The Alternatively Spliced V Region Contributes to the Differential Incorporation of Plasma and Cellular Fibronectins into Fibrin Clots

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Abstract. During blood clot formation in vivo, plasma fibronectin (pFN) is cross-linked to fibrin by coagulation factor XIIIa. Cellular FN (cFN), which localizes to connective tissue, is distinguished from pFN by the inclusion of alternatively spliced segments. To determine if these two FNs are functionally equivalent in blood clotting, the cross-linking of rat pFN and cFN to fibrin was compared in an in vitro clotting assay. Fibrinogen and FN were incubated at physiological ratios in the presence of thrombin and factor XIIIa. Cross-linking of FN to fibrin was monitored by SDS-PAGE and immunoblotting. Over 24 h, cFN was incorporated at a significantly slower rate than pFN and was not completely cross-linked to fibrin at a temperature that favors this interaction (0°C). This difference was observed with purified fibrinogens from human, rat, and bovine and with rat plasma and was maintained even after incubation of pFN with rat fibroblasts for several days. Using the same assay, purified recombinant V^+-V0 and V^+-V^+ FN dimers resembling pFN and cFN, respectively, showed a similar difference in cross-linking kinetics. These results suggest that the asymmetric distribution of the V region among pFN dimers plays a role in regulating its incorporation into blood clots.

In fibrin clots, cFN was converted into a set of cross-linked intermediates distinct from those of pFN. For example, while pFN was initially cross-linked into a pFN–fibrin α heterodimer, this product was not a major intermediate in clots formed with cFN. This finding, in conjunction with evidence for the formation of factor XIIIa–catalyzed cFN–cFN cross-links, indicated that cFN molecules interact with each other, and with fibrin, differently from pFN. Together, these results show an important functional distinction between pFN and cFN.

In tissue repair and remodeling, fibronectin (FN)[1] plays a crucial role in the assembly of specialized matrices important for these processes. When blood vessel injury occurs in an incisional skin wound, for example, leakage of plasma proteins into extravascular spaces activates zymogens of the clotting cascade to form a hemostatic plug, or blood clot (see Furie and Furie, 1988), containing fibrin, erythrocytes, platelets, and plasma FN (pFN) as major components. This structure is stabilized by covalent cross-links introduced between fibrin molecules (Lorand, 1972) and between fibrin and FN molecules by coagulation factor XIIIa (plasma transglutaminase) (Mosher, 1975, 1976). The fibrin-FN clot is adhesive and serves as a provisional matrix supporting the migration of epidermal cells during wound closure and the influx of inflammatory cells, fibroblasts, and endothelial cells into the wound bed as healing progresses (for reviews, see Clark and Henson, 1988; Mosher, 1989; Hynes, 1990).

In its genesis, a blood clot contacts interstitial tissue composed of collagens, proteoglycans, and cellular FN (cFN). Fibroblasts migrate from the stroma into the wound (Clark and Henson, 1988) to synthesize, secrete, and organize cFN into insoluble fibrils, providing a framework for subsequent collagen deposition (McDonald et al., 1982). While fibroblasts and most other cell types, including endothelial cells and macrophages, are sources of cFN, pFN is produced only in the liver (see Hynes, 1990). Although both forms are generated from a single gene, they are structurally differentiated by cell type–specific alternative splicing at three sites in the FN mRNA, namely, EIIIA, EIIIB, and the V (or variable) region (for review see Mosher, 1989; Hynes, 1990). In the rat, the pattern of splicing in the liver gives rise to pFN dimers of characteristic subunit composition: one subunit has the V region, the other lacks this segment (Schwarzbauer et al., 1989). In contrast, almost all cFN subunits are V^+. In addition, the two extra domains, EIIIA and EIIIB, are present in a small percentage of cFN subunits but are always excluded from pFN dimers (Paul et al., 1986; Schwarzbauer et al., 1987). Thus, differential splicing confers greater structural heterogeneity and complexity to cFN molecules

1. *Abbreviations used in this paper*: cFN, pFN, and recFN, cellular, plasma, and recombinant fibronectin, respectively.

than to pFN. These two forms of FN also differ in their in vivo localization. cFN is found as a fibrillar network within the tissue extracellular matrix. pFN, the more soluble form, circulates in the blood until immobilized by binding and cross-linking to fibrin during clot formation.

cFN is an integral component of the wound healing process, where it is present in the surrounding tissue and synthesized by cells that migrate into the wound area (Clark and Henson, 1988). However, it is not known whether cFN participates in one of the earliest stages of tissue repair, namely, cross-linking to fibrin. In this report, we have characterized the role of cFN during clot formation in vitro, both by comparing the native rat polypeptide with pFN, and by comparing cDNA-encoded recombinant dimers that differed in V-region complement. When cross-linked clots composed of purified fibrinogen and FNs were analyzed by solubilization, SDS-PAGE, and immunoblotting, we found that the rate of incorporation of cFN, as well as recombinant V^+-V^+ dimers, was significantly slower than that of pFN and its corresponding V^+-V0 dimers. In addition, factor XIIIa–mediated cross-linking between cFN, but not pFN, molecules could be detected in the absence of fibrinogen. Together, these results demonstrate that structural differences between pFN and cFN affect their interactions with each other and with fibrin during clot formation.

Materials and Methods

Formation of Fibrin Clots

Lyophilized human fibrinogen (98.8% clottable; American Diagnostica, Inc., Greenwich, CT) and bovine fibrinogen (96% clottable; Sigma Chemical Co., St. Louis, MO) were reconstituted in buffer to yield stock solutions of 6 mg/ml in 0.15 M NaCl and 10 mM Tris-HCl, pH 7.4. Reconstituted fibrinogens were either prepared fresh before an experiment or stored at −85°C to retain the activity potential of indigenous factor XIII. Bovine thrombin (Sigma Chemical Co.) was reconstituted with water to ~0.15 U/μl (in 37.5 mM NaCl, 1.25 mM Na citrate, pH 6.5) and stored in 100-μl aliquots at −20°C. At human fibrinogen concentrations ranging from 0.6 to 3.0 mg/ml, the factor XIIIa activity was sufficient for the conversion of fibrin γ chains into cross-linked dimers within an hour at 0°C, as assessed by SDS-PAGE (see Mosher, 1975). Cross-linking of the α subunits proceeded more slowly, as expected; these were not completely incorporated except in reactions incubated overnight at this temperature. However, α polymers were detectable within 15–30 min after the addition of thrombin.

To maintain the physiological 10:1 mass ratio of fibrinogen to pFN, clotting components were mixed in a volume of 50–100 μl to give the following concentrations: fibrinogen, 0.6–0.8 mg/ml; pFN or cFN, 60–80 μg/ml; thrombin, 1.5 U/ml; 20 mM $CaCl_2$ or EGTA, 0.15 M NaCl, 50 mM Tris-HCl, pH ~7.5. The concentration of fibrinogen in these reactions was fivefold lower than physiological, but in control experiments, the rates of fibrin–FN cross-linking were the same at 0.6 and 3.0 mg/ml. Reaction conditions were identical for recombinant FNs (recFNs), except the total FN concentration in each clot, including recFNs and endogenous SVT2 cFN, ranged from 2 to 10 μg/ml, as estimated by band intensities on silver-stained SDS gels. To determine if this variability had any effect on the rate of cross-linking to fibrin, fibrin–FN cross-linking was compared between clots prepared at 100:1 and 10:1 mass ratios of fibrinogen to FN. Increasing the ratio by an order of magnitude did not change the rate of FN incorporation.

In some reactions, human placental factor XIII (Calbiochem-Behring Corp., San Diego, CA), reconstituted from lyophilized powder to 10 U/ml (in 0.5% serum albumin, 22 mg/ml glycine, 7.2 mg/ml phosphate), was added to a final concentration of 1 U/ml (1 U of factor XIII is defined as the activity present in 1 ml of plasma). Before the addition of thrombin, the mixtures were equilibrated on ice for ≥5 min. Clots were solubilized by adding an equal volume of S buffer (8 M urea, 2% SDS, 2% 2-mercaptoethanol, 0.16 M Tris-HCl, pH 6.8), and boiling for 10 min.

