Covalent Cross-linking of Fibronectin to Fibrin Is Required for Maximal Cell Adhesion to a Fibronectin-Fibrin Matrix*

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In a blood clot, fibrin and plasma fibronectin (pFN) are covalently cross-linked by activated factor XIII (factor XIIIa) to form pFN-fibrin multimers. To determine the functional significance of covalent pFN-fibrin interactions, we have developed an in vitro model which allows the incorporation of recombinant FN (recFN) molecules into a covalently cross-linked recFN-fibrin matrix. Using the baculovirus expression system, we have expressed recFN monomers composed of the amino-terminal 70-kDa region and the first 11 type III repeats (WT) with mutations in the glutamines at positions 3 and 4 (Q2) or at 3, 4, and 16 (Q3). Examination of the covalent incorporation of these recFNs into fibrin clots confirms that glutamines 3 and 4 are major participants in FN-fibrin cross-linking as the mutation of these sites reduces cross-linking efficiency by 65%. Additional mutation of the glutamine at position 16, however, eliminates >99% of cross-linking suggesting that it also may be factor XIIIa reactive. When the Q3 recFN-fibrin clots were used as substrates for cell adhesion, there was a decrease in both cell attachment and spreading when compared with the WT recFN-fibrin clots. These data demonstrate that for maximal cell attachment to a FN-fibrin clot, FN must be cross-linked to fibrin by factor XIIIa.

Following tissue injury, formation of a blood clot serves both to restore vascular integrity and to provide a provisional matrix for the initiation of wound repair (1–3). The clot’s major protein components, fibrin and plasma fibronectin (pFN),⁴ are essential to these functions. Clot polymerization begins when soluble fibrinogen is converted by thrombin to fibrin (4). This proteolytic event is followed by spontaneous assembly of fibrin monomers into polymers (2, 4). Concurrently, soluble pFN is incorporated with fibrin into the clot. As the clot matures, intermolecular cross-linking between fibrin molecules and between pFN and fibrin proceeds, dependent on activated coagulation factor XIII (factor XIIIa, plasma transglutaminase) (5, 6). While covalent cross-linking between fibrin molecules is essential for the clot’s structural stability, the presence of pFN with its multiple adhesive domains is important to the cell adhesion and migration events required for the wound healing process. For example, FN is an absolute requirement for migration of fibroblasts into plasma clots in vitro, where it must be present prior to initiation of the clotting reaction (7). Furthermore, cross-linking of FN to soluble fibrin-coated dishes promotes fibroblast attachment and spreading (8) while fibrin adherence to a cross-linked FN-fibrin clot matrix results in unique cytoskeletal organization (9). Clearly, the association with FN improves the adhesive character of fibrin substrates and factor XIIIa-mediated covalent cross-linking appears to play a key role in this process.

Fibronectin, a multifunctional adhesive glycoprotein, plays an important role not only in hemostasis and tissue repair, but also in embryogenesis and oncogenic transformation (10, 11). Each FN subunit contains two major fibrin-binding sites which mediate noncovalent interaction with fibrin (12–15). These have been localized to the amino-terminal type I repeats 1–5 and the carboxyl-terminal type I repeats 10–12 (12, 14). Covalent FN-fibrin binding involves glutamine residues localized to a 27-kDa amino-terminal FN fragment containing repeats I₁₁–₅. Cross-linking at these sites is mediated by thrombin-activated factor XIII (factor XIIIa) which catalyzes the formation of covalent bonds between a glutamine (Gln) in FN and the ε-amino group of a lysine residue in the α-chain of fibrin (16, 17). Only a small subset of proteins are able to act as effective glutamine acceptors for factor XIIIa. The enzyme’s stringent specificity is determined by the amino acid sequence surrounding the reactive glutamine residue (18–20). Incubation of pFN with factor XIIIa and labeled lysine analogs has identified the primary factor XIIIa-reactive site as the glutamine in the third position from the amino terminus, but Gln-4 has also been shown to be factor XIIIa-reactive (18, 21).

