

Localization of a Cryptic Binding Site for Tenascin on Fibronectin*

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Fibronectin and tenascin are large extracellular matrix proteins that interact with each other and with integrin receptors to regulate cell growth and movement. They are both modular proteins composed of independently folded domains (modules) that are arranged in linear fashion. Fibronectin is a covalent dimer and tenascin is a hexamer. The site on tenascin to which fibronectin binds has been localized to type III modules 3–5. In this study we use surface plasmon resonance to examine the interaction between various fragments of fibronectin and tenascin to further characterize and localize the binding sites. We found that tenascin fragments that contain type III modules 3–5 bind primarily to the N-terminal 29-kDa hep-1/fib-1 domain, which contains the first five type I modules of fibronectin. The dissociation constant, K_D , is $\approx 1 \mu\text{M}$. The binding site on fibronectin appears to be cryptic in the whole molecule in solution but is exposed on the proteolytic fragments and probably when fibronectin is in the extended conformation.

Tenascin-C (TN)¹ and fibronectin (FN) are glycoprotein components of the extracellular matrix that interact with each other and with other matrix molecules including collagen and heparan sulfate proteoglycans. Both proteins are constructed from independently folded domains arranged in tandem to produce elongated structures with limited flexibility (dimeric in the case of FN and hexameric in the case of TN). FN is processed into an insoluble fibrillar matrix to which TN binds either directly or indirectly via proteoglycans that bridge the two proteins (1). Both proteins contain sites that are recognized by cell surface receptors whose occupation allows the extracellular matrix to regulate the adhesion, differentiation, growth, and migration of cells. Both proteins are subject to alternative splicing so that certain modules are differentially expressed during various stages of development and again during wound healing and tumorigenesis. TN is known to interfere with the cell adhesive function of FN either by binding to FN and restricting access of its integrin binding sites (2) or by binding to cell receptors and altering their responsiveness to FN (3, 4).

The potential for these proteins to interact *in vitro* was first inferred from the co-localization of TN on FN fibrils in tissue culture (5) and in amphibian embryos (6). Subsequently it was

reported that TN could bind to surfaces coated with FN or its cell-binding fragment but not those coated with a gelatin-binding fragment (7). The small alternative splice variant of TN bound better than the large one, and the binding was sensitive to high ionic strength and low concentrations of urea (7). FN and TN secreted into cell culture medium did not appear to form a complex in fluid phase (8). However, Chung *et al.* (9) found that TN sedimented faster in a glycerol gradient if plasma FN was present throughout, indicating a fluid phase association. This effect could be attenuated by including certain recombinant fragments of TN in the gradient, allowing the binding site on TN to be localized to a region near the third type III module (TN3), which by itself showed an effect. This was confirmed by solid phase binding measurements in which TN3-containing fragments were able to compete with TN for binding to FN-coated plastic wells. Fragment TN3–5 (the third through the fifth FN-III domains) was more potent than TN3 alone, suggesting that multiple modules are required for full affinity.

At the same time, the location of the TN binding site within FN has been uncertain. As mentioned above, TN was reported to bind to surfaces coated with the cell-binding fragment of FN but not with the gelatin-binding fragment (7). More recently, Huang *et al.* (18) reported an interaction of TN with the hep-2 region of FN localized to the 13th type III module. Neither study examined fragments from the 29-kDa N- or C-terminal regions of FN. Thus additional studies were needed to fully understand the nature of this interaction, to determine its affinity, and to more precisely define the location of the binding site(s) within FN.

MATERIALS AND METHODS

Fibronectin was prepared as described (10). Fragments of fibronectin (except the 70-kDa N-terminal fragment) were prepared as described (11). Tenascin fragments were prepared by bacterial expression and purified as described (9). Swine skin gelatin and sodium heparin (porcine intestinal mucosal) were obtained from Sigma. Recombinant fragments of tenascin were prepared as described (9). Absorbance at 280 nm corrected for light scattering was measured to calculate the concentration of the proteins using published extinction coefficients (9, 12).

