The NH$_2$-terminal Fibrin-binding Site of Fibronectin Is Formed by Interacting Fourth and Fifth Finger Domains

STUDIES WITH RECOMBINANT FINGER FRAGMENTS EXPRESSED IN *ESCHERICHIA COLI*

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(Received for publication, November 4, 1993, and in revised form, December 10, 1993)

The NH$_2$-terminal 29-kDa Fib-1 fragment consisting of the first five finger modules of fibronectin (F1-5) binds reversibly to fibrin and facilitates cross-linking by Factor XIII. To narrow down the fibrin-binding site within this region, we have used recombinant technology to express a number of individual fingers, rF1, rF2, rF3, rF4, and rF5, and their pairs, rF1-2 rF2-3, and rF4-5, as fusion proteins in *Escherichia coli*. These recombinant fragments were separated from the carrier maltose-binding protein by digestion with human factor Xa or other proteases, and their structural integrity was confirmed by spectroscopic and calorimetric methods. The recombinant F1 and F4-5 exhibited fluorescence-detected melting transitions of the same magnitude and with the same midpoint ($T_m$) as their natural analogues prepared from Fib-1 by proteolysis. Differential scanning calorimetry further demonstrated that these fragments are properly folded and have compact structures identical to the natural ones. Isolated rF4 melts at a much lower temperature than rF5 or the bimodular fragment rF4-5, indicating the loss of a stabilizing interaction between fingers 4 and 5. Comparison of fluorescence spectra of individual rF4 and rF5 with that of rF4-5 was also consistent with an interaction that affects the environment of Trp residue(s). rF2 also melts at a lower temperature than rF3 or rF2-3, suggesting a stabilizing interaction between the second and third fingers as well. When tested on fibrin-Sepharose, only the bimodular fragment rF4-5 was able to bind. All other fragments, including individual fingers 4 and 5, failed to bind. Thus, fibrin binding is not a common property of all fingers. The results indicate that a recognition site for fibrin is located within fingers 4 and 5. The interaction between these neighboring domains may play an important role in proper orientation of the residues forming this site.

Plasma fibronectin interacts reversibly with fibrin and is covalently incorporated into the fibrin matrix through the transglutaminase action of Factor XIII (1–3). The presence of fibronectin in the clot matrix affects its mechanical properties (4–7) and has important implications for the ensuing process of wound healing; fibrin matrices that contain fibronectin are better substrates for fibroblast adhesion and spreading than those without fibronectin (8). Both fibrinogen and fibronectin consist of two symmetrical subunits and are polyfunctional multidomain proteins whose individual domains are responsible for definite functions. Fibronectin seems to interact with the COOH-terminal parts of the fibrin α chains because their removal leads to abolishing of such an interaction (2, 9). Fibronectin contains two major fibrin-binding sites in each subunit whose localization was established by testing the ability of fibronectin fragments to bind fibrin-Sepharose. It was shown that the NH$_2$-terminal 29-kDa fragment (Fib-1) consisting of five finger modules and the COOH-terminal 19-kDa fragment (Fib-2) containing three finger modules both bind to fibrin-Sepharose (10, 11). Fibronectin-fibrin binding is easily detected at a low temperature, but it decreases with the increasing of temperature to physiological values (1, 2, 12).

Complexes between fibrinogen and fibronectin are stabilized by covalent cross-linking of glutamine and lysine residues with Factor XIII. The reactive glutamine residue of fibronectin is located in the third position from the NH$_2$ terminus (13). Thus, the Fib-1 region of fibronectin interacts with fibrin both non-covalently and covalently and seems to be responsible for incorporation of fibronectin into the fibrin clot at physiological temperatures. In an attempt to further localize the fibrin-binding site within the Fib-1 region of fibronectin, Sottile et al. (14) tested a number of recombinant 70-kDa NH$_2$-terminal fragments, from which one or more of the first five finger domains were deleted, while retaining the cross-linking site. It was found that deletion of all five fingers from the NH$_2$-terminal 70-kDa fragment did not prevent covalent incorporation into a fibrin clot or binding to fibrin-Sepharose at low temperature. The latter could be explained by the presence of the gelatin-binding region, which contains four additional fingers, and was shown previously to interact with fibrin at low temperature (10). Thus, the question about which fingers are most responsible for the non-covalent recognition of fibrin remains to be answered.

Binding sites in multidomain proteins may be localized indirectly by deletion of specific domains that reduce binding or directly by preparation of smaller fragments containing those domains and subsequent measurement of their binding properties. For example, preparation of subfragments from the 42-kDa gelatin-binding fragment allowed delineation of the gelatin-binding site in fibronectin (15). In all cases, physicochemical characterization of the fragments is required to check their structural integrity and, if possible, the correspondence of their structure to that in the parent protein. In the present study, all five individual fingers and several pairs of fingers from the Fib-1 region of fibronectin were expressed in *Escherichia coli*, and their structural integrity and fibrin binding properties were examined. It was found that all expressed
finger domains were properly folded, and domains 4 and 5 interacted with each other to form the fibrin recognition site.

