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Partial primary structure of bovine plasma fibronectin: Three types of internal homology

(plasmin digestion/gelatin and heparin binding/interchain disulfide bridge/phosphorylation/gene multiplication)

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ABSTRACT **Approximately one-half of the amino acid sequence (911 amino acid residues out of 1,880 expected) for bovine plasma fibronectin (cold-insoluble globulin) has been determined. Three types of internal homology were identified, showing that a number of partial gene duplications (multiplications) have occurred during the evolution of this protein. Digestion of fibronectin with plasmin results in major fragments with molecular masses of 29, 170, 23, and 6 kilodaltons (kDal). The NH_2-terminal 29-kDal fragment consists of 259 residues ordered as five mutually homologous domains (type I homology) with two disulfide bonds in each domain. The 170-kDal fragment shows two to three bands after $NaDodSO_4$ gel electrophoresis, indicating heterogeneity. This fragment contains the gelatin binding site and the strong heparin binding site present in fibronectin. Digestion of the 170-kDal fragment with chymotrypsin liberates a 45-kDal fragment that also binds to gelatin. This fragment contains at least one domain of type I homology and two domains of type II homology. Further digestion of the 170-kDal fragment with chymotrypsin results in the formation of a 30-kDal fragment that retains the heparin binding activity. This fragment contains sequences constituting type III homology. The 23-kDal fragment consists of 178 residues having three domains of type I homology. The 6-kDal fragment consists of two identical peptides of 26 residues, and these two peptides are linked to each other by two disulfide bonds that form the interchain bridges. Another one of the peptides for which the sequence was determined links the COOH-terminus of the 29-kDal fragment to the NH_2-terminus of the 170-kDal fragment. This and the fact that the COOH-terminal residue of the 6-kDal fragment is a glutamic acid residue order the four plasmin-digestion fragments as 29-, 170-, 23-, and 6-kDal in the intact fibronectin molecule.**

Fibronectin is a protein or a group of similar proteins found in blood plasma (1) and on the surface of different cell types (2) (for review, see refs. 3–5). The functions attributed to fibronectin, such as cell adhesion to substratum, cell spreading, opsonization of bacteria and other particulate matter, and wound healing, are all related to its affinity to cell surfaces. Binding to vastly different biological compounds, such as gelatin (6), glycosaminoglycans (7), DNA (8), fibrin (9), actin (10), sphingolipids (11), acetyl cholinesterase (12), amyloid P component (13), and complement factor C1q (14), has been demonstrated. Some of these binding sites have been found to be contained in specific fragments generated by limited proteolysis of fibronectin (15–24). Recently, a transformation-enhancing activity has been shown in the gelatin binding fragments of fibronectin (25).

Fibronectin is a substrate for blood coagulation factor $XIII_a$ (26) and can be covalently crosslinked to fibrin (26), collagen (21), and the surface of *Staphylococcus aureus* (22) by this transglutaminase. Fibronectin used to be known as cold-insoluble globulin (27) because it precipitates from plasma in the cold with fibrinogen and the factor VIII–von Willebrand factor complex.

Plasma fibronectin is composed of two polypeptide chains held together by a disulfide bond(s). The two chains have mobilities corresponding to molecular masses of 220 and 215 kilodaltons (kDal) when reduced samples of fibronectin are subjected to $NaDodSO_4$ gel electrophoresis (1). In this paper, we present the amino acid sequence of approximately half of one of the chains of bovine plasma fibronectin.

MATERIALS AND METHODS

Bovine plasma fibronectin was purified essentially as described for the human protein (6) by batch adsorption to gelatin/agarose followed by elution with a urea gradient. The generation of fragments by digestion with plasmin and the purification of these fragments have been described (28). An alternative purification scheme is mentioned in *Results*. The standard sequence analysis techniques used have been described in detail (29).