Freshly prepared rat plasma was passed over a gelatin-agarose column to remove endogenous pFN. Plasma clots were prepared essentially as described above except pFN-depleted plasma represented 30% of the reaction volume. These conditions gave a fibrinogen concentration of ~0.8 mg/ml, equivalent to the concentration used in the fibrin clot assays.

Construction of cDNA Encoding recV0

The construction of the pLJ-recV^+ plasmid was outlined previously, but under the designation I_{1-9}/C110 (Schwarzbauer, 1991). recV^+ in the vector pGEP-His (Schwarzbauer et al., 1989) was used to construct the recV0 plasmid. Sequences flanking and including the V region (residues 5536–6765) were removed by BstEII (New England Biolabs, Beverly, MA) cleavage and replaced with a BstEII fragment encoding the V0 variant. The absence of the 360 nucleotides that encode the V segment was verified by restriction analysis.

Cell Culture

G418-resistant SVT2 (SV-40–transformed 3T3) mouse fibroblasts expressing recV^+ were generated and grown as described previously (Schwarzbauer, 1991). For coexpression of recV^+ and recV0 polypeptides, these cells were infected with a Ψ_2-derived recombinant retrovirus carrying the bacterial hisD gene (Hartman and Mulligan, 1988) and sequences encoding recV0. Infected cells were selected with 3–4 mM histidinol buffered with 0.1 M Hepes, pH 7.3. Resulting colonies were ring cloned, expanded, and screened for expression of both recFNs as described before (Schwarzbauer et al., 1989). The F2408 Rat-1 cell line was cultured in DME supplemented with 5% calf serum (Hyclone Laboratories, Inc., Logan, UT). Hybridoma cells secreting the rat-specific anti-FN mAb VT1 were generously provided by Dr. K. Fukuda (University of Vermont, Burlington, VT), and were grown in DME plus 15% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Purification and Cross-linking of pFN and cFN

FN was purified from freshly drawn rat plasma, outdated human plasma, and the conditioned medium of a Rat-1 F2408 cell line by gelatin-agarose (Sigma Chemical Co.) chromatography (Engvall and Ruoslahti, 1977). Concentration of FNs was determined by optical density at 280 nm (ϵ = 1.3 [0.1%]). Purity was assessed by SDS-PAGE (Laemmli, 1970) and silver staining (Merril et al., 1984). When electrophoresed nonreduced, both pFN and cFN migrate primarily as dimers. FNs were stored at −85°C in cyclohexylaminopropanesulfonic acid (CAPS)-buffered saline (0.15 M NaCl, 0.01 M CAPS, pH 11). For several control experiments, purified human or rat pFN was added to the culture medium of subconfluent F2408 Rat-1 cells at a final concentration of 10 μg/ml. Cells plus pFN were then incubated for an additional 2–3 d. Culture medium was collected and pFN and rat cFN were copurified as described above. The yield of FN in the pFN + cFN preparation was twice that of cFN obtained from the same amount of culture medium.

Cross-linking of FN in the absence of fibrin was assayed by incubating rat pFN or cFN separately on ice at 80 μg/ml in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, with 1 U/ml placental factor XIII, 1.5 U/ml bovine thrombin, 20 mM $CaCl_2$, and 0.5 mg human serum albumin/ml. Reactions were terminated by adding EGTA to 25 mM and PMSF to 2 mM. Components were solubilized by addition of an equal volume of 8 M urea in CAPS-buffered saline. Approximately half of the total volume of each reaction was lyophilized in the SpeedVac (Savant Instruments, Farmingdale, NY), reconstituted in 10 μl water, electrophoresed reduced, and immunoblotted with the VT1 antibody.

Purification of recFNs

About 2 g sterile glass microcarrier beads (ICN-Flow Laboratories, McLean, VA) were seeded with cells trypsinized from four confluent 10-cm tissue culture dishes (~8×10^7 cells). Cells were allowed to attach 1 h to overnight in 100 ml DME plus 10% calf serum in a spinner flask (Techne, Inc., Princeton, NJ) at 37°C. Medium volume was increased to 350 ml the next day. On day 6, the medium was aspirated and beads were washed with 100 ml PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The beads were then resuspended in 350 ml DME containing 10% calf serum depleted of bovine pFN by a 40% ammonium sulfate precipitation and/or gelatin-agarose affinity chromatography. Cells were further incubated for 7–10 d, at which time the conditioned medium was harvested, clarified by centrifugation, and applied to a gelatin-agarose column after the addition of EDTA to 10 mM and PMSF to 2 mM for protease inhibition. In some experiments, the medium was first incubated batchwise with gelatin beads,

either overnight at 4°C or for several hours at room temperature. Columns were washed with PBS plus 1 mM EDTA; bound proteins were eluted with 8 M urea in CAPS-buffered saline. Fractions with the highest optical density at 280 nm were pooled and dialyzed into buffer. Several minor proteins were detected on silver-stained SDS-polyacrylamide gels of these pools and were the same in each preparation. recFN dimers were characterized by two-dimensional nonreduced/reduced SDS-PAGE as previously described (Schwarzbauer et al., 1989).

Separation and Identification of Cross-linked Products

Proteins were separated on 5% SDS-polyacrylamide minigels (Bio-Rad Laboratories, Cambridge, MA) unless indicated otherwise. For immunodetection, proteins were transferred to nitrocellulose (Sartorius Corp., Bohemia, NY) using the transfer apparatus (Mini-Protean II; Bio-Rad Laboratories) as suggested by the manufacturer. Filters blocked with 0.1% Tween 20 in TBS were probed with either the VT1 mAb or an anti-human FN mAb (clone III; Telios Pharmaceuticals, Inc., San Diego, CA). The VT1 mAb was normally used as a 1:10 dilution of hybridoma culture supernatant for the detection of ~0.5 μg FN per lane with biotinylated goat anti-mouse IgG, streptavidin-horseradish peroxidase conjugate (both from Gibco BRL, Gaithersburg, MD), and 4CN. To visualize amounts <0.5 μg, or the recFNs, immunoblots were developed with chemiluminescent reagents (Amersham Corp., Arlington Heights, IL) and exposed to film (X-omat; Eastman Kodak Co., Rochester, NY). With this detection system, VT1 culture supernatant was diluted 1:50 or 1:100; the anti-human FN mAb was used as a 1:10,000 dilution of reconstituted ascites fluid. For the detection of human fibrin cross-linked products (6 μg fibrin per lane), affinity-purified mAbs against the α and γ subunits (Matsueda, G., Bristol Myers-Squibb Pharmaceutical Research Institute, Princeton, NJ) were used at 1:500 and 1:20,000 dilutions, respectively. For staining with Coomassie brilliant blue (0.25% in 50% methanol, 10% acetic acid), fibrinogen and FN concentrations in clotting reactions were increased to 3 mg/ml and 0.3 mg/ml, respectively, and aliquots of solubilized clots containing 30 μg fibrin and 3 μg FN were electrophoresed.

Quantitation of Cross-linking

Solubilized clots were electrophoresed on 4 or 4.5% gels and bands were detected on immunoblots with the chemiluminescence system, using several exposure times. To determine the percentage of FN cross-linked at each time point, the amount of FN monomer remaining was measured using a technique developed by Suissa (1983). In this method, bands are excised from the film and placed in 1 N NaOH to elute the silver grains. The absorbance of the silver grain solutions at visible wavelengths reflects the amount of protein in the band at appropriate exposure times. To ensure that the absorbance was indeed linearly related to the amount of protein, bands representing 0.25 and 0.5 μg pFN on immunoblots were cut from film exposed to the filters for various times (between 5 and 60 s) and the silver grains were eluted. The absorbance of the solutions was measured and plotted against time of exposure. The lines obtained for these two masses were linear and parallel, except for very rapid exposures (≤5 s). Numbers calculated using the silver grain elution method correlated well with those derived by densitometric scanning of appropriately blackened bands.

We measured the amount of monomeric FN remaining in each reaction for silver grain suspensions with an absorbance between 0.45 and 0.05 at 500 nm; measurements <0.05 are less reliable because of limitations in the sensitivity of this method. Therefore, for light bands with an optical density <0.05, a combination of exposures was used to determine the amount of protein more accurately. These values were divided by that obtained for the noncross-linked control and were then converted to percent FN cross-linked and plotted as a function of time. For these experiments, time courses with the same or similar control absorbance values were compared.

