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IDENTIFICATION OF TWO FIBRIN-BINDING DOMAINS IN PLASMA FIBRONECTIN AND UNEQUAL DISTRIBUTION OF THESE DOMAINS IN TWO DIFFERENT SUBUNITS: A PRELIMINARY NOTE

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Summary: Fibronectin (FN) was cleaved into four functionally distinct domains with Mr=150,000-140,000 (150K-140K), 40,000 (40K), 32,000 (32K), and 21,000 (21K) by either sequential digestion with trypsin and thermolysin or by thermolysin alone according to the method as previously described (Ref. 7 of the text). Among them, the 32K and 21K domains bound to fibrin while the other two domains, the 40K domain which binds to gelatin and the 150K-140K domain which binds to heparin and promotes cell spreading, did not bind to fibrin. One of the fibrin-binding domains, the 32K domain, was present in both the larger and smaller subunits of intact FN, but the 21K domain was only present in the larger, but absent in the smaller, subunit.

Fibronectin (FN)[1], previously termed galactoprotein a (1), LETS (2), etc., is a macromolecular glycoprotein abundantly present in the pericellular matrix and in plasma although FN from these two sources is different (for recent reviews, see 3-5). FN is considered to be composed of several structural domains which are connected by flexible peptide segments (6). Recently, we have succeeded in *quasi*-quantitative separation of three major structural domains with Mr=150,000-140,000 (150K-140K), 40,000 (40K), and 32,000 (32K) by sequential digestion with trypsin and thermolysin (7). These domains were found to be distinct from each other in their binding specificity to collagen, glycosaminoglycans and in their ability to promote cell spreading (7).

Present communication describes the separation of a domain with Mr=21,000 (21K) in addition to the structural domains as previously described (7), and identification of two distinct fibrin-binding domains in FN. Plasma FN has been known to consist of two subunits, the larger (Mr=230,000) and the smaller

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1) Abbreviations: FN, fibronectin; K, 10^3; Mr, relative molecular weight.

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(Mr=210,000) polypeptides. This preliminary note also reports an unequal distribution of fibrin-binding domains in these two subunits; *i.e.*, one of the domains, the 21K domain, is present exclusively on the larger subunit.

MATERIALS AND METHODS

Materials: Thermolysin, gelatin (from swine skin, type I), bovine fibrinogen (type IV) were obtained from Sigma and trypsin from Worthington Biochemicals. Gelatin-Sepharose was prepared according to Cuatrecasas and Anfinsen (8).

Purification and Proteolytic Digestion of FN: FN was purified from hamster plasma by affinity chromatography on a gelatin-Sepharose column as described (7). Thermolysin digestion of FN was performed by incubating FN (approx. 1 mg/ml) with thermolysin (2.5 μg/ml) in the presence of 2 mM $CaCl_2$ at 22°C for 4 h. Thermolysin was inactivated by adding 5 mM EDTA. Trypsin digestion was carried out by incubating FN (approx. 1 mg/ml) with trypsin (1 μg/ml) at 22°C for 15 min. The 200K-180K tryptic fragments were isolated with gelatin-Sepharose as described (7) and further purified by gel filtration with Sephacryl S-200. Separation of the 200K fragment from the 180K was achieved through a fibrin-Sepharose column. The 32K tryptic fragment was purified by heparin-agarose chromatography.

Isolation of Fibrin-Binding Fragments: Fibrinogen was immobilized on Sepharose 4B according to Cuatrecasas and Anfinsen (8) and subsequently converted to fibrin by incubating with thrombin (1 unit/ml) for 30 min at 22°C. To isolate the fibrin-binding fragments, the proteolytic digest of intact FN was applied to a fibrin-Sepharose column (approx. 5 mg of protein per ml of packed gel) equilibrated with 25 mM Tris buffer containing 0.5 mM EDTA and 100 mM NaCl, pH 7.6. The column was extensively washed with the 25 mM Tris buffer. Bound materials were eluted from the column with the 25 mM Tris buffer containing 8 M urea.

SDS-Polyacrylamide Gel Electrophoresis: SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (9) with 9% polyacrylamide gels as described (7).

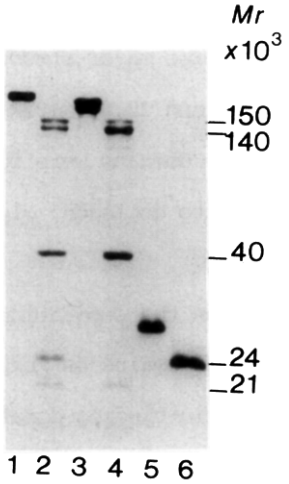

Fig. 1. Thermolysin digestion of intact FN and purified tryptic fragments.