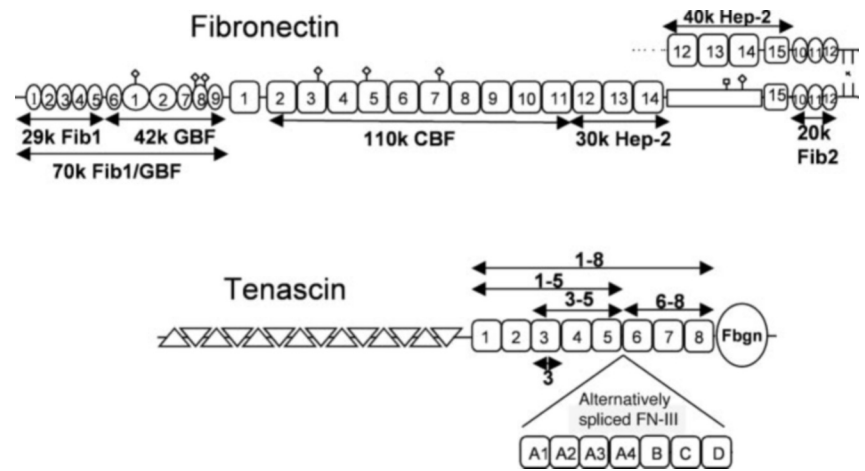
Fragments to be coupled to Biacore CM5 chips were dialyzed against the indicated coupling buffers, and fragments to be used as analytes were dialyzed against 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (HBS-EP). Surface plasmon resonance was used to detect interaction between recombinant tenascin fragments and fibronectin and its fragments with a Biacore 3000 instrument. Ligands were coupled to CM5 sensor chips with the wizard program for amine coupling chemistry according to the manufacturer's instructions. Excess sites were blocked with ethanolamine. TN1–8 and TN1–5 in 10 mM acetate, pH 4, were coupled to the flow paths of a CM5 sensor chip, each at levels of ~ 1000 resonance units. The 29- and 70-kDa N-terminal fragments of fibronectin

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¹ The abbreviations used are: TN, tenascin-C; FN, fibronectin; TN1–5, FN-III domains 1–5 of tenascin-C.

FIG. 1. Modular composition of plasma fibronectin and tenascin-C. Type I, II, and III modules of fibronectin are represented by *small ovals*, *circles*, and *rectangles*, respectively. Tenascin contains EGF-like modules (*triangles*), FN-III modules (*rectangles*), and a C-terminal fibrinogen-like module (*large oval*). Only one of six identical chains of TN are shown. Fragments used in this study are designated by *horizontal arrows*.



were coupled to a CM5 sensor chip in 10 mM maleate, pH 6, and 10 mM acetate, pH 4, respectively, at levels of ~2300 resonance units. Fibronectin in 10 mM acetate, pH 4.5, and swine skin gelatin (Sigma) in 10 mM borate, pH 8.5, were coupled to CM5 chips at ~3000 and 400 resonance units, respectively. A blank flow path lacking protein was derivatized with ethanolamine on each chip. All sensorgrams were corrected by subtracting the background runs of analytes over this flow path from corresponding runs of analytes over flow paths derivatized with ligands. The indicated concentrations of analytes in HBS-EP, which was also used as the running buffer, were injected at 30 μ l/min for 3 min. After a 2.5-min wash with HBS-EP, the flow paths were regenerated with 6 M urea in HBS-EP containing 1 M NaCl. Exposure to 6 M urea did not irreversibly affect the ability of immobilized proteins to bind analytes as duplicate runs gave essentially identical results, and the surfaces retained their binding properties over repeated use.

Using Biacore evaluation software the data were globally fit to the model for simple 1:1 interaction to obtain values for the forward (k_a) and reverse (k_d) reactions. The equilibrium dissociation constant was then calculated as $K_d = k_d/k_a$. Equilibrium binding was determined by plotting R_{eq} versus concentration (C) and fitting the data to the steady state model.

$$R_{eq} = R_{max} \times K_d \times C / (1 + K_d \times C) \quad (\text{Eq. 1})$$

RESULTS

Fig. 1 illustrates the modular composition of TN and FN and shows the fragments that were used in this study. The binding site for FN on TN was previously localized to type III modules 3–5 (9), whereas the binding site for TN on FN was the main subject of the present investigation. Three different recombinant fragments containing various type III modules of TN were immobilized on a sensor chip and used to test a battery of FN fragments spanning almost the entire polypeptide chain. As shown in Fig. 2, the only fragments that gave a significant response were the N-terminal 70-kDa Fib-1/gelatin-binding fragment and 29k-Da Fib-1 fragments (the latter is contained within the former). The 42-kDa gelatin-binding fragment that constitutes the other portion of the 70-kDa fragment failed to bind as did the central 110-kDa cell binding fragment, the 40-kDa hep-2 fragment, and the C-terminal 20-kDa fib-2 fragment. These results suggest that the primary binding site is located within the first five type I modules of FN that constitute the 29-kDa fragment.