RESULTS

Nomenclature. Fragments from bovine plasma fibronectin were obtained by limited proteolysis and cyanogen bromide degradation (see below). The amino acid sequences of 10 segments (S1–S10), totalling 911 residues, are shown in Fig. 1. Fragments S1 and S10 contain the NH_2-terminal and COOH-terminal sequences of fibronectin. Segments S2–S9 are numbered according to their probable relative position in fibronectin. These positions may change somewhat when the sequence analysis is complete.

Isolation of Fragments Obtained by Digestion with Plasmin. Digestion of bovine fibronectin with plasmin resulted in at least four fragments having molecular masses of 29, 170, 23, and 6 kDal. These fragments were purified by a combination of ion exchange chromatography and gel filtration. The mixture of fragments was applied to a column of DEAE-cellulose equilibrated with 0.05 M NH_4HCO_3. Under these conditions, the 29-kDal fragment was only partly adsorbed while the other fragments bound to the resin. The absorbed fraction of the 29-kDal fragment was eluted early in the NH_4HCO_3 gradient as two or three peaks and was followed by the 170-, 23-, and 6-kDal fragments, respectively. The reason for the apparent heterogeneity in the elution pattern of the 29-kDal fragment is not known, but one possibility is partial deamidation of the four asparagine-glycine sequences. A further gel filtration step separated the 170-, 23-, and 6-kDal fragments. The 6-kDal fragment was detected in the effluent by using the molybdate reaction for phosphate (31).

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Abbreviation: kDal, kilodalton(s).

```
             10        20        30        40        50
S1: <EAQQIVQPQSPLTVSQCKPGSYDNGKHYQINQQWERTYLGSALVCTCYGG
                                                     100
    SRGFNCESKPEPEETCFDKYTGNTYRVGDTYERPKDSMIWDCTCIGAGRG
                                                     150
    RISCTIANRCHEGGQSYKIGDTWRRPHETGGYMLECVCLGNGKGEWTCKP
                                                     200
    IAEKCFDQAAGTSYVVGETWEKPYQGWMMVDCTCLGEGSGRITCTSRNRC
                                                     250
    NDQDTRTSYRIGDTWSKKDNRGNLLQCICTGNGRGEWKCERHTSLQTTSA

    GSGSFTDVRTAIYQPQPHPQPPPYGHCVTDSGVVYSVGM

             10
S2: YRIGDQWDKQHDMGHMM

             10        20        30        40        50
S3: LCTCLGNGVSCQETAVTQTYGGNSNGEPCVLPFTYNGKTFYSCTTEGRQD
                                                     100
    GHLWCSTTSNYEQDQKYSFCTDHTVLVQTRGGNSNGALCHFPFLYNNHNY
                                                     CBH
    TDCTSEGRRDNMKWCGTTQNYDADQKFGFCPM

             10
S4: QWLKTQGNKQM

             10        20        30        40        50
S5: GGRPREDRVPPSRNSITLTNLNPGTEYVVSIVALNSKEESLPLVGQQSTV
                                                     100
    SDVPRDLEVIAATPTSLLISWDAPAVTVRYYRITYGETGGSSPVQEFTVP
                                                     150
    GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPVSINYRTEIDKPSQ

    M

             10        20        30        40        50
S6: TIEGLQPTVEYVVSVYAQNQNGESQPLVQTAVTTIPAPTNLKFTQVTPTS

    LTAQWTAPNVQLTGYRVRVTPKEKTGPM

             10
S7: KEINLAPDSSSVVVSGLM

             10
S8: VATKYEVSVYA

             10        20        30        40        50
S9: VREEVVTVGNSVDQGLSQPTDDSCFDPYTVSHYAIGEEWERLSDSGFKLS
                                                     100
    CQCLGFGSGHFRCDSSKWCHDNGVNYKIGEKWDRQGENGQMMSCTCLGNG
                                                     150
    KGEFKCDPHEATCYDNGKTYHVGEQWQKEYLGAICSCTCFGGQRGWRCDN

    CRRPGAEPGNEGSTAHSYNQYSQRYHQR

              10        20
S10: TNTNVNCPIECFMPLDVQADREDSRE
                           PO3
```

FIG. 1. Amino acid sequences of segments S1–S10 of bovine plasma fibronectin. Residues are indicated by the single-letter code (A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx) as recommended in ref. 30. CBH, carbohydrate; PO_3, phosphate.

