Interaction of Fibrin(ogen) with Fibronectin: Further Characterization and Localization of the Fibronectin-Binding Site†

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ABSTRACT: The interaction of fibronectin with fibrin and its incorporation into fibrin clots are thought to be important for the formation of a provisional matrix that promotes cell adhesion and migration during wound healing. However, it is still unclear whether fibronectin interacts with both fibrin and fibrinogen or fibrin only and whether fibronectin binds exclusively to the fibrin(ogen) αC domains. To address these questions, we studied the interaction of fibronectin with fibrinogen, fibrin, and their proteolytic and recombinant fragments. In both ELISA and surface plasmon resonance (SPR) experiments, immobilized fibrinogen did not bind fibronectin at all, but after conversion to fibrin, it bound fibronectin with high affinity. To test which regions of fibrin are involved in this binding, we studied the interaction of fibronectin with the fibrin-derived D−D:E_1 complex and a recombinant αC fragment (residues Aα221−610) corresponding to the αC domain that together encompass the whole fibrin(ogen) molecule. In ELISA, when fibronectin was added to the immobilized D−D:E_1 complex or the immobilized αC fragment, only the latter exhibited binding. Likewise, when fibronectin was immobilized and the complex or the αC fragment was added, only the latter was observed to bind. The selective interaction between fibronectin and the αC fragment was confirmed by SPR. The fibronectin-binding site was further localized to the NH_2 terminal connector region of the αC domain since in ELISA, the immobilized recombinant Aα221−391 sub-fragment bound fibronectin well while the immobilized recombinant Aα392−610 sub-fragment exhibited no binding. This finding was confirmed by ligand blotting analysis. Thus, the results provide direct evidence for the existence of a cryptic high-affinity fibronectin-binding site in the Aα221−391 region of the fibrinogen αC domain that is not accessible in fibrinogen but becomes exposed in fibrin.

The plasma proteins fibrin(ogen) and fibronectin play a prominent role in hemostasis and wound healing. Following vascular injury, fibrinogen is activated by thrombin to form an insoluble fibrin clot that prevents the loss of blood. The clot also serves as a provisional matrix for cell adhesion and migration into the injured tissue during subsequent tissue repair. These processes occur via interactions of cell surface receptors with fibrin as well as some other proteins, including fibronectin, which are incorporated into the fibrin clot upon fibrin polymerization.

Fibronectin is a multifunctional adhesive glycoprotein that contains multiple binding sites for a number of macromolecules and surface receptors on a variety of cells, including fibroblasts, neurons, phagocytes, and bacteria. Thus, being incorporated into the clot, it may affect the adhesion and migration of cells. Indeed, fibronectin was demonstrated to promote fibroblast adhesion and spreading and was found to be an absolute requirement for migration of fibroblasts into a plasma clot (*1*−*3*). On the other hand, incorporation of fibronectin into fibrin matrixes restricted the fibrin-mediated macrophage migration (*4*). Both the promoting and the inhibitory effects were maximal when fibronectin was cross-linked to the fibrin matrix by factor XIIIa (*2*−*5*).

Incorporation of fibronectin into a fibrin clot occurs in two stages. First, fibronectin binds reversibly to fibrin and then becomes covalently cross-linked to it by factor XIIIa. While the second stage is well characterized (*6*−*8*), the reversible stage is still not well understood. Early studies of cryoprecipitates or cryofibrinogens that represent complexes between fibrinogen, fibrin, and fibronectin suggested that the latter interacts with fibrin and to a lesser degree with fibrinogen and that this interaction is particularly evident in the cold (*9*−*12*). Affinity chromatography measurements revealed that fibrinogen−Sepharose displayed significant fibronectin binding at low temperature but very little at room temperature while fibrin−Sepharose bound significant quantities at both temperatures (*10*). It was also demonstrated that fibrin-binding fragments of fibronectin, Fib-1 and Fib-2, were able to bind to fibrin−Sepharose at low and physiological temperatures, although some differences were noticed with increasing temperature (*13*). Thus, the questions of whether fibronectin interacts only with fibrin or with both fibrin and fibrinogen and the extent to which this interaction is affected by temperature need further study.

Two separate regions in each fibronectin subunit have been implicated in the noncovalent binding, the NH_2 terminal Fib-1 region containing five type I domains, F1−5, and the COOH terminal Fib-2 region containing three type I domains,

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F10−12. (*12*, *14*, *15*). The fibrin-binding site within the Fib-1 region was further localized to type I domains 4 and 5 (*13*, *16*). Less is known about the location of complementary binding sites in fibrin(ogen). On the basis of the inability of fibrin(ogen) lacking COOH terminal portions of the Aα chains to form cryoprecipitates with fibronectin, it was suggested that this portion, which forms the αC domain in each of two fibrinogen subunits, is required for fibronectin binding (*9*, *10*). In agreement, a hementin-produced fibrinogen D_{hem} fragment containing this domain bound to immobilized fibronectin, while its truncated variant lacking this domain did not (*17*). At the same time, electron microscopy suggested that fibronectin can attach to two different sites in fibrin(ogen), namely, to its αC domains and to the terminal nodules formed by the COOH terminal regions of the γ chains (*12*, *18*), although it is not clear whether this attachment was noncovalent or factor XIIIa-mediated. It is also not clear which part of the αC domain, which is a large entity (40 kDa) consisting of two structurally different regions, a flexible NH_2 terminal connector and a compact COOH terminal region (*19*, *20*), is involved in binding of fibronectin.

