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Binding characteristics of complementary fibronectin fragments on artificial substrata

Kristine Lewandowska, Charlotte S. Kaetzel°, Luciano Zardi* and Lloyd A. Culp

Department of Molecular Biology and Microbiology, °Department of Pathology, Case Western Reserve University, School of Medicine, Cleveland, OH 44106, USA and **Cell Biology Laboratory, Istituto Nazionale per la Ricerca sul Cancro, 16132 Genoa, Italy*

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Various properties have been evaluated for the binding to tissue culture substrata of proteolytic fragments of human plasma or cellular fibronectins containing complementary sequences from the individual and alternatively spliced chains, since related fragments are known to yield differing adhesive responses from cells. These studies utilize ELISA methods and a polyclonal antiserum directed to human pFN for direct measurement or an occupancy test utilizing anti-albumin. Very related fragments (with or without an extra type III homology unit or extra domain$_{a \text{ or } b}$) have significantly different properties in substratum binding and such differences provide a partial explanation for alteration of cellular adhesive responses on such fragments.

Fibronectin; Proteolytic fragment; Substratum binding; Cell adhesion; ELISA

1. INTRODUCTION

Plasma and cellular fibronectins (pFN and cFN, respectively) mediate the adhesion and various physiological responses of cells by the activity of several mapped binding domains which recognize cell surface and extracellular matrix molecules [1,2]. Structure/function studies are complicated by alternative splicing of FN pre-mRNA at three sites into a variety of mature mRNAs for protein translation (e.g., in the human 20 species) [2,3]. An approach for analyzing adhesion-promoting roles of FN-binding domains utilizes proteolysis of pFNs (or in some cases cFNs) and purification of protease-resistant fragments containing one or two of these domains. Such studies require the binding of fragments to artificial substrata [4–10] or a collagen layer [11] with evaluation of cell responses. Only limited information has been available on the properties of the binding of any FN fragments to substrata in comparison to intact FN [12,13], although several laboratories have radiolabeled them to evaluate saturability [6,8,9,14]. Since adhesive responses of many cells are more 'normal' on intact FN than on mixtures of fragments containing the same binding domains [5–7], neighboring sequences in the FNs appear to modulate the function of the domains in unknown ways (possibly by altering protein conformation on such substrate) and this has been demonstrated recently for human neuroblastoma cells [10].

Structure/function studies have recently utilized a family of complementary fragments from individual pFN α- or β-chains + or − the alternatively-spliced IIICS region, respectively, as well as cFNs + or − the spliced extra domains (ED$_{a \text{ or } b}$) [3,15]. Fibroblasts and neural cells respond to sequence-differing, but related, fragments in significantly different ways [10,16,17]. One explanation for such differences, considering that related fragments have the same binding domains, is altered conformation of fragments on artificial substrata and/or differences in other binding properties. Some properties have been

Correspondence address: K. Lewandowska, Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, Cleveland, OH 44106, USA

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evaluated in this study by ELISA [18] of the complementary FN fragments binding to several substrata using a polyclonal antiserum raised to human pFNs [19]. ELISA methods have also described the binding of albumin and γ-globulins to various biomaterial surfaces [20].

