Interaction of Fibronectin and Its Gelatin-binding Domains with Fluorescent-labeled Chains of Type I Collagen

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Fluorescent probes have been used to obtain dissociation constants for the fluid-phase interaction of human plasma fibronectin and several of its gelatin-binding fragments with purified α chains of type I rat tail collagen, as well as with a cyanogen bromide fragment (CB7) of the α1 chain in 0.02 M Tris buffer containing 0.15 M NaCl at pH 7.4. Addition of fibronectin to fluorescent-labeled collagen chains caused a dose-dependent increase in the fluorescence anisotropy which continued over several logs of titrant concentration. Scatchard-type plots of the anisotropy response were biphasic indicating the presence of one or more weak sites \( K_d > 1 \, \mu M \) along the collagen chain in addition to a strong site characterized by \( K_d = 1.3 \times 10^{-8} \) M at 25 °C. Gelatin-binding fragments with \( M_r = 42,000, 60,000, \) and 72,000 also produced a biphasic response with \( K_d \) values for the high affinity site being 10- to 20-fold greater than for intact fibronectin. Binding of fibronectin and its fragments to fluorescent-labeled CB7 was essentially the same as to the whole α1 chain. In all cases, the anisotropy response could be reversed or prevented by addition of excess unlabeled gelatin or CB7, but not by synthetic peptides spanning the collagenase cleavage site of α1(I). Studies of the temperature dependence of \( K_d \) for binding of fibronectin to the high affinity site on α1 produced a value of +16 kcal/mol for the enthalpy of dissociation below 30 °C. Above this temperature, fibronectin appeared to undergo a subtle conformational transition characterized by a reduced affinity for collagen. This transition occurred in whole fibronectin but not in the gelatin-binding fragments and may involve disruption of intramolecular interactions between different domains.

Fibronectin is a 500-kDa adhesive glycoprotein found in plasma and other body fluids, in the extracellular matrix, and on the surface of various cells (for a review, see Refs. 1–4). Its two nearly identical polypeptide chains are each comprised of several distinct domains which can be isolated from proteolytic digests with retention of specific macromolecular recognition properties. Fibronectin binds to numerous macromolecules and mediates the attachment of many types of cells to surfaces containing those macromolecules. A thorough understanding of the numerous functions of this complex protein will require quantitative knowledge of its affinity for various substances. Here, we focus on the interaction with collagen.

a ubiquitous component of the extracellular matrix. Fibronectin is known to bind to several types of collagen and the binding site on fibronectin has been localized to a 30–40 kDa domain, near the N terminus of each polypeptide chain, where the unique type II homologous repeat units are located. The importance of the type II units for collagen binding was recently demonstrated by means of fusion proteins expressed in Escherichia coli (5). An intriguing aspect of this interaction is the fact that, with most types of collagen, the denatured forms exhibit a much stronger binding than the native forms, suggesting that the binding sites are at least partially masked in the native triple helix (6–9). A major site on type I collagen has been assigned to CNBr fragment 7 of the α1 chain (10, 11).

While much valuable qualitative information has accumulated about the interaction of fibronectin and its fragments with the collagens and their fragments, the quantitative picture is less clear. Most studies have focused on elucidating the regions of the various molecules that are involved in the recognition process with, at best, a comparison of the concentrations required for 50% inhibition in cell attachment or immunoprecipitation assays. The few dissociation constants available in the literature have been determined by solid-phase methods, with unfractionated gelatin as the substrate. No dissociation constants are available for purified α chains.

We have developed the use of fluorescent probes for the purpose of quantitating these interactions in the fluid phase under true equilibrium conditions (12, 13). Here, we present a systematic study of the binding of fibronectin and its fragments to purified chains of type I collagen and to the CB7 fragment of α1(I). In addition, we provide evidence that fibronectin undergoes a temperature-dependent conformational change near 33 °C which reduces its affinity for the α1 chain.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

The results show that binding of fibronectin to isolated chains of type I collagen and to the CB7 fragment of the α1 chain is a complex process; the anisotropy response extends over several logs of titrant concentration (Fig. 2) and Scatchard-type plots of the data are biphasic (Fig. 3). This was true for whole fibronectin as well as its fragments. Such heterogeneity can be interpreted in several ways. One possibility is that each collagen chain contains two (or more) high affinity sites which interact in such a way that occupation of the first somehow diminishes the affinity of the others, perhaps through steric interference. A second possibility is the...
formation of ternary or higher order complexes mediated by interactions between fibronectin molecules at high fibronectin concentration. The fact that the anisotropy response to fragments of fibronectin was qualitatively similar to that of the whole protein argues against this interpretation since self-association, to the extent that it has been characterized, appears to involve heterologous interactions between different domains widely separated in the polypeptide chains (14-17).