The availability of the recombinant αC domain and its truncated variants as well as various fibrin(ogen) fragments allowed addressing the above-mentioned questions. The major goals of this study were to characterize the noncovalent interaction between fibronectin and fibrin(ogen) and to further localize fibronectin-binding sites(s) in the latter.

EXPERIMENTAL PROCEDURES

Proteins. Fibronectin was purified from human plasma by affinity chromatography on gelatin−Sepharose as described in ref *21*. Human fibrinogen depleted from fibronectin, plasminogen, and von Willebrand Factor was purchased from Enzyme Research Laboratories (South Bend, IN). Prior to each binding experiment, fibrinogen was passed through a Superdex 200 column to remove traces of high molecular weight complexes. The recombinant αC fragment corresponding to the human fibrinogen αC domain (residues Aα221−610) and its truncated variants, the NH_2 and COOH terminal halves, including residues Aα221−391 and Aα392−610, respectively, were produced in *E. coli* using the pET-20b expression vector as described earlier (*22*). The D−D:E_1 complex was prepared from plasmin digest of factor XIIIa-cross-linked fibrin as described in ref *23*. Aprotinin and bovine serum albumin (BSA[1]) were purchased from Calbiochem, and thrombin was purchased from Sigma.

Antibodies. The rabbit antifibronectin polyclonal antibodies and the horseradish peroxidase-conjugated antirabbit IgG were purchased from Sigma. The sheep antifibrinogen polyclonal antibodies specific for the fibrinogen γ module (γ chain sequence 148−411) and the antibodies specific for the αC fragment (Aα chain sequence 221−610) were kindly provided by Dr. S. Cederholm-Williams (Oxford Bioresearch, Oxford, U.K.). Horseradish peroxidase-conjugated antisheep IgG was purchased from Sigma.

[1] Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PVDF, poly(vinylidene difluoride) membrane; SPR, surface plasmon resonance; TBS, 20 mM Tris, pH 7.4, buffer with 0.15 M NaCl.

Solid-Phase Binding Assay. Solid-phase binding was performed in plastic microtiter plates using an enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells (Fisher) were either treated with 2.5% glutaraldehyde for 2 h at 37 °C as in ref *24* or used without such treatment. The wells were coated overnight with 100 μL/well of 10 μg/mL fibronectin, fibrinogen, or fibrin(ogen) fragments (the recombinant αC fragment and its truncated variants, and the D−D:E_1 complex) in TBS (20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl). To convert fibrinogen into fibrin, we treated the wells with adsorbed fibrinogen with 100 μL/well of a mixture of thrombin (1 NIH u/ml) and aprotinin (400 u/ml) at 37 °C for 1 h. In some experiments, the wells were coated with fibrin that was made by the addition of fibrinogen and thrombin, both at the same concentration as that above, to the wells, followed by overnight incubation. The wells were then blocked with 3% nonfat milk in TBS containing 0.05% Tween-20 (TBS−Tween) at 37 °C for 1 h. Following washing with cold TBS−Tween, the indicated concentrations of fibronectin or fibrin(ogen) fragments in the same buffer were added to the wells and to the control wells coated with BSA and incubated for 2 h at indicated temperatures.

Bound fibronectin was measured by reaction with rabbit antifibronectin polyclonal antibodies, followed by peroxidase-conjugated antirabbit IgG. Bound αC fragment and the D−D:E_1 complex were measured by reaction with the sheep anti-αC fragment and anti-γ-module polyclonal antibodies, respectively, followed by peroxidase-conjugated antisheep IgG. A TMB Microwell Peroxidase Substrate (Kirkengaard & Perry Laboratories Inc.) was added to the wells, and the bound ligand was measured spectrophotometrically at 450 nm. Data were fitted by nonlinear regression analysis using eq 1:

$$B = B_{max}/(1 + K_d/[L]) \quad (1)$$

where B represents the amount of ligand bound, B_{max} is the concentration of ligand bound at saturation, [L] is the molar concentration of free ligand, and K_d is the dissociation constant.

