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DIFFERENCES IN DOMAIN STRUCTURE BETWEEN PERICELLULAR MATRIX AND PLASMA FIBRONECTINS AS REVEALED BY DOMAIN-SPECIFIC ANTIBODIES COMBINED WITH LIMITED PROTEOLYSIS AND S-CYANYLATION: A PRELIMINARY NOTE*

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Summary: Differences in domain structure between human fibronectins obtained from pericellular matrix and plasma have been revealed by limited proteolysis and S-cyanylation, followed by identification of each domain with domain-specific antibodies. Although the overall domain structure is similar between pericellular and plasma fibronectins, the fragments derived from the COOH-terminal region of these fibronectins, which were defined by specific antibodies, exhibited clear differences in their molecular weights and protease susceptibility, suggesting that the structure near the COOH-terminal region is significantly different between these two proteins.

Fibronectins (FNs[1]) are a group of high molecular weight glycoproteins present in the pericellular matrix and body fluids (for reviews, see Refs. 1-6). FNs purified from various sources share most chemical, biological, and immunological properties, yet there are some differences in their subunit size, their solubility, the structure of their carbohydrate units, and in some biological activities (see reviews above). The basis of the molecular heterogeneity of FNs is only poorly understood. FNs consist of at least four distinct domains that are referred to as Hep-1/Fib-1, Gel, Cell/Hep-2, and Fib-2 according to their biological activities (7). These domains are aligned

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[1]Abbreviations: FN, fibronectin; Hep-1/Fib-1, the NH_2-terminal 24K domain that binds to heparin and fibrin; Gel, the gelatin/collagen-binding 44K domain; Cell/Hep-2, the 155K/145K domain that binds to cell surface and heparin; Fib-2, the COOH-terminal 22K domain that binds to fibrin; Buffer A, 10 mM Tris/HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl; NTCB, 2-nitro-5-thiocyanobenzoic acid; PMSF, phenylmethylsulfonyl fluoride. Each fragment is designated by its apparent molecular weight. For example, a Mr=32,000 fragment is referred to as 32K fragment. The prefix "sc" specifies S-cyanylation-cleaved fragments.

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from NH_2- to COOH-terminus in the above order and can be easily separated from each other by mild protease treatment followed by affinity column treatment (7-9). In this communication, we compared the immunostaining pattern with domain-specific antibodies directed to various fragments released from human plasma FN and pericellular matrix FN (called hereafter "cellular" FN) by limited proteolysis and S-cyanylation. The results indicate that there are distinctive structural differences between plasma and cellular FNs near the COOH-terminal region.

MATERIALS AND METHODS

Human plasma FN was purified on gelatin-Sepharose (8). Human foreskin fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. The confluent monolayer was extracted with 10 mM Tris-buffered saline (pH 8.0) containing 0.5 % sodium deoxycholate and 1 mM PMSF according to Hedman et al. (10). In some experiments, the detergent-insoluble matrix was further extracted with 25 mM Tris buffer (pH 7.6) containing 1 M NaCl. The resulting matrix was washed with Buffer A (10 mM Tris.HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl) three times and then subjected to protease digestion or S-cyanylation. S-cyanylation by 2-nitro-5-thiocyanobenzoic acid (NTCB) was performed as described previously (9).

Antibodies specific to each domain were prepared by immunizing New Zealand white rabbits with the purified domain fragments emulsified with Freund's complete adjuvant (7). The fragments used for immunization are: 32K tryptic fragment for Hep-1/Fib-1 domain; 44K gelatin-binding thermolysin fragment for Gel domain; a 61K NH_2-terminal region of the 155K/145K thermolysin fragments for Cell/Hep-2 domain; 22K thermolysin fragment for Fib-2 domain. These fragments were purified by a series of affinity chromatography and DEAE-cellulose chromatography as described (7,8) and by subsequent preparative gel electrophoresis. Two monoclonal antibodies directed to human plasma FN, IST-1 and IST-4, were prepared as described previously (11). IST-1 is directed to COOH-terminal region of Cell/Hep-2 domain, i.e., Hep-2 subdomain, whereas IST-4 is directed to the NH_2-terminal region of the same domain (K.S., A.S., S.H., and L.Z., manuscript in preparation).