The third and simplest explanation for the observed heterogeneity in binding is that each chain contains two or more independent sites that differ widely in their intrinsic affinity for fibronectin. In most cases, the initial linearity of the Scatchard-type plots extended almost to the abscissa (see Figs. 3 and 4B) such that neglect of the weak sites introduced a negligible error into the estimate of $K_a$ for the strong site(s).

This same linearity also suggests that if multiple high affinity sites exist, they must be virtually identical. Studies by Kleinman and co-workers (10, 18) suggest that binding of fibronectin to the $\alpha_1$ chain of type I collagen is dominated by a single site about three-fourths of the distance between the N and C termini, near the cleavage site for mammalian collagenase. This site is contained within the CB7 fragment and our results confirm that binding of fibronectin to the $\alpha_1$ chain can be explained entirely in terms of the behavior of the CB7 fragment which appears to contain not only the strong site but one or more weak sites. However, synthetic peptides which span the collagenase cleavage site showed no binding or inhibitory activity in our system, indicating that further studies are required to define the precise location of the high affinity site.

Indirect evidence that each chain contains only a single high affinity site comes from a comparison of the apparent $K_a$ for whole fibronectin with those of its fragments. Since whole fibronectin is bivalent with respect to gelatin binding, the presence of more than one strong site on a given collagen chain would confer a much higher affinity for fibronectin relative to the isolated domains, since the flexible fibronectin molecule (19-21) could conceivably attach in two places to the flexible $\alpha$ chain. In fact, if $K_a$ for the isolated domains is taken as $3 \times 10^{-7}$ M at 25 °C (Table I), additivity of free energies would predict a value of $9 \times 10^{-14}$ M for whole fibronectin, i.e. if $K_{a,\text{mbl.}} = 10^{-4MRT}$ then $K_{a,\text{mbl.}} = 10^{-2MRT} = K_a^4$. Although configurational entropy changes would diminish this effect, the observed 10- to 20-fold difference in affinity is much too small to be compatible with simultaneous attachment of both gelatin-binding domains on one fibronectin molecule to two high affinity sites on a single $\alpha$ chain.

The 70,000 and 60,000 fragments, respectively, represent N-terminal and C-terminal extensions of the 42,000 fragment. The fact that they have similar affinities for FITC-$\alpha_1$ suggests that all three contain an intact collagen-binding domain which is located entirely within the 42,000 portion and has not been damaged by removal from its parental environment. How then to account for the observed 10- to 20-fold lower affinity relative to whole fibronectin? Here also the bivalency of intact fibronectin must be considered. Given the presence of a strong site and one or more weak sites on the same collagen chain or CB7 fragment, a model in which one chain of fibronectin binds to a strong site while the second chain binds to a weak site seems plausible, especially in view of the flexible nature of both reactants. However, additivity of free energies would still predict a much greater difference between the bivalent and monovalent species than is observed. It is possible that intramolecular associations between domains in fibronectin could affect the environment or conformation of the gelatin-binding domain in such a way as to increase its affinity for the strong site. Another possibility is that more distant domains of fibronectin have an affinity for collagen which is too weak to be detected independently but which contributes to the primary interaction when present as part of the same molecule.