**MATERIALS AND METHODS**

**Proteolytic Fragments—**NH$_2$-terminal 29-kDa fibrin-binding (Fib-1), 42-kDa gelatin-binding, 110-kDa cell-binding, a mixture of 30- and 40-kDa COOH-terminal heparin-binding, and COOH-terminal 19-kDa fibrin-binding (Fib-2) fragments (Fig. 1) were prepared by further digestion with pepsin and thermolysin, respectively.

**Fibrin-Sepharose—**This was prepared exactly as described by Heinze and Matthias (17) using fibrinogen that was prepared according to Varetsataya (18), taking care to minimize degradation of a chains. Several preparations of the affinity sorbent were made. Because the columns have a tendency to degrade with use, the Fib-1 fragment was applied to the column after each set of experiments to check preservation of its binding capacity.

**Affinity Chromatography Experiments—**These were performed on a 0.9 x 7-cm jacketed column of fibrin-Sepharose using the fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc.) with a flow rate of 0.5 ml/min. Samples were applied in 0.5 ml of TBS$^2$ (50 mM Tris-HCl buffer, pH 7.6, 150 mM NaCl). The flow was stopped and the samples were allowed to equilibrate with the column for 15 min. Flow was resumed and the loaded column was washed with 2 column volumes of TBS. Elution was effected with a linear gradient of 50 mM Tris-HCl buffer, pH 7.6, containing 3 M urea and 1 M NaCl, monitoring the absorbance at 280 nm during the entire process.

**Expression of Fusion Proteins—** All recombinant fragments were produced in E. coli using the pMAL-p2 expression vector (New England Biolabs Inc.), which generates fusion proteins containing the desired constructs separated from the carrier maltose-binding protein (MBP) by a segment containing the cleavage site for Factor Xa. To clone the designated domains we designed polymerase chain reaction primers shown in detail in Table 1. Both the forward and reverse primers contained 20-24 bases corresponding, respectively, to the 5' and 3' terminal sequences of the desired coding segment. Except for rec F1-2, which was the first construct prepared, all of the forward primers incorporated the BamHI restriction site immediately before the region coding for finger fragments. The reverse primers included a TGA stop codon immediately after the coding segment, followed by a HindIII site. Not shown in Table 1 is the presence of 5 or 6 additional bases at the 5' or 3' ends of each restriction site to facilitate binding of the restriction enzymes (19). The specific cDNA fragments were generated by the polymerase chain reaction using Pfu DNA polymerase (Stratagene) and a template consisting of full-length cDNA encoding human fibronectin kindly provided by S. Dufour (20). The reaction was cycled 15 times with denaturation at 94 °C, annealing at 50-54 °C (depending on $T_m$ of primers), and extension at 72 °C. All cDNA fragments were sequenced in both directions to confirm the integrity of the entire coding sequence. The amplified cDNA fragments were purified by electrophoresis in agarose gel, digested with appropriate restriction enzymes, and ligated into the pMAL-p2 expression vector.

pMAL-p2 was initially digested with XmnI and HindIII, while the polymerase chain reaction-generated cDNA fragment coding for F1-2

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**Table I**

| Recombinant protein name | Primers and NH$_2$-terminal sequence of recombinant finger domains* |
|--------------------------|---------------------------------------------------------------|
| rec F1-2                 | Residues 1-107                                               |
| Fwd: GAT ATC CAG GCT CAG CAA ATG GTT CAG | EcoRV |
| Rev compl: AAG GTC TCA GTC GAT GCA GCT TAT TC | HindIII |
| rec F1                   | Residues 1-61                                                |
| Fwd: GAT ATC CAG GCT CAG CAA ATG GTT CAG | BamHI |
| Rev compl: AAG GTC TCA GTC GAT GCA GCT TAT TC | HindIII |
| rec F2-3                 | Residues 60-151                                              |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | HindIII |
| Rev compl: AAG GTC TCA GAT GCT GAA GCT TAT TC | HindIII |
| rec F2                   | Residues 60-107                                              |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | BamHI |
| Rev compl: AAG GTC TCA GAT GCT GAA GCT TAT TC | HindIII |
| rec F3                   | Residues 105-151                                             |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | HindIII |
| Rev compl: AAG GTC TCA GAT GCT GAA GCT TAT TC | HindIII |
| rec F4-5                 | Residues 150-259                                             |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | HindIII |
| Rev compl: AAG GTC TCA GAT GCT GAA GCT TAT TC | HindIII |
| rec F4                   | Residues 150-197                                             |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | HindIII |
| Rev compl: AAG GTC TCA GAT GCT GAA GCT TAT TC | HindIII |
| rec F5                   | Residues 199-259                                             |
| Fwd: AGG ATC CCT GCA GGA GAG ACT TTC | HindIII |