We have developed an in vitro model that allows the incorporation of recombinant FN (recFN) molecules into a cross-linked recFN-fibrin matrix. The cross-linked products can then be quantified or alternatively, the recFN-fibrin clots can be used to form substrates for cell attachment. In this study, we sought to determine the role of covalent cross-linking in promoting cell interaction with a FN-fibrin clot. FN molecules composed of the amino-terminal 70-kDa region and the first 11 type III repeats (WT) with mutations in the glutamines at positions 3 and 4 (Q2) or at positions 3, 4, and 16 (Q3) were used to form recFN-fibrin matrices. RecFN-fibrin cross-linking was analyzed and cell adhesion assays were performed. These experiments have confirmed that Gln-3 and Gln-4 are the major participants in factor XIIIa-mediated FN-fibrin cross-linking, but suggest that Gln-16 may also be factor XIIIa-reactive. When Q3 recFN-fibrin clots were used to measure cell adhe-
sion, a decrease in both cell attachment and cell spreading were observed. Thus, these data provide strong evidence that factor XIIIa-mediated cross-linking of FN to fibrin is required for effective cell adhesion to a FN-fibrin matrix.

EXPERIMENTAL PROCEDURES

Cell Culture

Mouse NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (HyClone Laboratories). For cell attachment and spreading assays, cells were trypsinized, washed, and resuspended in serum-free DMEM for biologic assays as described (9). Where indicated, cells were labeled overnight with 25 μCi/ml [35S]methionine in DMEM-methionine supplemented with 10% calf serum and 1.5 μg/ml cold methionine. BTI-TN5B1-4 (High Five) insect cell line (Invitrogen Co.) was maintained in TMN-FH (Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone Laboratories). For purification of recombinant protein, High Five cells were grown in Express Five (Life Technologies, Inc.) serum-free medium supplemented with 18 μM t-glutamine.

Preparation of Mutants and Construction of FN cDNAs

70kDIII1–11—Construction of the 5′ end of 70kDIII1–11 has been previously described (22). A termination codon at position 5150 was inserted between the BamHI and XhoI sites in the baculovirus vector pVL1393. A 420-base pair fragment containing the factor XIIIa recognition site was cloned into pGEM-3Zf+ (Promega Biotec). A single-stranded DNA template was prepared and annealed to a 21-base pair oligonucleotide, 5′-CTGCACTATGGAGAGCTGGT-3′ (Synthesis and Sequencing Facility, Princeton University) complementary to the desired mutant sequence (5′-CAGCCTGCTGAATCGTG-CAG-3′). Substitution of T5 (underlined) for A5 in the native sequence changes amino acid residues 3 and 4 from <QAAQV> to <QL-LIQ> and also results in the loss of a Ddel restriction site (in bold). The mutant oligonucleotide was incorporated into the cDNA using Version 2 of the Oligonucleotide-directed In Vitro System (Amersham Corp.) as directed by the manufacturer. An XhoI-HoeII fragment containing the mutant sequence was used to generate 70kDIII1–11(Q3,4L) from pSP73 (pSP73-Q16A). This plasmid was then used to generate a 70kDIII1–11(Q3,4AA) fragment containing all three mutations into the 70kDIII1–11 cDNA in vector pVL1393.

Preparation of Mutants and Construction of FN cDNAs

70kDIII1–11—Construction of the 5′ end of 70kDIII1–11 has been previously described (22). A termination codon at position 5150 was generated by adding an XhoI linker to a blunted PstI site located at the end of type III repeat 11. This fragment of the rat FN cDNA (from previously described (22). A termination codon at position 5150 was primers, FNQQ34AA and FNQ16A (Synthesis and Sequenc- ing Facility, Princeton University), were used for polymerase chain reaction (PCR) amplification of the amino-terminal region. The primer sequence was 5′-CTGCACTATGGAGAGCTGGT-3′ (Synthesis and Sequencing Facility, Princeton University) complementary to the desired mutant sequence (5′-CAGCCTGCTGAATCGTG-CAG-3′). Substitution of T5 (underlined) for A5 in the native sequence changes amino acid residues 3 and 4 from <QAAQV> to <QL-LIQ> and also results in the loss of a Ddel restriction site (in bold). The mutant oligonucleotide was incorporated into the cDNA using Version 2 of the Oligonucleotide-directed In Vitro System (Amersham Corp.) as directed by the manufacturer. An XhoI-HoeII fragment containing the mutant sequence was used to generate 70kDIII1–11(Q3,4L) from pSP73 (pSP73-Q16A). This plasmid was then used to generate a 70kDIII1–11(Q3,4AA) fragment containing all three mutations into the 70kDIII1–11 cDNA in vector pVL1393.

