The Role of the Fibronectin IGD Motif in Stimulating Fibroblast Migration*

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Fibronectin (FN) is a multifunctional, multidomain adhesive glycoprotein that plays a prominent role in wound healing, embryogenesis, and hemostasis. It is found both in the extracellular matrix and in soluble form in blood plasma. The structure and diverse binding properties of FN have been much studied. FN contains a novel integrin binding motif in its N-terminal region (13). The importance of the N-terminal 70-kDa fragment was also emphasized recently when it was shown to initiate FN fibril formation in fibronectin-null mouse fibroblasts (14). These reports support the idea that fibrillogenesis...
migh be initiated through integrin cell attachment at the N terminus via a binding site other than the RGD region.

Here we set out to explore the structure-function relationships of the IGD motif sequence in the context of intact Fn1 domains. Of particular interest are the third and fourth IGD motifs in MSF, one in the seventh Fn1 (in this report these modules will be described using a superscript nomenclature: 7Fn1, etc.), and one in 9Fn1. Here we examine the role of the IGD motif by analyzing the properties of a series of fragments, namely the 8Fn1-9Fn1 module pair, each containing one IGD motif, and the 7Fn1-8Fn1 module pair, each containing two IGD motifs. The effects of site-specific mutations on migration and structure are examined using fibroblast migration assays and solution state NMR. Both IGD motifs are shown to have significant migration-stimulating activity while contained within fully folded fibronectin modules.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—The human fibronectin modules 8Fn1-9Fn1 (residues 485–577), 7Fn1-8Fn1 (residues 437–528), and 7Fn1-8Fn1-9Fn1 (residues 437–577) were cloned into the Pichia expression vector pPICZ Alpha (Invitrogen). All constructs included the conservative mutation R503K in the 8Fn1 module, to avoid cleavage during secretion by the endogenous Pichia protease KEX2 (15). This single point mutant will be referred to as 8Fn1*-9Fn1. A second conservative point mutation (N497Q) was introduced into 8Fn1-9Fn1 module pair to disrupt the IGD motif in 8Fn1 (16). The protein was initially passed through a SP-Sepharose cation-exchange chromatography column at pH 3.0 to partially purify and reduce the volume of the secreted protein. High mannose sugars were trimmed back to single N-linked acetylglucosamine (GlcNAc) residues with Endo H at pH 5.5. High performance liquid chromatography (RP-HPLC) on a C4 column with a gradient of 24–38% acetonitrile and 1% trifluoroacetic acid was used to achieve homogeneity. The purity of the protein at each stage was assessed with SDS-PAGE, and the identity and final purity were confirmed by electrospray ionization mass spectroscopy (ESI-MS).

**Cells**—Collagen gel migration experiments were performed with human skin fibroblasts FSF44. Stock cultures were maintained in Eagle’s minimal essential medium (MEM) containing 15% donor calf serum, as previously described (17).

**Migration Assays**—The collagen gel assay was performed in 30-mm plastic culture dishes containing preformed 2 ml of type I collagen gel, as previously described (5). 1 ml of serum-free MEM containing four times the desired final concentration of effector molecule and 1 ml of trypsinized fibroblasts (density 2 × 10^5 cells/ml) suspended in MEM containing 4% donor calf serum were plated in duplicate onto the collagen gel to give a final volume of 4 ml in 1% donor calf serum. After a 4-day incubation period at 37 °C, cell migration into the three-dimensional gel in response to the effector was determined as previously described (17). In short, this involved using an inverted phase microscope to count the cells on the surface of the gel and then focusing down through the gel to count the cells within the gel. This operation was repeated with 15 randomly selected areas on the gel surface.

**NMR Spectroscopy**—All NMR spectra were recorded at 25 °C using spectrometers built in-house, on samples of 1–2 mM protein in 150 mM NaCl, 20 mM sodium phosphate, 1 mM dioxane, 95% H2O, 5% D2O at pH 6.6. Backbone NH and Hα assignments have been deposited in the BioMagResBank under the accession number 15447 for 8Fn1-9Fn1 (8Fn1*-9Fn1 (R503K)).

**RESULTS**

The 8Fn1-9Fn1 (R503K) Protein Is Heterogeneously Glycosylated—Our earlier study concluded that the wild type 8Fn1-9Fn1 was proteolytically cleaved by the P. pastoris endoprotease KEX2 between residues Arg-503 and His-504. The R503K mutation was therefore introduced to alleviate this proteolysis problem (15). This 8Fn1-9Fn1 module pair expressed in Pichia was found to be heterogeneously glycosylated at two sites; at least one of the sites was glycosylated and the different fractions could be separated by increasing the retention time on a C4 RP-HPLC column. We previously concluded that glycosylation of Asn-511 was critical for optimal binding to gelatin whereas glycosylation of Asn-497 had no significant effect on binding activity (15). To increase the yield of homogeneous protein and to simplify the purification procedure, Asn-497 was mutated to remove the “non-essential” glycosylation site.

