IGD Motifs, Which Are Required for Migration Stimulatory Activity of Fibronectin Type I Modules, Do Not Mediate Binding in Matrix Assembly

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Introduction

Fibronectin (FN) is a large glycoprotein distributed widely in the body. It is an insoluble component of the extracellular matrix, where it plays a role in cell adhesion, migration, and embryogenesis [1,2]. It is also present at near micromolar concentrations in plasma [3], from which it is deposited in fibrin and assembled on the surface of platelets and thus contributes to the growth and stability of thrombi [4,5]. FN is a disulfide-linked dimer of subunits comprising 12 type 1 (FNI) modules, two type 2 (FNII), and 15–17 type 3 (FNIII) modules [2] (Fig. 1A). Nine of the FNI modules along with the two FNII modules are found in the N-terminal 70-kDa region of FN (70K) (Fig. 1A). This part of FN, as a proteolytic fragment or truncated FN splice variant that also includes part of the surface of platelets that is important for FN assembly. Further, FN fragment N-7FNIII, which does not stimulate migration, binds to assembly sites on FN-null fibroblast. The Ile-to-Ala mutations had effects on the structure of FNI modules as evidenced by decreases in abilities of 70K with Ile-to-Ala mutations to bind to monoclonal antibody 5C3, which recognizes an epitope in 5FNIII, or to bind to FUD, a polypeptide based on the F1 adhesin of Streptococcus pyogenes that interacts with 70K by the β-zipper mechanism. These results suggest that the picomolar interactions of 70K with cells that stimulate cell migration require different conformations of FNI modules than the nanomolar interactions required for assembly.

Abstract

Picomolar concentrations of proteins comprising only the N-terminal 70-kDa region (70K) of fibronectin (FN) stimulate cell migration into collagen gels. The Ile-Gly-Asp (IGD) motifs in four of the nine FN type 1 (FNI) modules in 70K are important for such migratory stimulating activity. The 70K region mediates binding of nanomolar concentrations of intact FN to cell-surface sites where FN is assembled. Using baculovirus, we expressed wildtype 70K and 70K with Ile-to-Ala mutations in 3FNI and 5FNI, 7FNI and 9FNI, or 3FNI, 5FNI, 7FNI, and 9FNI. Wildtype 70K and 70K with Ile-to-Ala mutations were equally active in binding to assembly sites of FN-null fibroblasts. This finding indicates that IGD motifs do not mediate the interaction between 70K and the cell-surface that is important for FN assembly. Further, FN fragment N-7FNIII, which does not stimulate migration, binds to assembly sites on FN-null fibroblast. The Ile-to-Ala mutations had effects on the structure of FNI modules as evidenced by decreases in abilities of 70K with Ile-to-Ala mutations to bind to monoclonal antibody 5C3, which recognizes an epitope in 5FNIII, or to bind to FUD, a polypeptide based on the F1 adhesin of Streptococcus pyogenes that interacts with 70K by the β-zipper mechanism. These results suggest that the picomolar interactions of 70K with cells that stimulate cell migration require different conformations of FNI modules than the nanomolar interactions required for assembly.
IGD Motifs Do Not Mediate Fibronectin Assembly

Results

Expression of 70K with mutations in IGD motif

Previous migration studies focused on the IGD motif of 7–9FNI or 8–9FNI in fibroblast migration [7]. The inability of N-3FNIII to stimulate fibroblast migration has been attributed to a salt bridge between Arg222 in 4FNI and residues in 5FNIII that causes N-3FNIII to fold back on itself, thus occluding IGD motifs in 3FNI and 5FNI [7]. To look for other differences between binding to cell surface assembly sites and MSF activity, we tested the ability of N-3FNIII to bind FN of fibroblasts. FN of fibroblasts adherent to 3FNIII-C EDA+ coated coverslips were provided 30 nM N-3FNIII or FN (molarity based on the subunit) and stimulated with 200 mM lysophosphatidic acid. After 3 h, cells were fixed and stained with mouse-anti human antibody 5C3, which recognizes 3FNIII [14]. As visualized by 5C3 staining, N-3FNIII or FN bound to FN-/- fibroblasts (Fig. 3).