Intact FN (lane 1 and 2), the purified 200K-180K tryptic fragments (lane 3 and 4), and the purified 32K fragment (lane 5 and 6) were digested with thermolysin (2.5 μg/ml) for 4 h at 22°C. (lane 1, 3, 5), before digestion; (lane 2, 4, 6), after digestion.

RESULTS AND DISCUSSION

The 21K fragment, an additional structural domain of plasma FN yielded by thermolysin treatment and its origin: FN from hamster plasma is composed of two subunit polypeptides with approx. Mr=230,000 (230K) and 210,000 (210K) (Fig. 1, lane 1). Thermolysin digestion of intact FN produced four functionally distinct fragments; (i) Mr=150,000 (150K) and 140,000 (140K) fragments which bound to heparin, and promoted cell spreading (7), (ii) Mr=40,000 (40K) fragment which bound to gelatin (7), (iii) Mr=24,000 (24K) fragment which bound to heparin (7), and (iv) Mr=21,000 (21K) fragment (Fig. 1, lane 2). In contrast, mild trypsin digestion only released a Mr=32,000 (32K) fragment from both 230K and 210K subunits which were concomitantly converted to Mr=200,000 (200K) and 180,000 (180K) fragments, respectively (7, also see Fig. 2 lane 6). The tryptic fragments, 200K-180K (Fig. 1, lane 3) and 32K (Fig. 1, lane 5) were separated by a gelatin-Sepharose column and each purified fragment was separately digested by thermolysin. The 21K fragment was only generated from the 200K-180K tryptic fragments together with the 150K-140K and 40K fragments (Fig. 1, lane 4). The 21K fragment was not generated from the tryptic 32K fragment (Fig. 1, lane 6), instead, the 32K fragment was quantitatively converted to a 24K fragment by thermolysin treatment. The results clearly indicated that both the tryptic 32K and the thermolysin-released 24K fragment represent an identical domain in intact FN. The results also indicated that the origin of the two thermolysin-released fragments, 21K and 24K, is entirely different as described above.

Two fibrin-binding fragments, 24K and 21K, produced by thermolysin digestion of intact FN and their origin: Since FN has been shown to bind to fibrin (10,11) as well as collagen (7,12,13) and heparin (14,15), the thermolysin digest of intact FN was fractionated with fibrin-Sepharose to identify the fibrin-binding fragment(s). Only the 24K and 21K fragments bound to fibrin, whereas the 150K-140K fragments (which bound to heparin and promoted cell spreading) and the 40K fragment (which bound to gelatin) failed to bind a fibrin-Sepharose column (Fig. 2, lane 1-4). This indicated that there were two distinct fibrin-binding

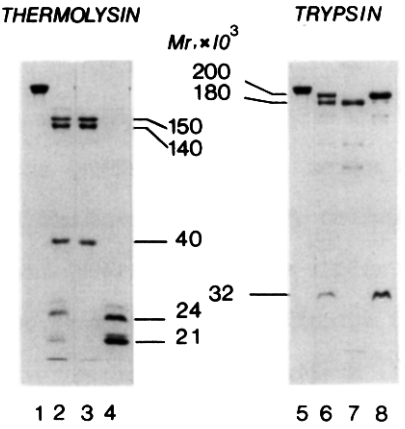

Fig. 2. SDS-polyacrylamide gel electrophoresis of fibrin-binding fragments.

FN was digested by either thermolysin (left) or trypsin (right) and the resulting fragments were chromatographed on a fibrin-Sepharose column. Detailed conditions were described under "Materials and Methods". Lane 1 and 5, intact FN; lane 2 and 6, the unfractionated digest; lane 3 and 7, fragments which passed through the column; lane 4 and 8, fragments which bound to the column. Protein bands were stained with Coomassie blue.

sites in intact FN, neither of which was on the gelatin-binding domain and this finding is contradictory to previous results (16).