* Forward (Fwd) and reverse complement (Rev compl) DNA sequences are indicated. The NH$_2$-terminal sequences determined for the final products are indicated above the forward primer; extra residues that are not part of the natural protein are indicated in boldface italics.
Fibronectin-Fibrin Interaction

RESULTS

Interaction of Major Proteolytic Fragments of Fibronectin with Fibrin-Sepharose—Fibronectin fragments (Fig. 1, top) used for subsequent ligation of the other cDNA fragments of fibronectin (Table I). The resulting plasmids were used for transformation of DH5α and then TB-1 E. coli host cells. For expression of finger fragments, TB-1 cells were grown at 37 °C in Luria broth medium containing ampicillin (50 ng/ml). Overnight cultures were diluted 1:100 with fresh Luria broth medium, grown for 2 h at 37 °C (midlog phase), induced with isopropyl-1-thio-β-D-galactopyranoside (0.9 mm) for 4 h, harvested by centrifugation, and lysed by the freeze/thaw method. According to SDS-polyacrylamide gel electrophoresis analysis, the fusion proteins were found in the soluble fraction. Despite the presence of a signal peptide, the majority of the fusion products were found in the cytoplasm. In all preparations described here, the total soluble fraction was used as the starting point for purification. The finger fragments were separated from MBP by digestion with Factor Xa, protease V8, or pepstatin, and their amino termini were confirmed by sequencing.

Fluorescence Measurements of Thermal Unfolding—These were performed with a protein concentration of 0.1 mg/ml by monitoring the ratio of the intrinsic fluorescence intensity at 350 nm to that at 320 nm formed with a protein concentration of 0.2 mg/ml in 0.1-cm path length cells. Data were expressed as mean residue ellipticity, units of degrees cm² dmol⁻¹. Melting curves were obtained by heating the solvent. The curves were analyzed using software provided by Dr. Filimonov (Institute of Protein Research, Pushchino, Russia).

Circular Dichroism Spectra—These were obtained with a Jasco-500C spectropolarimeter using protein concentration of 0.2 mg/ml in 0.1-cm path length cells. Data were expressed as mean residue ellipticity, [θ], in units of degrees cm² dmol⁻¹. Melting curves were obtained by monitoring the ellipticity at fixed wavelength while heating the jacketed cells at 1 °C/min with a circulating water bath.

Differential Scanning Calorimetry—These measurements were made with a DASM-1M or DASM-4M instrument (21) at a heating rate of 1 °C/min. Protein concentration was 1 mg/ml. The differential scanning calorimetry curves were corrected for an instrumental base line obtained by heating the solvent. The curves were analyzed using software provided by Dr. Filimonov (Institute of Protein Research, Pushchino, Russia).

was digested with EcoRV and HindIII and directly ligated into pMAL-p2, creating a unique BamHI restriction site in place of the original XmaI site. The resultant plasmid (pMAL-p2-F1-2) was used for expression of the MBP-F1-2 fusion protein as well as for the generation of all subsequent expression constructs. For the latter purpose pMAL-p2-F1-2 was digested with BamHI and HindIII, and the resulting 6.7-kilobase pair fragment was purified by agarose gel electrophoresis and

Fig. 1. Schematic representation of the modular structure of fibronectin (top), its Fib-1 region and all expressed fragments (middle), and SDS-polyacrylamide gel electrophoresis analysis of the expressed fragments in non-reduced and reduced conditions (bottom). Lanes 2, rF1-2; lanes 3, rF2-3; lanes 4, rF4-5; lanes 5, rF1; lanes 6, rF2; lanes 7, rF3; lanes 8, rF4; lanes 9, rF5; lanes 1 and 10, molecular weight standards, from bottom to top: 2.5, 6.2, and 8.2 unreduced, 14.4, 16.9, and 20.4 kDa. GBF, gelatin-binding fragments; CBF, cell-binding fragments; Hep, COOH-terminal hep-2 heparin-binding fragments.
were applied to fibrin-Sepharose at 4 °C and eluted with a
gradient of urea and NaCl. The results are shown in Fig. 2. The
29-kDa Fib-1 fragment bound to fibrin-Sepharose and was
eluted in a peak centered at 46% of elution buffer. The 19-kDa
Fib-2 bound and was eluted slightly earlier than Fib-1. The
42-kDa gelatin-binding fragment exhibited the weakest bind-
ing, with peak elution at 26% of the gradient buffer. Other
fragments including the 110-kDa cell-binding fragment and the
COOH-terminal hep-2 heparin-binding fragments, containing
no finger domains, failed to bind. Thus, at low temperature, all
finger-containing fragments exhibited fibrin binding proper-
ties; the affinity of Fib-1 and Fib-2 fragments seems to be
highest.