Results

Clotting and Cross-linking Assay

Formation of a blood clot is the culmination of a series of enzymatic reactions that result in the conversion of soluble fibrinogen, consisting of subunits Aα, Bβ, and γ, to spontaneously polymerizing fibrin molecules by thrombin-catalyzed cleavage of fibrinopeptides A and B (for review see Henschen and McDonagh, 1986). Noncovalent binding of pFN to fibrin occurs via at least one domain on each subunit of the FN dimer (Hormann and Seidl, 1980; Sekiguchi et al., 1981; see Fig. 2 *A*). In the presence of Ca^{2+}, thrombin-activated factor XIII (XIIIa) catalyzes formation of isopeptide bonds between glutamine and lysine residues on fibrin γ and α subunits to generate γ dimers and α polymers, respectively (Lorand, 1972). pFN is also cross-linked by this enzyme to lysine residues in the carboxy terminus of fibrin α chains (Iwanaga et al., 1978; Mosher and Johnson, 1983). The major factor XIIIa-reactive site in pFN has been mapped to a glutamine at the amino terminus (McDonagh et al., 1981; Parameswaran et al., 1990). This fibrin-FN cross-linked network can be assembled from purified components in vitro using a clotting and cross-linking assay (Fig. 1 *A*) developed by Mosher (1975, 1976). In this assay, fibrin formation and polymerization (clotting) are rapidly initiated by the addition of thrombin to a solution of fibrinogen, factor XIII, FN, and $CaCl_2$. Concomitantly, factor XIII is activated and covalently cross-links fibrin and FN in a Ca^{2+}-dependent reaction which is enhanced at low temperatures. At various times clots are solubilized with denaturing buffer. Cross-linked products are then separated by SDS-PAGE and analyzed either by Coomassie blue staining or immunoblotting.

Fig. 1 depicts the products of cross-linking of rat pFN to human fibrin at several time points. The Coomassie blue-stained gel (Fig. 1 *B*) shows the positions of the three fibrinogen subunits before clotting and cross-linking (lane *1*). Fibrin formation and cross-linking were induced in a mixture of pFN and fibrinogen by the addition of thrombin and $CaCl_2$. After thrombin cleavage of the fibrinopeptides, the α and β chains migrated ahead of their fibrinogen counterparts and the β and γ chains comigrated (compare lanes *1* and *2*). Fibrin-cross-linked γ dimers rapidly appeared, as well as several less intense α-polymer intermediates, as expected. With increased incubation times, the α chains, which are cross-linked more slowly than γ subunits (McKee et al., 1970), were completely incorporated into high molecular weight polymers (*asterisk*, lane *4*). In addition, pFN was converted into several cross-linked bands, particularly one migrating behind the monomer band (*arrow*). The composition of this band was determined by immunoblotting (Fig. 1 *C*). It was recognized both by a mAb to the human fibrin α subunit and by a rat-specific anti-FN mAb, VT1 (*arrow*), and therefore represents a heterodimer of one pFN subunit (~250 kD) and a fibrin α subunit (~65 kD). This pFN-fibrin α dimer is the major cross-linked intermediate at early time points. More complex polymers of FN and fibrin α chains were also visible with both antibodies (Fig. 1 *C*, lanes *2* and *3*) and migrated as a heterogeneous smear. With extended incubation (>18 h), FN, α subunits, and heterodimers were all completely converted into high molecular weight polymers seen at the top of the separating gel (Fig. 1 *B*, lane *4*) and in the well (not shown).

For these studies with rat FNs, we have primarily used a commercial human fibrinogen preparation that contains significant levels of factor XIII activity and is efficiently cross-linked during the clotting reaction. Human fibrinogen has been completely sequenced and is probably the most thoroughly characterized fibrinogen to date (for review see Henschen and McDonagh, 1986). Fibrinogen sequences, particularly the α chains, are somewhat heterogeneous among vertebrate species (for reviews see Doolittle, 1973, 1983); for example, while rat and human fibrinogen γ

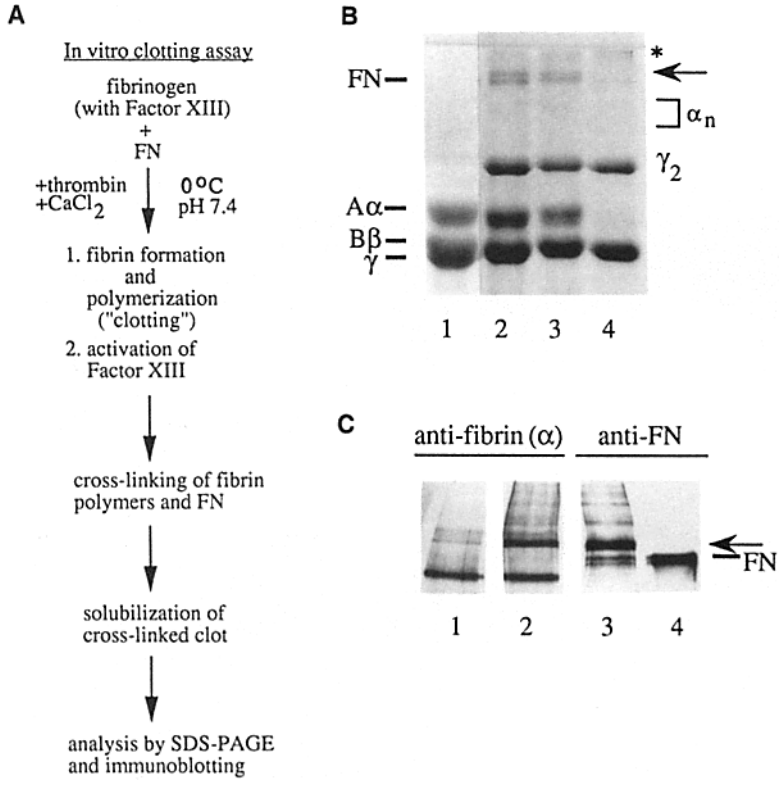


Figure 1. Formation of fibrin–FN clots in vitro. (*A*) The assay for clotting and cross-linking of purified fibrinogen and FN is outlined. (*B*) Clots were prepared with human fibrinogen and rat pFN at a mass ratio of 10:1. Clots wee solubilized under reducing conditions, electrophoresed in a 7% SDS–polyacrylamide gel, and stained with Coomassie blue. A short exposure of lane *1* shows the three subunits of fibrinogen (Aα, Bβ, and γ). Thrombin cleaves fibrinopeptides A and B, thus increasing the mobility of the α and β chains (compare lanes *1* and *2*). At 30 (lane *2*) and 60 min (lane *3*) after thrombin addition, Ca^{2+}-dependent cross-linking by factor XIIIa is evidenced by appearance of the fibrin γ dimer and concomitant conversion of fibrin α subunits into higher molecular weight bands (α_n and *asterisk*). FN is incorporated into some of these polymers; the most distinct cross-linked intermediate at 30 and 60 min is an FN–fibrin α heterodimer (*arrow*) migrating behind the pFN monomer band. Note the complete absence of fibrin α and FN monomers at 18 h (lane *4*), and the large polymers (*asterisk*) staining at the top of the separating gel. Not shown in this figure is staining of cross-linked material in the well. The fibrin β subunit does not participate in cross-linking. (*C*) Similar 60-min clotting reactions were compared by immunoblotting with mAbs to the α subunit of human fibrin (lanes *1* and *2*) and to rat FN (lanes *3* and *4*). Both antibodies recognize the heterodimer (*arrow*), which is not present if FN is omitted from the clotting reaction (lane *1*). The other band recognized by the antifibrin antibody is a cross-linked α trimer. Other α polymers are not shown in this panel. (Lane *1*) −FN, $+Ca^{2+}$; (lanes *2* and *3*) +FN, $+Ca^{2+}$; (lane *4*) +FN, $-Ca^{2+}$.

subunits are 83% homologous, the Aα chains show 64% identity (Crabtree and Kant, 1982; Crabtree et al., 1985). On the other hand, all mammalian FNs are extremely well conserved (for review see Hynes, 1990). In fact, the amino acid sequences of the amino-terminal fibrin-binding domains of human and rat FNs (type I repeats 1–5) are 96% identical. Furthermore, the amino-terminal type I repeats required for cross-linking of recombinant rat FN fragments to human fibrin have been identified (Sottile et al., 1991) and the alternatively spliced structures of rat FNs have been more extensively characterized than in other species. For these reasons, we elected to analyze the cross-linking of isoforms of rat FN to human fibrin.