Alteration of the epitope of mAb 5C3

Previous NMR studies comparing 8–9FNI structures with any of seven different mutations in the IGD motif in 8FNI showed minor changes in residues surrounding the mutations, and no changes in the folds of the modules [10]. As another probe of structural alterations, we used mAb 5C3, which binds Gly567 in the loop between the A and B strands, five residues away from Ile572 in the loop adjacent to the E-strand of 3FNIII [14]. In a competitive ELISA, increasing concentrations of 70K or 70K I150/242A decreased binding of 5C3 to coated FN; 30-fold higher concentrations of 70K I480/572A or 70K I150/242/480/572A were needed for the same effect (Fig. 4A). In contrast, when we tested mAb 7D5 to an epitope in N-3FNIII binds to the cell-surface

Previous studies have shown that 70K, but not N-3FNIII, stimulates fibroblast migration [7]. The inability of N-3FNIII to stimulate fibroblast migration has been attributed to a salt bridge between Arg222 in 4FNI and residues in 5FNIII that causes N-3FNIII to fold back on itself, thus occluding IGD motifs in 4FNI and 5FNI [7]. To look for other differences between binding to cell surface assembly sites and MSF activity, we tested the ability of N-3FNIII to bind FN of fibroblasts. FN of fibroblasts adherent to 3FNIII-C EDA+ coated coverslips were provided 30 nM N-3FNIII or FN (molarity based on the subunit) and stimulated with 200 mM lysophosphatidic acid. After 3 h, cells were fixed and stained with mouse-anti human antibody 5C3, which recognizes 3FNIII [14]. As visualized by 5C3 staining, N-3FNIII or FN bound to FN-/- fibroblasts (Fig. 3).

Mutations in IGD motif do not alter 70K binding

To determine the effect of mutations in IGD motifs on 70K binding to FN-null (FN-/-) fibroblasts, cells adherent to adsorbed FN were provided 40 nM FITC-70K, FITC-70K I150/242A, FITC-70K I480/572A, or FITC-70K I150/242/480/572A, a concentration of 70K that saturates ~80% of binding sites on the cell surface [22]. All proteins bound to the surface of FN-/- fibroblasts in fibrillar arrays as seen by microscopy (Fig. 2A). Western blotting corroborated the impression from microscopy that similar quantities of the proteins bound to the cell layers (Fig. 2B). These results indicate that intact IGD motifs are not required for binding of 70K to cell-surface assembly sites.

N-3FNIII binds to the cell-surface

Uses this mechanism to bind to the 70K region of FN with low nanomolar affinity and in so doing blocks FN assembly [14,21]. Here we compare binding to FN assembly sites and to FUD of wildtype 70K and 70K harboring isoleucine-to-alanine (ile-to-Ala) mutations in the IGD motifs of 3FNI and 5FNI, 7FNI and 9FNI, or 3FNI, 5FNI, 7FNI, and 9FNI modules. We conclude that mutations in IGD motifs do not grossly impair the nanomolar affinity interaction between 70K and assembly sites on fibroblasts. In contrast, the ile-to-Ala mutations alter binding of a monoclonal antibody (mAb) directed towards an epitope in 9FNI. These results contrast, the Ile-to-Ala mutations alter binding of a monoclonal antibody (mAb) directed towards an epitope in 9FNI. These results

Figure 1. Diagram of FN and FN fragments and location of IGD motifs in 70K. (A) The EDA+ V89 splice variant subunit of FN is shown consisting of 12 FNI modules (ovals), two FNII modules (diamonds), and 16 FNIII modules (squares). Plasma FN lacks EDA and one subunit contains a variable region and the other subunit lacks it. Modules are numbered to facilitate naming recombinant proteins according to modular content. MSF is the N-terminus through the sequence encoded by the 1FNIIIa exon and 10 intronic amino acids [6]. FNI modules containing IGD motifs are indicated with an *. (B) Sequence of FUD with presumptive binding sites for FNI modules in bold and underlined and N- and C-terminal tails in lower case. doi:10.1371/journal.pone.0030615.g001

Mutations in IGD motif

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We looked at the ability of soluble 70K and mutant 70Ks to compete for binding of 0.3 nM biotinylated FUD (b-FUD) to coated FN. Both I150/242A and 70K I480/572A had 2-fold decreased ability to compete for binding of b-FUD to coated FN as compared to wildtype 70K (Fig. 5A). 70K with all four Ile-to-Ala mutations had an even greater decrease in its ability to compete for b-FUD (Fig. 5A). To determine if the decreased affinity of b-FUD for mutated 70K was specific for Ile-to-Ala mutations, we compared the ability of human and rat 70K to compete for b-FUD binding to FN. There are 30 residues that differ in the expressed rat 70K protein as compared to the human protein [14]. However, increasing concentrations of human 70K or rat 70K had the same ability to compete for 0.3 nM b-FUD binding to coated FN (Fig. 5B). Further, human 70K in which Asn-Gly-Arg (NGR) motifs in 3FNI and 5FNI were mutated to Gln-Gly-Arg (QGR) sequences [23] competed equally well as 70K for b-FUD binding to coated FN (data not shown).