Recombinant Protein Production and Purification

Recombinant protein production was performed as described previously (22). Briefly, recombinant baculoviruses were generated by cotransfection of High Five cells with baculovirus vector pVL1393 and Baculogold DNA (Pharmingen). Single viral clones were obtained by limiting dilution cloning of transfection supernatants. High titer stocks were then amplified as described by Summers and Smith (24). High Five cells grown in serum-free Express Five medium (Life Technologies, Inc.) in 175-cm² flasks were infected with high-titer viral stock. The culture medium was collected 3 days post-infection at which time protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM) and EDTA (10 mM) were added. Recombinant FN (refFN) and rat plasma FN (pFN) were purified by gelatin-agarose chromatography (25) and stored at −80°C in CAPS-buffered saline (0.1 M CAPS, 0.15 M NaCl, pH 11.0). Purity of the preparations was confirmed by silver stain analysis after SDS-polyacrylamide gel electrophoresis (26, 27).

Formation and Analysis of FN-Fibrin Clots

Lyophilized human fibrinogen (98.8% clottable, American Diagnostics) and bovine thrombin were reconstituted as described previously (9). Endogenous human plasma FN contaminating the fibrinogen was removed by batch incubation with gelatin-agarose. To assess the cross-linking efficiency of the refFNs versus pFN, the clotting reactions were composed of the following components mixed in a 1:10:1 physiologic ratio of fibrinogen to FN: fibrinogen (600 μg/ml), pFN/refFN (60 μg/ml), thrombin (2 units/ml), 0.02 M CaCl₂, 0.15 M NaCl, 0.05% Tris-HCl, pH 7.5. Human coagulation factor XIII (Calbiochem-Novabiochem Corp.) was added at 6 μg/ml (28). The clots were formed in a volume of 0.05 ml in microcentrifuge tubes and incubated on ice for various time periods as indicated. To prepare ligand-coated dishes, the clotting components were mixed in a volume of 0.06 to 0.25 ml to give concentrations of fibrinogen (and FN of 2.4 μg/ml) and thrombin of 0.12 μg/ml, respectively. As the remainder of the clotting components were at the concentrations as described above. After the addition of thrombin, the clotting reaction was rapidly pipetted into nontissue culture dishes and allowed to incubate overnight at 4°C. Care was taken to ensure that the mixture completely covered the bottom of the dish.

To solubilize the clots, an equal volume of S buffer (8 M urea, 2% SDS, 2% 2-mercaptoethanol, 0.16% Tris-HCl, pH 6.8) was added to each reaction. Separation and identification of cross-linked products was performed as described in Wilson and Schwarzbauer (29). Briefly, cross-linked products were separated on 5% polyacrylamide minigels and, after transfer to nitrocellulose, FN was detected using hybridoma culture supernatant containing a rat-specific monoclonal antibody, 504, at a dilution of 1:100. Immunoblots were then developed using biotinylated goat anti-mouse IgG and streptavidin horseradish peroxidase (Life Technologies, Inc.) followed by peroxide and 4-chloro-1-naphthol substrate.

