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# Differences in Domain Structure between Human Fibronectins Isolated from Plasma and from Culture Supernatants of Normal and Transformed Fibroblasts

STUDIES WITH DOMAIN-SPECIFIC ANTIBODIES*

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**The domain structure of human fibronectins isolated from plasma and from the conditioned medium of normal and transformed fibroblasts was analyzed by limited proteolysis and *S*-cyanylation followed by immunostaining of released fragments with five kinds of antibodies, each specific for one functional domain. The results indicate that all three human fibronectins are composed of the same set of functional domains aligned in the same topological order. However, the following clear differences were found in specific fragments released from plasma fibronectin (pFN) and those released from fibronectin of normal (N-cFN) and transformed fibroblasts (T-cFN). (i) Two fragments ($M_r$ = 70,000 and 60,000) were released from the COOH-terminal region of pFN by cathepsin D. These fragments represent the COOH-terminal heparin-binding (*Hep-2*) and fibrin-binding (*Fib-2*) domains. The corresponding fragments released from both N-cFN and T-cFN by cathepsin D had much larger molecular weights ($M_r$ = 100,000 and 83,000–74,000) than those from pFN. The fragments from the *Fib-2* domain alone, however, did not show any difference among all three FNs. (ii) The internal region, from the gelatin-binding (*Gel*) domain through the *Hep-2* domain, of N-cFN and T-cFN was released as a $M_r$ = 210,000 fragment upon mild trypsin digestion. The corresponding fragment from pFN was released as a $M_r$ = 185,000 fragment. (iii) The COOH-terminal half, including the *Hep-2* domain, of both N-cFN and T-cFN was released by *S*-cyanylation as $M_r$ = 160,000–145,000 fragments, which are 25,000–20,000 larger than the corresponding fragments of pFN. These results clearly indicate that the *Hep-2* domain of N-cFN and T-cFN is 30,000–20,000 daltons larger than the same domain of pFN.**

**Although various fragments released from N-cFN and T-cFN showed a similar pattern, there were minor differences. (i) Thermolysin fragments derived from the *Hep-2* domain of N-cFN were clearly distinguishable from those from T-cFN. Three groups of fragments with $M_r$ = 40,000, 35,000–32,000, and 30,000 were released from N-cFN, while only the 35,000–32,000 fragment was released from T-cFN. (ii) The $M_r$ = 44,000/60,000 thermolysin fragments representing the *Gel* domain and the $M_r$ = 210,000/165,000 tryptic fragments representing the internal domains of T-cFN were slightly, but consistently, larger than those of N-cFN.**

**A panel of domain-specific antibodies combined with well-characterized proteolytic and chemical fragmentation provides a highly sensitive and specific procedure to study the domain structure of various types of fibronectins. This procedure requires only a small quantity of protein, without prior purification, and is applicable to both soluble and insoluble fibronectins.**

Fibronectins are a group of high molecular weight adhesive glycoproteins present in extracellular matrices and plasma (reviewed in Refs. 1–8). FNs[1] are produced by many types of cultured cells including fibroblasts, epithelial and endothelial cells, myoblasts, glial cells, and macrophages (1–8). Oncogenic transformation of these cells causes a marked decrease in the biosynthesis of FN and its deposition in the extracellular matrix (9–11).

FNs isolated from plasma and from cells in culture are very similar in their chemical, biological, and immunological properties, yet there are clear differences between them in subunit size (12, 13), carbohydrate structure (14–16), disulfide-dependent polymerization (17–19), solubility (17), ability to agglutinate sheep erythrocytes (12), and ability to restore normal fibroblast morphology to transformed cells (12). FNs produced by transformed cells may also be different from those produced by normal cells (20, 21). The basis for the molecular and functional polymorphism of FNs is poorly understood.

FNs are composed of multiple domains which differ in their biological activities (reviewed in Refs. 1–8). These domains can be separated by limited proteolysis and subsequent affin-

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[1] The abbreviations used are: FN, fibronectin; pFN, plasma fibronectin; cFN, cellular fibronectin; N-cFN, cellular fibronectin produced by normal fibroblasts; T-cFN, cellular fibronectin produced by transformed fibroblasts; *Hep-1/Fib-1*, the $NH_2$-terminal domain that binds to heparin and fibrin; *Gel*, the gelatin-binding domain; *Cell/Hep-2*, the cell- and heparin-binding domain which can be further separated into *Cell* and *Hep-2* domains, which represent the $NH_2$-terminal and COOH-terminal regions of the *Cell/Hep-2* domain, respectively; *Fib-2*, the COOH-terminal fibrin-binding domain; CAPS, cyclohexylaminopropane sulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PBS, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4, sc, the *S*-cyanylation-cleaved fragments.

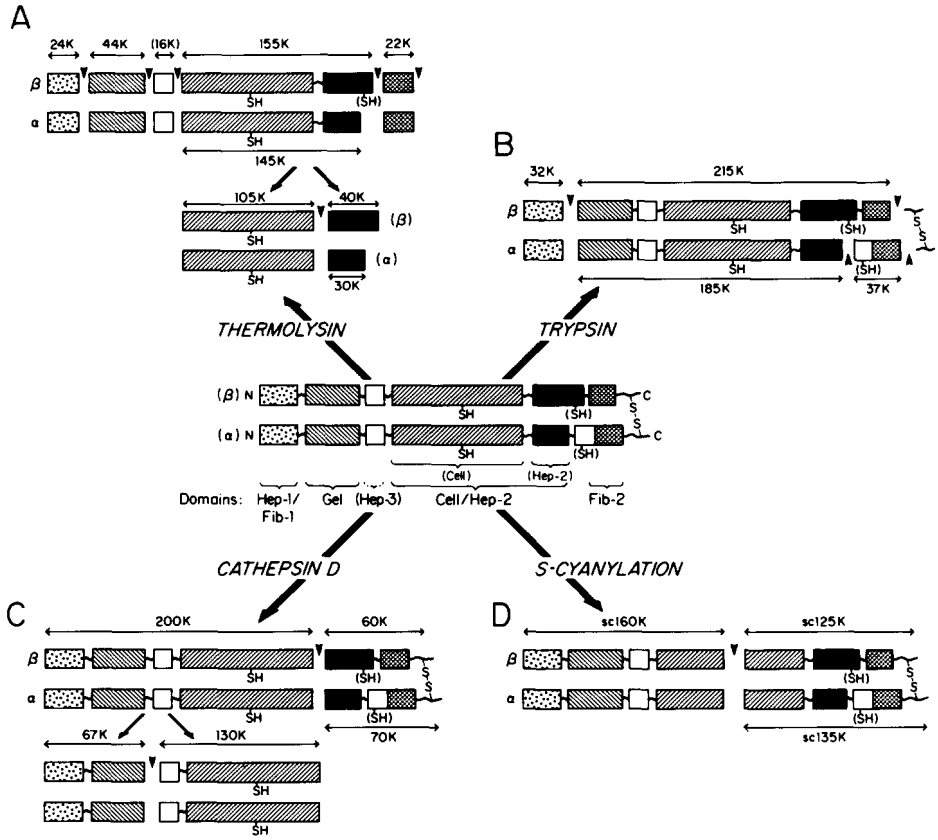


FIG. 1. **Proposed model for the proteolytic and chemical fragmentation of plasma fibronectin.** Fragmentation pathways of pFN by limited proteolysis with thermolysin (*A*), trypsin (*B*), and cathepsin D (*C*), and by *S*-cyanylation (*D*) are schematically presented based on previous reports (Refs. 22, 23, 33, 34, 36, 48) and the results reported here. The nomenclature of the functional domains is taken from Refs. 22 and 49. The molecular weights of cathepsin D fragments are based on the present study and are slightly different from the previous reports by Richter *et al.* (23, 48). The location of the *Fib-2* domain within the tryptic 37K fragment is tentative. The *arrowheads* indicate the major cleavage sites.

ity chromatography using different ligands. For example, human plasma FN can be cleaved into at least four functionally distinct fragments with $M_r$ = 155,000–145,000 (*Cell/Hep-2* domain), 44,000 (*Gel* domain), 24,000 (*Hep-1/Fib-1* domain), and 22,000 (*Fib-2* domain) by mild thermolysin digestion (22) (also see Fig. 1). The domain structure of FNs produced by cells in culture is, however, less well-characterized than that of pFN. Comparison of the domain structure of different FNs may provide a molecular basis for understanding FN polymorphism. In an extensive comparative study (37) of the domain structure of chicken FNs, thermolysin fragments of pFN and cFN separated by various affinity columns were compared. In another study (38), differences between pFN and cFN in the COOH-terminal domain were suggested by a monoclonal antibody which reacts preferentially with cFN.