Biosensor Assay. The interaction of fibronectin with fibrinogen, fibrin, and the αC fragment was studied by surface plasmon resonance (SPR) using the IAsys biosensor (Fisons, Cambridge, UK), which measures the association/dissociation of proteins in real time (*25*). Fibrinogen or the αC fragment was covalently coupled to the activated carboxymethyldextran-coated sensor surface at a coupling density of 9−15 ng/mm^2 by the procedure recommended by the manufacturer. To convert immobilized fibrinogen into fibrin, we treated the former with a mixture of thrombin (1 NIH u/ml) and aprotinin (400 u/ml) for 30 min. Binding experiments were performed in TBS containing 0.05% Tween 20 and 0.1 mM PMSF (binding buffer). The association between the immobilized and the added proteins was monitored as the change in the SPR response. To regenerate the surface, complete dissociation of the complex was achieved by adding 3 M guanidinium chloride for 0.5 min, followed by re-equilibration with binding buffer. The traces of the association processes were recorded, and the data were analyzed using the FASTfit kinetics analysis software as previously described in detail (*26*). Briefly, the association

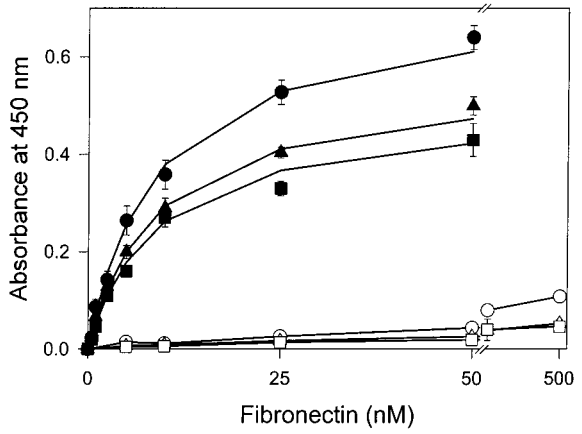

FIGURE 1: Analysis of binding of fibronectin to immobilized fibrinogen and fibrin at different temperatures by ELISA. Increasing concentrations of fibronectin were incubated with microtiter wells coated with fibrinogen (open symbols) or fibrin (closed symbols) made by treatment of fibrinogen with thrombin (see Experimental Procedures). The incubation was performed at +4 (circles), +22 (triangles), and +37 °C (squares). Bound fibronectin was detected with anti-fibronectin polyclonal antibodies as described in Experimental Procedures. Error bars reflect the standard deviation of three independent determinations. The curves represent the best fit of the data to eq 1 to obtain K_d's.

Table 1: Dissociation Constants (K_d, nM) for the Interaction of Fibronectin with Fibrin, the αC Fragment, and Its Truncated Variants

protein	+4 °C	+22 °C	+37 °C
fibrin	7.3 + 1.7[a]	7.7 ± 2.3[a] 8.5 ± 1.9[b]	7.2 ± 1.9[a] 8.0 ± 1.7[b]
Aα 221−610	15 ± 4[a]	56 ± 7[a] 9 ± 6[b]	83 ± 14[a] 3 ± 2[b]
Aα 221−391	11 ± 9[a]	59 ± 13[a]	92 ± 15[a]
Aα 392−610	no binding[a]	no binding[a]	no binding[a]

[a] Obtained by ELISA. [b] Obtained by SPR. Values are means ± S.D. of three independent experiments.

curves at each concentration of ligand were fitted to the pseudo-first-order equation to derive the observed rate constant, k_{obs} (termed on-rate constant in FASTfit). Then the concentration dependence of k_{obs} was fitted to eq 2:

$$k_{obs} = k_{diss} + k_{ass}[\text{ligand}]$$

to derive the association rate constant (k_{ass}) from the slope and the dissociation rate constant (k_{diss}) from the intercept. The dissociation equilibrium constant (K_d) was calculated as $K_d = k_{diss}/k_{ass}$. The values were examined for self-consistency of the data as described (*27*).

Ligand Blotting Assay. Detection of fibronectin bound to fibrinogen, fibrin, or the αC fragment variants was performed using NuPAGE BisTris electrophoretic system (Invitrogen). The proteins were subjected to SDS electrophoresis and electrotransferred to a polyvinyliden difluoride (PVDF) membrane (Invitrogen). To check the effectiveness of the protein transfers, the membrane was stained with 0.5% Ponceau S for 1 min following washing with deionized water and destaining with 5% acetic acid. The membrane then was blocked for 1 h with Casein Blocker (Pierce) containing 1% casein in TBS−Tween, followed by incubation with 20 μg/mL fibronectin at +4 °C for 1 h. Bound fibronectin was detected by reaction with rabbit antifibronectin polyclonal antibodies and peroxidase-conjugated antirabbit IgG. Visualization of the peroxidase-labeled protein bands was performed by the procedure recommended by the manufacturer using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