Quantitation of cross-linking was performed according to Secher et al. (23). Immunoblots of cross-linked products were blocked overnight at 4°C in 10% bovine serum albumin in TBS (200 mM Tris-HCl, 200 mM NaCl) and then incubated with 504 monoclonal antibody at a dilution of 1:100 in 5% bovine serum albumin in TBS for 1 h. After three washes in TBS, the filter was incubated with rabbit anti-mouse IgG (Pierce) diluted to 1 mg/ml in 5% bovine serum albumin in TBS for 1 h at room temperature, and then washed four times in TBS. Approximately 2 μCl of 35S-Protein A (NEN Life Science Products, Inc.) was added to 10 μl of 504 monoclonal antibody in TBS. The 125I-Protein A was incubated with the blot for 1 h at room temperature and then washed four times with Buffer A until the background signal was minimal. Washed blots were exposed to a phosphor storage screen and analyzed using a Molecular Dynamics PhosphorImager. Uncross-linked monomer was calculated for each of four time points using Image Quant software and the percent of refFN monomer incorporated into high molecular weight multimers over time was determined. After exposure to the phosphor storage screen, immunoblots were exposed to film (X-Omat; Eastman Kodak).

Cell Attachment and Spreading Assay

To quantitate cell attachment to clots containing different FNs, well-controlled non-tissue culture dishes were coated with FN-fibrin clots as detailed above. After the overnight incubation, the clots were washed with serum-free DMEM. Mouse NIH 3T3 cells were labeled with [35S]methionine as described above. A total of 4 × 10⁴ cells in serum-free DMEM were added per well and then allowed to attach for 30 min. Wells were gently washed twice with phosphate-buffered saline, and the cells were fixed for 20 min in 3.7% formaldehyde in phosphate-buffered saline. The wells were allowed to dry at 37°C for 2 h and then exposed for 20 min to a PhosphorImager screen (Molecular Dynamics) as described by Dalton et al. (30). Total counts per well were determined using Image Quant software. For each experiment, clots containing either pFN or refFN were formed in quadruplicate. The results are representative of one of three experiments.

To determine cell area, 4 × 10⁴ cells were applied to clots formed in 48-well non-tissue culture dishes. Attached and spread cells were examined using inverted phase-contrast optics. Photographic images were captured using an NEC video camera (NEC Corp.) connected to a
The FN monomers used for the experiments reported here lack the collagen-binding region of FN, they can be purified by gelatin-agarose chromatography to yield protein preparations that are 90% pure (data not shown).

RESULTS

Production of Recombinant FN Monomers Using the Baculovirus Insect Cell System—We have developed an in vitro model that allows the covalent incorporation of recFN molecules into a three-dimensional FN-fibrin clot. Using this model, we can both quantify transglutaminase-mediated FN-fibrin cross-linking and determine its functional significance in terms of cell-ligand interaction. To generate sufficient quantities of recFN for incorporation into clots, we have used the baculovirus insect cell system. Expression of FN dimers using this system has been previously described and has the advantage of yielding recFN molecules in which the type I, II, and III repeats are properly folded (23). Fig. 1 illustrates the recFN constructions used in this study. 70kDIII1–11 (WT) is composed of the 70-kDa region, known to contain the primary factor XIIIa reactive site(s), connected to the first 11 type III repeats terminating at indicated times (2, 6, and 18 h) were compared by immunoblotting with 504, a rat specific monoclonal antibody against FN. The control samples in lane C were incubated with EGTA which prevents cross-linking. Cross-linked FN-fibrin heterodimers (arrow) increase with time in a similar fashion in both pFN and the WT recFN.

pFN when compared with the truncated monomeric WT recombinant protein (Fig. 2B). This demonstrates that the carboxyl-terminal fibrin-binding region plays a limited role in mediating the noncovalent intermolecular associations required for the action of factor XIIIa in this model.