2. MATERIALS AND METHODS

2.1. *Fibronectin fragments*

pFN from human plasma and cFN from cells were purified by gelatin-Sepharose affinity chromatography [10,15]. Fragments from thermolysin digests were purified by affinity chromatography on columns of gelatin-, heparin-, and/or monoclonal antibody-Sepharose [15]. cFN from normal or transformed cells generated fragments enriched in ED_a or ED_b, respectively [3]. The following family of fragments are obtained from adjacent sequences with minimal loss of amino acids at the cleavage point (see fig.1 from [10]). From the α-chain of pFN, F145 (145 kDa) contains an RGDS-dependent cell-binding domain ($Cell_I$), the C-terminal heparin-binding domain (Hep_{II}), and five amino acids of IIICS. Cleavage of F145 generates F110 containing $Cell_I$ but not Hep_{II}; the complementary F29 from F145 cleavage contains Hep_{II} and five amino acids of IIICS at its C-terminus. Comparable fragments from the β-chain of pFN are F155 with both $Cell_I$ and Hep_{II} domains, containing an additional type III homology unit but no IIICS sequence. Fragments from F155 are F110 with $Cell_I$ (identical to F145-derived F110) and F38 with Hep_{II}, as well as the additional type III homology unit. From normal cell FN, a mixture of 44 kDa and 47 kDa fragments was obtained retaining Hep_{II}, ED_a, and five amino acids of IIISC at the C-terminus. F120 was derived from cFN of transformed cells and contains all the sequence of F110 plus ED_b; this fragment is cleaved into F35 containing $Cell_I$ and F90 containing ED_b.

2.2. *Protein adsorption*

Tissue culture cluster dishes (96-well; Costar, Cambridge, MA) were rinsed once with phosphate-buffered saline (PBS) and adsorbed for 1 h (or as indicated) at 37°C with the indicated proteins at varying concentrations diluted in serum-free medium. Wells were rinsed three times with PBS and post-adsorbed with attachment medium (250 μg/ml of heat inactivated BSA or myoglobin [see below] in medium) for 1 h at 37°C.

2.3. *Enzyme-linked immunosorbent assay [18]*

Goat antiserum to human pFN has been described [19] and does not react with BSA (see below) used to block available substratum sites. Rabbit antisera to BSA were used for comparison (gift of Dr Abram Stavitsky of this Department). Sera are diluted in 1% (w/v) BSA (for anti-pFN) or myoglobin (for anti-BSA) in PBS and added to wells treated with test proteins. Wells were incubated for 1 h at 37°C, rinsed three times with 0.1% BSA or myoglobin (w/v) in PBS, and incubated for 1 h at 37°C with an alkaline phosphatase conjugate of rabbit anti-goat IgG (Zymed Laboratories, San Francisco, CA) [or, in some cases, goat anti-rabbit IgG conjugate to evaluate anti-BSA reactivity]; wells were rinsed three times again. Enzyme substrate (*p*-nitrophenyl phosphate; Sigma Chemical Co.) in 50 mM glycine buffer, pH 9.6, with 1 mM $MgCl_2$ was diluted to 1 mg/ml and added at 100 μl/well. $A_{405\ nm}$ values on a Perkin-Elmer ELISA reader were used within the linear range and routinely gave standard errors in multiple determinations of between ± 2 and 8%.

3. RESULTS AND DISCUSSION

Antisera were tested with the ELISA method against pFN- or BSA-coated substrata at varying dilutions as shown in fig.1 to determine antibody-excess conditions of reactivity; as demonstrated previously [12,13], the 20 μg/ml of pFN or BSA used to coat wells saturates the substratum. Anti-pFN reacted effectively with pFN but not BSA substrata (fig.1A) and a dilution of $10^{-2.5}$ was chosen for the experiments. Anti-BSA demonstrated specificity for the BSA substratum above dilutions of 10^{-3} and this dilution was chosen as the antibody-excess condition.

Reactivity of pFN or cFN fragments were then tested at this anti-pFN dilution for comparison with intact pFN (fig.2). Fragments may contain only some of the epitopes represented in the whole molecule, yielding less saturable reactivity in the ELISA; they may also contain unique epitopes masked by folding of intact FN. α-chain fragments of pFN give differing saturation reactivities when the concentration of the adsorbing fragment is varied (fig.2A). F145 yields reactivity almost indistinguishable from pFN, even though it represents only 60–65% of the sequence of the entire molecule. This also indicates that on a weight basis F145 (containing both $Cell_I$ and Hep_{II} domains) binds to the substratum as effectively as the intact molecule. Interestingly, F110 (containing the $Cell_I$ domain) derived from F145 does not saturate the substratum below an adsorbing concentration of 50 μg/ml while the complementary Hep_{II}-containing fragment F29 appears to be saturable (to be tested below) at 20–50 μg/ml but yields a much lower reactivity with the antiserum, suggesting that most of the epitopes in F145 in this goat antiserum are localized in sequences found in F110 or that much higher concentrations of F29 are required to drive the substratum binding to completion. When the β-chain fragments of pFN are tested (fig.2B), similarities and differences in ELISA reactivity are noted; F155 containing both domains binds as effectively as F145, while the