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (12). Electrotransfer of proteins from polyacrylamide gels to nitrocellulose was carried out by the method of Towbin et al. (13). Immunostaining of the nitrocellulose blots with the antibodies and ^{125}I-protein A was performed as described previously (7).

RESULTS

Detergent-insoluble matrix as a source of cellular FN: The sodium deoxycholate-insoluble matrix contains three major components, FN, 200K, and 45K proteins. The latter two proteins appear to correspond to myosin and actin, and can be mostly removed by subsequent washing with 1 M NaCl (compare the protein staining of the matrix of Fig. 1A and 2A). These matrices, with or without a 1 M NaCl wash, were used as a source of cellular FN throughout the present study.

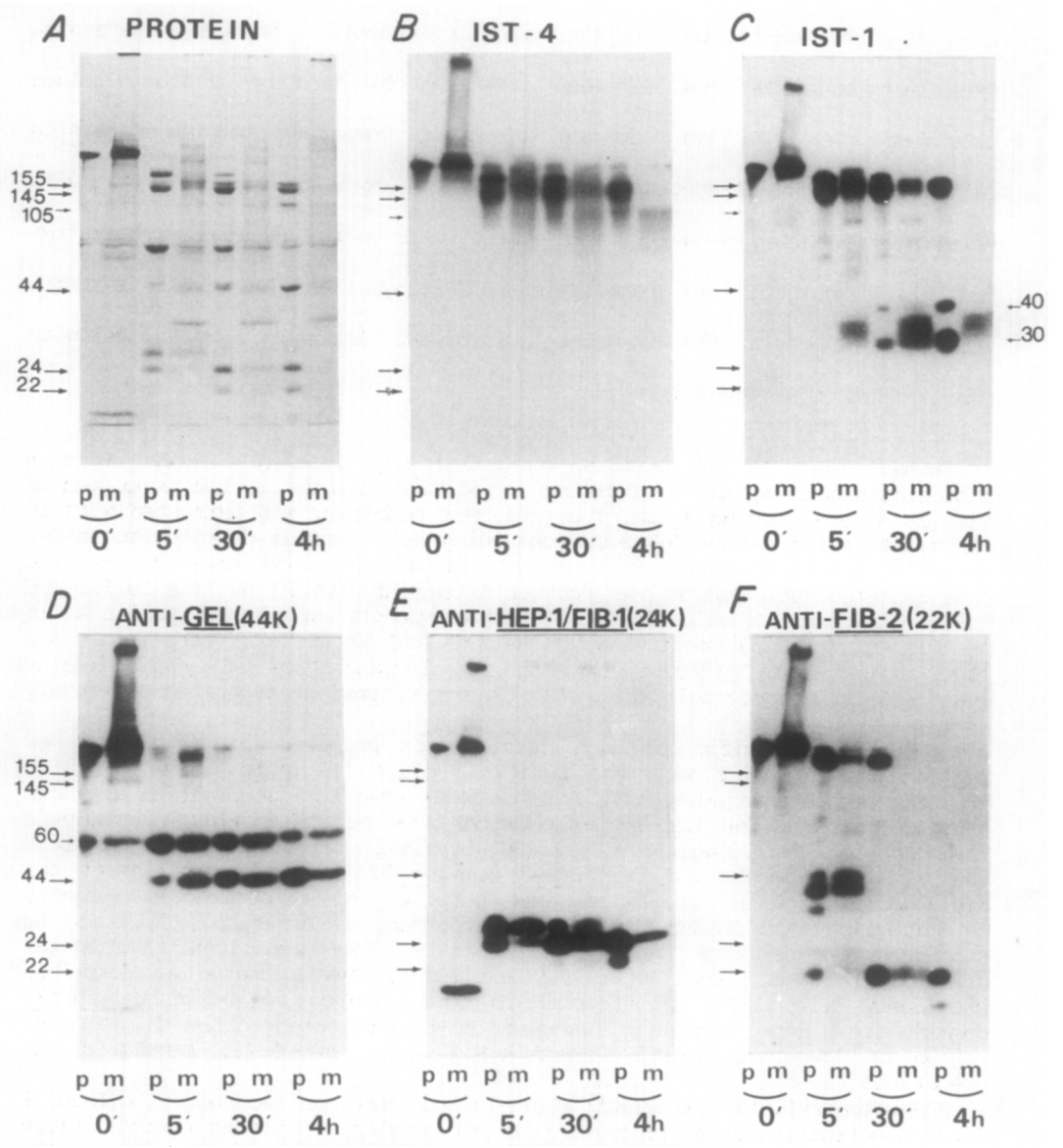

Figure 1: Immunostaining of the thermolysin digest of plasma FN (p) and the detergent-insoluble matrix (m) with domain-specific antibodies. Plasma FN (1.0 mg) and the matrix on the 15 cm culture dishes were digested by thermolysin (5 μg/ml) in 2 ml of Buffer A containing 2.5 mM $CaCl_2$ at 22° for different periods of time. The digests were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent immunostaining of the nitrocellulose blots with domain-specific antibodies. A, protein staining with Coomassie blue; B-F, immunostaining of the nitrocellulose blots with IST-4 (B), IST-1 (C), anti-Gel antibodies (D), anti-Hep-1/Fib-1 antibodies (E), and anti-Fib-2 antibodies (F).

Thermolysin fragments: Cellular FN in the detergent-insoluble matrix was cleaved by thermolysin into the same set of fragments as those obtained from plasma FN (Fig. 1). The Cell/Hep-2 domain of both FNs was obtained as