FN itself gave a relatively small response. This was surprising because the surface plasmon resonance signal tends to be proportional to the molecular weight of the substance that binds. Because FN was applied at the same concentration as all of the fragments (1 μ M), this means that much less FN is bound

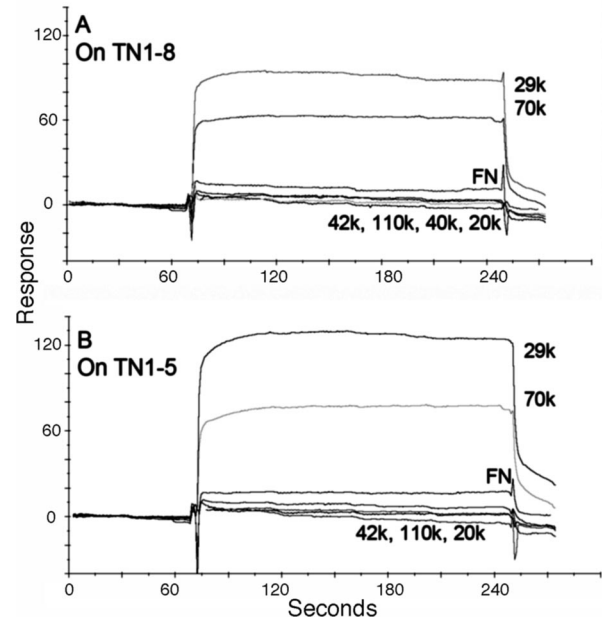


FIG. 2. Binding of FN and its fragments to immobilized TN fragments at 25 °C, pH 7.4, in HBS-EP. Proteins were applied at a concentration of 1 μ M and a flow rate of 30 μ l/min. Binding was detected by surface plasmon resonance using a Biacore 3000 instrument as described under "Methods and Materials." Only the N-terminal 70- and 29-kDa fragments show a significant response.

to the surface, implying a significantly lower affinity. Note that the response with the 70-kDa fragment was also lower than that of the 29-kDa fragment. This pattern suggests that the binding site is largely masked in the intact protein and partially masked in the 70-kDa fragment.

Next we compared a collection of TN fragments for binding to the immobilized 29- and 70-kDa fragments of FN. As shown in Fig. 3A, all TN fragments that contain FN-III modules 3–5 gave a good response with the fragment of 29-kDa FN; those lacking these modules gave a poor response. Module TN3 by itself gave a slight response, suggesting that it may participate but that it needs additional modules 4 and/or 5 for strong binding. Again, the response of the 70-kDa fragment (Fig. 3B) was consistently weaker than that on the 29-kDa fragment, and the response of the 42-kDa fragment (not shown) was negligible.

FN was immobilized on the biosensor surface and tested for binding to TN fragments. Under conditions where gelatin gave a strong response, TN1–5 and TN1–8 gave a poor response (Fig. 4A). Likewise, when fluid phase FN was tested on immo-

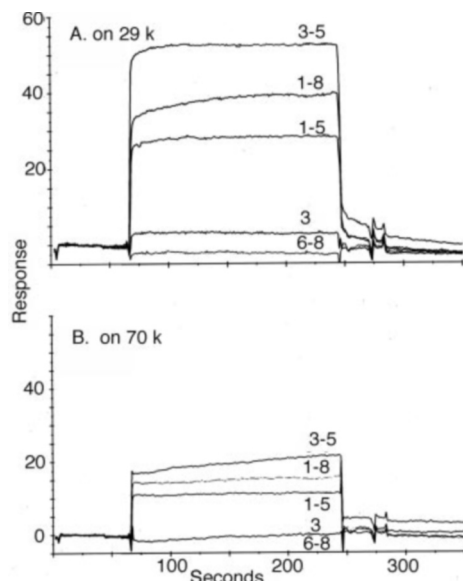


FIG. 3. Binding of TN fragments to immobilized N-terminal fragments of FN under the same conditions described for Fig. 2. Fragments were injected at a concentration of 1 μM .