Comparison of pFN and cFN Covalent Incorporation into Clots

The FNs are a family of dimeric, modular proteins composed of repeating units, some of which confer binding to fibrin (Fig. 2 *A*). Rat plasma and cellular FNs share the same fibrin-binding and cross-linking sequences (Patel et al., 1987), and Mosher has shown for human FN that each form is a substrate for the factor XIIIa–catalyzed addition of lysine analogues such as dansyl cadaverine (1975, 1976, 1977). Therefore, to determine if cFN, like pFN, can be cross-linked to fibrin, we compared the direct incorporation of the purified rat proteins into human and bovine fibrin clots and clots formed from rat plasma using a rat-specific mAb (Fig. 2 *B*). cFN was incorporated into these clots, but, in comparison to pFN, there was a striking difference in the extent of cross-linking to fibrin. While the majority of pFN was converted into the FN–fibrin α heterodimer by the first time point, cFN was only partially cross-linked. In fact, cFN subunits could still be detected in clotting reactions incubated >18 h, whereas all pFN subunits, and most of the pFN–fibrin α heterodimers, were converted into high molecular weight polymers. The incorporation of FNs was slower with bovine than with human fibrinogen or rat plasma. Comparatively lower amounts of bovine fibrin γ dimers were detected on Coomassie blue–stained gels at early time points, indicating that the difference was most likely due to the amount or activity of factor XIII copurifying with bovine fibrinogen. Addition of purified placental transglutaminase to fibrinogens with lower indigenous factor XIII activity than that of human fibrinogen did increase the rate of cross-linking, but the difference between the extent of incorporation of pFN and cFN was still observed for these supplemented reactions (data not shown). A similar difference between pFN and cFN was found with purified rat fibrinogen (data not shown); however, the rat fibrinogen preparation was not used further since these fibrin clots contained very little active factor XIII.

The major structural differences between pFN and cFN are generated by tissue-specific alternative splicing (Schwarzbauer et al., 1985; Paul et al., 1986) and could explain the cross-linking differences. Two other explanations for these results, differential glycosylation and solubility, were eliminated by the following control experiments. In both molecules, the major carbohydrate group is the biantennary complex asparagine-linked side chain. Unlike pFN, cFN side

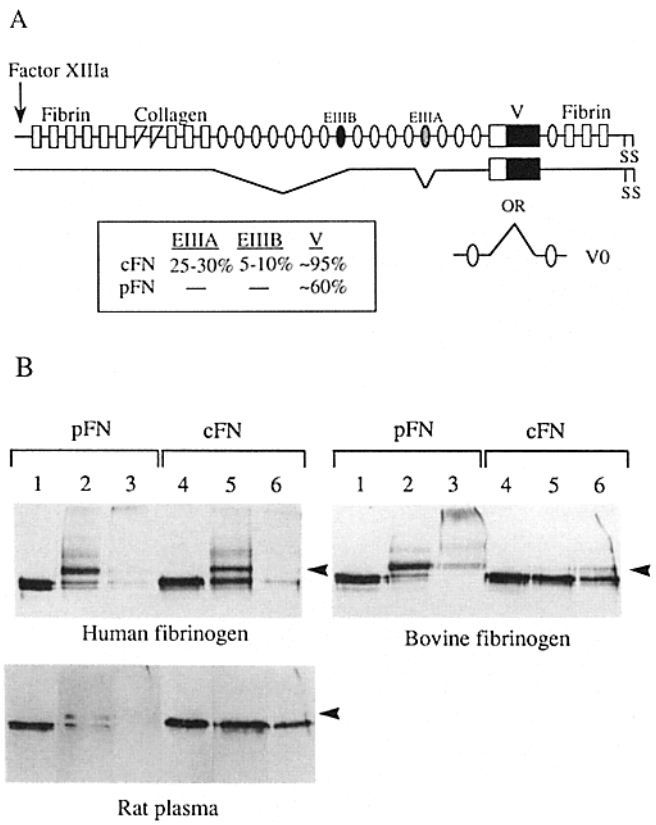


Figure 2. Differences in structure and cross-linking kinetics of rat pFN and cFN. (*A*) This schematic of an FN subunit depicts the domains for binding to fibrin and collagen (gelatin), as well as the amino-terminal factor XIIIa–reactive site. The domains are composed of three types of repeating units: type I (*boxes*), type II (*triangles*), and type III (*ovals*). EIIIA and EIIIB are cFN-specific type III repeats. Almost all Rat-1 cFN subunits in these experiments are V^+, whereas this region is completely excluded (*V0*) in ~40% of rat pFN subunits. Subunits are disulfide bonded into dimers via a pair of cysteines at the carboxy terminus (*SS*). Below this subunit is a diagram of the cDNA-encoded recFN used in the clotting assay. This 180-kD recFN lacks repeats III_{1-7}, EIIIA, and EIIIB, and either includes or excludes (*V0*) the V region. This recFN was used for these experiments because we have been unable to produce sufficient quantities of full-length recFN by expressing cDNAs in mouse fibroblasts. (*B*) Equal masses of rat pFN and cFN were added to clotting reactions containing either human or bovine fibrinogen or rat plasma depleted of endogenous FN. Immunoblots of reactions terminated at the given time points were probed with the VT1 mAb. (Lanes *1* and *4*) $^-Ca^{2+}$, >18 h; (lanes *2* and *5*) $+Ca^{2+}$, 2 h; (lanes *3* and *6*) $+Ca^{2+}$, >18 h. Arrowheads indicate FN–fibrin α heterodimer. pFN migrates as a doublet corresponding to V^+ and V0 subunits, whereas monomeric cFN migrates as a diffuse band.

chains have been reported to contain fucose, and comparatively less sialic acid (Vuento et al., 1977; Carter and Hakomori, 1979). To discern if carbohydrate organization has any role in the interaction of FN with fibrin during clotting, we metabolically labeled Rat-1 fibroblasts in the presence of tunicamycin to inhibit asparagine-linked glycosylation, purified cFN from the conditioned medium, and analyzed its incorporation into clots. Because there was no difference in the rate of cross-linking when compared with native cFN, asparagine-linked carbohydrate groups must have no effect on the incorporation of FN into fibrin clots (data not shown).

The two forms also reportedly differ in solubility (Hynes, 1990). During clotting, nonspecific aggregation of cFN molecules could decrease accessibility of the protein to factor XIIIa, thereby reducing the rate at which cFN is incorporated. Accordingly, the solubilities of pFN and cFN were examined by incubating them at neutral pH with $CaCl_2$ and either BSA or factor XIII–depleted fibrinogen. The mixtures were centrifuged at various time points to pellet any protein aggregates. A sample of each supernatant was removed and compared with controls. Because the band intensities for pFN and cFN were the same as the controls, we conclude that both FN forms remained soluble under these conditions (data not shown).

Time Course of Incorporation

To monitor the progression of cross-linking early after fibrin clot formation, we assayed the incorporation of pFN and cFN into fibrin gels using a wider range of time points (Fig. 3 *A*). This time course shows the same kinetic differences in cross-linking of the FNs, but also emphasizes a difference in the nature of the cross-linked products obtained. Cross-linking of pFN to fibrin α subunits was clearly visible as early as 5 min after the addition of thrombin, while cFN–fibrin cross-linked products appeared at significantly later times. While the FN–fibrin α heterodimer (*arrow*) was a major intermediate in pFN–fibrin clots, this was not the case for fibrin gels containing cFN. Instead, the incorporation of cFN was characterized primarily by the production of high molecular weight polymers (*asterisks*). In a parallel experiment, the extent of cross-linking was quantitated by measuring the percentage of monomeric FN remaining at each time point (Fig. 3 *B*). By 45 min, pFN was essentially completely incorporated into the clot, whereas only 40–45% of cFN subunits were cross-linked. Although cFN continued to be gradually cross-linked, it was not entirely incorporated even after 24 h. The graph also shows values at several early time points for reactions carried out at 37°C. Plots of these values closely follow those of reactions performed on ice, indicating these differences persist under variable conditions.