155K/145K fragments that were specifically recognized by two monoclonal antibodies, IST-1 and IST-4 (Fig. 1, panel B and C). However, most of this domain derived from cellular FN was further degraded upon prolonged digestion into a 105K fragment which was specifically stained with IST-4 (Fig. 1, panel B) but not with IST-1 (Fig. 1, panel C). Fragments with smaller molecular weights released from this domain upon prolonged thermolysin digestion were also strikingly different between cellular and plasma FNs. Two fragments, 40K and 30K, were released from plasma FN and a single broad 34K fragment was released from cellular FN. These fragments were all stained by IST-1 (Fig. 1. panel C) but not by IST-4 (Fig. 1, panel B). The 40K and 30K fragment has been shown to be derived from the heparin-binding region of the Cell/Hep-2 domain, i.e., Hep-2 subdomain, located at the COOH-terminal region of the Cell/Hep-2 domain (K.S., A.S., S.H., and L.Z., manuscript in preparation). These results indicate that the structure around Hep-2 subdomain is significantly different between cellular and plasma FNs.

The Gel domain of both FNs was recovered as 44K fragment and its precursor, 60K (Fig. 1, panel D). The NH_2-terminal Hep-1/Fib-1 domain of both FNs was also obtained as 24K fragment and its precursor 27K (Fig. 1, panel E), indicating that the domain structure of the NH_2-terminal portion of the plasma and cellular FNs are very similar, if not identical.

The COOH-terminal Fib-2 domain of both FNs was obtained as a 22K fragment and its precursor fragments with Mr=40,000-44,000 (Fig. 1, panel F). However, this domain of cellular FN appears to be less stable than those from plasma FN, because the 22K fragment from cellular FN almost disappeared after prolonged digestion.

Tryptic fragments: Human plasma FN was cleaved by trypsin into 215K/185K, 37K, and 32K fragments, each of which can be specifically detected by Anti-Gel, Anti-Fib-2, and anti-Hep-1/Fib-1 antibodies, respectively (Fig. 2, panel A-D, lane 4). Cellular FN was also cleaved by trypsin into a similar sets of fragments (Fig. 2, panel B-D, lane 3), except that the anti-Gel antibodies predominantly stained a broad band with Mr=200,000 instead of