The 7Fn1-8Fn1**-9Fn1*, 8Fn1**-9Fn1** and 7Fn1-8Fn1**-9Fn1 Mutants (R503K,N497Q) Are Singly Glycosylated—The mutation N497Q was introduced into all constructs used in this study (with the exception of 8Fn1*-9Fn1, which was used as a fractional fashion to that described previously for the 4F1-5F1 module pair (16). The protein was initially passed through a SP-Sepharose cation-exchange chromatography column at pH 3.0 to partially purify and reduce the volume of the secreted protein. High mannose sugars were trimmed back to single N-linked acetylglucosamine (GlcNAc) residues with Endo H at pH 5.5. High performance liquid chromatography (RP-HPLC) on a C4 column with a gradient of 24–38% acetonitrile and 1% trifluoroacetic acid was used to achieve homogeneity. The purity of the protein at each stage was assessed with SDS-PAGE, and the identity and final purity were confirmed by electrospray ionization mass spectroscopy (ESI-MS).
control) as it was judged to be a suitable conservative mutation to remove the Asn-497 glycosylation site. Approximately 80 mg/liter of 8Fn1**-9Fn1 was expressed, as judged by SDS-PAGE of crude fermentation supernatant under non-reducing conditions (data not shown). This initial yield was comparable with that of the wild type and single point mutant (R503K) protein but the purification yield was greatly enhanced as the target protein was homogenously glycosylated. SDS-PAGE showed that the molecular mass was reduced from 16 to 11 kDa after treatment with Endo Hf, indicating the removal of a single high mannose sugar chain. N-terminal sequencing gave a single sequence DQCIVDDIY, and ESI-MS confirmed the expected molecular mass of 11,075 Da, indicating the presence of the complete module pair with a single GlcNAc sugar residue.

The 8Fn1**-9Fn1 module pair was analyzed by NMR, both to compare the fold of this protein to that of the 8Fn1*-9Fn1 single point mutant and to study the sugar attachment site. The module pairs were isotopically labeled by replacing the sole nitrogen source in the fermenter vessel with (15NH4)2SO4. Both module pairs were expressed, purified, and characterized by ESI-MS, which gave an increased mass of 131 Da, corresponding to label incorporation of 98.5%. The cross-peaks of the 8Fn1**-9Fn1-GlcNAc511 and 8Fn1*-9Fn1-GlcNAc511 [1H-15N]-HSQC spectra (a single N-linked GlcNAc sugar residue on Asn-511) were shown to overlap as expected, with the exception of mutated residue Asn-497 (data not shown). Direct comparison of the 8Fn1**-9Fn1 spectrum and the 8Fn1*-9Fn1 spectrum allowed assignment of the 8Fn1**-9Fn1 cross-peaks. After this comparison, a C-terminal His tag was engineered onto the 8Fn1**-9Fn1 to aid identification of the module pair by Western blot. This addition did not significantly affect the NMR spectra other than by obvious addition of new peaks from mobile residues.

The 8Fn1**-9Fn1 Module Pair Contains Typical Fn1 Folds and the IGD Motif Is Located in a Rigid Loop between Strand B and Strand C—The solution structures of several Fn1-type module pairs have already been calculated by NMR (18, 19). The secondary structure of the 8Fn1**-9Fn1 was mapped here using inter-strand NOEs. Both modules were found to have a typical type I module fold (Fig. 2A), and the observed hydrogen bonding pattern agrees well with existing structures. The position of the IGD motif in the 9Fn1 can be mapped to a tight turn between the B strand and the C strand, and a model based on the structure of the 2Fn1-3Fn1 pair confirms that the loop is accessible to the solvent (Fig. 2B). The heteronuclear 1H-15N NOE was used to map the internal motion of residues on a subnanosecond time scale, showing the module pair to be relatively rigid, especially in the short linker between modules and in the IGD loop of the 9Fn1. Those values lower than 0.65 indicate regions with fast internal mobility, the majority falling within the loop regions of the protein.