Another important difference between whole fibronectin and its gelatin-binding fragments is the dependence of the binding on temperature. In the range of 10-30 °C, van't Hoff plots had similar slopes for fibronectin and 42,000 reflecting similar enthalpies of dissociation of about 16 kcal/mol (Fig. 5). This indicates that the weaker binding of the fragment in this temperature range arises from a larger entropy of dissociation. Above 30 °C, fibronectin, but not the fragment, underwent a transition to a form characterized by a 10-fold weaker affinity for the $\alpha_1$ chain such that the difference in affinity between fibronectin and 42,000 was diminished above this temperature. Triple-helical collagen structures are known to unfold near this temperature and the existence of such structures could explain a discontinuity in fibronectin binding. Although isolated $\alpha$ chains and even CNBr fragments of type I collagen are capable of reforming triple helical structures, the required concentrations and incubation times are orders of magnitude greater than those involved here (22-25). Furthermore, the effect seems to be in the wrong direction since it is well-established that denatured collagen has much higher affinity for fibronectin than does native triple-helical collagen (6-9). Finally, as shown in Fig. 1, the anisotropy of FITC-$\alpha_1$ varied smoothly with temperature over the entire range investigated with no evidence for unfolding of the kind easily detected by the same method when FITC-labeled native collagen was heated (9). We therefore conclude that the observed discontinuity in the van't Hoff plot reflects a change in fibronectin and not in the $\alpha$ chains. If the $\alpha$ chains were responsible, one would expect to see the effect with the 42,000 fragment as well as with fibronectin.

Studies of the thermal stability of fibronectin and its fragments have focused primarily on the gross denaturation which occurs above 50 °C (26, 27, and Refs. therein). However, several observations in the literature support the possibility of a more subtle thermal transition near physiological temperature. Lai and Tooney (28) examined the dependence of the ESR spectrum of a spin-labeled derivative of fibronectin on temperature; their published spectra show new features appearing above 30 °C. Brown et al. (29), interpreted differences in monoclonal antibody binding, surface binding, and trypsin sensitivity as evidence for a conformational change in fibronectin between 4 and 37 °C. Williams et al. (30) observed a break near 25 °C in the Perrin plot of dansylcadaverine-labeled fibronectin, similar to the one we have seen near 33 °C with 1-anilino-8-naphthalenesulfonate-labeled fibronectin.

| Titration | FITC-$\alpha_1$ | FITC-CB7 |
|-----------|---------------|----------|
| Fibronectin | $10^{-8}$ M, 25 °C |
| 70,000 | 1.3 (0.5-2.2, $n = 6$) | 3.5 (0.9-6.0, $n = 4$) |
| 60,000 | 18.0 ($n = 1$) | 30.0 (9.0-40, $n = 5$) |
| 42,000 | 42.0 (35-50, $n = 2$) | 24.0 (11-38, $n = 2$) |
Fibronectin/Collagen Interaction

SUPPLEMENTAL MATERIAL TO INTERACTION OF FIBRONECTIN AND ITS CELATION BINDING DOMAINS WITH FLUORESCENT-LABELED CHAINS OF TYPE I COLLAGEN

METHODS

Human plasma Fn was purified by affinity chromatography on gelatin-Sepharose and characterized as previously described (33). Peptide extraction chromatography of the purified protein on a Sepharose 6-cm column (Pharmacia) produced a single symmetrical peak with no evidence of polymers and covalent conjugates of FITC-Fn, with the chromatographic material produced a peak which was identical with authentic Fn by immunoblotting.

For the interaction studies, FITC-labeled Fn was used as the ligand. The molecular weight of FITC-Fn was determined to be 90,000 by Sephadex G-75 gel filtration. Equal molar concentrations of FITC-Fn and type I collagen were incubated for 16 hr at 25°C. The reaction mixtures were then centrifuged at 10,000 g for 30 min to remove any undissolved Fn or collagen. The supernatants were then dialyzed against PBS (pH 7.4) containing 0.3 M NaCl and 0.5 M NaCl, respectively. The dialyzed supernatants were then split into two equal parts, one for the gel filtration and one for the gel electrophoresis.

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where $K_0$ is the dissociation constant, $A$ is the change in anisotropy, $\Delta A_{high}$ is the maximum change at saturation, and $[F]_a$ is the molar concentration of free $F_a$ (or fragment). In the experiment reported here, the concentration of labeled reactant was always kept low compared to the concentration of reactant required to produce a response of $100\%$, so the reactant was approximately equal to the total. For a single class of sites, it is reasonable to assume that $A$ is proportional to the specific affinity. If additional weaker sites are present, the anisotropy will continue to increase after the high-affinity sites are filled and saturation may not be obtained. In that case, data, plotted according to equation 1 will exhibit upward curvature. Without knowledge of the multiconstituents and $A_{max}$ values for each class of site, it would be impossible to extract $K_0$ for each class of site. However, if there is sufficient difference between the $K_0$s for the high- and low-affinity sites, this curve can be obtained and a reasonable estimate of $K_0$ for the high-affinity site can be derived from the limiting slope at low $A$, as determined by linear regression.