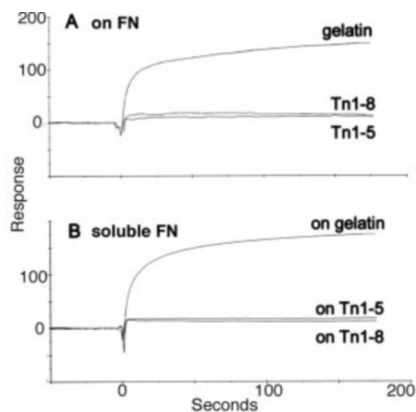


FIG. 4. Binding of TN fragments and gelatin to immobilized FN (*top*) and binding of FN to immobilized gelatin and TN fragments (*bottom*) under the conditions described for Fig. 2. The results confirm the cryptic nature of the TN-binding site within FN.

bilized TN1-5 and TN1-8, very little response was obtained under conditions where it gave a strong response on immobilized gelatin (Fig. 4B). The same was true on TN3-5 (not shown). These results support the above suggestion that the TN binding site is cryptic in whole FN and becomes more accessible after proteolysis. They also illustrate that the FN used in this study was functional, at least in terms of gelatin binding.

An additional experiment was conducted to determine whether the binding of FN to gelatin, which occurs through the 42-kDa region, would render the TN-binding site in the adjacent 29-kDa region more accessible. Whole FN was first loaded onto a gelatin surface, and the solvent was then switched to buffer alone to initiate slow dissociation. After a few seconds, the solvent was switched again to buffer containing a TN fragment. There was no increase in the resonance signal, indicating that the binding of FN to gelatin does not expose the TN-binding site (data not shown). Likewise, heparin when added to TN fragments had little or no effect on their binding to immobilized 29-kDa or 70-kDa fragments, and heparin by itself did not appear to bind to either FN fragment, *i.e.* it gave no plasmon resonance signal.

Fig. 5 presents a typical example of the concentration de-

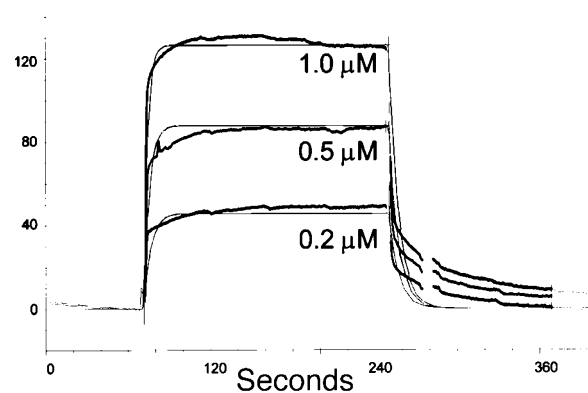


FIG. 5. Example of binding of several concentrations of N-terminal 29-kDa FN fragment to immobilized TN fragment TN1-5. Smooth curves show the global best fit to a 1:1 binding isotherm. The conditions were the same as those described for Fig. 2.

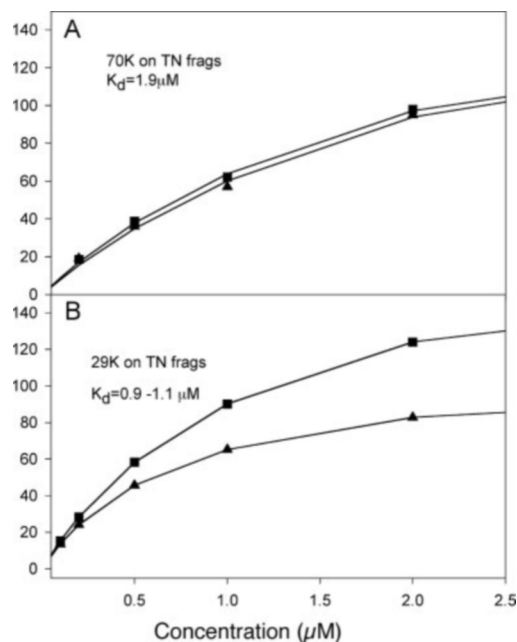


FIG. 6. Steady state binding results for interaction of FN 29-kDa and 70-kDa fragments with fragments of TN (*squares*, TN1-5; *triangles*, TN1-8). Equilibrium values of the surface plasmon resonance response (*e.g.* plateaus in Fig. 5) were plotted and fit to a 1:1 binding isotherm (*solid lines*). The resulting K_d values are given on the figure.