Because the binding of fibronectin to immobilized fibrin is
reportedly sensitive to variations in temperature (1, 2, 12), all
three fibrin-binding fragments were tested for their ability to
bind fibrin-Sepharose at 4 °C, room temperature, and 37 °C.
The results are presented in Fig. 3. The 42-kDa gelatin-binding
fragment, which exhibited the weakest affinity at low tempera-
ture (Fig. 2), failed to bind at all at room temperature (Fig. 3A).
By contrast, the Fib-1 (Fig. 3B) and Fib-2 (Fig. 3C) fragments
bind at all three temperatures, although the percentage bound
diminished slightly with increasing temperature. Also the elu-
tion profile became broader with increasing temperature and,
especially in the case of Fib-2, the concentration of eluting
buffer required for elution appeared to increase with increasing
temperature. Thus, among the major finger-containing frag-
ments, only Fib-1 and Fib-2 were able to bind to fibrin-Sepha-
rose at physiological temperatures.

Preparation of Recombinant Finger Fragments—To further
localize the fibrin-binding site in the Fib-1 region, we expressed
all five individual fingers and some pairs of fingers (Fig. 1) as
fusion proteins with a carrier, MBP. The fusion proteins were
completely soluble after cell lysis and were purified by one-step
affinity chromatography on amylose resin. With the exception
of MBP-F1, all of the purified fusion proteins contained varying
amounts of dimeric and multimeric forms as detected by SDS-
polyacrylamide gel electrophoresis. The ratio of monomers to
multimers was similar in the periplasmic and cytoplasmic frac-
tions. Upon addition of reducing agent, all forms were con-
verted to monomers, indicating the presence of intermolecular
disulfide bonds. The proportion of monomers was 70–80% for
MBP-F2, -F3, -F4, and -F5, and 20–30% in the case of MBP-
F1-2, -F2-3, and -F4-5. The monomers were isolated by fast
reversed-phase HPLC and were characterized by fluorescence
spectroscopy. All fragments except rF2 and rF3 contained 2 extra
residues, and the pepsin-generated rF5 contained 6 extra NH2-
terminus residues including the recognition site for Factor Xa. Only the V8-liberated rF2 was devoid of extra residues.

Structural Characterization of the Recombinant Fragments—
Since the recombinant fragments contained disulfides and
were expressed in a bacterial system, it was important to char-
acterize their structural integrity, i.e. the presence of compact
structure, and to compare them with their natural proteolyti-
cally generated analogues where possible. Fig. 4 presents melt-
ing curves obtained by heating natural and recombinant frag-
ments F4-5 and F1 at neutral pH while monitoring the change in
fluorescence ratio. All of the fragments exhibit highly reversible
denaturation transitions with midpoints near 75 °C, indicat-
ing that the recombinant fragments are folded similarly to
their natural counterparts. These same two recombinant frag-
ments were also analyzed by differential scanning calorimetry.
The measurements were made at pH 3.2 where the fragments
are still stable, but their melting remains reversible at the
higher concentrations required for differential scanning calori-
metry. The excess heat capacity peak for rF1 was well de-
scribed by a single two-state transition with an enthalpy of 35
kcal/mol (Fig. 5A). The peak for rF4-5 has an enthalpy of 66
kcal/mol, consistent with the melting of two finger domains,
and was well described by 2 two-state transitions (Fig. 5B). The
enthalpies and deconvolution patterns are similar to those pre-
presented elsewhere with the natural F1 and F4-5 fragments.1
Thus, both of these recombinant fragments are folded into com-
plex structures similar to those of the same domains in plasma
fibronectin.

Melting properties of other recombinant proteins were studied
by fluorescence spectroscopy. All fragments except rF2 and
rF4 exhibited well defined highly reversible transitions in the
high temperature region (Fig. 6) demonstrating their struc-

![Fig. 4. Comparison of the denaturation/rehydration processes of proteolytically obtained natural (curves n) and recombinant (curves r) F1 (panel A) and F4-5 (panel B) fragments of fibronectin. Reversibility is shown by the dashed curves obtained upon cooling.](image-url)
Fig. 5. Differential scanning calorimetric analysis of recombinant F1 (panel A) and F4-5 (panel B) fragments of fibronectin in 50 mM Gly, pH 3.2, buffer. Smooth curves drawn through the experimental excess heat capacity curves represent the best fits of the data to one (rF1) or two (rF4-5) two-state transitions.