Discussion

The 70K fragment of FN binds to cell-surface FN assembly sites with the same nanomolar affinity as full-length FN, and at a thousand-fold lower picomolar concentration, 70K stimulates migration of fibroblasts into type I collagen [7,22]. Full-length FN and the N-3FNIII construct lack MSF activity, probably because...
the sites required for the activity are obscured in the quaternary structure [7,12]. The structure/function relations of the MSF activity compared to FN assembly activity are not known. Because migration stimulating ability of 70K has been shown to be dependent on the IGD motifs [10,11], we investigated binding of wildtype 70K and 70K with Ile-to-Ala mutations in 3FNI and 5FNI; 7FNI and 9FNI; or 3FNI, 5FNI, 7FNI, and 9FNI to the cell surface as well as to mAbs and FUD polypeptide that bind to 70K [14].

Ile-to-Ala mutations in either two or four IGD motifs in 70K did not affect the binding of 70K to FN$^{+/−}$ fibroblasts and had minimal effects on binding of FUD. These results contrast with loss of MSF activity when isoleucine residues in the IGD motifs of 7FNI and 9FNI were mutated in a FN fragment containing 7–9FNI [10]. The contrasting results indicate that the interactions with cell surfaces that stimulate migration and enable FN assembly are different. Additional evidence supporting the idea that the interactions are different is provided by experiments showing that N-3FNIII binds to the cell-surface of FN$^{+/−}$ fibroblasts, whereas N-3FNIII does not stimulate migration [7]. Finally, a polypeptide highly homologous to FUD has been demonstrated to enhance rather than inhibit the ability of N-3FNIII to stimulate migration whereas FUD is an inhibitor of binding of 70K to fibroblast assembly sites [19,24].

Signaling mediating MSF activity seems to involve inhibition of AKT [25]. The cell-surface molecules that initiate such signaling remain to be identified. Experiments blocking $\alpha_v\beta_3$ integrins with antibodies indicate a role for this integrin in migration [11,12], but whether $\alpha_v\beta_3$ interacts with MSF to initiate signaling or engages binding sites in the supporting collagen gel to mediate migration is not known. We are not aware of $\alpha_v\beta_3$ interacting with any ligands with pico-molar affinity. In addition, there is no evidence that the nanomolar binding of 70K to the cell-surface sites is dependent on $\alpha_v\beta_3$. A cyclic RGD peptide that inhibits $\alpha_v\beta_3$ cell adhesive activity does not block binding of 70K at nanomolar concentrations to the cell-surface [23]. Further, although NGR motifs in 3FNI and 5FNI spontaneously convert to integrin-binding isoDGR sequences, and isoDGR can interact with $\alpha_v\beta_3$ integrins [26], the conversion of NGR to isoDGR is incomplete, and

![Figure 4. Structural alterations in 70K with IGD mutations.](http://www.plosone.org/adaptive-risk-assessment-system/) Binding relative to no competitor of 1:30,000 5C3 ascites (A) or 1:50,000 7DS ascites (B) to coated FN in the presence of increasing concentration of 70K (□), 70K I150/242A (△), 70K I150/242A (▼), or 70K I150/242/480/572A (■). Values are mean plus/minus standard deviation of 3 experiments.
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![Figure 5. 70K with Ile-to-Ala mutations compete less well for binding of b-FUD to adsorbed FN.](http://www.plosone.org/adaptive-risk-assessment-system/) (A) Binding relative to no competitor of 0.3 nM b-FUD to coated FN in the presence of increasing concentrations of 70K (□), 70K I150/242A (△), 70K I150/242A (▼), or 70K I150/242/480/572A (■). (B) Binding relative to no competitor of 0.3 nM b-FUD to FN in the presence of increasing concentrations of human 70K (□) or rat 70K (▼). Values are mean plus/minus standard deviation of 3 experiments.
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mutagenesis experiments indicate that the sequences are not responsible for binding of 70K to assembly sites [23].