Expression, Purification, and Characterization of 8Fn1**-9Fn1 IGD Mutants—Conservative and non-conservative point mutations were introduced into 8Fn1**-9Fn1 to interrupt the
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The IGD Motifs in 7Fn1-8Fn1** and 7Fn1-8Fn1**-9Fn1—The 7Fn1-8Fn1** and 7Fn1-8Fn1**-9Fn1 fragments were judged by SDS-PAGE to express with yields of ~80 and 40 mg/liter, respectively. The lower yield of the module triple is presumably due to some misfolding, and therefore degradation, of this larger recombinant fragment. The identity of the proteins was confirmed by N-terminal sequencing and ESI-MS (expected molecular masses of 12,061 and 18,393 Da). The recombinant material was isotopically labeled with 15N and examined by NMR. The dispersion of cross-peaks in [1H,15N]-HSQC spectra was indicative of a fully folded protein.

Following the results from the 8Fn1**-9Fn1 mutations the relative roles of the IGD motifs in 8Fn1 and 9Fn1 were explored by replacing IGD with RGD in 7Fn1-8Fn1** and 7Fn1-8Fn1**-9Fn1. The proteins were successfully expressed, purified to homogeneity, and characterized by Western blotting and ESI-MS. The two IGD motifs in 8Fn1-9Fn1**-9Fn1 were mutated in turn by introducing single point mutations I449R and I541R before introducing both mutations to create a double mutant. The structure of the mutants, before and after the Ile to Arg mutation, was judged not to have changed significantly by comparing resolved up-field methyl peaks in one-dimensional NMR spectra (data not shown).

The IGD Motifs in 7Fn1 and 9Fn1 Can Stimulate Fibroblast Migration Independently—The 7Fn1-8Fn1** with the intact IGD stimulated fibroblast migration at the same concentration as the 8Fn1**-8Fn1 (Fig. 4). The 7Fn1-8Fn1**-9Fn1 with both IGD motifs stimulated fibroblast migration with a factor of 100 higher potency with maximal activity at 1 pmol/IGD. Mutation of the 7Fn1 IGD motif in 7Fn1-8Fn1** caused significant loss of bioactivity. Independent mutation of the seventh and the ninth IGD motifs in 7Fn1-8Fn1**-9Fn1 reduced the maximal bioactivity to the level of the 7Fn1-8Fn1**, but the activity was absent in the double mutant (Fig. 3B).

An Intact IGD Motif Is Required in 8Fn1**-9Fn1 for Stimulation of Fibroblast Migration—The effect of each module pair on the behavior of fibroblast cells was assessed in a collagen gel migration assay. Increasing concentrations of the module pairs were added to cells plated on the surface of three-dimensional gels of native type I collagen fibers. In a similar manner to that of MSF and IGD peptides (8), 8Fn1**-9Fn1 with an intact IGD motif stimulated fibroblast migration into this matrix, in a bell-shaped dose-response fashion. Maximal bioactivity was expressed at concentrations of 100 pmol (Fig. 3A). As predicted from peptide studies (8), the module pairs at mutation at Ile-541 (VGD, AGD, RGD) did not affect cell migration (Fig. 3A). The DGI mutant was also unable to stimulate migration (Fig. 3B). In contrast, those module pairs carrying a mutation at Asp-543 (IGE and IGA) showed some bioactivity, but this was significantly reduced with respect to the intact IGD at the same protein concentrations (Fig. 3B). It has also been suggested that the fourth amino acid in the IGD loop could affect migration (8). The amino acid immediately following the IGD was also mutated from Ser to Gln in 8Fn1**-9Fn1 but did not change the extent of cell migration (Fig. 3B).

The 8Fn1**-9Fn1 pair (R503K,N497Q) was shown to be as active as 8Fn1**-9Fn1 (R503K), indicating that the removal of the glycosylation site does not affect the bioactivity of the protein (Fig. 3B). 8Fn1**-9Fn1 (His) was also shown to exhibit the same bioactivity as the module pair without the His tag (Fig. 3B).

The Relative Roles of IGD in 7Fn1-8Fn1** and 7Fn1-8Fn1**-9Fn1—The 7Fn1-8Fn1** and 7Fn1-8Fn1**-9Fn1 fragments were judged by SDS-PAGE to express with yields of ~80 and 40 mg/liter, respectively. The lower yield of the module triple is presumably due to some misfolding, and therefore degradation, of this larger recombinant fragment. The identity of the proteins was confirmed by N-terminal sequencing and ESI-MS (expected molecular masses of 12,061 and 18,393 Da). The recombinant material was isotopically labeled with 15N and examined by NMR. The dispersion of cross-peaks in [1H,15N]-HSQC spectra was indicative of a fully folded protein.
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**DISCUSSION**

In this study we have been able to extend the earlier work on the bioactivity of soluble synthetic IGD peptides (8) to that of IGD motifs in the context of folded Fn1-type modules. Various Fn1 module fragments, including several IgG mutants, were produced in *P. pastoris*, and their ability to stimulate fibroblast migration was assessed in a collagen gel migration assay. All of the proteins produced had a similar fold, as judged by NMR. A 10,000-fold increase in potency was observed in moving from peptides to recombinant module pairs.