In our previous study (33), we developed a highly sensitive and specific procedure for the analysis of the domain structure of FNs using a panel of antibodies specific for each functional domain. The method is now applied to three human FNs isolated from plasma and from the culture supernatants of normal and transformed fibroblasts, which were cleaved by limited proteolysis with thermolysin, trypsin, and cathepsin D as well as by *S*-cyanylation, followed by identification of each domain with specific antibodies. The fragmentation patterns of human pFN by these treatments have been well-characterized (13, 22–24, 33–37) and are illustrated schematically in Fig. 1. The results indicate that the COOH-terminal heparin-binding domain, *Hep-2*, of both N-cFN and T-cFN is significantly larger than the same domain of pFN. A significant difference in the fragments derived from the *Hep-2* domain and from the gelatin-binding domain between N-cFN and T-cFN has also been demonstrated by this new procedure.

## EXPERIMENTAL PROCEDURES

*Purification of FNs*—Plasma FN was purified from human plasma by gelatin-Sepharose affinity chromatography as previously described (22) and further purified by heparin-Sepharose chromatography. pFN bound to heparin-Sepharose was eluted with PBS supplemented with 0.5 M NaCl, dialyzed against 2 mM CAPS, 0.5 mM EDTA (pH 11), and stored at −85 °C. The pFN thus purified was found to contain a trace amount of the tryptic 32K fragment[2] which was detectable by immunostaining with anti-*Hep-1/Fib-1* antibodies. The contaminant fragment came from the heparin-Sepharose, which had been used for the purification of the tryptic 32K fragment. cFNs produced by normal human lung fibroblasts WI-38 and their SV-40 transformants WI-38/VA13 were isolated from the conditioned culture medium as follows. Cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum which had been depleted of bovine pFN by passing through a large capacity gelatin-Sepharose column twice. After the cells became confluent, fetal calf serum in the medium was replaced with a mixture of growth hormones including epidermal growth factor (1 ng/ml), insulin (4 μg/ml), transferrin (2.5 μg/ml), and bovine serum albumin (0.1 mg/ml) to avoid possible contamination of bovine FN. Approximately 4 liters of conditioned medium were harvested from 40 plastic roller bottles (Falcon No. 3027) every 2 days, clarified by centrifugation at 10,000 × *g* for 15 min, and applied to a gelatin-Sepharose column (40 ml of bed volume) equipped with a precolumn of underivatized Sepharose. The gelatin-Sepharose column was washed sequentially with PBS containing 1 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA, PBS supplemented with 0.5 M NaCl, and PBS containing 0.8 M urea. Bound FNs were eluted with PBS containing 6 M urea, dialyzed against 2 mM CAPS, 0.5 mM EDTA (pH 11) unless otherwise stated, and stored at −85 °C. The protein concentration of purified FNs was estimated spectrophotometrically by assuming that the absorption coefficient of 0.1% protein solution at 280 nm is 1.28 $cm^{-1}$ (50). FNs thus purified from the condition medium contained a variable amount of contaminant proteins as judged from the protein-staining patterns of these FNs after SDS-polyacrylamide gel electrophoresis. Since these contaminants do not affect the immunoblot analysis, these FNs were used without further purification.

*Fragmentation of FNs*—Thermolysin and trypsin digestion of FNs

[2] 32K, 155K, 145K fragments, etc., fragments with $M_r$ = 30,000, 155,000, 145,000, etc.

was performed as described previously (22). Cathepsin D digestion was performed according to Richter *et al.* (23). *S*-Cyanylation was carried out using 2-nitro-5-thiocyanobenzoic acid as described previously (24). Detailed fragmentation conditions are described in the legend for each figure.

*Subfragmentation of the Thermolysin 155K and 145K Fragments*—The thermolysin-cleaved 155K/145K fragments were purified by DEAE-cellulose chromatography as described previously (13). The purified 155K/145K fragments (3.6 mg) were further digested with thermolysin at an increased protease concentration (15 μg/ml) in 2.7 ml of 25 mM Tris·Cl (pH 7.6) containing 0.5 mM EDTA, 135 mM NaCl, 2.5 mM $CaCl_2$ at 22 °C for 4 h. Digestion was terminated by the addition of EDTA to give a final concentration of 5 mM. The digest was applied to a heparin-Sepharose column (bed volume, 4 ml) equilibrated with 25 mM Tris·Cl (pH 7.6) containing 0.5 mM EDTA and 135 mM NaCl. The bound fragments were eluted with 25 mM Tris buffer containing 1 M NaCl. The unbound and bound fragments were separately dialyzed against 10 mM Tris·Cl (pH 7.6) containing 0.5 mM EDTA, lyophilized, and analyzed by SDS-polyacrylamide gel electrophoresis.

*Domain-specific Antibodies*—Antibodies directed to *Hep-1/Fib-1*, *Gel*, and *Fib-2* were raised in New Zealand white rabbits by immunization with purified domain fragments, *i.e.*, the tryptic 32K fragment (22) for *Hep-1/Fib-1* domain, the thermolysin-cleaved 44K fragment (13) for *Gel* domain, and the thermolysin 22K fragment (13) for *Fib-2* domain. These fragments were purified as described (13, 22) and further purified by preparative gel electrophoresis. Two monoclonal antibodies directed to human pFN, IST-1 and IST-4, were obtained as previously described (25, 26).

*SDS-Polyacrylamide Gel Electrophoresis and Immunoblots*—Proteolytic or *S*-cyanylation-cleaved fragments were separated on 9.5% SDS-polyacrylamide gels by the method of Laemmli (27). Samples were reduced with 2% 2-mercaptoethanol. Gels were either directly stained with Coomassie blue or subjected to electrotransfer of the fragments to nitrocellulose sheets according to Towbin *et al.* (28). After the transfer of proteins, the nitrocellulose sheets were stained with 0.1% Fast green (29) to visualize proteins, then blocked with 2% BSA, and immunostained with domain-specific antibodies and $^{125}$I-protein A as previously described (30). When monoclonal antibodies were used, the nitrocellulose sheets were incubated with rabbit anti-mouse immunoglobin antibodies between the incubation with primary antibodies and $^{125}$I-protein A. The nitrocellulose sheets were then washed with PBS containing 0.05% Triton X-100, briefly blotted with paper towels, and exposed to Kodak XAR-5 X-ray films.

*Materials*—Thermolysin, cathepsin D, pepstatin A, gelatin, soybean trypsin inhibitor, BSA (Fraction V), transferrin, and insulin were obtained from Sigma; trypsin was from Worthington; epidermal growth factor was from Collaborative Research (Lexington, MA); rabbit antimouse immunoglobulin antibodies (light and heavy chains specific) was from Cappel Laboratories (Cochranville, PA); 2-nitro-5-thiocyanobenzoic acid was from Eastman Kodak; Na$^{125}$I was from New England Nuclear; ultra pure urea was from Schwarz/Mann (Orangeburg, NY). Gelatin-Sepharose was prepared according to Cuatrecasas and Anfinsen (31). Iodination of protein A was performed by a chloramine-T method (32). Heparin-Sepharose was a generous gift of Dr. Kazuo Fujikawa (Department of Biochemistry, University of Washington).