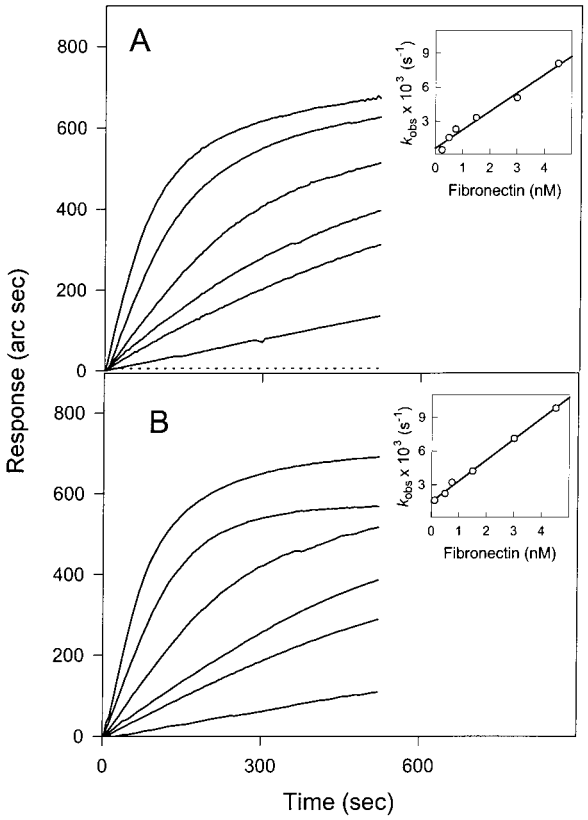

FIGURE 2: Analysis of binding of fibronectin to immobilized fibrin at different temperatures by surface plasmon resonance. Increasing concentrations of fibronectin were added to immobilized fibrin, and their association was monitored in real time at +22 (panel A) and +37 °C (panel B) while registering the resonance signal (response). The dotted curve in panel A shows interaction of fibronectin at 100 nM with immobilized fibrinogen prior its conversion to fibrin. The concentrations in panel A were 0.25, 0.5, 0.75, 1.5, 3.0, and 4.5 nM; those in panel B were 0.125, 0.5, 0.75, 1.5, 3.0, and 4.5 nM. The inset in each panel shows a plot of the values of k_{obs} determined for each association curve vs ligand concentration to derive k_{ass} and k_{dis} and thus determine the dissociation equilibrium constant, K_d, presented in Table 1. Here and in other figures, a signal of 200 arc sec corresponds to 1 ng of protein bound per mm^2 of the sensor surface.

RESULTS

Interaction of Fibronectin with Fibrinogen and Fibrin at Different Temperatures. Interaction of fibronectin with fibrinogen and fibrin at different temperatures was tested by two methods, ELISA and surface plasmon resonance (SPR). In ELISA experiments, to make adsorption of fibrinogen and fibrin to microplate wells more uniform, all wells were first coated with fibrinogen, and then some of them were treated with thrombin to convert fibrinogen into fibrin (see Experimental Procedures). To avoid a possible influence of fibrinogen heterogeneity on binding, fibrinogen was routinely purified by size-exclusion chromatography prior to coating. Such fibrinogen did not bind fibronectin at any studied temperature, but after being converted into fibrin, it bound fibronectin with high affinity (Figure 1). The affinity at +4,

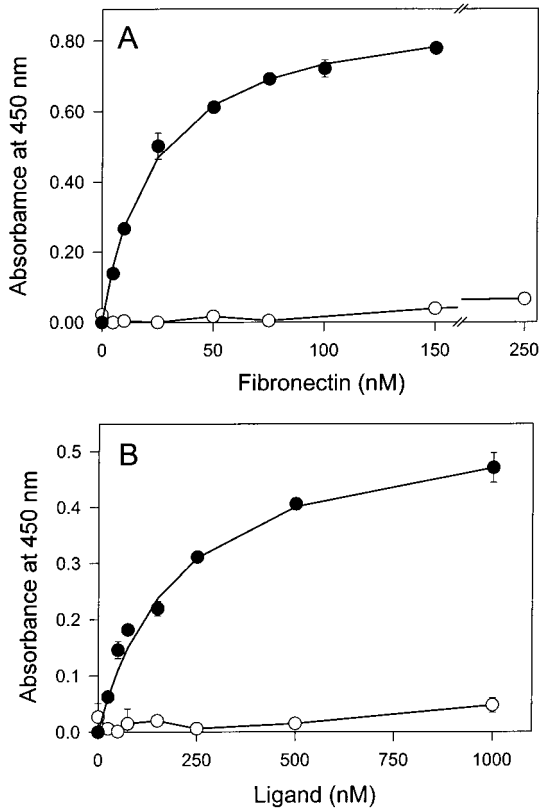

FIGURE 3: Analysis of interaction between fibronectin and fibrin(ogen) fragments by ELISA. (panel A) Increasing concentrations of fibronectin were incubated with microtiter wells coated with the αC fragment (closed circles) or the D−D:E_1 complex (open circles), and bound fibronectin was detected with antifibronectin polyclonal antibodies. (panel B) Increasing concentrations of the αC fragment (closed circles) or the D−D:E_1 complex (open circles) were incubated with microtiter wells coated with fibronectin, and bound species were detected with anti-αC fragment or anti-γ-module polyclonal antibodies, respectively (see Experimental Procedures). All experiments were performed at +4 °C. Error bars reflect the standard deviation of three independent determinations. The curves for the αC fragment in both panels represent the best fit of the data to eq 1.