The 29-kDal fragment. The sequence of the 29-kDal fragment has been completely determined and corresponds to residues 1–259 in segment S1. This fragment is from the NH_2-terminal end of fibronectin and contains a pyroglutamate in position 1 (28, 32). Putrescine is covalently linked to glutamine (position 3 in segment S1) in a reaction catalyzed by factor $XIII_a$ (28). The amino acid sequence of the 29-kDal fragment can be aligned in five mutually homologous domains (type I homology), as shown in Fig. 2 (lines 1–5). Within each of the five domains, the half-cystine residues in group 1 are linked by a disulfide bond to the half-cystine residues in group 3. These bonds link residues 110–138, 155–184, and 200–229. Similarly, the half-cystine residues in group 2 are linked to the half-cystine residues in group 4. These bonds link residues 45–56, 92–104, 136–148, 182–194, and 227–239. With the exception of residues 17–47 and 66–94, all the disulfide bonds in the 29-kDal fragment have been identified by isolation and performic acid oxidation of small peptides linked by disulfide bonds. The 29-kDal fragment has a +2 net charge and shows a weak affinity for heparin/agarose.

The 170-kDal fragment. After $NaDodSO_4$/polyacrylamide gel electrophoresis of reduced and unreduced samples, the 170-kDal fragment migrated as a doublet or occasionally as a triplet. Only one NH_2-terminal sequence has been found beginning at threonine residue 260 in segment S1 (Fig. 1). After degradation of the 170-kDal fragment with cyanogen bromide, fragments corresponding to the following residues were isolated and their sequences were determined: residues 260–289 in S1, residues 1–13 and 14–17 in segment S2, residues 1–112 and 113–132 in segment S3, and segments S4, S5, S6, and S7. Segment S8 is the NH_2-terminal sequence of a larger cyanogen bromide fragment. The residues in segment S2 form part of a type I homology domain as indicated in Fig. 2 (line 6). Residues 1–13 in segment S3 have tentatively been aligned with part of a type I homology domain (Fig. 2, line 7) while another type of homology (type II homology) has been found between residues 14–73 and 74–132 in segment S3 (Fig. 2, lines 11 and 12). The type II homology domains are each composed of 60 residues with four half-cystines. The location of their disulfide bonds has not yet been determined. The degree of identity between the two type II homology domains (Fig. 2) is 50% with no insertions or deletions. A carbohydrate chain is attached to asparagine-99 in segment S3.

The sequences of segments S5–S8 can be aligned according to a third type of homology (type III homology) (Fig. 2, lines 13–17). In type III homology, a domain consists of 90 residues with no insertions or deletions. The degree of identity is approximately 30%. The beginning and termination of the type III homology domain are not obvious from the sequence but evidence from limited proteolysis (see below) indicates that the residues connecting two type III homology domains are located around residues 50 and 140 in segment S5 and residue 34 in segment S6. The type III homology sequences lack half-cystine and histidine residues and contain only two tryptophan and two phenylalanine residues. Sequence analysis of the fragment corresponding to segment S5 was difficult because of the resistance of the fragment to proteolytic enzymes, suggesting that this cyanogen bromide fragment may retain some tertiary structure.

The 170-kDal fragment has a strong affinity for both gelatin/agarose and heparin/agarose. Partial digestion of the 170-kDal fragment with chymotrypsin resulted in a 45-kDal fragment that bound to gelatin/agarose with the same affinity as the 170-kDal fragment as judged by the concentration of urea necessary to elute it from the gelatin. The NH_2-terminal sequence of this fragment starts at threonine-260 in segment S1, showing that the gelatin-binding 45-kDal fragment constitutes the NH_2-terminal part of the 170-kDal fragment. The complete sequence of the 45-kDal fragment has not yet been established but residues 260–289 in segment S1 and fragments corresponding to segments S2 and S3 are contained within the 45-kDal fragment, showing that both type I and type II homologies occur in the gelatin-binding fragment.