## RESULTS

### *Domain Specificities of Antibodies*

Three polyclonal and two monoclonal antibodies were used as specific probes for four functionally distinct FN domains: *Hep-1/Fib-1*, *Gel*, *Cell/Hep-2*, and *Fib-2* (summarized in Table I). Two monoclonal anti-FN antibodies, IST-1 and IST-4, were found to recognize different regions of the *Cell/Hep-2* domain (Fig. 2). Among the fragments of pFN cleaved by *S*-cyanylation, IST-1 bound to sc135K and sc125K fragments, whereas IST-4 bound to the sc160K fragment (Fig. 2). The former fragments (*i.e.*, sc135K and sc125K) were derived from the COOH-terminal halves of α and β subunits of pFN, whereas the latter fragment was derived from the $NH_2$-terminal half (33, 34) (also see below). IST-1 also bound to 40K and 30K thermolysin fragments in addition to the 155K/145K (Fig. 2*B*). In contrast, IST-4 recognized a 105K fragment, but not the 40K nor 30K fragments (Fig. 2*C*). This indicates that the 40K and 30K fragments originated from the COOH-terminal region of the *Cell/Hep-2* domain, which is considered to be the heparin-binding domain.

TABLE I

*Summary of the domain-specific antibodies used in the present study*

| Antibodies | Domain specificity | Polyclonal/ monoclonal | Antigen used for immunization |
|---|---|---|---|
| Anti-*Hep-1/Fib-1* | *Hep-1/Fib-1* | Polyclonal | Tryptic 32K[a] |
| Anti-*Gel* | *Gel* | Polyclonal | Thermolysin-cleaved 44K[b] |
| IST-4 | *Cell* | Monoclonal | pFN |
| IST-1 | *Hep-2* | Monoclonal | pFN |
| Anti-*Fib-2* | *Fib-2* | Polyclonal | Thermolysin-cleaved 22K[b] |

[a] Prepared as described in Ref. 22.
[b] Prepared as described in Ref. 13.

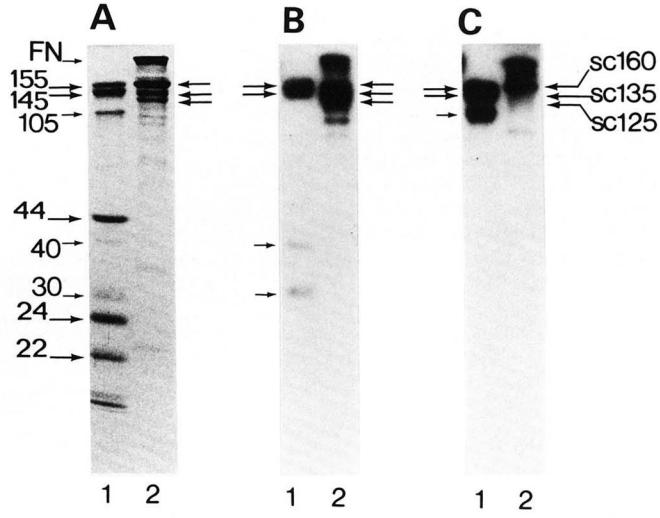


FIG. 2. **Domain specificity of two monoclonal antibodies, IST-1 and IST-4.** The binding specificity of two monoclonal anti-human pFN antibodies, IST-1 and IST-4, was examined using thermolysin fragments (*lane 1*) and *S*-cyanylation-cleaved fragments (*lane 2*) of pFN by immunoblots. *A*, protein staining; *B* and *C*, immunostaining with IST-1 and IST-4, respectively. The pFN used here was dialyzed against 10 mM Tris·Cl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl instead of 2 mM CAPS buffer, pH 11. pFN (1.0 mg/ml) was digested with thermolysin (2.5 μg/ml) for 2 h at 22 °C. The enzyme/substrate ratio was thus 1:400. *S*-Cyanylation was performed as described under "Experimental Procedures." The apparent molecular weights of the fragments of interest are shown in the margin.

In support of this conclusion, purified 155K/145K fragments were further degraded into 105K, 40K, and 30K fragments upon prolonged thermolysin digestion (Fig. 3*A*, *lane 1*). Fractionation of these fragments on heparin-Sepharose indicated that the 40K and 30K fragments were capable of binding to heparin, but that the 105K fragment was not (Fig. 3*A*, *lanes 2* and *3*). IST-1 selectively bound to the heparin-binding 40K and 30K fragments (Fig. 3*B*), confirming that IST-1 is directed to the *Hep-2* domain located at the COOH-terminal region of the *Cell/Hep-2* domain. In contrast, IST-4 bound to the heparin-unbound 105K fragment, which is considered to be derived from the $NH_2$-terminal portion of the *Cell/Hep-2* domain, *i.e.*, the *Cell* domain. Specificities of IST-1 and IST-4 were further confirmed by the binding of these antibodies

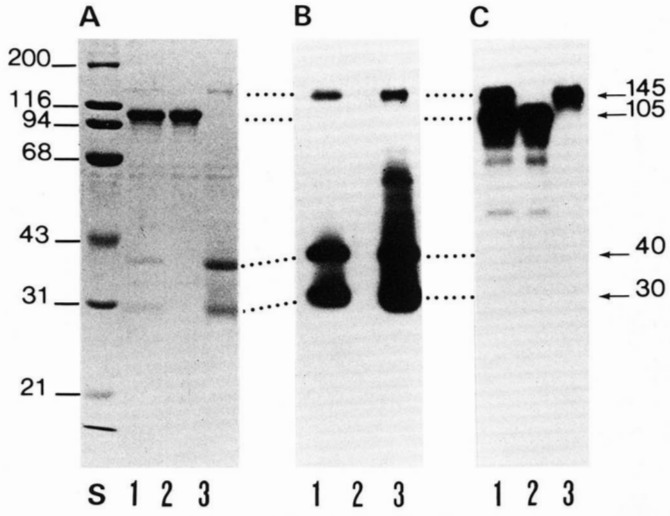


FIG. 3. **Subdivision of the 155K/145K thermolysin fragments.** The purified 155K/145K fragments were cleaved further by thermolysin using an increased enzyme concentration (15 μg/ml). The digest was fractionated on a heparin-Sepharose column as described under "Experimental Procedures." The unfractionated digest (*lane 1*), heparin-unbound fragments (*lane 2*), and heparin-bound fragments (*lane 3*) were analyzed by immunoblots. *A*, protein staining of the gel; *B* and *C*, immunostaining with IST-1 and IST-4, respectively. Molecular weights of the standard proteins (*A*, *lane S*) are shown in the *left margin*. A small amount of the 145K fragment remained undigested under these conditions.

to the cathepsin D fragments of the three different FNs as described below.

### *Comparison of Domain Structure of pFN, N-cFN, and T-cFN by Limited Proteolysis and S-Cyanylation*

*Thermolysin Digestion*—Thermolysin has been shown to dissect FNs into their constitutive domains (13, 22, 35) (also see Fig. 1*A*). Fig. 4 shows the thermolysin digestion patterns of pFN, N-cFN, and T-cFN. Fragments containing each domain were identified by immunostaining with domain-specific antibodies (Fig. 4, *B–F*). The *Hep-1/Fib-1* domain of all three FNs was released as a 24K fragment and its 27K precursor (Fig. 4*B*). These fragments were qualitatively identical irrespective of the FN type. The *Gel* domain of these FNs was obtained as a 44K fragment and its 60K precursor (Fig. 4*C*); however, the 44K/60K fragments of N-cFN were slightly smaller than those of pFN (Fig. 4*C*, *lanes 4–6*). In contrast, the 44K/60K fragments of T-cFN were consistently slightly larger than those of pFN. The *Fib-2* domain was released as the same 22K fragment from all three FNs (Fig. 4*F*), although immunostaining of this fragment from T-cFN was consistently weaker than that from N-cFN or pFN. These results indicate that pFN, N-cFN, and T-cFN consist of the same *Hep-1/Fib-1*, *Gel*, and *Fib-2* domains, although the *Gel* domain of T-cFN is slightly larger than that of N-cFN or pFN.