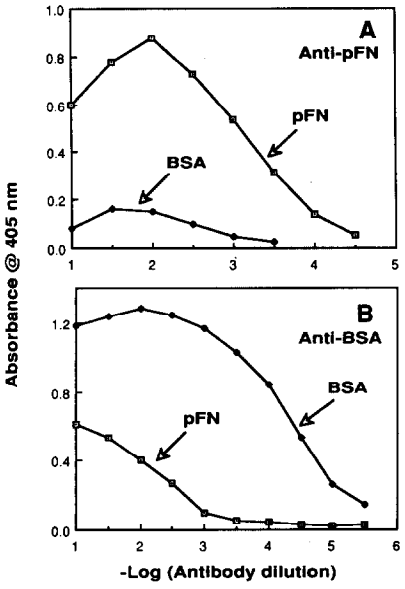

Fig.1. ELISA reactivity of pFN or BSA substrata as a function of antisera dilution. Anti-pFN (A) or anti-BSA (B) were diluted with PBS as indicated (antibody dilution) and tested for reactivity in wells coated with 20 μg/ml pFN or 250 μg/ml BSA with the ELISA method ($A_{405\ nm}$ at 30 min).

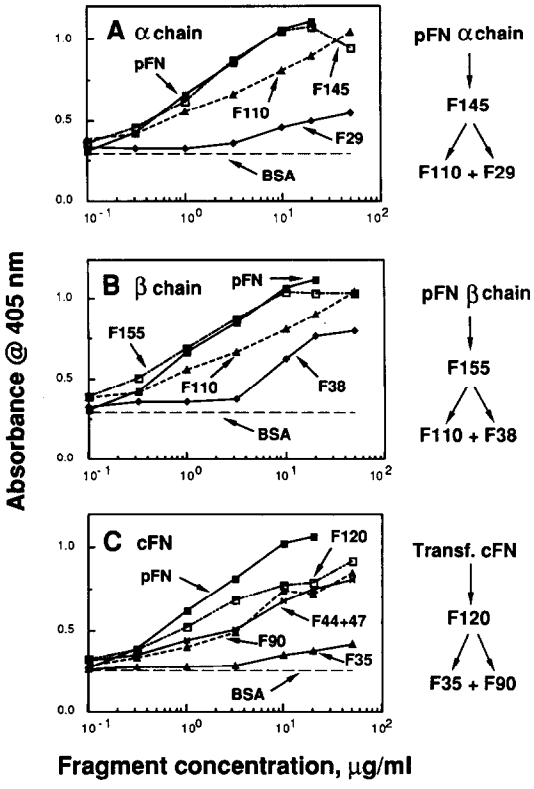

Fig.2. Concentration dependence of ELISA reactivity with anti-pFN of pFN, pFN α-chain fragments (A), pFN β-chain fragments (B), or cFN fragments (C). The origin of each class of fragments is displayed at the right of the appropriate panel. All data were obtained from the same experiment for direct comparison.

Hep$_{II}$-containing F38 saturates the substratum at approx. 20 μg/ml and yields better reactivity with the antiserum than F29 (in fig.2A).