Extensive digestion of the 170-kDal fragment with chymotrypsin resulted in a 30-kDal fragment that bound to heparin/agarose with essentially the same affinity as the 170-kDal fragment. This fragment was eluted from the heparin/agarose with 0.4 M NH_4HCO_3. The NH_2-terminal sequence of the 30-kDal fragment starts at threonine-34 in segment S6. The sequence of the 30-kDal fragment has not been completed but, after degradation with cyanogen bromide, sequences corresponding to segments S7 and S8 have been identified as parts of this fragment.

The 23-kDal fragment. The sequence of the 23-kDal fragment has been completely determined and corresponds to segment

```
                                Group                                             Groups        Group
Homology   Segment                1                                                2&3            4                                                                      Line
                          11      ↓                                               ↓  ↓  50        ↓                                                                      Number
Type I     S1             PLTVSQCKPGSYDNGKHYQINQQWER-TYLGSAL-VCTCYGGSRG-FNCESK-                                                                                           1
                                                                                  100
                          PEPEETC-FDKY-TGNTYRVGDTYERPKDS--MIWDCTCIGAGRGRISCTIA-                                                                                           2
                                                                                        150
                              NRC----HEGGQSYKIGDTWRRPHETGGYMLECVCLGNGKGEWTCKPI-                                                                                           3

                             AEKC-FDQ-AAGTSYVVGETWEKP-YQGWMMVDCTCLGEGSGRITCTSR-                                                                                           4
                              200
                              NRC-NDQ-DTRTSYRIGDTWSKKDNRG-NLLQCICTGNGRGEWKCERH...                                                                                         5
                                                  10
Type I     S2                                 YRIGDQWDKQHDMGHMM                                                                                                           6
                                                                                     10
Type I     S3                                                        L-CTCLGNG---VSCQE...cont. type II                                                                    7
                          18                                         50
Type I     S9             QPTDDSC-FDPY-TVSHYAIGEEWERLSDSGFK-LSCQCLGFGSGHFRCDSS-                                                                                           8
                                                                              100
                              KWC----HDNGVNYKIGEKWDRQGENGQMM-SCTCLGNGKGEFKCDPH-                                                                                           9
                                                                                      150
                             EATC----YDNGKTYHVGEQWQK-EYLG-AICSCTCFGGQRG-WRCDNC...                                                                                        10
                          14                                           50
Type II    S3             TAVTQTYGGNSNGEPCVLPFTYNGKTFYSCTTEGRQDGHLWCSTTSNYEQDQKYSFCTDH-                                                                                  11
                                                        100
                          TVLVQTRGGNSNGALCHFPFLYNNHNYTDCTSEGRRDNMKWCGTTQNYDADQKFGFCPM                                                                                    12
                          1                                                          50
Type III   S5             GGRPREDRVPPSRNSITLTNLNPGTEYVVSIVALNSKEESLPLVGQQSTVSDVPRDLEVIAATPTSLLISWDAPAVTVRYYRITYGETGG-                                                    13
                                100                                                      150
                          SSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPVSINYRTEIDKPSQM                                                                                  14
                                          1                                                                          50
Type III   S6                             TIEGLQPTVEYVVSVYAQNQNGESQPLVQTAVTTIPAPTNLKFTQVTPTSLTAQWTAPNVQLTGYRVRVTPKEKTGPM                                         15
                                     10
Type III   S7                 KEINLAPDSSSVVVSGLM                                                                                                                         16
                                                  10
Type III   S8                                 VATKYEVSVYA                                                                                                                17
```

FIG. 2. Alignment of parts of the sequence shown in Fig. 1 in three types of homology. Half-cystine residues are underlined.