In contrast, the *Cell/Hep-2* domain of pFN, N-cFN, and T-cFN gave similar, yet significantly different, fragments upon thermolysin digestion. The *Cell/Hep-2* domain of pFN was released as 175K and 145K fragments after brief thermolysin digestion (Fig. 4, *D* and *E*, *lane 4*). The 175K fragment contained both *Cell/Hep-2* and *Fib-2* domains and subsequently degraded into 155K (*Cell/Hep-2*) and 22K (*Fib-2*) fragments (Fig. 4, *D* and *F*, *lanes 7* and *10*). The 155K and 145K fragments were further degraded into a 105K fragment (reactive with IST-4) and 40K/30K fragments (reactive with IST-1) as shown in Fig. 3.

The *Cell/Hep-2* domain of N-cFN and T-cFN, however, was released as 155–160K fragment(s) upon brief thermolysin digestion (Fig. 4, *D* and *E*, *lanes 5* and *6*). These fragments were further degraded into a 105K fragment reactive with IST-4 (Fig. 4*D*, *lanes 8*, *9*, *11*, and *12*) and multiple 30–40K fragments reactive with IST-1 (Fig. 4*E*, *lanes 8*, *9*, *11*, and *12*). The 105K fragment of T-cFN was slightly larger than the 105K fragment of N-cFN.

A clearer difference among pFN, N-cFN, and T-cFN was found in the 30–40K *Hep-2*-derived fragments reactive with IST-1. The *Hep-2* domain of N-cFN gave three distinct fragments, 40K, 35–32K, and 30K (Fig. 4*E*, *lane 11*). The 35–32K fragment gave a broad band upon immunostaining with IST-1. In contrast, the *Hep-2* domain of T-cFN gave only the 35–32K fragment, but not the 40K or 30K fragments (Fig. 4*E*, *lane 12*). Since the *Hep-2* domain of pFN gave only 40K and 30K fragments (Fig. 4*E*, *lane 10*), the 35–32K fragment appears to be unique to cFNs. These results indicate that the structure of the *Hep-2* domain is significantly different among pFN, N-cFN, and T-cFN.

*Trypsin Digestion*—Gross domain structure of these three FNs was compared further by trypsin digestion (Fig. 5). Mild trypsin digestion of pFN produced four major fragments, 215K, 185K, 37K, and 32K (Fig. 5*A*, *lane 4*). Immunostaining with a panel of domain-specific antibodies showed that the 32K fragment was derived from the $NH_2$-terminal *Hep-1/Fib-1* domain (Fig. 5*B*, *lane 4*), whereas the 37K fragment was from the COOH-terminal *Fib-2* domain (Fig. 5*F*, *lane 4*). Both 215K and 185K fragments contained the internal *Gel* and *Cell/Hep-2* domains (Fig. 5, *C–E*, *lane 4*). The 215K, but not the 185K, fragment also contained the *Fib-2* domain (Fig. 5*F*, *lane 4*). These results are in good agreement with the previous finding (22, 36) that the 185K and 37K fragments are derived from the α subunit, whereas the 215K fragment is from the β subunit of pFN (see also Fig. 1*B*).

The 215K and 185K fragments were further degraded to 145K, 115K, and 80K fragments at higher trypsin concentration (Fig. 5*A*, *lane 7*). Both the 145K and 115K fragments were immunostained by IST-1 and IST-4 antibodies, but not by anti-*Gel* antibodies (Fig. 5, *C–E*, *lane 7*). The 145K fragment, but not the 115K fragment, was also stained by anti-*Fib-2* antibodies (Fig. 5*F*, *lane 7*). In contrast, the 80K fragment was stained only by anti-*Gel* antibodies (Fig. 5*C*, *lane 7*). These results indicate that the 215K and 185K fragments were degraded to the 145K and 115K fragments, respectively, upon release of the *Gel* domain as the 80K fragment.

Mild trypsin digestion of both N-cFN and T-cFN also gave four major fragments, 210K, 165K, 37K, and 32K (Fig. 5*A*, *lanes 5* and *6*). The 210K and 165K fragments derived from T-cFN were slightly larger than those from N-cFN. Immunostaining with domain-specific antibodies showed that the 32K fragment was derived from the *Hep-1/Fib-1* domain (Fig. 5*B*, *lanes 5* and *6*), and the 37K fragment was derived from the *Fib-2* domain (Fig. 5*F*, *lanes 5* and *6*), as was the case for pFN. A 43K fragment that was barely detectable by protein staining was also identified by anti-*Fib-2* antibodies in the trypsin digests of all three FNs (Fig. 5*F*, *lanes 4–9*). This fragment may be a precursor form of the 37K fragment or may reflect a minor polymorphism in the *Fib-2* domain. Neither the 210K nor 165K fragment was immunostained by anti-*Hep-1/Fib-1* or anti-*Fib-2* antibodies. Instead, both were stained by anti-*Gel* and IST-4 antibodies (Fig. 5, *C* and *D*, *lanes 5* and *6*). The 210K fragment, but not the 165K fragment, was also stained by IST-1 (Fig. 5*E*, *lanes 5* and *6*). IST-1 also stained multiple 58–64K fragments. These results suggest that the 210K fragment degrades into the 165K fragment upon release of the *Hep-2* domain as 58–64K fragments. The