Previous NMR studies showed little structural alteration in 3FNII with mutations in the IGD motif; these changes involved Ser575 and the disulfide connecting the A- and D-strands [10]. Our results showed that the I572A mutation results in decreased binding of mAb 5C3 to its epitope, which contains Gly567 five amino acids away from Ile572. Further, mutations in the IGD motif, but not the 30 differences between rat and human 70K or mutations in NGR motifs, affected the ability of 70K to interact with the bacterial peptide FUD. As with other FN-binding sequences from bacteria [13,15,16], FUD appears to bind to the E-strand of FNII modules [14]. Because the IGD sequence is in the loop connecting B and C strands, it is unlikely that mutations in IGD motifs interfere directly with FUD binding [12]. Instead, because the conserved isoleucine is part of the hydrophobic core of FNII modules [9], we hypothesize that the Ile-to-Ala mutations disrupt the hydrophobic core of the FNII module which in turn deforms the modules and alters the ability of FUD to bind. The IGD motif is part of the sequence XY/IVGD/E found in nine of 12 FNII modules. It is noteworthy that mutation of the tyrosine, which is found in all 12 FNII modules and contributes to the hydrophobic core [9], to serine in any of the five N-terminal FNII modules is deleterious to secretion of 70K and binding of 70K to matrix assembly sites or to Staphylococcus aureus [27], an interaction that requires β-zipper formation.

The literature and present results present a conundrum. The interaction leading to migration is not only orders of magnitude tighter than described integrin-ligand interactions, but also orders of magnitude tighter than the interaction of 70K to FUD or assembly sites. Nevertheless, the MSF interaction apparently involves recognition of a binding surface that can be presented by any of four different FNII modules whereas the binding to assembly sites or FUD involves simultaneous interactions with multiple FNII modules. The attribute required for MSF activity may be the display of the side chains of the IGD motifs on the surface of the modules, as suggested by the MSF activity of micromolar concentrations of IGD-containing tetrapeptides [11], or a common structural feature of IGD-containing FNII modules that requires the isoleucine.

Materials and Methods

Expression of recombinant proteins, purification of FN, and antibodies

Recombinant human 70K (N-3FNIII) with I150/242A, I480/572A, or I150/242/480/572A mutations and rat 70K were produced using a baculovirus expression system with either High 5 or SF9 cells as described before [14,28]. Proteins were purified over nickel-nitrilotriacetic acid agarose as previously described [28,29]. Residues in 70K proteins are numbered starting with the initiating methionine of FN. Thus, the residue that we call Ile572 would be called Ile541 if numbering is of mean ±/− standard deviation of the mean of triplicates from 3 separate experiments.

Enzyme-linked assay

Enzyme-linked assays were done using high binding plates (Corning 3590) coated overnight at 4°C with 50 μL of 10 μg/mL FN. Assays were preformed as previously described [14]. Graphs are of mean ±/− standard deviation of the mean of triplicates from 3 separate experiments.

References

1. George EL, Georges-Laboureur EN, Patel-King RS, Rayburn H, Hynes RO (1993) Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development 119: 1079–1091.
2. Pankov R, Yamada KM (2002) Fibronectin as a glancer, J Cell Sci 115: 3861–3863.
3. Zerlauth G, Wolf G (1964) Plasma fibronectin as a marker for cancer and other diseases. Am J Med 77: 685–689.
4. Cho J, Mosher DF (2006) Role of fibronectin assembly in platelet thrombus formation. J Thromb Haemost 4: 1461–1469.
5. Maurer LM, Tomkins-Johansson BR, Mosher DF (2010) Emerging roles of fibronectin in thrombosis. Thromb Res 125: 287–291.
6. Schor SL, Ellis IR, Jones SJ, Baillie R, Seneviratne K, et al. (2003) Migration-stimulating factor: a genetically truncated onco-fetal fibronectin isoform sequence [7]. 70K proteins were stored in 10 mM Tris, 300 mM NaCl, 1 M NaBr. Recombinant N-3FNIII was expressed and purified as described previously [14]. 3FNII-C EDA+ was expressed previously [29]. Expression of FUD was described previously [14]. The sequence of FUD is shown in Fig 1B.

Human plasma FN was prepared by anion exchange chromatography of a fibrinogen-rich fraction as described previously [30]. Mouse anti-human mAbs 7D3 and 5C3 were described previously [14].

Labeling of 70K and FUD

70K was labeled with fluorescein-5-isothiocyanate (FITC) (Invitrogen) as previously described [23]. FUD was biotinylated with N-hydroxysulfosuccinimide-biotin (Pierce) as previously described [14].