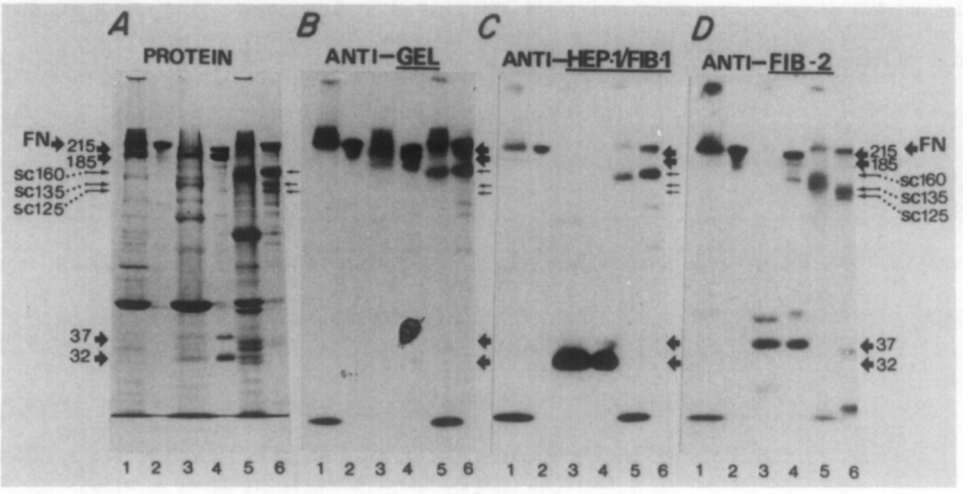

Figure 2: Immunostaining of the tryptic and S-cyanylation-cleaved fragments with domain-specific antibodies. Detergent-insoluble matrix and plasma FN were cleaved by trypsin (1 μg/ml, 30 min) or by S-cyanylation. The resulting fragments were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent immunostaining of the nitrocellulose blots with domain-specific antibodies. A, protein staining, B-D, immunostaining of the nitrocellulose blots with anti-Gel (B), anti-Hep-1/Fib-1 (C), and anti-Fib-2 (D) antibodies. Lane 1, intact matrix; lane 2, intact plasma FN; lane 3, the trypsin digest of the matrix; lane 4, the trypsin digest of plasma FN; lane 5, S-cyanylation-cleaved fragments of the matrix; lane 6, S-cyanylation-cleaved fragments of plasma FN.

215K/185K fragments. The Fib-2 domain of the entire cellular FN was quantitatively degraded into a 37K and its possible precursor 43K fragments, whereas these fragments were only released from the Fib-2 domain of α , but not β, subunit of plasma FN (7). This indicates that cellular FN is more related to α subunit than β subunit of plasma FN in terms of trypsin susceptibility. It is also noted that the 43K precursor fragment from cellular FN is slightly smaller than the corresponding fragment from plasma FN (Fig. 2, panel D, lane 3 and 4).

S-cyanylation fragments: S-cyanylation of human plasma FN produced three major fragments, sc160K, sc135K, and sc125K (Fig. 2, panel A, lane 6). The sc160K fragment was stained by anti-Gel and anti-Hep-1/Fib-1 antibodies (Fig. 2, panel B and C, lane 6) and the sc135K and sc125K fragments were stained by anti-Fib-2 antibodies (Fig. 2, panel C, lane 6). This indicates that the sc160K and sc135K/125K fragments are, respectively, derived from the NH_2-terminal half and the COOH-terminal half of α and β subunits.

S-cyanylation of cellular FN produced similar fragments, but the apparent molecular weights of these fragments are slightly different from those of plasma FN. Both anti-Gel and anti-Hep-1/Fib-1 antibodies stained a fragment that migrated slightly below the scl60K fragment (Fig. 2, panel B and C, lane 5), whereas the anti-Fib-2 antibodies detected fragments with Mr=155,000-145,000 that are significantly larger than the scl35K/125K from plasma FN (Fig. 2, panel D, lane 5).