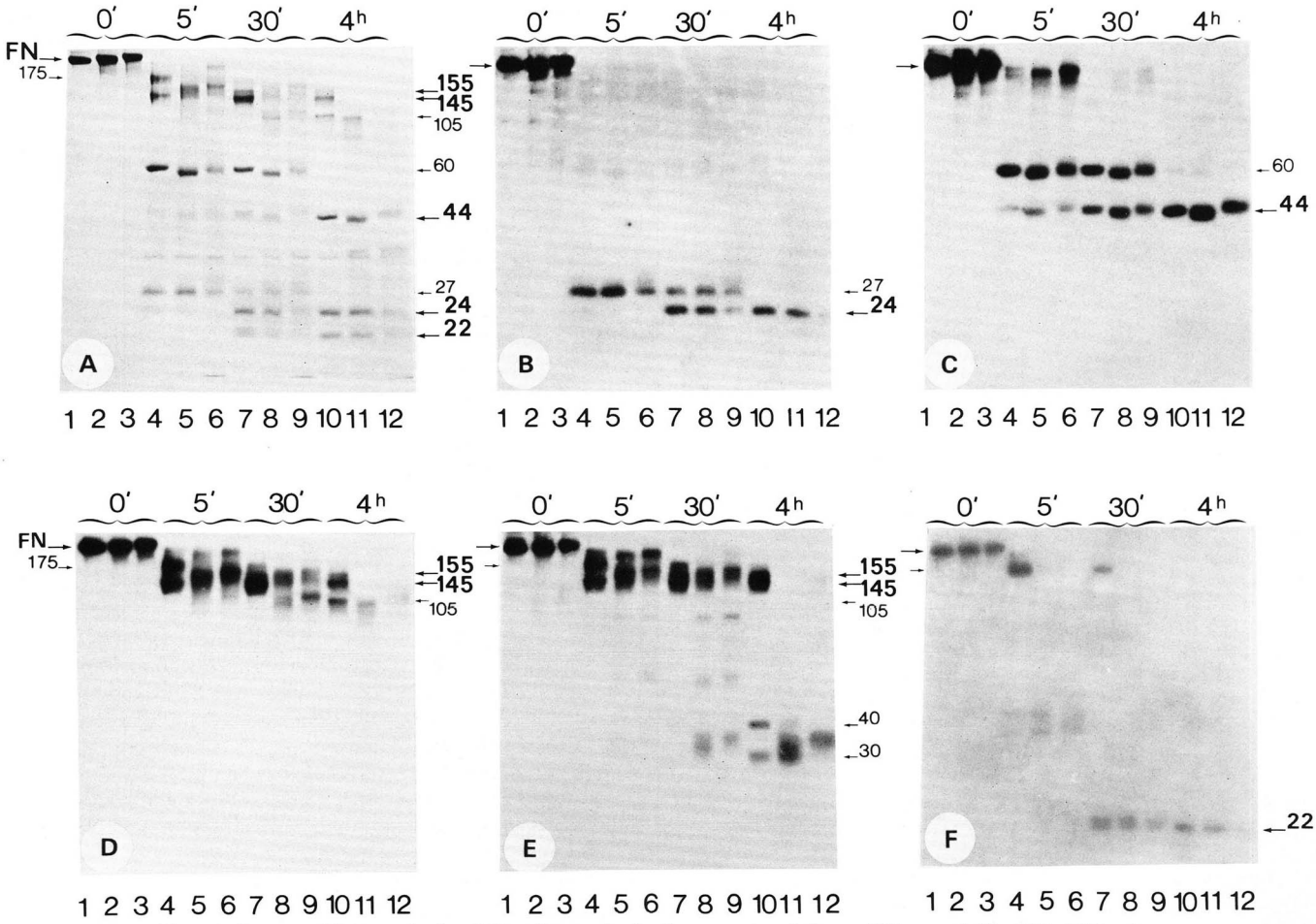


FIG. 4. **Immunoblot analysis of the thermolysin fragments of three different FNs.** FNs (100 μg) were digested with thermolysin (2.5 μg/ml) in 1 ml of 20 mM Tris·Cl (pH 7.8) containing 1.9 mM CAPS, 50 mM NaCl, 0.5 mM EDTA, and 2.5 mM $CaCl_2$ at 22 °C for 0 min (*lanes 1–3*), 5 min (*lanes 4–6*), 30 min (*lanes 7–9*), and 4 h (*lanes 10–12*). The enzyme/substrate ratio was 1:40. Digestion was terminated by the addition of EDTA to give a final concentration of 5 mM. Five micrograms of protein were applied to *lanes 1–3* and 10 μg to *lanes 4–12. Lanes 1, 4, 7,* and *10*, pFN; *lanes 2, 5, 8,* and *11*, N-cFN; *lanes 3, 6, 9,* and *12*, T-cFN. *A*, protein staining of the gel with Coomassie blue; *B* through *F*, immunostaining of the nitrocellulose blots with anti-*Hep-1/Fib-1* (*B*), anti-*Gel* (*C*), IST-4 (*D*), IST-1 (*E*), and anti-*Fib-2* (*F*) antibodies. Molecular weights of the fragments of interest are shown in the *right margin* of each *panel.*

210K fragment appears to correspond to the 185K fragment of pFN, because these fragments share the same internal *Gel, Cell,* and *Hep-2* domains. Since the *Gel* and *Cell* domains of pFN and the two cFNs were obtained as similar 44K/60K and 105K fragments, respectively, the difference between pFN and the cFNs appears to be in the *Hep-2* domain.

*Cathepsin D Digestion*—Cathepsin D has been shown to release the COOH-terminal region of pFN by retaining the interchain disulfide bond(s) (23). Short cathepsin D digestion of pFN produced three fragments, 200K, 70K, and 60K, among which the latter two fragments were weakly detectable by protein staining (Fig. 6*A*, *lane 4*). The 200K fragment was immunostained by anti-*Hep-1/Fib-1*, anti-*Gel*, and IST-4 antibodies, but not by IST-1 nor anti-*Fib-2* antibodies (Fig. 6, *B–F*, *lane 4*). Conversely, the 70K and 60K fragments were stained by IST-1 and anti-*Fib-2* antibodies, but not by anti-*Hep-1/Fib-1*, anti-*Gel*, nor IST-4 antibodies (Fig. 6, *B–F*, *lane 4*). The 70K and 60K fragments were either connected to each other or to the uncleaved subunit chain by disulfide bond(s) (23) (data not shown) and they appear to correspond to the 75K and 65K cathepsin D fragments reported previously by Richter *et al.* (23) to be derived from the COOH-terminal region of pFN.

In contrast, the COOH-terminal region of both N-cFN and T-cFN gave much larger fragments than the 70K/60K fragments of pFN. Both IST-1 and anti-*Fib-2* antibodies specifically stained a 100K fragment after a 3-h digestion (Fig. 6, *E* and *F*, *lanes 5* and *6*), whereas the $NH_2$-terminal and central portion of both cFNs were released as a 200K fragment as was the case for pFN (Fig. 6, *B–D*, *lanes 5* and *6*). The 200K fragment derived from T-cFN migrated slightly above the corresponding fragments produced from pFN and N-cFN (Fig. 6, *A–C*, *lanes 4–6*).

Prolonged cathepsin D digestion of pFN gave additional 130K, 90K, and 67K fragments (Fig. 6*A*, *lane 7*). The 67K fragment was immunostained by anti-*Hep-1/Fib-1* and anti-*Gel* antibodies (Fig. 6, *B* and *C*, *lane 7*), indicating that this fragment represents the $NH_2$-terminal region of pFN. The 130K and 90K fragments were stained by IST-4 antibody (Fig. 6*D*, *lane 7*). These results indicate that the 200K fragment generated at the early phase of cathepsin D digestion was degraded to the $NH_2$-terminal 67K and central 130K fragments upon prolonged digestion, whereas the 130K fragment was further degraded into a 90K fragment.

Prolonged cathepsin D digestion of N-cFN and T-cFN produced additional 74–83K fragments reactive with IST-1