S9 in Fig. 1. The net charge is $-5/2$ in agreement with its affinity for DEAE-cellulose. The fragment is composed of 178 amino acid residues and the alignment (Fig. 2, lines 8–10) shows three domains of type I homology. Two "extra" half-cystines are found at positions 135 and 151 in segment S9. No free SH group was detected in the 23-kDal fragment, suggesting that an extra disulfide bond connects these two half-cystines. The exact location of the disulfide bond, however, has not been determined.

The 6-kDal fragment. After reduction and alkylation of the 6-kDal fragment, the amino acid sequence corresponding to segment S10 was determined (Fig. 1). This peptide has a COOH-terminal glutamic acid, indicating that it is derived from the COOH-terminal end of fibronectin. The sequence has half-cystine residues at positions 7 and 11 in segment S10. The 6-kDal fragment is eluted in the void volume from Sephadex G-50, in agreement with a dimeric structure, whereas the reduced and alkylated 6-kDal fragment is eluted much later, in a position corresponding to a monomeric structure. The location of the disulfide bonds in the dimer has not been determined. High-voltage paper electrophoresis of a partial acid hydrolysate of the 6-kDal fragment showed that this fragment contains *O*-phosphoserine. After tryptic digestion, the peptide Glu-Asp-Ser-Arg-Glu was found in both a phosphorylated and a nonphosphorylated form in roughly equal amounts. This indicates that serine-24 in segment S10 is approximately 50% phosphorylated in bovine plasma fibronectin.

Alignment of Fragments. From a cyanogen bromide digest of intact fibronectin, a fragment corresponding to residues 180–289 in segment S1 has been isolated (Fig. 1). After further digestion with chymotrypsin, the peptide Thr-Asp-Val-Arg-Thr-Ala-Ile-Tyr (residues 256–263 in segment S1) was isolated. This peptide links the COOH-terminus of the 29-kDal fragment to the NH_2-terminus of the 170-kDal fragment. Since the 6-kDal fragment is the COOH-terminal fragment of the protein, it follows that the overall order of the plasmin-digestion fragments is 29, 170, 23, and 6 kDal. Overlapping peptides linking the 170- and the 23-kDal fragments and the 23- and 6-kDal fragments have not been found thus far.

DISCUSSION

From the sequence information accounting for approximately half of one or most likely both chains of fibronectin (Fig. 1) and the alignment of the peptides shown in Fig. 2, it is clear that at least three different types of internal homology occur in fibronectin. At present, nine regions of type I homology, two of type II homology, and four of type III homology have been identified. When comparing the type I homology domains one by one, the degree of identity ranges from 18% to 60%. As mentioned above, the degrees of identity within types II and III are 50% and 30%, respectively.

Fibronectin is a structural protein with a number of specific binding characteristics (3–5). The amino acid sequence shows that these binding regions have developed by successive multiple gene duplications of at least three different segments of DNA. A similar diversification of specificity by gene multiplication has occurred in other proteins, such as albumin (33), ovomucoid (34), and plasminogen (35). Fibronectin, however, is a remarkably complex example.

A proposed model of a fibronectin chain is shown in Fig. 3. The solid lines correspond to the 911 residues presented in Fig. 1, while the dots symbolize the remaining residues (approximately 970). Our results indicate, in agreement with those of others (3–5), that the two chains in fibronectin are essentially identical, with the 29-kDal fragment as the NH_2-terminal fragment and the 6-kDal fragment as the COOH-terminal fragment. The two-chain structure of the 6-kDal fragment shows that this COOH-terminal part must be identical in the two chains except possibly for a difference in the degree of phosphorylation. Nevertheless, some of the sequences in Fig. 1 may be derived partly from one chain and partly from the other. After digestion of hamster fibronectin with thermolysin, Sekiguchi *et al.* (24) identified a 21-kDal fragment apparently originating near the COOH-terminus of only one of the chains. This fragment may be similar to our 23-kDal fragment but we do not know whether

