 13 kDa*   | Ala (1.0) | Val (1.1) | Tyr (0.88) | Gln (0.46) | Pro (0.44) | Gln (0.32) |
|           | Val (0.5) | Tyr (0.26) | Gln (0.17) | Pro (0.12) | Gln (0.10) | Pro (0.17) |
| 16 kDa    | Val (0.7) | Gln (0.49) | Thr (0.07) | Arg (0.36) | Gly (0.28) | Gly (0.36) |
|           | Met (0.75) | Arg (0.45) | Cys (ND) | Thr (0.09) | Cys (ND) |         |

* Heterogeneous, two sequences differing by 1 amino acid, present in 2:1 ratio.

FIG. 3. Affinity fractionation of pepsin digest of 42-kDa GBF and analytical affinity chromatography of the fractionated peptides. A, 24 mg of 42-kDa GBF was digested with a 1:200 ratio of pepsin for 10 min at 35°C in 0.01 M H3PO4, pH 2.5. The reaction was stopped by adjusting the pH of the sample to 7.4 with concentrated Tris base. The sample was immediately applied to a 50-ml column of gelatin-Sepharose equilibrated with TBS. After washing the column with TBS until the A280 of the effluent was <0.05, the bound protein was eluted with 6 M urea in TBS (arrow). Three pools were made: non-binding fraction (1), weak-binding retarded fraction (2), and binding fraction (3). B, rechromatography of 12 pg of non-binding pool 1, 200 pg of weak-binding pool 2, and 300 pg of binding pool 3 from A on a 2.3-ml column of gelatin-Sepharose. The samples were injected onto the column equilibrated in TBS and subsequently washed with TBS at a flow rate of 0.5 ml/min. A urea gradient from 0 to 6 M in TBS was applied at 1 ml/min. Elution was monitored by fluorescence at 340 nm with excitation of 280 nm.

FIG. 4. Titration of a fluorescein-labeled 70-kDa CNBr peptide of type I collagen (0.044 μM) with 42-kDa GBF (A), 21-kDa GBF (C), and with 42-kDa GBF in the presence of 36 μM 21-kDa GBF (D). Fluorescence anisotropy was monitored at 524 nm with excitation at 493 nm. Curves represent theoretical best fits to Equation 1.
kDa GBF is rapidly cleaved by pepsin to produce two fragments, one of which retains the ability to bind tightly to gelatin-Sepharose. Titration experiments showed that the affinity of this 21-kDa fragment for a fluorescent collagen peptide was about 10-fold lower than that of the parent 42-kDa GBF, which itself binds such peptides 10–20-fold weaker than whole fibronectin. It is possible that the recognition site has a different conformation in the smaller fragment or that residues not present in 21-kDa GBF contribute to the stronger binding of 42-kDa GBF. A hint of the latter possibility was provided by the observation that a 13-kDa fragment derived from the opposite end of 42-kDa GBF appeared to interact weakly with the gelatin-Sepharose column. Using the expression \( \Delta G = -RT \ln K \), one can show that 80% of the free energy associated with binding of 42-kDa GBF to gelatin in solution can be accounted for by the 21-kDa fragment. An additional site which by itself bound gelatin with a \( K \) of only 30 nM would be sufficient to restore the difference. This additional binding would presumably involve a separate site in close proximity to that occupied by the 21-kDa fragment. The possibility of multiple contact points between a collagen chain and the larger gelatin-binding fragments of fibronectin would be consistent with the extensive internal sequence homology in both reactants. There is ample evidence in the literature for multiple sites of varying affinity on collagen chains (Ref. 9, and references therein).

The 21-kDa GBF is comprised of two type I finger modules, the first of which contains two carbohydrate moieties (5). This is consistent with the observation that increased glycosylation of placental fibronectin weakens the affinity for gelatin (16). Such carbohydrate may also account for the aberrant behavior of 21-kDa GBF on SDS-PAGE. The apparent mass of about 3 kDa, despite the lack of evidence for a disulfide-linked peptide, i.e. only one NH2-terminal sequence was obtained for both the thermolytic and the pepsin-generated 21-kDa fragments. This decrease, also observable in the 42-kDa precursor, is not a general feature of type I finger containing fragments since the NH2-terminal 29-kDa heparin/fibrin-binding fragment displays a substantial increase in apparent mass upon reduction (14). It is possible that disruption of disulfide bonds in 21-kDa GBF alters the interaction between the carbohydrate groups and the protein in such a way as to alter the amount of bound detergent during SDSPAGE.

The most surprising result of our study was the finding that the type II modules are not essential for gelatin binding activity. These modules are unique to the gelatin binding region of fibronectin and are commonly thought to be responsible for the interaction with collagen. This notion is reinforced by the observation of similar type II modules in two other proteins that are devoid of type I fingers and can be purified by affinity chromatography on gelatin-Sepharose. These are type IV collagenase (18, 19) and bovine seminal plasma protein (20, 21), whose type II modules respectively exhibit approximately 50 and 35% identity with those of fibronectin. Type II modules are also found in coagulation factor XII (22) and mannose-6-P04 insulin-like growth factor II receptor (23), for which gelatin binding properties, if any, have not been documented. In our hands, factor XII had no activity when tested by assays of the kind in Fig. 4 (not shown). Nor do we have a problem with factor XII contamination of fibronectin prepared from plasma by affinity chromatography on gelatin-Sepharose. Thus, the mere presence of type II modules does not guarantee gelatin-binding activity, as first concluded by Owens and Baralle based on the expression in E. coli of fusion proteins containing fibronectin type II modules (6). However, our 21-kDa GBF is one finger removed from the region reported by these workers to be critical (see Fig. 1). We have no obvious explanation for this discrepancy. The conclusions of Owens and Baralle (6) were based on the ability of fusion proteins to bind to gelatin-Sepharose. The yield of active protein in these experiments was very low and problems of proteolytic degradation and improper folding could produce false-negative results. Furthermore, constructs coding for the region identified here as being important were not part of their series. Future work utilizing site-directed mutagenesis to delineate specific residues involved in gelatin-binding should not ignore the 21-kDa region, which itself binds such peptides 10-20-fold weaker than 42-kDa GBF, and perhaps beyond, are required to confer maximum affinity for gelatin.

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