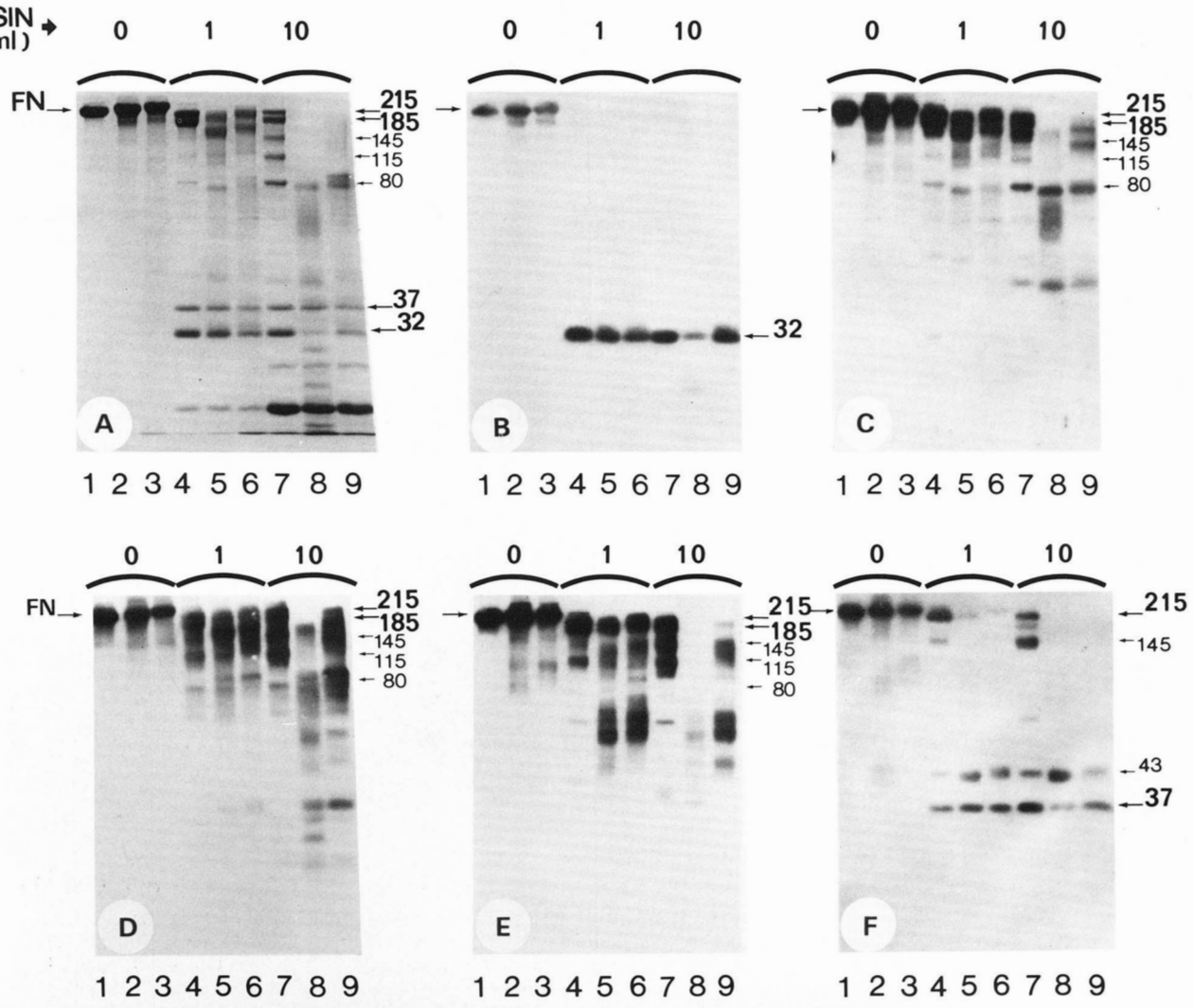


FIG. 5. **Immunoblot analysis of the tryptic fragments of three different FNs.** FNs (100 μg) were digested with different concentrations of trypsin in 20 mM Tris·Cl (pH 7.8) containing 1.9 mM CAPS, 50 mM NaCl, and 0.5 mM EDTA at 22 °C for 10 min. Digestion was terminated by the addition of soybean trypsin inhibitor to give a final concentration of 2.5 μg/ml. Trypsin concentrations used were: 0 μg/ml (*lanes 1–3*), 1 μg/ml (*lanes 4–6*), and 10 μg/ml (*lanes 7–9*). Ten micrograms of protein were applied to each lane. *Lanes 1*, *4*, and *7*, pFN; *lanes 2*, *5*, and *8*, N-cFN; *lanes 3*, *6*, and *9*, T-cFN. *A*, protein staining of the gel; *B* through *F*, immunostaining of the nitrocellulose blots with anti-*Hep-1/Fib-1* (*B*), anti-*Gel* (*C*), IST-4 (*D*), IST-1 (*E*), and anti-*Fib-2* (*F*) antibodies.

and anti-*Fib-2* antibodies (Fig. 6, *E* and *F*, *lanes 8* and *9*). These fragments often gave a broad band upon immunostaining, but appeared to consist of two fragments with approximately $M_r$ = 74,000 and 83,000. The $NH_2$-terminal portion of both cFNs was released after prolonged digestion as a 67K fragment, which was strongly reactive with anti-*Hep-1/Fib-1* and anti-*Gel* antibodies (Fig. 6, *B* and *C*, *lanes 8* and *9*). Longer digestion of cFNs also produced 130K and 90K fragments which were recognized by the IST-4 antibody (Fig. 6, *D*, *lanes 8* and *9*). These results indicate that the $NH_2$-terminal and central regions of cFNs are very similar, if not identical, to that of pFN, whereas the COOH-terminal region of both cFNs was significantly larger than that of pFN. However, there was no clear difference between N-cFN and T-cFN even at the COOH-terminal region.

*S-Cyanylation*—A difference in the COOH-terminal region of pFN and cFNs was further demonstrated by *S*-cyanylation. *S*-Cyanylation of human pFN has been shown to produce three major fragments, sc160K, sc135K, and sc125K (33, 34) (also see Figs. 1*D* and 7*A*, *lane 1*). The sc160K represents the $NH_2$-terminal half of both α and β subunits as revealed by specific immunostaining with anti-*Hep-1/Fib-1* and anti-*Gel* antibodies (33) (Fig. 7, *B* and *C*, *lane 1*). The sc135K and 125K fragments represent the COOH-terminal halves of the α and β subunits (33) and were immunostained by anti-*Fib-2* antibodies (Fig. 7*D*, *lane 1*).

*S*-Cyanylation of both N-cFN and T-cFN apparently produced only one major fragment with $M_r$ = 155,000 (Fig. 7*A*, *lanes 2* and *3*). Immunostaining with anti-*Hep-1/Fib-1* and anti-*Gel* antibodies detected a sc155K fragment as the $NH_2$-terminal half of both cFNs (Fig. 7, *B* and *C*, *lanes 2* and *3*). The COOH-terminal half of N-cFN, however, was identified by anti-*Fib-2* antibodies as a broad 160–145K band (Fig. 7*D*, *lane 2*). Although the resolution of the closely spaced bands on the autoradiogram was limited, the COOH-terminal half of N-cFN appears to consist of two discrete bands, sc160K and sc145K. This may indicate that N-cFN consists of two nonidentical subunit chains (see also under "Discussion"). In contrast, the COOH-terminal half of T-cFN gave a sc160K fragment, but almost no sc145K fragment, stainable by anti-*Fib-2* antibodies (Fig. 7*D*, *lane 3*).