The pFN and cFN preparations used in these experiments were purified by gelatin–agarose affinity chromatography from two different sources, fresh blood plasma and fibroblast-conditioned medium, respectively. Since no difference in fibrin–fibrin cross-linking between clots containing pFN or cFN could be detected, it appears that thrombin activation of factor XIII proceeds at the same rate (data not shown). To determine if there were elements from conditioned medium that were copurifying with cFN and affecting its cross-linking to fibrin, several control experiments were carried out. Human pFN and rat cFN were mixed together in clotting reactions. Solubilized clots were immunoblotted with a mAb against human FN and compared with reactions containing pFN alone (data not shown). In a second mixing experiment, human pFN purified from outdated plasma was added to rat fibroblast cultures and incubated for 2 d. Human pFN was then isolated from the culture medium along with rat cFN and compared to nonincubated pFN in the clotting assay (Fig. 4 *A*). In both types of mixing experiment, the rates and patterns of cross-linking of human pFN to fibrin were similar in the presence and absence of rat cFN, show-

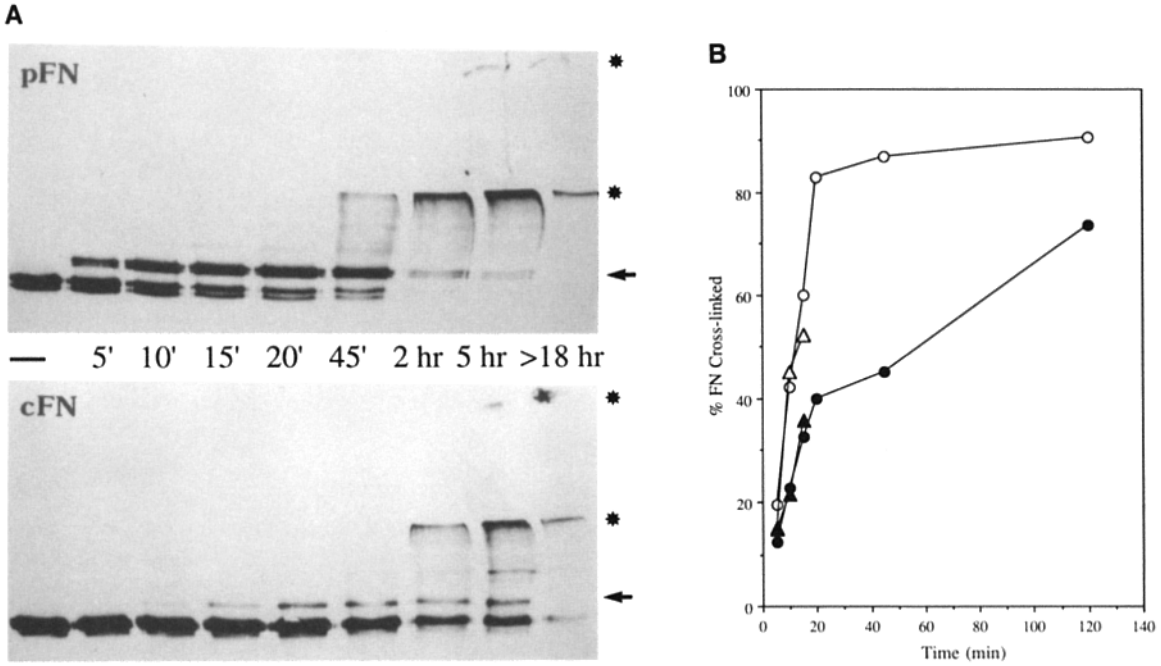


Figure 3. Time course of incorporation of rat pFN and cFN into human fibrin clots. (*A*) Clotting reactions were terminated at the indicated time points and analyzed by immunoblotting with the VT1 mAb. The dash denotes the noncross-linked control. Stacking gels were retained to show high molecular weight material migrating at the top of the separating gels and in the wells at later time points (*asterisks*). Arrows indicate FN–fibrin α heterodimer. (*B*) The rate of cross-linking of pFN (○, 0°C; △, 37°C) and cFN (●, 0°C; ▲, 37°C) was quantitated at various time points as described in Materials and Methods.

ing that contaminating components of the fibroblast culture medium are not responsible for the differences between pFN and cFN.

In a more direct control experiment, rat pFN was incubated with rat fibroblasts and the mixture of rat pFN + cFN was copurified from the culture medium. Cross-linking of the mixture was compared with cross-linking of cFN purified from a parallel culture. Although the VT1 antibody recognizes both pFN and cFN equally well, cross-linking of the two forms could be distinguished by monitoring the levels of the FN–fibrin α heterodimer, which is a major intermediate in pFN–fibrin reactions. In the pFN + cFN clotting reactions (Fig. 4 *B*, *top*), this cross-linked intermediate had the doublet appearance characteristic of pFN and was evident well before any cross-linking of cFN had occurred (Fig. 4 *B*, *bottom*). Furthermore, a direct comparison of the two sets of reactions clearly shows the pFN-specific cross-linking above the cFN background. Significant differences in the amounts of high molecular weight cross-linked products were also observed after overnight incubations (compare Fig. 4 *B*, lanes *5*). Together, the results of these control experiments indicate that the difference in the rate of cross-linking of the two forms must be inherent in the FN molecule itself.

Formation of cFN–cFN Cross-linked Intermediates

A comparatively slow rate of cross-linking, in conjunction with reduced formation of cFN–fibrin α heterodimers, suggests that the mechanism of cFN incorporation into fibrin clots differs from that of pFN. We hypothesized that if cFN dimers could self-associate and were cross-linked to each other by factor XIIIa, this interaction might alter binding and cross-linking of cFN to fibrin. In fact, when incubated with $CaCl_2$ and placental factor XIIIa, a small fraction of cFN molecules was cross-linked into FN–FN dimers and conjugates migrating at the top of the separating gel (Fig. 5, *arrowheads*). As expected, the cross-linked dimers comigrated with purified dimeric cFN (data not shown). Cross-linking of pFN molecules to each other was not detected in this experiment (Fig. 5), in agreement with Mosher's results (1975). Homotypic interactions and/or cross-linking of cFN during clot formation could result in reduced efficiency of incorporation into the clot and decreased incidence of the cFN–fibrin α heterodimer on gels. Indeed, it is possible that polymers of cross-linked cFN, rather than native dimers, are involved in the interaction with fibrin. The time course of cFN incorporation shown in Fig. 3 *A* reveals at least one additional cross-linked intermediate in the 5-h reaction that was not present in fibrin–pFN clots.

Characterization of Recombinant V^+-V0 and V^+-V^+ Dimers

Differential incorporation of pFN and cFN appears to be due to structural differences between the two forms. Alternative splicing of the V region is one feature that distinguishes pFN from cFN dimers. Since native FNs are a population of splicing variants, we needed an approach that would allow analysis of these regions independently of each other. Therefore, SVT2 cells were engineered to produce recFN dimers varying only in the number of V segments per dimer, as illustrated in Fig. 6 *A*. Compared with a native FN subunit (Fig. 2 *A*), the recombinant versions lack the first seven type III repeats, as well as the alternatively spliced segments EIIIA and EIIIB, and either contain (recV+) or lack (recV0) the