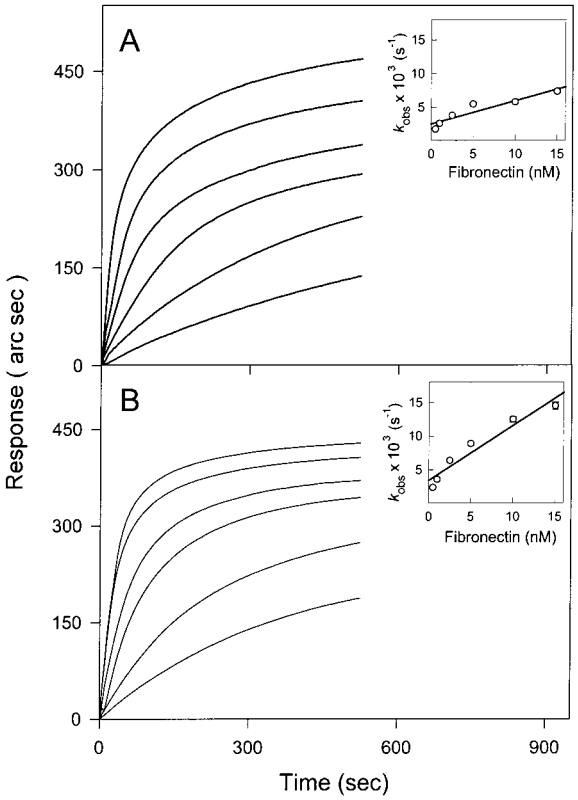

FIGURE 4: Analysis of binding of fibronectin to the immobilized αC fragment at different temperatures by surface plasmon resonance. Increasing concentrations of fibronectin were added to the immobilized αC fragment, and their association was monitored in real time at +22 °C (panel A) and +37 °C (panel B) while registering the resonance signal (response). The concentrations in both panels were 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 nM. The inset in each panel shows a plot of the values of k_{obs} determined for each association curve vs ligand concentration to derive k_{ass} and k_{dis} and thus determine the dissociation equilibrium constant, K_d, presented in Table 1.

+22, and +37 °C was essentially the same with K_d's in the range of 7.2−7.7 nM (Table 1). It should be noted that when fibrin was prepared in a different way, namely, by incubation of fibrinogen with thrombin in microtiter plate wells (see Experimental Procedures), the affinity tested at +4 °C was similar. These findings were confirmed by SPR experiments in which fibronectin even at 500 nM failed to bind to immobilized fibrinogen while it bound to immobilized fibrin with an affinity similar to that determined by ELISA (Figure 2 and Table 1). These results clearly indicate that fibronectin-binding sites are cryptic in fibrinogen and become exposed in fibrin and that the fibronectin-fibrin interaction is rather insensitive to temperature, at least in the studied temperature range.

Interaction of Fibronectin with Various Regions of Fibrin(ogen). The fibrin-derived noncovalent D−D:E_1 complex and the recombinant αC fragment (residues Aα221−610) corresponding to the isolated αC domain are the best available models mimicking fibrin since they seem to preserve the activity and conformation of the corresponding regions of fibrin (*22*, *23*). In addition, the individual components of the complex, D−D and E_1, and the αC fragment together cover the entire structure of the molecule. To test directly which regions of fibrin interact with fibronectin, we studied the binding of the latter to the D−D:E_1 complex and the αC fragment. In ELISA experiments, the immobilized αC fragment bound fibronectin with K_d = 30 nM, while the immobilized complex exhibited no binding (Figure 3A). Since the D−D:E_1 complex may alter its conformation upon immobilization, we performed the reverse experiment, in which immobilized fibronectin was titrated with increasing concentrations of D−D:E_1 or the αC fragment. Again, the D−D:E_1 complex exhibited no binding even at 1 μM, while the αC fragment bound well (Figure 3B), although the affinity was lower (K_d = 210 nM). The interaction between fibronectin and the immobilized αC fragment was confirmed in SPR experiments (Figure 4).