**RESULTS**

Effect of heat on FITC-$\alpha_1$.

In contrast, collagen chains would have the potential to reform heat-stable triple-helical structures under appropriate conditions, either through intermolecular association or through intramolecular interactions resulting from a folding back of the chain against itself. The extent to which this may be lower in such structures, their formation and destruction would be expected to produce changes in the relative population of the fluorescent proteins in solution in place of anisotropy vs. temperature. However, when 1 to 50 mM samples of FITC-$\alpha_1$ bearing a reactive side chain in the procollagen region to a value close to that 25 to 30 deg, the anisotropy decreased smoothly with no evidence of a melting transition. The anisotropy remained smooth to the starting values (not shown). The lower anisotropy of the highly purified preparations results from energy transfer between different fluorophores on the same chain (16). The parallel response to heat and the small difference between anisotropyH buffer or titration experiments were done at 5 nM and 250 nM in the presence or absence of 1 mM NaCl and NaHCO$_3$. These require further investigation and have been done in the absence of heat and temperature solutions. The fluorescence anisotropy of the labeled protein was done in the presence of 1 mM NaCl and NaHCO$_3$. These have been done in the absence of heat and temperature solutions.

Figure 2 shows the effect on the fluorescence anisotropy of the labeled protein at 10-fold higher than that of the sample with 1 mM NaCl and NaHCO$_3$. These have been done in the absence of heat and temperature solutions.

**Additional for a temperature-induced conformational change in Fn**

Addition of unlabelled protein to a mixture of FITC-$\alpha_1$ (4.8 kDa) and Fn (0.1 kDa) results in a precipitous decrease in anisotropy to a value close to that observed in the absence of Fn (not shown but see Fig 2C) below. Similar results were observed at addition of a mixture of FITC-$\alpha_1$ and Fn. These observations indicate a low degree of specificity and show that the binding of these labeled chains and fragments to Fn is not mediated by the metal

Additional for a temperature-induced conformational change in Fn**

**Lack of inhibition by antibodies**

Two peptides whose sequence overlap the collagenous sequence Gly58-Arg77 bound to $\alpha_1(1)$ were used for their ability to inhibit the anisotropy response of FITC-$\alpha_1$ to Fn. The first was a commercially available collagenous substrate, Pro-Gly-Gly-Pro-Gly-Lys-Ala-Pro-Gly-Lys-Ala, which was not included in the reaction to anisotropy at concentrations up to 1 mM, under conditions where unlabeled $\alpha_1$ caused 50% inhibition at 0.8 mM. The second peptide was a 20-mer recognizing sequence Gly58-Arg77 (Fn) (Kennon et al. 1985: Gly-Pro-Gly-Lys-Ala-Pro-Gly-Lys-Ala). This peptide gave no detectable inhibition at concentrations up to 1200 nM, the approximate limit of its solubility in TBS. When labeled with FITC, the 20-mer showed no anisotropy response in Fn at concentrations up to 1.5 mM and failed to bind to a column of immobilized 42K gelatin-binding fragment of Fn under conditions where FITC-$\alpha_1$ bound tightly. When currently coupled to Sepharose, the 20-mer failed to bind the 42K gelatin-binding fragment under conditions where that fragment bound readily to cyanogen bromide-treated collagen. Complexes of $\alpha_1$ and $\alpha_2$ Chains

An alternative approach to preparing fluorescent-labeled chains in quantities sufficient for extensive analytical studies was to label FITC and subjected to SDS-PAGE (Method). Fluorescent bands corresponding to the two chains were visualized from the gel, dialyzed extensively to remove SDS, and cleaved with Fn. As shown in Fig. 4A, the anisotropy response was almost identical for the two chains. Spectral properties of the fluorescein-labeled Fn, measured at low concentrations of unlabeled Fn. In this case, the response was reversed with respect to that of the labeled proteins with the same concentration of unlabeled protein for each chain (Fig. 4C). Thus, with the presence of these measurements, there is no difference between the two chains with respect to their fluorescent interactions with Fn. Temperature Dependence of Fibronectin Binding to FITC-$\alpha_1$