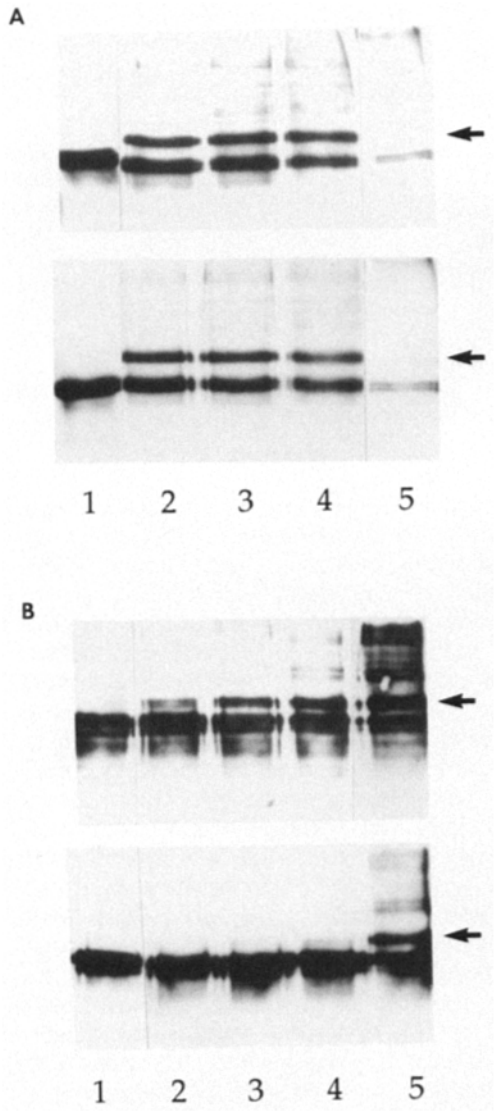


Figure 4. Cross-linking of pFN after incubation with rat fibroblasts. (*A*) Human pFN was purified from outdated plasma and used in the clotting assay without further manipulations (*top*) or after incubation with rat fibroblast cultures (*bottom*). Equal amounts of human pFN were used in the two sets of reactions. Reactions were terminated at various times after thrombin addition: (lane *1*) $-Ca^{2+}$ control; (lane *2*) $+Ca^{2+}$, 30 min; (lane *3*) 1 h; (lane *4*) 2 h; (lane *5*) 20 h. Aliquots containing 0.25 μg human pFN from each clot were immunoblotted with an anti-FN mAb recognizing only the human form and detected using chemiluminescence. Arrows indicate FN–fibrin α heterodimer. (*B*) Purified rat pFN was added to rat fibroblast cultures and incubated for several days. Rat pFN and cFN were copurified and cFN alone was purified from a parallel culture. Fibrin clots were prepared using either the rat pFN + cFN mixture (*top*) or rat cFN alone (*bottom*) with equal quantities of cFN in each reaction. Cross-linked products were detected with the rat-specific VT1 antibody. Times after thrombin addition (lanes *1–5*) are as in *A*. Arrows indicate FN–fibrin α heterodimer. The pFN + cFN panel is a shorter exposure than the cFN alone to provide better visibility of individual bands.

entire V segment. Details of the cDNA construction and characterization of $recV^+$ have been previously described (I_{1-9}/C110; Schwarzbauer, 1991). To generate recombinant V^+-V0 dimers, the G418-resistant cell line producing $recV^+$

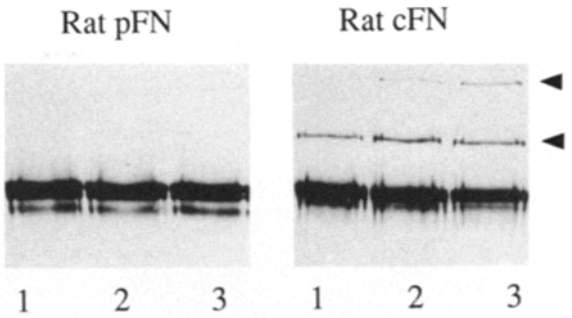


Figure 5. Formation of FN–FN cross-links. Rat pFN and cFN were incubated separately in clotting buffer plus thrombin and placental factor XIII but with serum albumin in place of fibrinogen. Reactions were terminated at the times indicated and components were solubilized and immunoblotted with the VT1 mAb. (Lanes *1*) 15 min; (lanes *2*) 1 h; (lanes *3*) 23–24 h. Arrowheads indicate cross-linked products.

was infected with a second retrovirus carrying sequences encoding recV0 and a different selectable marker, hisD (Hartman and Mulligan, 1988). Cells able to grow in the presence of G418 and histidinol were screened for secretion of $recV^+$ and recV0 polypeptides. recFNs from one clone expressing

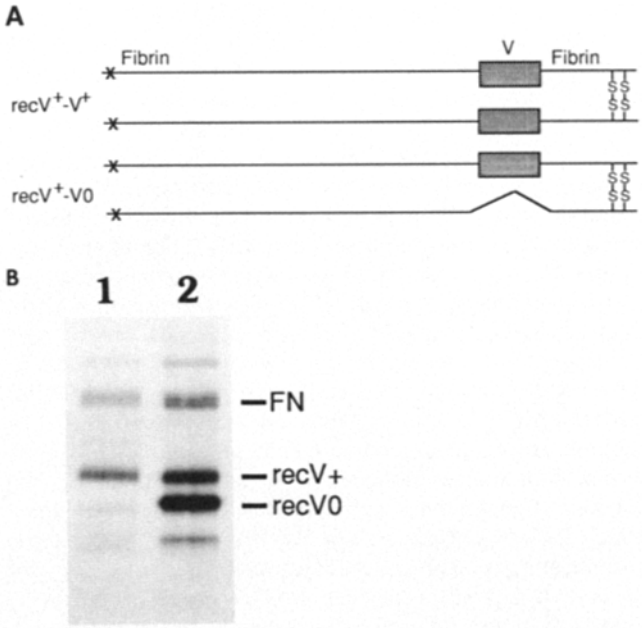


Figure 6. Analysis of recombinant FN dimers. (*A*) The recFN dimers engineered for these studies, V^+-V^+ by expression of a single cDNA, V^+-V0 by coexpression of two cDNAs. The major recFN dimers secreted by the SVT2 cell lines are illustrated here. With regard to V-region splicing pattern, V^+-V^+ dimers are like cFN, whereas V^+-V0 dimers resemble pFN. The domain structure of the nearly full-length recombinant subunits comprising these dimers is diagrammed in Fig. 2 *A*. The X denotes the amino-terminal factor XIIIa–reactive site. (*B*) The fluorogram shows FNs immunoprecipitated with a polyclonal anti-FN Ab from the parental $recV^+$ (lane *1*) and the $recV^+$-recV0–secreting SVT2 clone resistant to G418 and histidinol (lane *2*). The position of the recombinant subunits relative to endogenous cFN is indicated. The molar ratios of $recV^+$ and recV0 to endogenous cFN were 3.3 and 5.0, respectively. In the double infectant, there is a band of unknown origin migrating ahead of recV0 that is recognized by the VT1 mAb. Less protein was loaded in lane *1* than lane *2*.

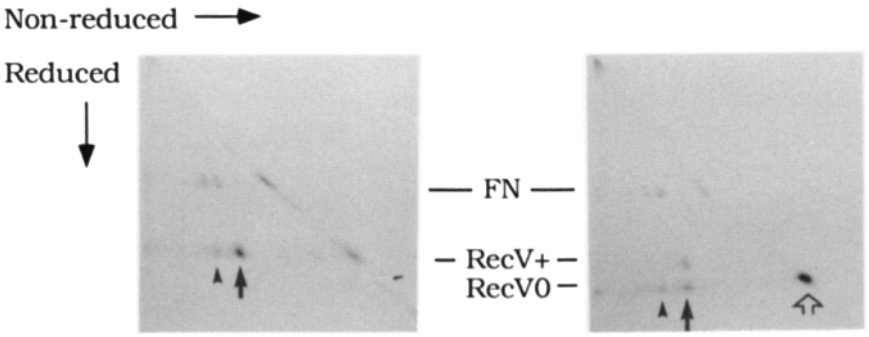


Figure 7. Two-dimensional nonreduced/reduced SDS-PAGE of recFN dimers. FNs were isolated by gelatin–agarose affinity chromatography after labeling cells with [^{35}S]methionine for 24 h. Dimers were separated without reduction (*left to right*), reduced in situ, and electrophoresed in the second dimension (*top to bottom*). These fluorograms show the composition of dimers secreted from cells producing recV$^+$ subunits singly (*left*) or simultaneously with recV0 (*right*). In each panel, the arrows point to the recombinant dimers and the arrowheads indicate recFN–cFN heterodimers. The recV0 monomer is denoted by the open arrow. The positions of the subunits were verified by electrophoresing the same samples reduced only (not shown). In both cases, very few cFN homodimers are secreted. In the parental recV$^+$ cell line, we calculate that no more than 35% of this recFN dimerizes with endogenous cFN.

both recV$^+$ and recV0 (recV$^+$-recV0) are compared with the parent recV$^+$ in Fig. 6 *B*.