Mutation of the Major Factor XIIIa-reactive Site and Its Effect on Cross-linking—Factor XIIIa catalyzes the coupling of ε-NH₂ groups of lysine residues on the fibrin α-chain to γ-carbamoyl groups of specific glutamine residues on FN, forming an isopeptide bond that is stable and resistant to proteolysis (6, 16, 17). McDonagh et al. (21) have reported that the glutamine at position 3 in the amino terminus of bovine FN is labeled by the lysine analogue, putrescine, indicating that this residue is the primary acceptor site for factor XIIIa. Others have suggested that the glutamine at position 4 may also be factor XIIIa reactive (18). To determine the role of the glutamines at positions 3 and 4 in factor XIIIa-mediated FN-fibrin cross-linking, mutations were introduced at these sites and Q2 recFN was compared with WT recFN in cross-linking assays. Like the WT recFN, Q2 forms the major FN-fibrin heterodimer and larger oligomers (Fig. 3, A and B). However, quantification of the residual monomer over time revealed that cross-linking is significantly diminished. Eighty percent of WT is incorporated into high molecular multimers by 18 h compared to 27% of Q2, a 65% decrease in cross-linking efficiency (Fig. 4). These results confirm that glutamines 3 and 4 are major participants in FN-fibrin cross-linking but suggest that an additional site(s) may also play a role in the cross-linking process.
sequence corresponding to residues 161 through 175 of β-casein (19, 20). Fig. 5 illustrates the consensus sequence for the factor XIIIa-reactive site in β-casein and compares this sequence to residues 10 through 18 in the amino terminus of FN. The asterisk denotes the lysine at position 169 in β-casein, the loss of which causes a significant decrease in factor XIIIa labeling of Gln-167 in this peptide. Clearly, there is significant homology between the β-casein consensus sequence and residues 10 through 18 in FN suggesting that this may be an additional site for factor XIIIa recognition.

To determine if the glutamine at position 16 plays a role in factor XIIIa-catalyzed FN-fibrin cross-linking, a construction was made in which mutations were introduced into the codons at positions 3, 4, and 16 (Q3) changing the glutamines to alanines (Fig. 1). The Q3 protein was then used in cross-linking assays under identical conditions as WT recFN. Fig. 3 demonstrates a significant difference in the cross-linking pattern between Q3 and the WT and Q2 recombinants. The elimination of Q16 results in a virtually complete loss of cross-linking. Cross-linked recFN-fibrin heterodimers (arrow) increase with time (A and B). When Q2-fibrin cross-linking is analyzed, the formation of the recFN-fibrin heterodimer is decreased (B). No recFN-fibrin heterodimer or high molecular weight multimers are observed with Q3 and fibrin (C).

Cross-linking of FN to Fibrin Is Required for Maximal Cell Attachment and Spreading—FN-fibrin clots composed of either pFN or recFN were prepared in 96-well dishes. Fibrin clots served as the negative control. Equal numbers of NIH 3T3 cells metabolically labeled with [35S]methionine were allowed to attach to the clots for 30 min. The number of cells attached to pFN-fibrin clots when compared with the WT-fibrin clots was similar (Fig. 6). While cell attachment to the Q2-fibrin clots was diminished, these results were not statistically different from WT (p = 0.19). In contrast, when clots were formed with the Q3 protein, cell attachment was significantly decreased to 43% that observed on pFN- or WT-fibrin clots (Fig. 6). These results show that despite the presence of an equal amount of FN in the WT and Q3 clots, cross-linking of the FN to fibrin must occur for the efficient exposure of ligand-binding sites.

The complete process of cell attachment to an adhesive substrate involves initial attachment followed by cell spreading and cytoskeletal organization. While cell adhesion to a Q3-fibrin matrix is significantly decreased, some attachment does occur. However, the attached cells appear smaller than cells adhering to WT-fibrin clots (Fig. 7). To determine whether cell spreading on this substrate is similarly affected by the absence of cross-linking, cell areas on WT- and Q3-fibrin clots were determined and the measurements were then grouped according to size. On the WT-fibrin clots, the greatest numbers of cells had areas between 500 and 700 μm² (Fig. 8A). In contrast, cells attached to the Q3-fibrin clots spread to a reduced degree with...
the greatest number of cells found between 300 and 500 μm² (Fig. 8B). Even when the cells were allowed to attach to the Q3-fibrin clots for an extended time, cell areas never reached that seen on WT recFN (data not shown). These results provide further evidence of the requirement for factor XIIIa-mediated FN-fibrin cross-linking for efficient cell-FN interaction. Thus, for maximal cell attachment and spreading on a FN-fibrin clot, FN must be cross-linked to fibrin by factor XIIIa.