Fig. 6. Fluorescence-detected thermal denaturation of recombinant finger fragments. All experiments were performed in TBS. The scale on the ordinate corresponds to the lower curve; the other curves have been arbitrarily shifted along the vertical axis to improve visibility. Broken lines indicate reversibility on cooling.

Fig. 7. Denaturation of individual recombinant finger fragments and their pairs detected by CD. The solvent was 0.02 M phosphate, 0.15 M NaCl, pH 7.4. In each panel, the ordinate scale corresponds to the bimodular fragment; the other curve was shifted down to improve visibility. The insets show the CD spectra obtained with the bimodular fragments (solid curves) and unimodular fragments (dashed curves) at 10°C (positive amplitude) and 95°C (negative amplitude). Denaturation was monitored at 228 nm for rF4-5 and rF4, and 226 nm for rF2-3 and rF2.

Tural integrity. Recombinant fragments rF2 and rF4 exhibited only a hint of a transition that did not allow an unambiguous conclusion about their compactness and stability. Their fluorescence maxima occurred at rather long wavelengths, indicating that most of the fluorescence originates from Trp residues that are highly accessible to solvent even before denaturation (see below). This would decrease the change in fluorescence ratio that accompanies unfolding. We therefore examined these fragments by circular dichroism spectroscopy, an alternative method for monitoring structural changes upon denaturation. The CD spectrum of recombinant fragment rF4 showed a positive band at 228 nm. As shown in Fig. 7A, heating of this fragment up to 95°C abolished this feature. Registration of the change of ellipticity at 228 nm during heating revealed a broad transition with a midpoint near 45°C. A similar transition with a midpoint at about 66°C was observed for the rF2 fragment (Fig. 7B). Thus, the above results indicate that all recombinant finger fragments formed a compact structure.

Interaction Between Finger Domains in Bimodular Recombinant Fragments—Because interaction between domains in the parent protein may alter their stability and functional properties, we compared also the denaturation of individual recombinant fragments with that of their recombinant pairs. The CD-detected transition for rF4 occurs at a much lower temperature in comparison with that of rF4-5 (Fig. 7A) and rF5 (Fig. 6). This suggests the loss of a stabilizing interaction between the fourth and fifth finger domains. Additional evidence for such an interaction came from a comparison of the fluorescence spectra of the three fragments (Fig. 8). The maximum of fluorescence of the bimodular rF4-5 fragment occurs at 337 nm, whereas the separate fingers rF4 and rF5 both fluoresce at longer wavelengths. Also the fluorescence intensity of separated rF4 is much higher than that of the rF4-5. These results suggest that
Fibronectin residues on one protein to \( \gamma \)-carbamoyl groups of specific glutamine residues on the other protein to form an isopeptide bond. The action of \( \text{fXIIIa} \) involves coupling of \( \text{E-NH}_2 \) groups of lysine residues on one protein to \( \gamma \)-carbamoyl groups of specific glutamine residues on the other protein to form an isopeptide bond between them. However, by circular dichroism, isolated \( \text{rF2} \) melted at a lower temperature than bimodular fragment \( \text{rF2-3} \) (Fig. 7B) or \( \text{rF3} \) (Fig. 6). Although destabilization of the \( \text{rF2} \) fragment is not as dramatic as that of the \( \text{rF4} \), it could still suggest the loss of an interaction with the neighboring finger.

Fibrin Binding Properties of the Recombinant Fragments—The results of analytical affinity chromatography experiments at room temperature with all expressed fragments are presented in Fig. 9. Recombinant fragment \( \text{rF4-5} \) bound to the column and was eluted with a gradient of urea and sodium chloride. Similar binding was observed at 37°C. At the same time neither \( \text{rF4} \) nor \( \text{rF5} \) bound to the column, suggesting that both finger domains are required to form the fibrin-binding site. None of the other recombinant fingers, \( \text{rF1} \), \( \text{rF2} \), or \( \text{rF3} \), bound to the column. Their recombinant pairs, \( \text{rF1-2} \) and \( \text{rF2-3} \), also failed to bind. Affinity chromatography of all expressed fragments at 4°C gave essentially the same results (not shown) suggesting that neither individual finger domains nor the pairs, \( \text{rF1-2} \) and \( \text{rF2-3} \), bind fibrin even at low temperature. Thus, the fibrin-binding site of the Fib-1 region of fibronectin is formed by the two contiguous interacting finger domains 4 and 5.