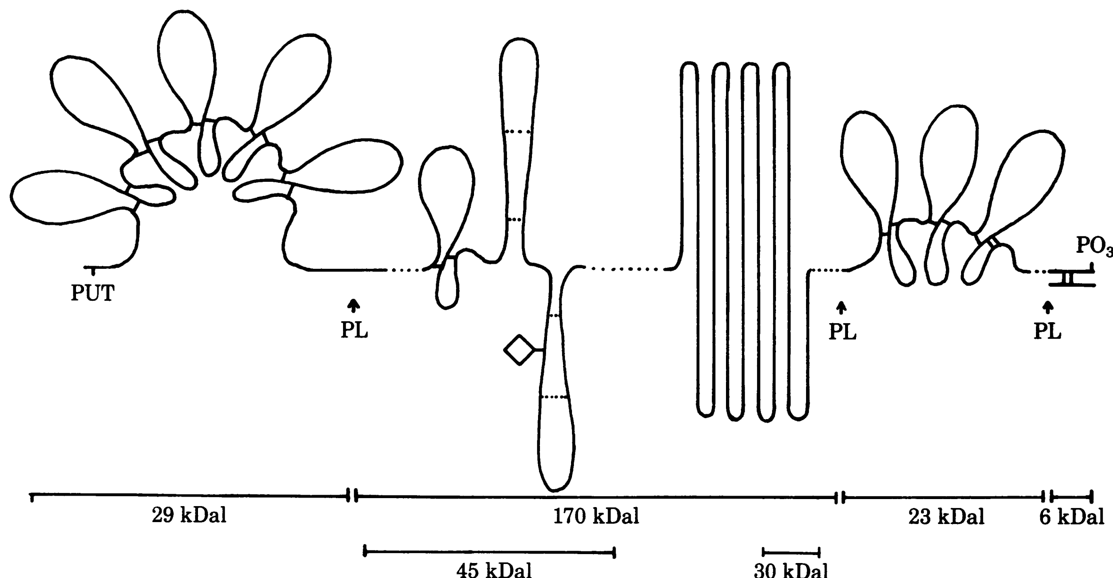

FIG. 3. Proposed model of one of the chains of fibronectin. The 29-, 170-, 23-, and 6-kDal fragments were obtained by digestion with plasmin (PL). Each of the halves of the 6-kDal fragment belongs to one of the two chains of fibronectin. The 45- and 30-kDal fragments are obtained by digestion with chymotrypsin. The 45-kDal fragment binds to gelatin and the 30-kDal fragment binds to heparin. The solid line corresponds to the 911 residues shown in Fig. 1, and the dotted line corresponds to the remaining residues (approximately 970). The dots between the two loops indicate disulfide bonds, but their positions are unknown. The domains containing the type I homology have been called finger domains. PUT, position to which putrescine can be linked by factor $XIII_a$; PO_3, a phosphorylation site; ◇, the carbohydrate chain.

the 23-kDal fragment occurs in both or only one of the chains.

Fibronectin is phosphorylated at serine-24 in segment S10. The functional significance of this phosphorylation is not clear. It is interesting to note that Ali and Hunter (36) have found that fibronectin from transformed cells is more extensively phosphorylated than fibronectin from normal cells.

Fibronectin is very sensitive to proteolytic enzymes (3–5). Two areas are particularly sensitive, the region connecting the 29-kDal fragment to the 170-kDal fragment and a second region located on the NH_2-terminal side of the 6-kDal fragment. Plasmin hydrolyzes the peptide bond between arginine-259 and threonine-260 in segment S1 and also the arginine linked to threonine-1 in segment S10. In the first case, the 29-kDal fragment containing the potential crosslinking site (glutamine-3 in segment S1) becomes separated from the rest of the fibronectin molecule and, in the second case, the 6-kDal fragment with the two interchain disulfide bonds is released. The hydrolysis of these two plasmin-susceptible bonds may be of functional importance in such reactions as the dissolution of blood clots and invasive growth.

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