**DISCUSSION**

In this report, we have described an in vitro model that allows the incorporation of recFN molecules into a cross-linked fibrin matrix. In the presence of activated factor XIII, these recFNs are covalently bound to fibrin in a reaction that mimics in vivo clotting events. The resulting matrix can then be analyzed to quantify FN-fibrin cross-linking or used as a substrate for cell attachment. We have used this model to determine the functional significance of covalent FN-fibrin interactions. FN molecules composed of the 70-kDa region and the first 11 type III repeats (WT) with mutations in the glutamines at positions 3 and 4 (Q2) or at positions 3, 4, and 16 (Q3) were used to form recFN-fibrin clots. These experiments demonstrate a 65% decrease in the cross-linking efficiency of the Q2 recFN confirming that Gln-3 and -4 are major factor XIIIa-reactive sites in the amino terminus of FN as has been previously described (18, 21). Greater than 99% of cross-linking was then eliminated by the additional mutation of Gln-16. When the Q3-fibrin clots were formed as substrates for cell adhesion, there was a significant decrease in both cell attachment and spreading when compared with the WT-fibrin clot. This provides strong evidence that factor XIIIa-mediated cross-linking of FN to fibrin is required for efficient cell-FN interactions.

Site-specific labeling of the factor XIIIa-reactive glutamines in FN has been performed using lysine analogs such as putrescine or monodansylcadaverine and has shown that Gln-3 near the amino terminus of FN is a major factor XIIIa-reactive residue (21). Factor XIIIa displays a preference for the first glutamine in an X-Q-Q sequence, not only in FN, but also in the γ-chains of fibrin (31), in α₂-plasmin inhibitor (32), and in α₂-macroglobulin (33). The second glutamine can also be labeled but to a much lesser degree (18). Our results confirm that Gln-3 and Gln-4 are primary sites for FN-fibrin cross-linking, but in their absence cross-linking still occurs, indicating that another glutamine residue is susceptible to factor XIIIa. To determine a possible candidate residue, the structural features of transglutaminase specificity were considered.

Transglutaminases are a highly conserved family of enzymes with widespread tissue distribution (16, 17). They catalyze the formation of e-(γ-glutamyl)lysine isopeptide bonds either within or between polypeptide chains creating cross-linked multimeric structures (6, 16, 17). In general, these enzymes have a broad specificity for donor amines. However, only a small subset of proteins are able to act as effective glutamine acceptors and this depends on their capacity to form acyl-enzyme intermediates with the enzyme’s active site. Activated factor XIII (plasma transglutaminase) has particularly stringent structural requirements for glutamine residues (18–20). For example, Gorman and Folk (18) found β-casein to be an excellent substrate for factor XIIIa but a poor substrate for liver transglutaminase. Furthermore, they noted that the determinants for enzyme recognition of β-casein were contained...
in the linear sequence surrounding the glutamine at position 167 (19, 20). Amino acid residues 10 through 18 in FN have significant homology to this sequence with Gln-16 corresponding to the reactive glutamine in the β-casein sequence. Mutation of this residue in conjunction with Gln-3 and Gln-4 eliminated >99% of cross-linking. These data suggest that Gln-16 may function as an additional acceptor site which seems likely given its homology to the β-casein sequence. However, it does not rule out that the mutation may disrupt the tertiary structure of the amino terminus so that factor XIIIa recognition of an alternate site is affected.