Preliminary experiments suggested an essentially linear decrease in the affinity of Fn for gelatin upon raising the temperature from 25 to 35C. We therefore titrated FITC-$\alpha_1$ with Fn at several temperatures between 10 and 40C and determined the Kd for the highest-affinity site as described above. The results are summarized in Fig. 4. Panel A shows that the concentration of Fn required to produce an equivalent change in anisotropy increases with increasing temperature. However, the shift between 10 and 20C is remarkably smaller than that between 20 and 35C. The solid lines are theoretical curves generated with the values of $K_0$ and $\Delta A_{high}$ for the high-affinity site, determined from the slopes of the theoretical-fit points as before. The Fn curves are plotted in panel B (open circles) according to the van't Hoff equation. There is a sharp decrease in the curve between 30 and 35C, suggestive of a change in conformation of one of the reactants. The absence of an effect in the plot of anisotropy vs. temperature for FITC-$\alpha_2$ (Fig. 1) suggests that the change occurs in Fn as opposed to the collagen chains. The filled circles in panel B illustrate the temperature dependence of the binding of the 42 kDa fragment in gelatin (13). No break in the curve is evident in this case. The slope of the curve for Fn in the temperature range between 10 and 20C is identical to that of the fragment over the entire range and represents an enthalpy of displacement of 16 kcal/mole. The absence of a break in the curve for the fragment suggests that the unusual behavior seen with whole Fn is a property of the intact protein and is not intrinsic to the gelatin-binding domain. It also further excludes the collagen chains from responsibility for this effect.

**Temperature-induced Conformational Change in Fibronectin**

Additional for a temperature-induced conformational change in Fn when a fluorescent-labeled derivative bearing several sulfonamide-substituted amino moieties was attached to the free sulfonamide groups (method). The accessibility of the exposed tyrosines to the probes was monitored as a function of temperature to produce a data set represented by the circles in Fig. 5, plotted according to the Arrhenius equation. There is a dramatic increase in fluorescence intensity near 25C, suggesting a transition at a state of greater flexibility above that temperature. With similar experiments were conducted with labeled-BSA 150 kDa and 40 kDa fragments, the break was either absent or drastically reduced. This suggests the presence of a white Fn, noted in that in a broad conformation change around the vicinity of the probe to more global changes involving interactions between different domains.
Fibronectin/Collagen Interaction

Figure 3. Scatchard-type plots of the titration data from Figure 2. The upper panels refer to FITC-CBF and the lower panels to FITC-α3. Symbols are the same as in Figure 2. Dissociation constants for the high-affinity site(s) were calculated from the limiting slopes (dashed lines) and are summarized in Table I.

Figure 4. Titration of fluorescein-labeled α1 and α2 chains of type I collagen with fibronectin. Type I collagen was labeled with FITC and the individual chains were isolated by polyacrylamide gel electrophoresis as described in Methods. Titrations were done in TBS at 25°C. Panel A: anisotropy as a function of Fn concentration; Panel B: Scatchard-type plots of the data in A; Panel C: inhibition of the response by addition of increasing gelatin to the final samples (see panel A). Dissociation constants calculated from the slopes in panel B are 3 x 10^-7M for α1 (n = 4) and 8 x 10^-7 M for α2 (n = 5).

Figure 5. Effect of temperature on the titration of FITC-α1 with fibronectin. Panel A: increasing amounts of Fn were added to a solution of FITC-α1 (5 nM) in TBS and allowed to equilibrate at the indicated temperature until the anisotropy stabilized. The solid lines are theoretical curves corresponding to the Kd and ΔAmax values obtained from limiting slopes of Scatchard-type plots of the type shown in Figure 3. Panel B: Van't Hoff plots illustrating the temperature dependence of the binding of Fn to FITC-α1 (α1) and the binding of gelatin to FITC-43K (α2). The latter data are taken from (679), supplemented with a few additional points at the higher temperatures. Brackets around the 25°C point for Fn correspond to the range of values reported in the first line of Table I.

Figure 6. Perrin plot of the effect of temperature on the fluorescence anisotropy of AME-labeled fibronectin and AME-labeled fibronectin fragments in TBS at a concentration of 0.1 μM. The samples were heated at approximately 0.07°C/sec while measuring the anisotropy every 10 sec; not all data points are shown (0). Fn (A), 45K gelatin-binding fragment (D), 48K heparin-binding fragment.