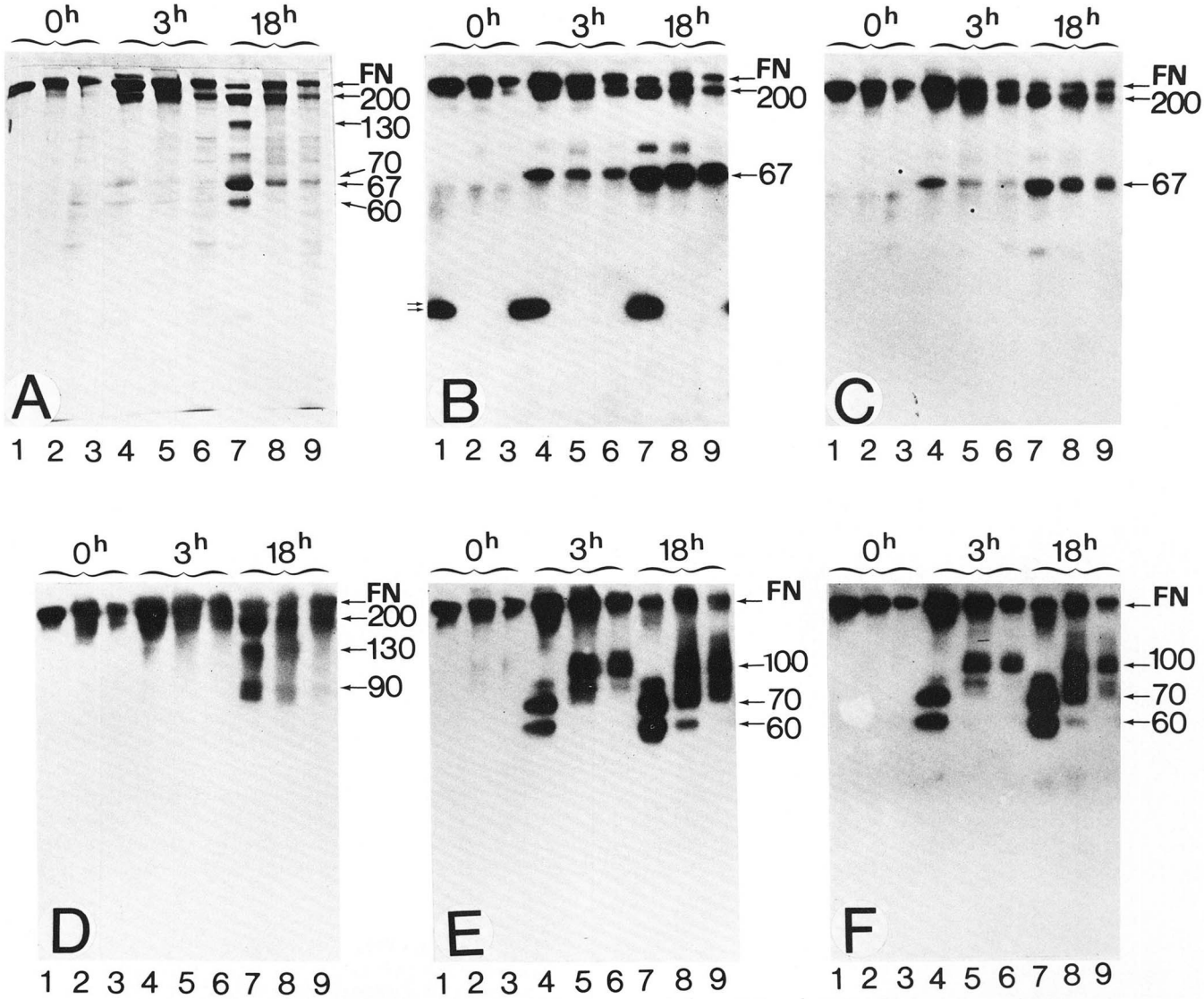


FIG. 6. **Immunoblot analysis of cathepsin D fragments of three different FNs.** FNs (80 μg) were digested with cathepsin D at an enzyme/substrate ratio of 1:250 in 1 ml of 50 mM sodium acetate (pH 3.5) containing 1.8 mM CAPS, 0.5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride at 37 °C for 0 h (*lanes 1–3*), 3 h (*lanes 4–6*), and 18 h (*lanes 7–9*). Digestion was terminated by the addition of 10 times molar excess of pepstatin A and 28 μl of 2.5 M Tris·Cl (pH 8.8) to bring the pH to approximately 7.5. Five micrograms of protein were applied to *lanes 1–3* and 7.5 μg to *lanes 4–9*. *Lanes 1*, *4*, and *7*, pFN; *lanes 2*, *5*, and *8*, N-cFN; *lanes 3*, *6*, and *9*, T-cFN. *A*, the protein staining of the gel; *B* through *F*, show the immunostaining of the nitrocellulose blots with anti-*Hep-1/Fib-1* (*B*), anti-*Gel* (*C*), IST-4 (*D*), IST-1 (*E*), and anti-*Fib-2* (*F*) antibodies. The *small double arrows* in *B* indicate the contaminant tryptic 32K fragment in pFN.

## DISCUSSION

The domain structure of three different FNs, pFN, N-cFN, and T-cFN, was compared by limited proteolysis and *S*-cyanylation followed by identification of each domain by a panel of domain-specific antibodies. This approach is highly sensitive, specific, and capable of picking up small quantities of fragments derived from specific domains. Fig. 8 shows a proposed model of the domain structure of cFNs and their fragmentation patterns upon proteolytic and chemical degradation. The corresponding patterns of pFN, based on previous studies (13, 22–24, 33–37), are shown in Fig. 1. The results indicate clearly that the overall composition and alignment of the functional domains of all three FNs are essentially identical. However, one major difference between pFN and the two cFNs was found at the COOH-terminal region, *i.e.*, the *Hep-2* domain, detectable by monoclonal antibody IST-1. Although the differences between N-cFN and T-cFN were minor, a clear distinction in the thermolysin fragments representing the *Gel* and *Hep-2* domains and in the tryptic fragments representing internal domains were observed.

*Difference between pFN and cFNs*—Several lines of evidence indicate that the region showing the major difference between pFN and cFNs is localized within the *Hep-2* domain. (i) Limited proteolysis with thermolysin and trypsin showed that pFN and both cFNs consist of almost identical *Hep-1/Fib-1*, *Gel*, *Cell*, and *Fib-2* domains. However, the thermolysin fragments representing the *Hep-2* domain of pFN were clearly different from those of cFNs. The *Hep-2* domain of cFNs, identified by IST-1 antibody, gave a characteristic 35–32K fragment, whereas the same domain of pFN was obtained as 40K and 30K fragments. (ii) The COOH-terminal *Hep-2* and *Fib-2* domains of pFN were released by cathepsin D as 70K

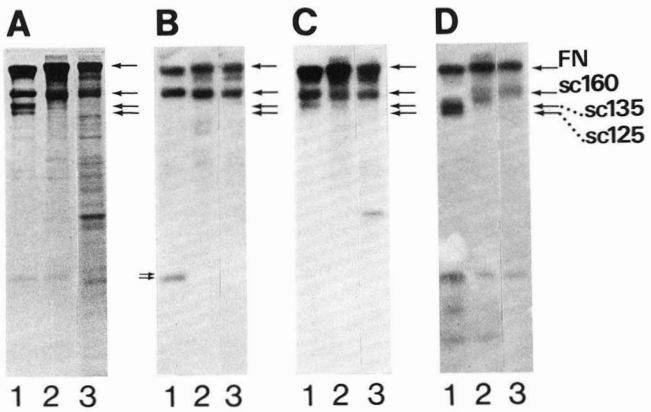


FIG. 7. **Immunoblot analysis of *S*-cyanylation fragments of three different FNs.** FNs (200 μg) were *S*-cyanylated with 0.5 mM 2-nitro-5-thiocyanobenzoic acid in 1 ml of 0.1 M Tris/acetate (pH 8.0) containing 0.5 mM EDTA and 0.1% SDS. The modified FNs were dialyzed against 20 mM Tris/acetate (pH 9.0) containing 0.1 mM EDTA and 0.1% SDS for the cleavage. The cleaved FNs were dialyzed against 10 mM Tris·Cl (pH 7.0) containing 0.5 mM EDTA, lyophilized, and subjected to immunoblot analysis. *Lane 1*, pFN; *lane 2*, N-cFN; *lane 3*, T-cFN. *A*, the protein staining of the gel; *B* through *D*, the immunostaining with anti-*Hep-1/Fib-1* (*B*), anti-*Gel* (*C*), and anti-*Fib-2* (*D*) antibodies. Twelve micrograms of protein were applied to each lane. The *small double arrows* in *B* indicate the contaminant tryptic 32K fragment in the purified pFN.

and 60K fragments (23), whereas the same region of both N-cFN and T-cFN was obtained as 100K and 83K-74K fragments by cathepsin D digestion. Since there was no significant difference in the *Fib-2* domain between pFN and cFNs, the difference in the cathepsin D-derived, COOH-terminal fragments could be mapped to the *Hep-2* domain. Thus, the *Hep-2* domain of cFNs appears to be 30,000–20,000 daltons larger than the *Hep-2* domain of pFN. In support of this conclusion, the major *Hep-2* derived fragments obtained from pFN and cFNs by tryptic digestion or *S*-cyanylation also showed clear differences. The *Hep-2* domain of cFNs was recovered within a 210K fragment, which spans from the *Gel* through the *Hep-2* domain, upon mild trypsin digestion. The corresponding region of pFN was released as a 185K fragment. The COOH-terminal half of cFNs, including *Hep-2*, *Fib-2*, and a part of *Cell* domains, was obtained as sc160K–145K fragments upon *S*-cyanylation, whereas the corresponding COOH-terminal half of pFN was obtained as sc135K/125K fragments.

Conversely, the $NH_2$-terminal half of cFNs (the sc155K fragment) was approximately 5,000 daltons smaller than the $NH_2$-terminal half of pFN (the sc160K fragment). This difference may be attributable to differences in the carbohydrate moieties, which have been shown to cluster in the $NH_2$-terminal half (35). Similarly, the 44K/60K thermolysin fragments derived from the *Gel* domain showed a small difference in electrophoretic mobility between pFN and N-cFN; the 44K/60K fragments of N-cFN, but not T-cFN, appeared to be slightly smaller than those of pFN. A similar difference has been observed between chicken pFN and cFN (37).

Previously, Hayashi and Yamada (37) compared the polypeptide structure of chicken pFN and cFN by limited proteolysis with thermolysin followed by fractionation of the resulting fragments on various affinity columns. Three regions showed differences between pFN and cFN. One difference was in the *Gel* domain and the other two differences were in the *Cell/Hep-2* domain where pFN had an 11,000-dalton deletion in one site and a 1,000-dalton extrasegment in the other site (37). These authors mapped the 11K deletion site in pFN at the $NH_2$-terminal region of the *Cell/Hep-2* domain. Our data, however, strongly suggest that the major deletion site in human pFN is in the COOH-terminal region of the domain, *i.e.*, *Hep-2*. This apparent discrepancy may be the result of species differences in the domain structure of FNs.