pendence of binding of the N-terminal 29-kDa fragment of FN to immobilized TN1-5. The data were fit globally to a Langmuir binding isotherm to obtain an equilibrium dissociation constant of 0.85 μM . Although the fit of the kinetics is poor (especially in the region of dissociation), the concentration dependence of the equilibrium plateau level is well reproduced by the global fit. It is apparent that the bulk of the binding and dissociation occur at too high of a rate for meaningful analysis of the kinetics. However, the plateau values still provide a valid measure of the relative concentration of analyte bound at equilibrium. Therefore, we plotted the plateau values from these and other titrations as a function of concentration and fit the data to the binding isotherm (Fig. 6). The K_d values for the binding of the 70-kDa fragment to TN fragments TN1-8 and TN1-5 were identical at 1.9 μM . The values for the 29-kDa fragment were only slightly smaller (0.9 and 1.1 μM) in good agreement with the value of 0.85 μM obtained from the global fit in Fig. 4.

All of the binding experiments shown above were done at a NaCl concentration of 0.15 M. Divalent cations, calcium and magnesium, at concentrations of 1 mM had no significant effect on the interaction with 29-kDa fragments, and they did not disclose the cryptic binding site in FN (not shown). When the NaCl concentration was increased to 0.5 M, no binding was detected with any of the fragment combinations (not shown), suggesting that the interaction is dominated by electrostatic forces.

DISCUSSION

Our results show that the primary TN-binding site on FN is located in the N-terminal 29-kDa region, which is composed of the first five type I modules. The K_d for the interaction is $\sim 1 \mu\text{M}$.

Additional binding sites have been reported in other regions of FN, namely the central cell binding region (7) and the hep-2 region (18). However, fragments containing those regions were inactive in our assays. We note that neither study (7, 18) tested the 29-kDa N-terminal fragment of FN, which is most active in our assays, so it is not known if this would be active in their systems. Both studies utilized whole TN (whereas we used recombinant TN segments) raising the possibility of an additional binding site that could account for the discrepancy. In the end, however, we cannot explain why Huang *et al.* (18) found significant binding to the hep-2 segment of FN, whereas we found negligible binding to this segment.

Among the various TN fragments tested, only those containing type III modules 3–5 were able to bind. This is consistent with previous studies that localized the FN-binding site to this region of TN (9).

The sensitivity of the interaction to high salt suggests that electrostatic effects play a major role. Both domains have a large number of charged surface residues that could mediate the interaction. The 29-kDa fragment binds to heparin-Sepharose, which is also an electrostatic interaction. However, it binds poorly to heparin in the fluid phase, and heparin binds poorly to the immobilized 29-kDa fragment (13). In the present study we also failed to observe a plasmon resonance signal when heparin was flowed over a Biacore chip to which the 29-kDa fragment was coupled, and the presence of heparin did not affect the binding of TN fragments to that chip. These data consistently indicate that binding of heparin to the 29-kDa fragment is very weak.

The TN-binding site appears to be cryptic in FN because whole FN when coupled to the Biacore chip failed to bind any of the TN fragments. Moreover, FN in solution failed to bind to immobilized TN fragments. FN in solution in physiological salt is known to adopt a compact conformation in which the two subunits of the dimer are thought to be folded upon each other (14). One possibility is that the TN binding site may be buried

in the compact conformation. This could explain the apparent high affinity interaction observed between whole TN and FN when the latter was adsorbed to microtitre wells (9). Several studies have reported a change in FN conformation upon binding to plastic (15, 16), and it is possible that this change exposes the cryptic TN-binding site as well as others. TN did appear to interact with soluble FN when sedimented through a glycerol density gradient (9). However, glycerol has been shown to favor the extended conformation of FN (17) and may have exposed the TN-binding site. We note in addition that this previous solution phase assay involved dimeric FN and hexameric TN, giving the possibility of enhanced apparent affinity through multivalent binding.

The binding reaction measured here is likely to be relevant in tissues where FN exists in a stretched fibrillar form that is thought to expose other cryptic binding sites (19, 20). TN concentrations in tissues may reach 0.2–2.0 mg/ml (21), which corresponds to the low μM range. Thus, if the site is exposed, significant interaction could be expected based on the K_d values reported here. Moreover, because TN is hexameric, it may have substantially enhanced binding through multivalent attachment to two or more FN molecules in FN matrix fibrils.

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