70K binding

FN−/− mouse fibroblasts were derived from stem cells of FN knockout mice as previously described [14,29]. For assembly experiments, coverslips coated with 10 μg/mL FN (20 nM) or 3.8 μg/mL (10 nM) 3FNII-C EDA+ were provided 70,000 cells in 0.5 mL Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) plus 0.2% bovine serum albumin (BSA) as described previously except that cells were allowed to spread for 1 h before the addition of 40 nM FITC-70K proteins for 1 h [14]. In experiments comparing the deposition of N-3FNII or FN, cells adherent to 3FNII-C EDA+ were provided 30 nM N-3FNII or 30 nM FN (monomer, 13 nM dimer) with 200 nM lysophosphatidic acid (LPA) for 3 h. Cells were fixed and immunostained with 2 μg/mL 5C3 followed by rhodamine red-x donkey anti-mouse IgG (Jackson ImmunoResearch). For Western blot, cells were treated as above except that cells were grown in 24-well tissue culture treated plates (Costar 3524) coated with 10 μg/mL FN. Instead of fixation, cells were provided 40 μL SDS-PAGE sample buffer containing 10% β-mercaptoethanol, incubated for 10 min, and sample buffer was collected after forceful scraping. This was repeated with 10 μL of sample buffer also containing 10% β-mercaptoethanol. Samples were run on an 8% SDS-PAGE gels and Western blot was done with rabbit anti-FITC (Molecular Probes) and peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) according to standard procedure [29,31].

Author Contributions

Conceived and designed the experiments: LMM DSA DFM. Performed the experiments: LMM. Analyzed the data: LMM DSA DFM. Contributed reagents/materials/analysis tools: DSA. Wrote the paper: LMM DFM.
expressed by carcinoma and tumor-associated stromal cells. Cancer Res 63: 8827–8836.
7. Vakonakis I, Staunton D, Ellis IR, Sarkies P, Flanagan A, et al. (2009) Motogenic sites in human fibronectin are masked by long range interactions. J Biol Chem 284: 15660–15675.
8. Solinas G, Sillaire S, Lipori M, Fabbri M, Pesci S, et al. (2010) Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. J Immunol 185: 642–652.
9. Potts JR, Campbell ID (1996) Structure and function of fibronectin modules. Matrix Biol 15: 313–320; discussion 321.
10. Millard CJ, Ellis IR, Pickford AR, Schor AM, Schor SL, et al. (2007) The role of the fibronectin IGD motif in stimulating fibroblast migration. J Biol Chem 282: 35530–35533.
11. Schor SL, Ellis I, Banyard J, Schor AM (1999) Motogenic activity of IGD-containing synthetic peptides. J Cell Sci 112(Pt 22): 3979–3988.
12. Ellis IR, Jones SJ, Staunton D, Vakonakis I, Norman DG, et al. (2010) Multifactorial modulation of IGD motogenic potential in MSF (Migration Stimulating Factor). Exp Cell Res 316: 2466–2476.
13. Bingham RJ, Rudino-Pinera E, Meenan NA, Schwarz-Linek U, Turkenburg JP, et al. (2008) Crystal structures of fibronectin-binding sites from Staphylococcus aureus FnBPA in complex with fibronectin domains. Proc Natl Acad Sci U S A 105: 12254–12258.
14. Maurer LM, Tomasini-Johansson BR, Annis DS, Mosher DF (1991) Five type I modules of fibronectin form a functional unit that binds to fibroblasts and Staphylococcus aureus. J Biol Chem 266: 12840–12843.
15. Notte J, Schwarz-Bauer J, Selegue J, Mosher DF (1991) Five type I modules of fibronectin form a functional unit that binds to fibroblasts and Staphylococcus aureus. J Biol Chem 266: 12840–12843.
16. Mosher DF, Hasdier KG, Miethingauer TM, Annis DS (2002) Expression of recombinant matrix components using baculoviruses. Methods Cell Biol 69: 69–81.
17. Xu J, Bae E, Zhang Q, Annis DS, Erickson HP, et al. (2009) Display of cell surface sites for fibronectin assembly is modulated by cell adherence to (1)F3 and C-terminal modules of fibronectin. PLoS One 4: e1113.
18. Mosher DF, Johnson RB (1983) In vitro formation of disulfide-bonded fibronectin multimers. J Biol Chem 258: 6595–6601.
19. Annis DS, Murphy-Ullrich JE, Mosher DF (2006) Function-blocking antithrombospondin-1 monoclonal antibodies. J Thromb Haemost 4: 459–460.