**DISCUSSION**

Incorporation of fibronectin into the fibrin clot can be envisioned to occur in two steps. The first step is a reversible non-covalent interaction between the two proteins, and the second is their covalent cross-linking by activated Factor XIII (\( \text{fXIIIa} \)). The action of \( \text{fXIIIa} \) involves coupling of \( \text{e-NH}_2 \) groups of lysyl residues on one protein to \( \gamma \)-carbamoyl groups of specific glutamine residues on the other protein to form an isopeptide bond between them. The reaction is quite specific; of the hundreds of proteins in plasma, only a limited number have been identified as substrates for \( \text{fXIIIa} \), and only those with a known tendency to self-associate or to associate with another protein are likely to be cross-linked. Efficient cross-linking requires that the reacting partners associate in such a way that the acyl donors and acceptors are brought into proximity. In the case of fibronectin/fibrin, the association seems to involve type I finger domains on fibronectin, whereas the cross-linking involves Gln\(^3\) near the end of a 20-residue extension preceding the first finger. The non-covalent binding of tissue plasminogen activator to fibrin also appears to involve a finger domain. In our hands, all of the major finger-containing fragments of fibronectin were able to bind fibrin-Sepharose, but only at 4°C. These include the 29-kDa Fib-1 fragment (F1-5), the 19-kDa Fib-2 fragment (F10-121), and the 42-kDa gelatin-binding fragment, which contains F6-9. However, only the former two were able to bind at physiological temperatures, and of these, Fib-1 is likely to be most important for the cross-linking reaction.

Expression was performed in a bacterial system that allows one to produce substantial quantities of protein but is often inefficient for proteins that contain disulfide bonds. Each finger module contains four cysteines which form two disulfide bonds; MBP is devoid of cysteine. Analysis of the fusion proteins revealed a tendency for the proportion of disulfide-linked multimers to increase with an increasing number of fingers in the construct. In the case of whole Fib-1, which contains five fingers, all of the fusion protein was in the multimeric form (results not shown). Bimodular fragments contained 70–80% multimers while single modules gave only 20–30% multimers. In other words, while the yield of monomeric fusion protein correlated inversely with the number of cysteines, the results presented here clearly indicate the feasibility of producing properly folded finger fragments in prokaryotic cells. The specificity of cleavage of fusion proteins with Factor Xa has been discussed (26–28). In our case, cleavage of the purified monomeric fusion proteins with Factor Xa was successful in six out of eight cases. The resistance of MBP-F5 to proteolysis with Factor Xa may be due to the presence of only 2 residues separating the expected cleavage site from the following Cys that is part of the compact structure of the finger module. MBP-F2 presented a different kind of problem in that cleavage occurred not only at the expected site but at two additional sites in each disulfide loop of the F2 module. This was not due to improper folding of the module since digestion with protease V8 produced an intact finger. Interestingly, cleavage of MBP-F2-3 proceeded with no difficulty, suggesting that the sites that were vulnerable in MBP-F2 were masked in the bimodular fragment.
ger fragments confirmed that each was folded into a compact structure that underwent a cooperative unfolding transition when heated. In two cases, rF1 and rF4-5, it was possible to show that their melting temperatures at neutral pH, detected by fluorescence, and their thermodynamic properties obtained by differential scanning calorimetry at lower pH were similar to those of their natural analogues. The heat absorption peaks for these recombinant fragments were deconvoluted into one or two transitions, respectively, with enthalpies similar to those expected for finger domains. This clearly indicates that all of the molecules in each preparation were properly folded. All but two of the recombinant products showed a well defined transition by fluorescence. The exceptions were rF2 and rF4 which, when examined by CD, exhibited a positive heat-sensitive band near 225 nm that seems to be a prominent feature of fibronectin and has been used by others to monitor denaturation of fibronectin fragments (29). This feature was absent in the internally cleaved rF2 that had been liberated from its carrier protein with Factor Xa (not shown). This is consistent with the tenet of small-amplitude folding of their compact structure when cleaved within a disulfide loop (22).

Comparison of the melting behavior of the recombinant individual fingers with their pairs revealed a stabilizing interaction between some of them. Individual rF4 was much less stable by itself than in rF4-5, suggesting a strong interaction between this pair of modules. Additional evidence came from the observation that the fluorescence of rF4-5 was shifted to shorter wavelengths compared with that of rF4 and rF5. This suggests involvement of Trp residue(s) in the interdomain interaction. A recent NMR study of rF4-5 that had been expressed in yeast also demonstrated an interaction between these two modules via a well defined hydrophobic interface in which the nonconserved Trp<sup>177</sup> in F4 plays a major role (30). It seems likely that this residue could be responsible for the short wavelength fluorescence in rF4-5. In the absence of F5, this residue would be exposed to the solvent where it could account for the long wavelength fluorescence of isolated rF4. Note that rF5 was also slightly less stable when isolated. This could be due to the absence of Arg<sup>179</sup>-Asn<sup>180</sup> in the linker region immediately preceding F5 (Table I). These residues were shown to have several nuclear Overhauser effects with the turn between the last two β strands of F5 (30).