The major finding of this work is that the factor XIIIa-mediated formation of covalent bonds between FN and fibrin is required for maximal cell adhesion to a FN-fibrin matrix. The presence of pFN in the fibrin clot is essential to its function as a provisional matrix as it provides important adhesive sites for cell attachment and migration into the wound (1, 3). Our results now demonstrate that the mere presence of FN is not sufficient for efficient cell attachment and spreading. Rather, FN must be covalently cross-linked to fibrin to provide an effective adhesive substrate. These data support the suggestion by others that FN-fibrin cross-linking can influence cell adhesion. Grinnell et al. (7) demonstrated that FN cross-linked by factor XIIIa to soluble fibrin-coated dishes was required for fibroblast attachment and spreading. Other work determined that fibroblast migration into plasma clots required pFN-fibrin interaction during clot formation (8). When FN was adsorbed to FN-depleted plasma clots, cell migration was not observed, supporting a role for factor XIIIa-mediated FN-fibrin cross-linking in these events. Thus, it is apparent that covalent interaction between FN and fibrin can modulate FN’s adhesive properties in some fashion.

One possible explanation for the cross-linking requirement may be that cross-linking of FN to fibrin induces a conformational change in the molecule that influences cell recognition of its adhesive domains. Soluble FN is a poor ligand for cell surface receptors as it adopts a compact conformation which is maintained by hydrophobic interactions (34). When FN is immobilized onto a surface, however, it appears as though the molecule unfolds (35–37). Recently, Ugarova et al. (38) assessed the availability of cell-binding sites in repeat III10 of pFN using monoclonal antibodies and showed that there was limited accessibility of the epitope in soluble pFN while immobilization of the protein on a surface increased antibody binding, suggesting that the cell-binding domains are exposed by this process. Thus, immobilization may induce a conformational change in FN that is an important determinant of cell recognition. One mechanism for immobilization in vivo is by the covalent cross-linking of FN to fibrin. Our experiments which mimic in vivo clotting events indicate that covalent incorporation of FN into a fibrin matrix may improve the access of cell surface receptors such as those of the integrin superfamily, to the cell-binding site. This may occur by allowing the molecule to unfold into an “extended” conformation. In addition to receptor occupancy, cell adhesion requires receptor clustering (39). The structural constraints of a FN-fibrin matrix may fix cell-binding sites in proximity so that organization of cell surface receptors occurs in a defined manner. Efficient cell adhesion therefore, could be dependent on cross-linking not only for conformation but also for the proper architecture of the matrix.

When FN is cross-linked to fibrin, high molecular weight multimeric forms of the protein are formed while the Q3 recFN remains as a monomer. This may have functional significance as structurally distinct forms of the protein seem to have different effects on cell adhesion. For example, treatment of soluble pFN with a fragment from the first type-III repeat induces spontaneous disulfide cross-linking of the molecule into multimers which resemble matrix fibrils (40). This “superfibronectin” has greatly enhanced adhesive properties when compared with the dimeric form of the protein. The mechanism by which this effect is mediated is unknown, however, differences in the adhesive characteristics of monomeric and polymeric forms of collagen have also been demonstrated (41). Thus, the decreased ability of the Q3-fibrin clot to support cell attachment may reflect the absence of more adhesive FN multimers.

Recent evidence has demonstrated that cells may be able to sense the rigidity of the extracellular matrix and respond by a localized strengthening of cytoskeletal linkages (42). These data suggest that the degree of tension in the extracellular matrix can determine adhesion site characteristics which are important not only to cell migration but also to initial cell attachment and spreading. Thus, if the cells can sample the resistance of the extracellular matrix anchoring site, then they may be able to detect whether molecules are free or whether they are bound to a fibrillar network and respond by strengthening their contacts with the bound molecules. This offers an alternate explanation as to why factor XIIIa-mediated cross-
linking of FN and fibrin is required for efficient cell adhesion. Cells contacting the Q3 recFN within the fibrin clot may be unable to generate sufficient tension/resistance as they spread because the protein is not covalently bound to the fibrin within the clot. Cytoskeletal linkages are not reinforced to the same degree as if the protein were bound to the matrix thus affecting both cell attachment and cell spreading.

In summary, we have synthesized recFN molecules with mutations in the amino-terminal fibrin cross-linking region of FN. The Q2 recFN-fibrin cross-linking is significantly reduced, confirming that Gln-16 may also be factor XIIIa-reactive. Finally, we have demonstrated conclusively that factor XIIIa-mediated cross-linking of FN to fibrin is required for effective cell adhesion to a FN-fibrin matrix.

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