Cellular FN fragments were then tested for comparison with the pFN-derived fragments (note that the antiserum was raised to human pFN). As shown in fig.2C, F120 from transformed cFN and comparable to F110 from pFN (with the addition of the alternatively spliced ED$_b$ sequence) binds to the substratum with a dose dependence indistinguishable from that of F110. However, cleavage of F120 yields F90 which contains none of the well-characterized binding domains but which reacts almost as effectively in the ELISA while the complementary F35 containing the Cell$_I$ domain yields the lowest reactivity in the ELISA (fig.2C). Fragments 44 + 47 from normal cFN containing the Hep$_{II}$ domain and the Ed$_a$ sequence bind in a similar manner to F38 (fig.2C), demonstrating that the ED$_a$ sequences do not affect binding and antibody reactivity as much as the extra type III homology unit found in F38 and F44 + 47 but not in F29. Therefore, the larger fragments appear to bind more effectively than the smaller fragments with the caveat that small fragments with related sequences can have very different binding properties (e.g., F38 or F44 + 47 compared to F29 or F35).

Another approach for testing whether fragments saturate the substratum utilizes bovine serum albumin to bind to available sites, after prior adsorption of FN fragments, and then testing reactivity of the substratum to anti-BSA by ELISA, i.e., testing substratum occupancy by the FN fragments and their ability to block BSA binding. As shown in fig.3A for pFN α-chain fragments, F145 blocks subsequent albumin binding at concentrations >50 μg/ml (identical to pFN; not shown). In contrast, F110 adsorption blocks the substratum from BSA occupancy to 10 μg/ml and then occupancy becomes poorer at higher F110 concentrations as if F110 is facilitating BSA bind-

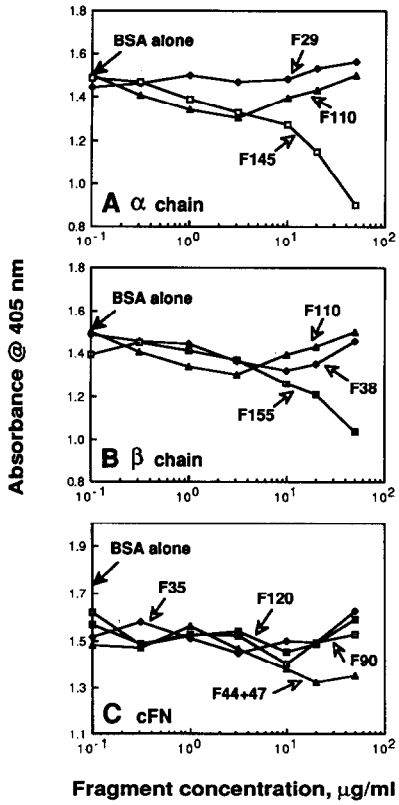

Fig.3. FN fragment occupancy on substratum as determined by BSA post-adsorption and anti-BSA ELISA. Fragments from pFN α-chain (A), pFN β-chain (B), cFN (C), or 250 μg/ml BSA alone as a control (A,B,C) were adsorbed to wells at increasing concentrations and rinsed out of wells, which were then post-adsorbed with BSA. ELISA reactivity with anti-BSA was then tested. All data were obtained from the same experiment for direct comparison.

ing or is being labilized off the substratum. F29 occupancy was poor at all concentrations, consistent with the anti-pFN ELISA data of fig.2A. For pFN β-fragments (fig.3B), F155 occupancy was very good while F38 occupancy increased to 10 μg/ml and then facilitated BSA replacement at higher concentrations. For cFN fragments (fig.3C), F44 + 47 occupied the substratum more effectively than pFN-derived F29 or F38 while both F120 and its derivatives (F90 and F35) gave biphasic plots. Therefore, larger fragments occupy the substratum more effectively than the smaller fragments which display complex relationships with subsequent BSA binding.