A different situation was found in the rF2-3 pair, which also contains an unconserved Trp<sup>60</sup> in the second finger. There was no noticeable difference in the maximum of its fluorescence spectra compared with that of the individual fragments rF2 and rF3. However, the stability of rF2 was significantly lower in the absence of neighboring F3 (Fig. 7B). As with rF4-5, this destabilization could reflect an interaction between the second and third finger domains in the rF2-3 pair. The absence of a spectral shift in this pair suggests that the environment of nonconserved Trp<sup>60</sup>, the only Trp in F2, would not be affected by that interaction. Trp<sup>60</sup> in F2 is not exactly homologous with Trp<sup>177</sup> in F4, being separated by 2 as opposed to 4 residues from the following conserved Cys. Thus, at least two pairs of domains in the fibronectin Fibr-1 region interact with each other based on the observed shifts in <i>T<sub>m</sub></i>. By this criterion, there were no obvious interactions between the first and second or third and fourth fingers, but one cannot exclude weaker interactions that would not be detected by this approach. That such interactions might exist is suggested by the fact that the fluorescence maximum of natural 29-kDa Fibr-1 occurs at a lower wavelength than any of the available subfragments (data not shown).

Affinity chromatography experiments revealed that among all recombinant fragments, rF4-5 alone was able to bind to fibrin-Sepharose at all temperatures tested. It is possible that the interaction between these two fingers creates a proper orientation of side chains, which may come from residues on both finger domains to form the fibrin-binding site. Alternatively, the binding site could reside on a single finger whose conformation is affected by its neighbor. It is interesting that F2 also interacts with F3, but this pair did not exhibit any fibrin binding activity, nor did rF1-2 or any of the individual fingers possess such properties even at 4 °C. This was unexpected based on published work suggesting that binding to fibrin in the cold is a general property of all fingers (Ref. 14, and references therein). This impression was reinforced by the observation that deletion of any one or all of the first five fingers from a recombinant 70-kDa NH<sub>2</sub>-terminal fragment of fibronectin had no effect on binding to fibrin-Sepharose at 4 °C; this was attributed to the fact that the remaining gelatin-binding portion of the construct also contains four finger domains (14). We show here that the 42-kDa gelatin-binding fragment fails to bind fibrin-Sepharose at 25 or 37 °C. Thus, in the context of the two-step mechanism of fibrinolysis action previously discussed, it is not clear how the Gap 1-5 derivative of Sottile et al. (14), lacking fingers 1-5 but retaining the NH<sub>2</sub>-terminal segment bearing the Gln<sup>2</sup> donor, is able to be cross-linked to fibrin at 37 °C. On the other hand, the work of Lorand and co-workers (31) suggests that mere peptides based on the first five amino acids of fibronectin, when presented at micromolar concentrations, can be cross-linked to fibrin by FXIIa without the need of any recognition by fingers. It appears that the "recognition" step discussed above might have the effect of accelerating the reaction without being absolutely required. A rigorous test of the effects of deleting fingers from the 29-kDa region on its cross-linking to fibrin would then require a kinetic analysis.

The major conclusions of this work are presented schematically in Fig. 10. Strong interactions are depicted by the intersection between pairs of modules 2-3 and 4-5. Although no evidence was obtained for additional interactions, the possibility cannot be excluded. The fibrin-binding site is formed by interacting fingers 4 and 5 and should be separated by a "spacer" module from the cross-linking site in the NH<sub>2</sub>-terminal extension preceding the first finger (13). Such separation of non-covalent and covalent binding sites suggests that binding of the fibrin aC domain to the complementary site formed by 4-5 creates a proper orientation of the donor and acceptor residues of both proteins to accelerate cross-linking by FXIIa. This also implies that complementary non-covalent and covalent binding sites of the aC domain would be spatially separated. Otherwise the first five finger domains should not be arranged linearly but would have to form a "closed" structure in order to bring the amino terminus containing Gln<sup>2</sup> close to the fourth/fifth finger domains containing the non-covalent fibrin-binding sites. Localization of the complementary sites in the aC domain may help to select between these two alternatives.

![Fig. 10. Schematic representation of domain-domain interactions and localization of the fibrin-binding site in the NH<sub>2</sub>-terminal region of fibronectin.](image-url)
Fibronectin-Fibrin Interaction

Acknowledgments—We thank Dr. S. Dufour for providing full-length cDNA for human fibronectin. Thanks also to Drs. Scott Argraves and David Mann for helpful advice about the expression work.