It should be noted that the above-mentioned ELISA experiments were performed at +4 °C to reduce possible proteolytic activity that may result in dissociation of the noncovalent D−D:E_1 complex and influence the final results. When ELISA experiments were performed at +22 and +37 °C, the interaction between the immobilized αC fragment and fibronectin was not observed (not shown). However, the binding at these temperatures was restored when the wells were treated with glutaraldehyde (see Experimental Procedures) prior to adsorption of the αC fragment (Figure 5 and

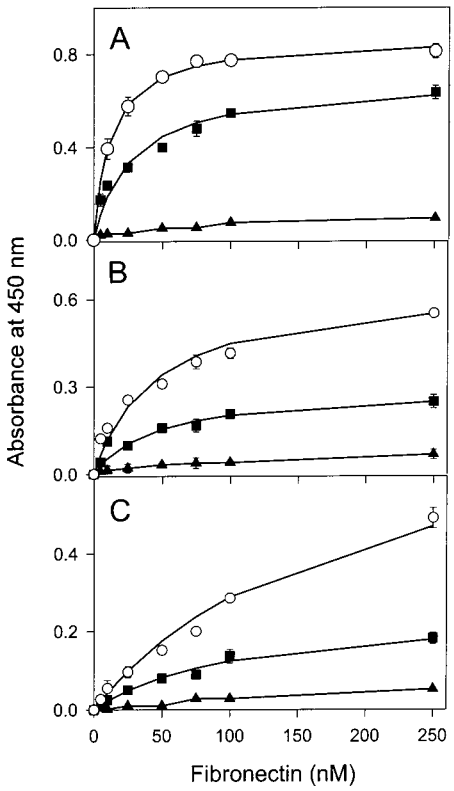

FIGURE 5: Analysis of binding fibronectin to the immobilized αC fragment and its truncated variants by ELISA. Increasing concentrations of fibronectin were incubated with the glutaraldehyde-treated microtiter wells coated with the αC fragment (circles), the Aα221−391 fragment (squares), and the Aα392−610 fragment (triangles) at +4 (panel A), +22 (panel B), and +37 °C (panel C). Bound fibronectin was detected with anti-fibronectin polyclonal antibodies. Error bars reflect the standard deviation of three independent determinations. The curves for the αC fragment and the Aα221−391 fragment represent the best fit of the data to eq 1.

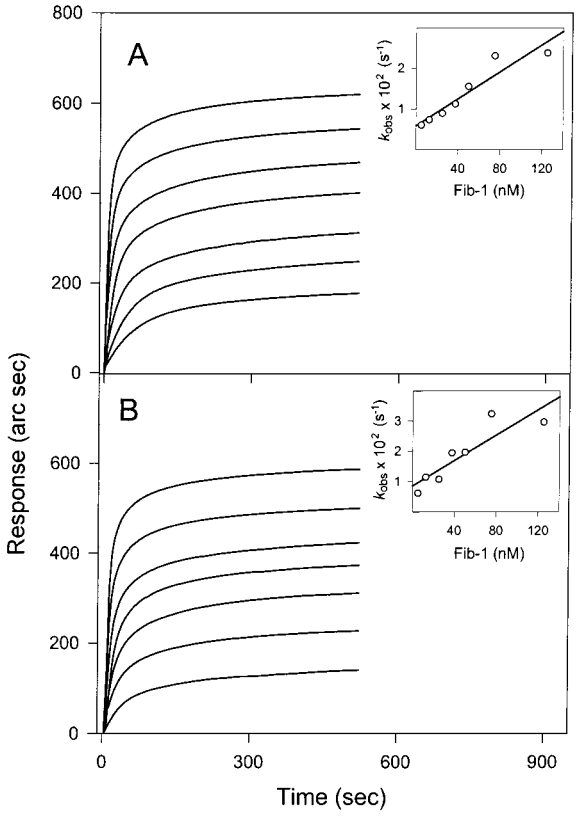

FIGURE 6: Analysis of binding of the fibronectin Fib-1 fragment to the immobilized αC fragment at different temperatures by surface plasmon resonance. Increasing concentrations of Fib-1 were added to the immobilized αC fragment, and their association was monitored in real time at +22 (panel A) and +37 °C (panel B) while registering the resonance signal (response). The concentrations in both panels were 5, 12.5, 25, 37.5, 50, 75, and 125 nM. The inset in each panel shows a plot of the values of k_{obs} determined for each association curve vs ligand concentration to derive k_{ass} and k_{dis} and thus determine the dissociation equilibrium constant, K_d. The K_d's at room and physiological temperatures were found to be 36 and 42 nM, respectively.

Table 1). More so at low temperature, the affinity of the αC fragment immobilized on such wells was also higher with $K_d = 15$ nM (Table 1). One can speculate that such treatment may provide covalent immobilization of the αC fragment, which prevents its loss from the wells' surface upon repeated washing or may provide a covalent spacer that facilitates the exposure of the fibronectin-binding sites in the αC fragment. Whatever the reason, the above results provide direct evidence for the interaction between fibronectin and the isolated αC domain. They also indicate that in fibrin only the αC domains are involved in the binding of fibronectin.