To confirm the presence of two different fibrin-binding sites in FN, the trypsin digest of intact FN was fractionated with a fibrin-Sepharose column (Fig. 2, lane 5-8). Of the two tryptic fragments (200K-180K), only the 200K fragment bound to fibrin, unexpectedly, the 180K fragment failed to bind to a fibrin-Sepharose column. The 32K tryptic fragment, from which the 24K thermolysin-released fragment was derived, was also capable of binding to fibrin. Since the 21K fragment, another fibrin-binding fragment released by thermolysin, was only generated from the 200K-180K tryptic fragments (see Fig. 1, lane 5), the "21K domain" was conceived to be present only in the 200K, but absent in the 180K fragment. To substantiate this possibility, each of the 200K and 180K fragments was separated from each other through a fibrin-Sepharose column as above, and separately digested by thermolysin. As shown in Fig. 3, the 21K fragment was generated only from the 200K tryptic fragment together with 150K-140K and 40K fragments, but it was not generated from 180K fragment. This result confirmed the presence of 21K in 200K tryptic fragment, but the absence of it in the 180K tryptic fragment. Time course analysis of the thermolysin digestion of the 200K

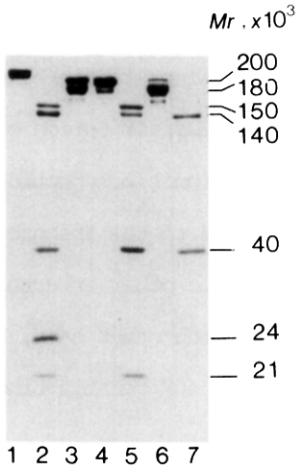

Fig. 3. Thermolysin digestion of 200K and 180K tryptic fragments.

Each of the 200K and 180K fragments was isolated as described under "Materials and Methods" and subsequently digested with thermolysin. Lane 1, intact FN; lane 2, thermolysin digest of intact FN; lane 3, 200K and 180K tryptic fragments purified on a Sephacryl S-200 column; lane 4, isolated 200K fragment; lane 5, thermolsyin digest of the 200K fragment; lane 6, isolated 180K fragment; lane 7, thermolysin digest of the 180K fragment.

fragment showed that, of the 150K-140K fragments, the 150K fragment was predominantly produced in the early stage of digestion and gradually converted to the 140K (data not shown), suggesting that the 150K and 140K fragments were respectively derived from the 230K and 210K subunits.

A model showing unequal distribution of fibrin-binding sites among two subunits of plasma FN: Figure 4 summarizes the proposed scheme of proteolytic fragmen-

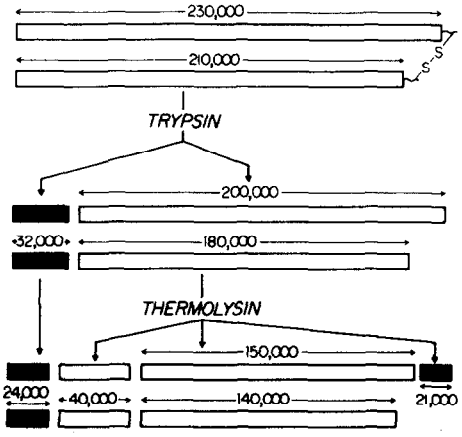

Fig. 4. Model for the sequential fragmentation of intact FN with trypsin and thermolysin.
Two fibrin-binding domains, which are recovered as 32K tryptic (or 24K thermolysin-released) and 21K thermolysin-released fragments are shown as solid bars. For details, see the text.

tation of intact FN by sequential digestion with trypsin and thermolysin, emphasizing the presence of two distinct fibrin-binding fragments. Most important feature of this model is the unequal distribution of the 21K fibrin-binding domain between two non-identical subunit polypeptides. Heterogeneity of the subunits can be basically attributed to the absence of the 21K domain in the smaller (210K) subunit, although some other structural differences also seem to exist in the 150K-140K domain. On the other hand, both the 32K domain which binds to heparin and fibrin and the 40K gelatin-binding domain are assumed to be equally present on both subunits.

Previously, Engvall *et al* (16) proposed that the fibrinogen- (fibrin-) binding site was localized on the collagen-binding domain, mainly based upon their finding that gelatin strongly inhibited the binding of FN to fibrinogen. Our present data clearly indicate that (i) the 40K gelatin-binding fragment did not bind to fibrin, and (ii) neither the 24K nor the 21K fibrin-binding fragments bound to gelatin. Since the 32K domain has been shown to be in the juxtaposition of the 40K domain (17-19), the binding of gelatin to the 40K domain may hinder the subsequent binding of fibrinogen to FN. In support of our conclusion, treatment of intact FN with 10 mM dithiothreitol, which strongly inhibited the binding of FN to gelatin, did not affect the binding of FN to fibrin (data not shown).

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