REFERENCES

1. Ruoslahti, E., and Vaheri, A. (1975) J. Exp. Med. 141, 497–501
2. Stathakis, N. E., Moseson, M. W., Chen, A. B., and Galanakis, D. K. (1976) Blood 51, 1211–1222
3. Mosher, D. F., and Johnson, R. B. (1983) Ann. N. Y. Acad. Sci. 408, 582–593
4. Chow, T. W., McIntire, L. V., and Peterson, D. M. (1983) Thromb. Res. 29, 243–248
5. Carr, M. E., Gabriel, D. A., and McDonagh, J. (1987) J. Exp. Med. 160, 1811–1820
6. Seidl, M., and Hormann, H. (1983) Hoppe-Seyler’s Z. Physiol. Chem. 364, 83–92
7. Seelig, K., and Hakomori, S. (1983) J. Biol. Chem. 258, 3967–3973
8. Hormann, H. (1985) in Plasma Fibronectin, Structure, and Function (McDonagh, J., ed) pp. 99–120, Marcel Dekker, Inc., New York
9. McDonagh, B. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorsten-gaard, K., Settrup-Jensen, L., Magnusson, S., Dell, A., and Morris, H. R. (1981) FEBS Lett. 127, 174–178
10. Botttete, J., Schwartzbauer, J., Selegue, J., and Mosher, D. F. (1991) J. Biol. Chem. 266, 12840–12843
11. Ingham, K. C., Brew, S. A., and Migliorini, M. M. (1989) J. Biol. Chem. 264, 16977–16980
12. Borsi, L., Castellani, P., Balza, E., Siri, A., Pellecchia, C., De Scalzi, P., and Zardi, L. (1986) Anal. Biochem. 155, 335–345
13. Heene, D. L., and Matthias, F. R. (1973) Thromb. Res. 2, 137–154
14. Voresthaya, T. V. (1961) Ukr. Biochem. J. IU. S. S. R.) 32, 13676
15. Seidl, M., and Hormann, H. (1983) Hoppe-Seyler’s Z. Physiol. Chem. 364, 109-123
16. Heene, D. L., and Matthias, F. R. (1973) Thromb. Res. 2, 137–154
17. Voresthaya, T. V. (1961) Ukr. Biochem. J. IU. S. S. R.) 32, 13676
18. Stathakis, N. E., Mosesson, M. W., Chen, A. B., and Galanakis, D. K. (1976) Blood 51, 1211–1222
19. Mosher, D. F., and Johnson, R. B. (1983) Ann. N. Y. Acad. Sci. 408, 582–593
20. Mosher, D. F. (1976) J. Biol. Chem. 251, 1639–1645
21. van Zonneveld, A.-J., Veerman, H., and Pannekoek, H. (1986) J. Biol. Chem. 261, 14214–14219
22. Nagai, K., and Thogersen, H. C. (1987) Methods Enzymol. 153, 461–481
23. Heene, D. L., and Matthias, F. R. (1973) Thromb. Res. 2, 137–154
24. Seidl, M., and Hormann, H. (1983) Hoppe-Seyler’s Z. Physiol. Chem. 364, 83–92
25. Seelig, K., and Hakomori, S. (1983) J. Biol. Chem. 258, 3967–3973
26. Hormann, H. (1985) in Plasma Fibronectin, Structure, and Function (McDonagh, J., ed) pp. 99–120, Marcel Dekker, Inc., New York
27. McDonagh, B. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorsten-gaard, K., Settrup-Jensen, L., Magnusson, S., Dell, A., and Morris, H. R. (1981) FEBS Lett. 127, 174–178
28. Botttete, J., Schwartzbauer, J., Selegue, J., and Mosher, D. F. (1991) J. Biol. Chem. 266, 12840–12843
29. Ingham, K. C., Brew, S. A., and Migliorini, M. M. (1989) J. Biol. Chem. 264, 16977–16980
30. Borsi, L., Castellani, P., Balza, E., Siri, A., Pellecchia, C., De Scalzi, P., and Zardi, L. (1986) Anal. Biochem. 155, 335–345
31. Seidl, M., and Hormann, H. (1983) Hoppe-Seyler’s Z. Physiol. Chem. 364, 83–92
32. Seelig, K., and Hakomori, S. (1983) J. Biol. Chem. 258, 3967–3973
33. Hormann, H. (1985) in Plasma Fibronectin, Structure, and Function (McDonagh, J., ed) pp. 99–120, Marcel Dekker, Inc., New York
34. McDonagh, B. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorsten-gaard, K., Settrup-Jensen, L., Magnusson, S., Dell, A., and Morris, H. R. (1981) FEBS Lett. 127, 174–178
35. Botttete, J., Schwartzbauer, J., Selegue, J., and Mosher, D. F. (1991) J. Biol. Chem. 266, 12840–12843
36. Ingham, K. C., Brew, S. A., and Migliorini, M. M. (1989) J. Biol. Chem. 264, 16977–16980