Polylysine coatings on surfaces facilitate adhesion responses of some cell types. Therefore, the binding of pFN and fragments was evaluated in plastic wells or in wells precoated with polylysine (20 μg/ml for 1 h) using anti-pFN in the ELISA (fig.4). In most cases, the amounts bound were indistinguishable on the two substrata, demonstrating that adhesion differences cannot be due to quantitative differences in FN products bound; however, their conformation could be quite different, although not detected here by antibody binding. Two exceptions were noted. Both F155 from the β-chain and F145 from the α-chain bound comparably to plastic while the latter bound less well on polylysine (by Student's t-test, $p < 0.02$), indicating that the extra type III homology unit in F155 confers greater interaction with a positively charged matrix.

Two different experiments were designed to test the kinetics and stability of binding of fragments to plastic wells. First, the time of adsorption of fragments to wells was varied prior to washing excess fragment out of wells for testing immediately with anti-pFN by ELISA (not shown). For pFN, F155, F145, and F29, binding was immediate and maximal. However, for F110, F90, and to some extent F44 + 47, binding increased during the initial 20 min, suggesting cooperative interaction in the binding of these fragments to substrata. Second, the stability of binding was evaluated over a 24 h period (fig.5). pFN-derived fragments (fig.5A) appeared quite stable, with the exception of F38

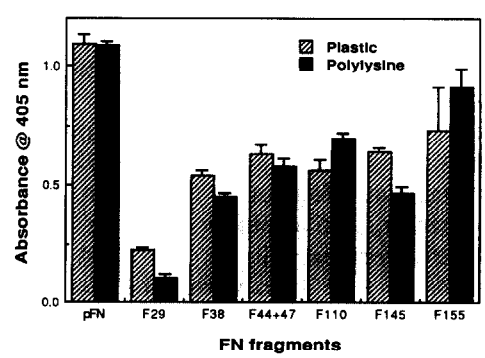

Fig.4. Adsorption of fragments to polylysine-coated substrata. Wells were left untreated (plastic) or coated with 20 μg/ml poly-L-lysine (polylysine) for 1 h, rinsed, then adsorbed with 50 μg/ml of the indicated fragments or pFN for 1 h, rinsed, post-adsorbed with BSA, and finally rinsed with PBS. ELISA reactivity was tested with anti-pFN.

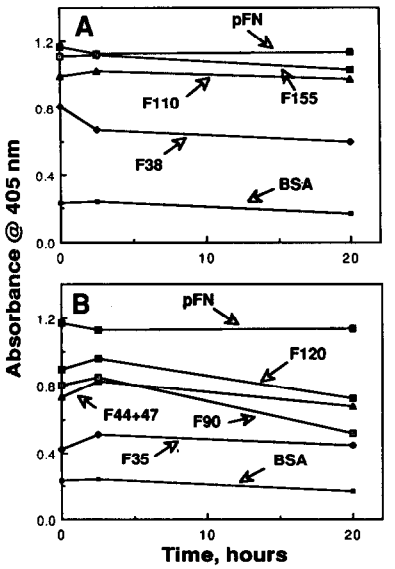

Fig.5. Stability of fragment binding to substrata. Plastic wells were coated with the indicated fragments, pFN, or BSA and then rinsed with PBS. Adhesion medium (250 μg/ml BSA in DMEM) was added to wells for varying periods of time and ELISA reactivity tested with anti-pFN. All data were obtained from the same experiment for direct comparison.

which decreased slightly during the initial several hours. cFN fragments (fig.5B) F120 and F90 were stable initially but then lost reactivity on the substratum during the subsequent 20 h. These results show that there is no massive loss of fragment from substrata in the absence of attaching cells in adhesion medium but that some of the fragments are more labile than others.

In summary, ELISA methods were used to demonstrate differing amounts and/or conformation of related FN fragments on artificial substrata. An additional type III homology unit or alternatively spliced sequence in FN chains can have significant effects on substratum-binding properties and, ultimately, on the adhesive responses of cells as mediated by binding domains represented in these various fragments.

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