Interaction of the αC *Fragment with the Fib-1 Fragment of Fibronectin*. Since the αC domains are the only regions of fibrin which interact with fibronectin, both NH_2 terminal Fib-1 and the COOH terminal Fib-2 fibrin-binding regions of the latter should interact with them. Here we focused on the Fib-1 region that contains both the noncovalent recognition site and the covalent cross-linking site involved in the first and second stages of interaction, respectively. To confirm directly its interaction with the αC domain, we performed SPR experiments in which increasing concentrations of the Fib-1 fragment were added to the immobilized αC fragment. Fib-1 exhibited a dose-dependent binding at both room and physiological temperatures with the K_d's of 36 and 42 nM, respectively (Figure 6). Although these dissociation constants were higher than those obtained by SPR for the interaction of fibronectin with the αC fragment (Table 1), the affinity of interaction was still high, indicating that the Fib-1 region of fibronectin interacts strongly with the fibrin αC domains.

Further Localization of the Fibronectin-Binding Site(s) in the αC *Domain.* To further localize the fibronectin-binding site within the αC domain, we tested the interaction of fibronectin with the truncated recombinant variants of the αC fragment, Aα221−391 and Aα392−610, corresponding to its NH_2 and COOH terminal halves. In ELISA, when increasing concentrations of fibronectin were added to the αC fragment variants immobilized on glutaraldehyde-treated wells, the COOH terminal half of αC exhibited no binding while the NH_2 terminal half bound in a dose-dependent manner with the same affinity as the full length αC fragment (Figure 5 and Table 1). To further document this specificity, we used ligand blotting assay. As shown in Figure 7, fibronectin bound to the NH_2 terminal half and the full length αC fragment that were immobilized on a PVDF membrane after being electrophoresed in 10% polyacrylamide gel, while no binding was observed with the COOH terminal half. Altogether, these results indicate that the fibronectin-binding site is located in the NH_2 terminal connector region of the

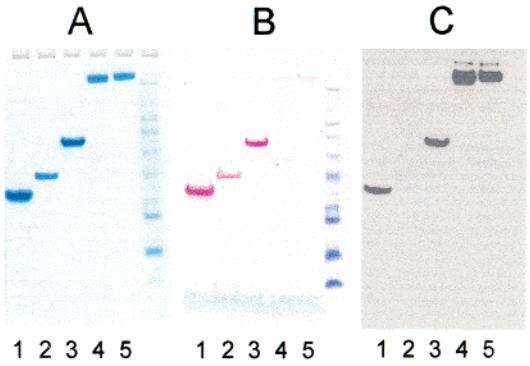

FIGURE 7: Analysis of binding of fibronectin to fibrinogen, fibrin, and the αC fragment and its variants by ligand blotting. The Aα221−391 NH_2 terminal and the Aα392−610 COOH terminal halves of the αC fragment (lanes 1 and 2, respectively), the αC fragment (lanes 3), fibrin (lanes 4), and fibrinogen (lanes 5) were electrophoresed in 10% polyacrylamide gels that were subsequently stained with Coomassie Blue (panel A) or electrophoretically transferred to PVDF membrane, stained with Ponseau S (panel B), and probed with fibronectin after destaining (panel C). Bound fibronectin in panel C was detected with antifibronectin polyclonal antibodies (see Experimental Procedures). The right outer lanes in panels A and B contain molecular mass markers.

αC domain, including residues 221−391. It should be noted that in ligand blotting assay, fibronectin bound to both fibrinogen and fibrin, suggesting that not only conversion of fibrinogen into fibrin but also denaturation of fibrinogen by SDS results in the exposure of its fibronectin-binding sites. This reinforces our previous conclusion that these sites are cryptic in native fibrinogen.

DISCUSSION

Fibrinogen is usually inert in circulation, however upon conversion into fibrin it undergoes conformational changes resulting in the exposure of cryptic sites that enable its polymerization and interaction with a number of proteins and cell types during wound healing and some other processes. It was not clear from the previous studies whether fibronectin-binding sites are cryptic in fibrinogen since the latter was reported to also bind fibronectin (*10*). This study clearly demonstrates that fibrinogen does not bind fibronectin, i.e., fibronectin-binding sites are cryptic even in surface-immobilized fibrinogen and become exposed only upon its conversion to fibrin. The previously reported interaction of fibronectin with fibrinogen (*10*) could be explained by the quality of fibrinogen, whose conformation is quite labile and could be partially altered, for example, by freeze-drying or treatment with EDTA (*28*). Indeed, in some of our ELISA experiments in which commercially available fibrinogen was used without additional purification, we observed some fibronectin-binding activity (not shown). However, this activity was abolished when fibrinogen was additionally purified prior to the experiments. A similar situation was previously observed with some fibrin(ogen) fragments whose stimulating effect toward plasminogen activation disappeared after removal from the starting material of the fraction with altered conformation (*29*). These observations emphasize the necessity to use for binding assays highly purified fibrinogen preparations.