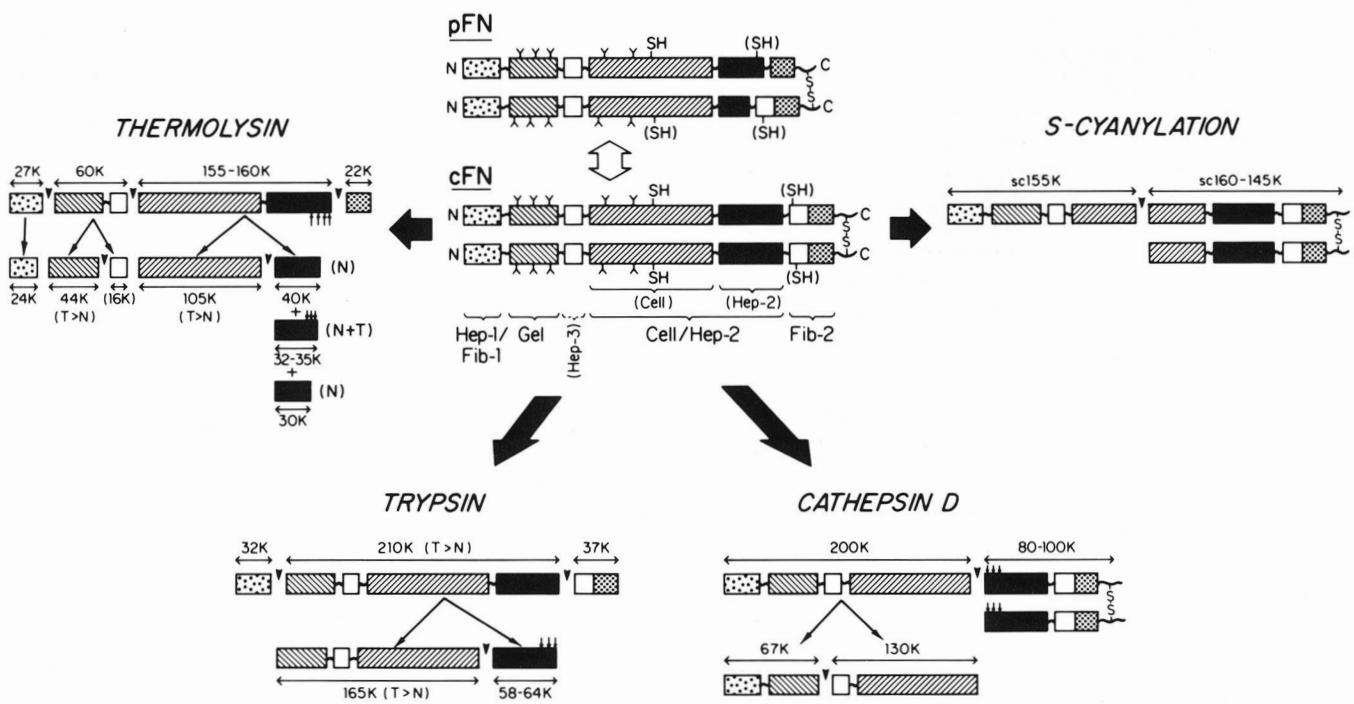


FIG. 8. **A proposed model for the domain structure of cFNs and their fragmentation patterns.** The corresponding model for pFN is shown in Fig. 1. The (*Y*) attached to intact FNs (*center*) represents the carbohydrate units attached to polypeptide chains. *Small arrows* (↓↓) indicate the multiple cleavage sites, which generate several fragments with slightly different molecular weights. (*N*) and (*T*) stand for N-cFN and T-cFN, respectively. (*T* > *N*) indicates that the fragments derived from T-cFN are slightly larger than those from N-cFN.

Alternatively, the topological mapping of the fragments based on their binding specificities to various ligands may not be exclusive of other possibilities, since there are multiple binding sites in FNs for heparin, DNA, and *Staphylococcus aureus* (37); mapping of the 11K deletion site in chicken pFN was based essentially on the binding of thermolysin fragments to these ligands. A structural difference near the COOH-terminal end between pFN and cFN has also been suggested by Atherton and Hynes (38) using an anti-FN monoclonal antibody that preferentially reacted with cFN.

Recently, several groups have reported the isolation of cDNA clones for chicken, human, and rat FNs (39–43). Schwarzbauer *et al.* (42) reported that rat liver contained three different mRNAs, among which two shorter mRNAs had 75 and 360 base deletions within the region encoding the COOH-terminal heparin-binding domain. These findings are consistent with our present observations. Kornblihtt *et al.* (43) also found two different mRNAs in human breast carcinoma cells; one of them had a deletion of 270 bases in the region encoding the *Hep-2* domain. This 270 nucleotide sequence was found to correspond to the so-called "type III homology" which is repeated more than six times in the *Cell/Hep-2* domain (44, 45).

*Differences between N-cFN and T-cFN*—In spite of the clear difference between pFN and cFNs described above, the domain fragments released from N-cFN and T-cFN defined by antibodies to *Hep-1/Fib-1*, *Gel*, and *Fib-2* domains were essentially identical. There was, however, a clear difference between N-cFN and T-cFN in the *Hep-2* domain defined by the IST-1 antibody, *i.e.*, three fragments (40K, 35–32K, and 30K) were released from N-cFN, while only the 35–32K fragment was released from T-cFN on extensive thermolysin digestion. In addition, the following minor differences between N-cFN and T-cFN in size of domain fragments were observed. (i) The 44K/60K *Gel* domain fragments from T-cFN were consistently larger than those from N-cFN; (ii) the tryptic 210K and 165K fragments of T-cFN were slightly larger than those of N-cFN; (iii) the 200K cathepsin D fragment of T-cFN was slightly larger than that of N-cFN; and (iv) the COOH-terminal half of N-cFN was obtained as a mixture of sc160K and sc145K fragments, whereas the COOH-terminal half of T-cFN was obtained only as a sc160K fragment. Since human carcinoma cells have been shown to contain two different FN mRNAs (43), the sc160K and sc145K fragments of N-cFN may represent the COOH-terminal halves of two different subunit polypeptides. The structural basis for the differences between N-cFN and T-cFN described above is unknown. It may well be due to the degree of phosphorylation (20) and to differences in carbohydrate structure detectable between N-cFN and T-cFN (21). Although glycosylation occurs densely at the *Gel* domain (35), it also occurs in the COOH-terminal half.[3] Further extensive study in domain distribution of carbohydrates is obviously important to clarify the domain polymorphism of the various cFNs and their matrix forms. These differences in post-translational modification may have important functional significance.

The FN produced by cultured fibroblasts is partly deposited in the pericellular matrix and partly released into the culture medium. Recently, we analyzed the domain structure of the cFN in the pericellular matrix of normal human fibroblasts (33). The proteolytic and chemical fragmentation profiles of the matrix form of cFN were very similar to those of released form of cFN reported here. Further detailed studies are needed to substantiate the identity of matrix form and released form of cFN. It is possible, however, that matrix form and released form of cFNs share the same polypeptide structure, but differ in their degree of disulfide bond-dependent polymerization (17–19). It should be noted that the cFNs so far analyzed for domain structure were all produced *in vitro* by cultured fibroblasts, whereas pFN was obviously produced *in vivo*, probably by liver cells (46, 47). It will be important to study tissue FN; however, it has been difficult to obtain tissue FN in an intact form. The protocol used in the present study does not require purification of these proteins and has been successfully applied to the structural analysis of cFN in the pericellular matrix (33). The same method should be applicable for the analysis of cFN in tissue.

[3] E. Nichols and S. Hakomori, unpublished observation.

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