DISCUSSION

Most of cellular FN in the pericellular matrix exists as disulfide-bonded multimer and is difficult to solubilize without strong denaturants and reducing agents (14,15). We developed a novel method to study the domain structure of the matrix FN by *in situ* fragmentation followed by identification of each domain by blotting on nitrocellulose and immunostaining with domain-specific antibodies. The results clearly indicate that cellular FN is composed of the same set of structural domains as plasma FN, *i.e.*, Cell/Hep-2, Gel, Hep-1/Fib-1, and Fib-2 that can be obtained as 155K/145K, 44K, 24K, and 22K thermolysin fragments. Nevertheless, there are several clear differences between these FNs in the following aspects: i) Hep-2 subdomain of these FNs were recovered as clealy different thermolysin fragments, 40K and 30K from plasma FN in contrast to a broad 34K from cellular FN. ii) The Fib-2 domain of cellular FN is less stable than those of plasma FN and, upon trypsin digestion, is partly released as 43K fragment that is slightly smaller than the corresponding fragment from plasma FN. iii) COOH-terminal half of cellular FN obtained by S-cyanylation is significantly larger than that from plasma FN. All of these results strongly indicate that the structure of the COOH-terminal region including Hep-2 and Fib-2 domains is significantly different between cellular and plasma FNs. These differences appears to be due to the differences in the polypeptide portion rather than the carbohydrate, because the majority of the carbohydrate units are localized in the Gel domain (16,17), and this domain did not show any apparent difference. Previously, Atherton and Hynes obtained a monoclonal

antibody against hamster cellular FN which selectively binds to cellular, but not plasma, FN (18). Immunoprecipitation of various proteolytic fragments with this antibody indicates that it is directed to the COOH-terminal region of cellular FN, being consistent with the present observation.

Hayashi and Yamada compared the structure of chicken cellular and plasma FNs by thermolysin digestion followed by fractionation of the fragments on various affinity columns (17). They found that there are at least three regions which are different between the two FNs; one is in the Gel domain and others are in and/or around the Cell/Hep-2 domain. Although we did not detect any difference in the Gel domain between human FNs, this could be due to the species difference. Interestingly, the structural difference between α and β subunits of plasma FN is also localized at the juctional region between Hep-2 and Fib-2 domains (7,19). Further studies on the primary structure as well as the gene organization of these domains and their junctional region will provide a clue to understanding the basis for the molecular heterogeneity of FNs.

REFERENCES

1. Mosesson, M. W., and Amrani, D. L. (1980) Blood 56, 145-158.
2. Mosher, D. F. (1980) Prog. Hemostasis Thromb. 5, 111-151.
3. Ruoslahti, E., Engvall, E., and Hayman, E. G. (1981) Coll. Res. 1, 95-128.
4. Hynes, R. O., and Yamada, K. M. (1982) J. Cell Biol. 95, 369-377.
5. Furcht, L. T. (1983) Modern Cell Biology 1, 53-117.
6. Yamada, K. M. (1983) Annu. Rev. Biochem. 52, 61-99.
7. Sekiguchi, K., and Hakomori, S. (1983) J. Biol. Chem. 258, 3967-3973.
8. Sekiguchi, K., Fukuda, M., and Hakomori, S. (1981) J. Biol Chem. 256, 6452-6462.
9. Sekiguchi, K., and Hakomori, S. (1983) Biochemistry 22, 1415-1422.
10. Hedman, K., Kurkinen, M., Alitalo, K., Vaheri, A., Johannson, S., and Höök, M. (1979) J. Cell Biol. 81, 83-91.
11. Zardi, L., Carnemolla, B., Siri, A., Santi, L., and Accolla, R. (1980) Int. J. Cancer 25, 325-329.
12. Laemmli, U. K. Nature (Lond.) 227, 680-685.
13. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.
14. Hynes, R. O., and Destree, A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2855-2859.
15. Carter, W. G., and Hakomori, S. (1981) J. Biol. Chem. 256, 6953-6960.
16. Sekiguchi, K., and Hakomori, S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2661-2665.
17. Hayashi, M., and Yamada, K. M. (1981) J. Biol. Chem. 256, 11292-11300.
18. Atherton, B. T., and Hynes, R. O. (1981) Cell 25, 133-141.
19. Hayashi, M., and Yamada, K. M. (1983) J. Biol. Chem. 258, 3332-3340.