Because the recombinant subunits were made in SVT2 fibroblasts, which synthesize their own FN, albeit at low levels, a relatively complex array of dimers was possible. To characterize the composition of dimers secreted by these cells, two-dimensional nonreduced/reduced SDS-PAGE was performed. Samples were first electrophoresed nonreduced in SDS–polyacrylamide tube gels, then reduced in situ, electrophoresed in the second dimension, and processed for fluorography. Fig. 7 displays the two-dimensional profiles obtained for both cell lines. From the recV$^+$-producing clone, the majority of recombinant subunits were dimerized with each other (*arrow, left*) as previously shown on nonreduced one-dimensional gels (Schwarzbauer, 1991). On the other hand, the recV$^+$-V0–producing clone synthesizes recV0 subunits at higher levels than recV$^+$; therefore, these subunits were secreted in three forms (*right*): as a heterodimer with endogenous FN (cFN-recV0, *arrowhead*), as a heterodimer with all the recV$^+$ subunits (recV$^+$-V0, *arrow*), and the majority as a monomer (*open arrow*). recV0-V0 dimers were not detected, as expected (Schwarzbauer et al., 1989), nor were recV$^+$-V$^+$ dimers. Thus, because virtually all cFN subunits are V$^+$, all dimers in the conditioned medium from the recV$^+$-producing clone are V$^+$-V$^+$ and resemble cFN. In contrast, the dimers secreted by the recV$^+$-V0 cell line, which include cFN-recV0 and recV$^+$-V0, contain the V region in only one subunit and are like pFN.

For the large-scale production of recombinant polypeptides, cells were grown on microcarrier beads in spinner flasks. The conditioned medium was harvested and subjected to gelatin–agarose affinity chromatography. This procedure resulted in purification of both endogenous cFN and recFNs.

Cross-linking of recFN Subunits to Fibrin

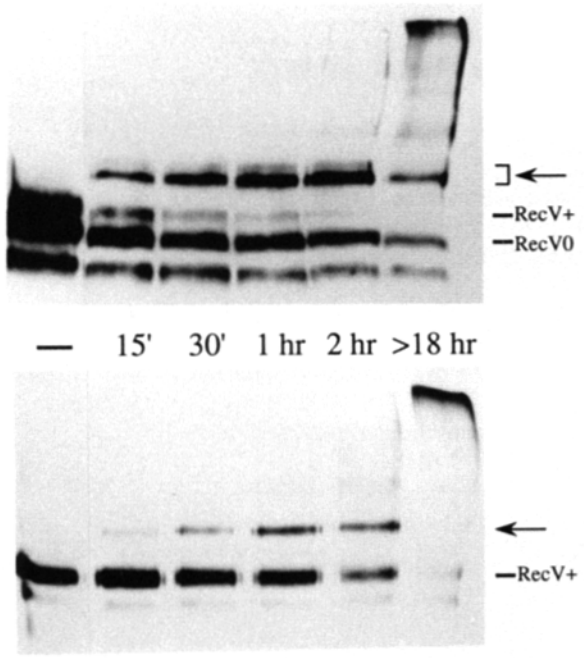
Using the clotting assay, we compared the rates of incorporation of purified recV$^+$-V0 and recV$^+$-V$^+$ dimers. After solubilization, cross-linked products were analyzed by SDS-PAGE and immunoblotting with the VT1 mAb, which detects only the recFNs. The time course in Fig. 8 *A* shows that these recV$^+$ and recV0 FN polypeptides were competent for factor XIIIa–mediated incorporation into fibrin clots. Cross-linking was evidenced by Ca^{2+}-dependent disappearance of monomer bands and formation of new products, including prominent intermediates (*arrows*) and high molecular weight complexes. These cross-linked products were also recognized by the mAb against the fibrin α chain (data not shown). The recV$^+$-V$^+$ dimers were cross-linked relatively slowly, with some noncross-linked recV$^+$ remaining after overnight incubation (Fig. 8 *A*, *bottom*). In comparison, immunoblots of clots formed with recV0-containing FNs showed more complex patterns due to the existence of multiple recV0 forms (recV$^+$-V0, cFN-recV0, recV0 monomer). We were primarily interested in monitoring the fate of the recV$^+$ subunits in these reactions, since these subunits were always present as recV$^+$-V0 dimers (Fig. 7). Cross-linked intermediates of both recV$^+$ and recV0 proteins with fibrin were formed early in the time course and the majority of the recV$^+$ subunits was cross-linked by 2 h (Fig. 8 *A*, *top*). Quantitation of the recV$^+$ band in each time course showed that it was not incorporated at the same rate (Fig. 8 *B*). As a heterodimer with recV0, 83% of recV$^+$ was cross-linked into the fibrin clot by 1 h. In contrast, when paired with another V$^+$ subunit, only 35% was incorporated at this time point and, as observed for cFN, recV$^+$-V$^+$ was not completely cross-linked over the 24-h time period.

To demonstrate that the factor XIIIa activity was equivalent in both sets of reactions, rat pFN was mixed with recFN preparations and monitored for its incorporation into clots. The pattern of pFN–fibrin cross-linking was identical when mixed with preparations containing recombinant proteins or analyzed by itself (data not shown). This experiment eliminated the possibility of a contaminant specific to one of the preparations as a factor in the differential incorporation of the recFNs. In fact, in multiple experiments, we consistently observed an accelerated rate of recV$^+$-V0 cross-linking. Data from several experiments showing the difference between percent recV$^+$ cross-linked at two time points are presented in Table I. Although the 30-min values were more variable, the differences between cross-linking of the two kinds of recombinant dimers were almost 50% at the 1-h time points.

Discussion

Cross-linking of pFN to fibrin within the blood clot matrix is required for cell adhesion and migration (Grinnell et al., 1980; Knox et al., 1986). In vivo, recruitment of some cell types (neutrophils, monocytes, epidermal cells, fibroblasts)

A



B

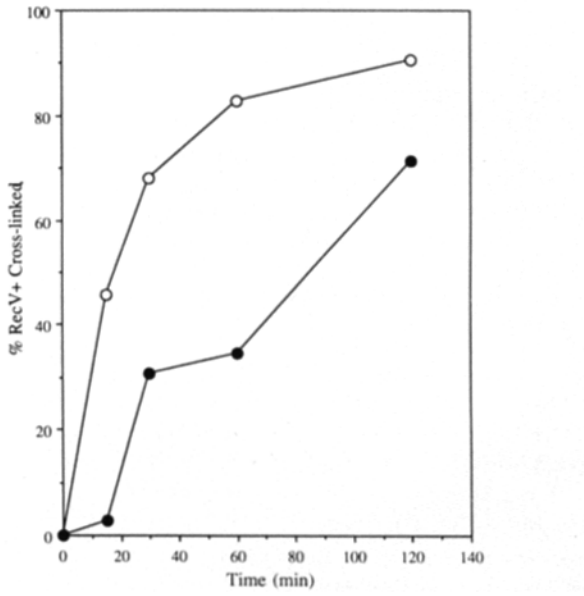


Figure 8. Time course of incorporation of recV$^+$ subunits into clots. (*A*) FN polypeptides were purified by gelatin–agarose chromatography from the conditioned medium of the two SVT2 cell lines. Proteins were added to clotting reactions containing human fibrinogen. Clots were solubilized and rat-specific proteins detected by immunoblotting with the VT1 mAb to follow the cross-linking of recV$^+$-V0 dimers (*top*) and recV$^+$-V$^+$ (*bottom*). Blots were developed using the chemiluminescence system. The recV0 band is more intense than the recV$^+$ since its expression level is higher, but most of it is present as a monomer. The arrows point to cross-linked recFN–fibrin α intermediates—a single recV$^+$–fibrin α band (*bottom*), as well as both recV$^+$–fibrin α and recV0–fibrin α bands (*top, bracket*). (*B*) Bands representing recV$^+$ (○, in V$^+$-V0 dimers; ●, in V$^+$-V$^+$ dimers) were excised from films exposed to the blots in *A* and processed for quantitation as described in Materials and Methods.