In light of the above-mentioned finding, the interaction of fibronectin with soluble fibrinogen-fibrin complexes to form cryoprecipitates should occur via their fibrin component. It was assumed that fibronectin−fibrin(ogen) interaction is most efficient in the cold since precipitation occurred most efficiently at low temperatures. Our study does not confirm this assumption. Although interaction of fibronectin with the fibrinogen αC fragments exhibited some temperature dependence, there was no substantial influence of temperature on the interaction of fibronectin with fibrin (Table 1). The dissociation constants, K_d's, determined at low, room, and physiological temperatures by two independent methods, ELISA and SPR, were quite similar, in the range of 7.2−8.5 nM. These K_d's were also similar to that reported for fibrin−fibronectin interaction at room-temperature based on ELISA data (*30*). Thus, our data clearly indicate that the fibronectin−fibrin interaction is rather temperature-independent, at least in the studied temperature range. They also reinforce the previous finding that fibronectin interacts with fibrin with high affinity (*30*). Given that the concentration of fibronectin in plasma is ∼0.5 μM (*12*), one can expect that all soluble fibrinogen−fibrin complexes that occur in blood in certain pathological states (*10*) would also contain fibronectin. Such high affinity also should provide an effective adsorption of fibronectin on fibrin clots, even in the absence of covalent cross-linking, to promote cell attachment and migration.

For a long time, the high susceptibility of the fibrinogen αC domains to proteolysis was a major obstacle for their isolation by limited proteolysis. That is why most of the activities localized in these domains have been deduced by comparing the behavior of intact fibrin(ogen) with its proteolytic derivatives, for example, fibrinogen fractrion I-9, lacking these domains. The involvement of the fibrin(ogen) αC domains in binding of fibronectin was suggested on the basis of comparisons of such fibrin(ogen) and its derivatives as well as hementin-produced fibrinogen fragments D containing and lacking these domains (*9*, *10*, *17*). In the present study, we expressed the αC domain and its variants in a bacterial system and tested directly their interaction with fibronectin. This enabled us to obtain direct evidence for the involvement of the αC domain in binding of fibronectin and to further localize its fibronectin-binding site to its NH_2 terminal half (connector region). In addition, our study with the fibrin-derived D−D:E_1 complex, which mimics the remaining portion of the molecule, revealed that the αC domains are the only structures in fibrin that bind fibronectin. In agreement, our additional SPR experiments revealed no interaction between fibronectin and fibrin prepared from fibrinogen fraction I-9 (not shown). It should be noted that this finding does not contradict the electron microscopic observation of fibronectin attached to the terminal nodule of fibrin(ogen) (*18*). Indeed, the fibronectin-binding connector region of the αC domain originates from the terminal nodule and seems to be flexible (*19*). Such flexibility implies that it could adopt various locations, including those in close proximity to the terminal nodules.

Our previous study revealed that the noncovalent fibrin-recognition site of the Fib-1 region of fibronectin is formed by its type I domains F4−5 (*13*) while reactive Gln residues involved in factor XIIIa-catalyzed covalent cross-linking with fibrin were located in a flexible region NH_2 terminal to the

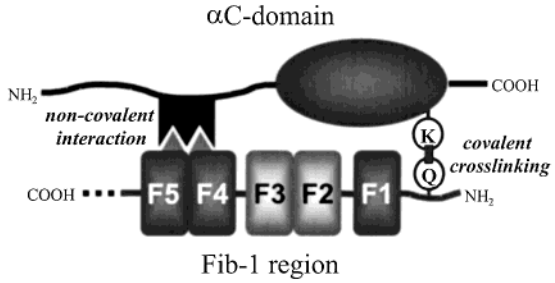

FIGURE 8: Schematic representation of the noncovalent and covalent interactions between the αC domain and the NH_2 terminal Fib-1 region of fibronectin. The αC domain consisting of the NH_2 terminal flexible connector and the COOH terminal compact part is presented on the top while the Fib-1 region consisting of five fingerlike domains, F1−5, is presented on the bottom. The noncovalent interaction via the recognition sites is presented on the left, while the covalent cross-linking via reactive Lys and Gln residues (K−Q) is on the right.

first type I domain (*6*). A similar situation was found in the αC domain whose reactive Lys residues involved in the cross-linking were found exclusively in its COOH terminal half, (*8*) while the noncovalent recognition site localized in this study is situated in its NH_2 terminal half. Such spatial separation of noncovalent interaction sites may provide proper orientation of the cross-linking sites to facilitate the covalent stage of the interaction. This is presented schematically in Figure 8. The role of the fibrin-fibronectin interaction via the Fib-2 fibrin-binding site needs to be further clarified.

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