Table I. Differential Incorporation of recV$^+$-V0 and recV$^+$-V$^+$ Dimers

	Difference in % cross-linked	
Experiment	30 min	60 min
1	25.8	46.2
2	37.2	48.2
3	23.5	47.6

FNs derived from the recV$^+$- and recV$^+$-V0–producing cells were clotted with human fibrinogen for the indicated times in three independent experiments. Immunoblots probed with the VT1 mAb were developed and bands quantitated as described in Materials and Methods. Difference in % cross-linked = difference between recV$^+$-V0 and recV$^+$-V$^+$ values at the indicated time points.

into the wound area begins very early after clot deposition; therefore timely incorporation of FN into the clot would benefit those cells that are dependent on its presence in a provisional matrix (Grinnell et al., 1981; Fujikawa et al., 1981; Clark et al., 1982; Lanir et al., 1988). The experiments reported here show that compared with pFN, cross-linking of cFN to fibrin by factor XIIIa is less efficient. In the comparison presented in Fig. 3, the majority of pFN was cross-linked to human fibrin within 30 min, while <50% of cFN was cross-linked at this time. A difference in the cross-linking of the two FNs was maintained with fibrins from human, bovine, and rat and in the context of FN-depleted whole blood plasma. In addition, differential incorporation was observed at 37°C and 0°C, the latter a temperature that favors fibrin–FN interactions.

Along with differences in rate of cross-linking, cFN incorporation into fibrin clots proceeds through a more complex spectrum of cross-linked intermediates. The major intermediate in pFN–fibrin reactions is a heterodimer of pFN cross-linked to the α subunit of fibrin. With time, this heterodimer is converted into high molecular weight cross-linked polymers. Compared to pFN, much lower levels of cFN–fibrin α heterodimer are formed, both at 0°C and 37°C. Instead, cFN is apparently incorporated directly into the large polymers. This could be merely a consequence of the delay in cross-linking to fibrin, such that when cFN is finally incorporated, significant cross-linking has already occurred between the α chains, and therefore complexes larger than the heterodimer are formed. Another possibility is that cFN preferentially interacts with a network of cross-linked α polymers rather than single subunits. Okada et al. (1985) found that pFN does not become cross-linked to fibrin until the gel point and proposed that some form of three-dimensional structure is necessary for the reaction. Invoking their model, it may be that cFN has a more stringent requirement for cross-linked multimeric α chains than pFN.

That cFN can serve as both donor and acceptor in factor XIIIa–catalyzed reactions might also explain the low level of cFN–fibrin α heterodimer. A cFN–cFN cross-linked dimer was detected as early as 15 min after the addition of thrombin to a solution containing purified cFN, placental factor XIII, and $CaCl_2$. Under our experimental conditions, this reaction was not extensive, which may be a consequence of either the activity of the factor XIII preparation or the accessibility of appropriate glutamines and lysines. However, our results show that cFN molecules interact with each other differently from pFN. It is possible that cFN–cFN cross-linking occurs during clot formation as well. In fact, one of the intermedi-

ates present in cFN–fibrin clots migrates at the position expected for a cFN–cFN–fibrin α complex. Interactions between cFN molecules could prevent fibrin binding and result in a slower rate of incorporation into the clot. Three domains for noncovalent binding of FN to fibrin have been identified: the major amino-terminal binding domain, the weaker carboxy-terminal region (Sekiguchi et al., 1981; Hayashi and Yamada, 1983), and a third region spanning the last type I repeat of the gelatin-binding domain and at least the first type III repeat (Seidl and Hormann, 1983). Interestingly, each of these sites has also been implicated in FN–FN interactions (Ehrismann et al., 1981; Homandberg and Erickson, 1986; Chernousov et al., 1991; Schwarzbauer, 1991; Morla and Ruoslahti, 1991).

The major structural differences between cFN and pFN are due to the presence or absence of alternatively spliced segments. pFN has a relatively simple dimer structure in which one subunit contains the V region and the other lacks this segment. cFN is composed of a more complex repertoire of subunits with variation at three sites—EIIIA, EIIIB, and the V region. Because the V region is present in both subunits of cFN dimers but in only one subunit of the pFN dimer, we speculated that this segment might have some effect on the cross-linking of cFN during clot formation, despite its distance from the amino-terminal factor XIIIa–reactive site. To analyze the effects of this segment apart from other variations in the protein, the V region splicing patterns of pFN and cFN were recapitulated in recombinant V^+-V0 and V^+-V^+ dimers, respectively. Aside from the inclusion or exclusion of the 120-amino acid V region, these recombinant polypeptides were identical. Because the V region is required for FN dimer secretion (Schwarzbauer et al., 1989), recV0 homodimers are not secreted and could not be tested.

When recFN polypeptides were tested in the clotting assay, we found that the context in which $recV^+$ was presented markedly influenced its rate of cross-linking to fibrin. $recV^+$ cross-linking as a dimer with recV0 was similar to that of pFN. Furthermore, endogenous cFN (V^+) subunits were also cross-linked more rapidly when paired with recV0 (Wilson, C. L., and J. E. Schwarzbauer, unpublished observations). $recV^+$ homodimers, on the other hand, were cross-linked more slowly, reminiscent of cFN–fibrin cross-linking. Although the rates of incorporation differed, we did not observe a consistent variation in the types of intermediates as we had with pFN and cFN. Taken together, these findings implicate the V region of FN as a factor in determining the efficiency of incorporation into fibrin clots.

How does a region downstream of the amino-terminal factor XIIIa–reactive site exert an effect on this kind of reaction? One possibility is that the V segment alters the folding of subunits to modify interactions with fibrin via the adjacent carboxy-terminal fibrin-binding domain. The extent of fibrin binding by fragments containing this domain can vary depending on the buffer conditions used and the molecular context (Sekiguchi et al., 1981; Hayashi and Yamada, 1983). Fragments from the carboxy terminus of the B (V0) subunit of pFN appeared to interact more weakly with fibrin–Sepharose than fragments from the A (V^+) chain, suggesting that the V region can modulate fibrin–FN interactions. Similarly, the juxtaposition of two carboxy-terminal fibrin domains in a disulfide-bonded FN dimer could have an effect on fibrin binding. In any case, interactions between the carboxy-terminal region and fibrin would probably put constraints on binding and cross-linking at the amino-terminal site. Perhaps the absence of the V region in one of the subunits is sufficient to allow binding at the amino terminus to predominate.

It is also possible that the V region contributes residues involved in cross-linking by factor XIIIa. Several groups have demonstrated the existence of factor XIIIa-reactive sites near the carboxy terminus of FN using radiolabeled lysine analogues (Jilek and Hormann, 1977; Richter et al., 1981; Williams et al., 1982; Molnar et al., 1983; Fesus et al., 1986). There is a conserved glycine–glutamine–glutamine sequence in the V segments of human, bovine, rat, and chicken FNs and this tripeptide sequence is analogous to the amino-terminal factor XIIIa–reactive site. V region participation in transglutaminase-mediated cross-linking to itself or to fibrin would further affect interactions via the primary site at the amino terminus.

The difference in the rate and pattern of cross-linking of pFN and cFN during clotting demonstrates that these forms have temporally and structurally specific roles in the tissue repair process. An asymmetric distribution of the V segment in blood FN is important for its specific role in the coagulation pathway, whereas the V^+-V^+ forms comprising the majority of FNs in vivo are specialized for assembly into insoluble fibrils in the extracellular matrix. The alternatively spliced EIIIA and EIIIB segments, which are absent in pFN but predominate in newly synthesized cFN in the wound (ffrench-Constant et al., 1989), may also play some role in modulating the interactions and functions of cFN. Involvement of alternatively spliced regions in the different phases of blood clotting and tissue repair would provide a satisfying explanation for the evolution of the splicing patterns of pFN and cFN. The experiments described here provide a foundation for further work to define the importance of other regions in the differential cross-linking of pFN and cFN, and to determine the role of these segments in cell adhesion and migration on fibrin clots.

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