The conservative I541V mutation was designed to mimic the VGD of the first Fn1-type module; it was unable to promote cell migration in the context of 8Fn1**-9Fn1. The I541R mutation was designed to introduce an RGD sequence, a motif that promotes cell adhesion when in other regions of fibronectin, e.g. 10Fn3 (9). This mutation did not, however, promote cell migration here; neither did I541A. These results suggest that the isoleucine is critical for the observed bioactivity.

The bioactivity of 7Fn1-8Fn1** is very similar to that of 8Fn1**-9Fn1 (Figs. 3A and 4). Substitution of I449R in the 7Fn1-8Fn1 module pair also resulted in a protein devoid of migration-stimulating activity.

About half the bioactivity remained in the protein with the conservative D543E mutation. Some activity also remained for the D543A mutation but it was significantly lower than the wild type 8Fn1**-9Fn1. These results suggest that the aspartate residue in the IGD is required for optimal bioactivity but is less critical than the isoleucine. The “reverse” mutant DGI was also devoid of motogenic activity.

It has been suggested that the IGDS of 7Fn1 is more active than the IGDQ in 7Fn1 (8), but the mutation S544Q did not change the bioactivity of the protein. This finding suggests that the fourth amino acid is less important in the context of the 8Fn1**-9Fn1 module pair than in the unconstrained peptide form.

The increased potency of the protein in comparison to the peptide is remarkable. Constraining the ends of the IGD motif in the 7Fn1 module may favor the orientation of ligand binding that accounts for migration stimulation at much lower concentrations. A cyclic IGD peptide containing 20 amino acids from around the IGD site is also more potent than a similar length unconstrained IGD peptide (data not shown). Cyclic RGD peptides can inhibit cell attachment at a 20-fold lower concentration than linear peptides (20). The insertion of the RGD motif into mutant lysozyme significantly increased the cell adhesion activity (21), again implying that the introduction of conformational constraints can increase affinity to the integrin receptor, although a constrained RGD sequence in the IGD loop of Fn1 modules does not enhance cell migration. In the current study, the IGD motif as presented by the 9Fn module structure has markedly increased the bioactivity compared with free peptides.

The heteronuclear {1H-}15N NOE data on 8Fn1**-9Fn1 here, as well as previous dynamics studies of 2Fn1-3Fn1 and 4Fn1-5Fn1 (18), show that the IGD motif is in a relatively immobile part of the module. This inflexibility in the IGD loop is quite different from the considerable flexibility of the RGD loop observed in 10F3 (22).

| TABLE 1 |
|---|
| Relative potency of IGD motifs in different contexts |
| The peptide/protein concentration (pmol) showing maximal motogenic activity in the fibroblast migration assay was divided by the number of IGD motifs in that construct. GBD, gelatin-binding domain; MSF, migration-stimulating factor. |

| IGD peptides | 8Fn1**-9Fn1 | 7Fn1-8Fn1**-9Fn1 | GBD | MSF |
|---|---|---|---|---|
| 10^-6 | 10^-2 | 1 | 10^-4 | 10^-6 |

We also explored possible cooperativity by studying the effect of two IGD motifs in one protein using a triple module construct (7Fn1-8Fn1**-9Fn1) containing an IGD motif in both 7Fn1 and 8Fn1. The maximal activity of two IGD motifs occurred at significantly lower concentrations than a single IGD (the concentration dependence of the MSF activity of different peptides and proteins is summarized in Table 1). It is interesting to note that the gelatin-binding domain, which contains the same two IGD motifs, is some 1000-fold more potent than the triple module (6). Migration-stimulating activity was only lost from the triple module protein after mutation of both of the IGD sites, confirming that they are both able to stimulate migration independently as well as by acting together.

The profile of cell migration at different concentrations takes a bell shape, suggesting that a threshold concentration is required to initiate cell migration and that a negative feedback mechanism occurs at higher concentrations. This is somewhat similar to a previous observation (23) that a 21-kDa fragment of FN stimulated the migration of fibroblasts in a bell-shape dose-response pattern. We have previously shown (8) that fibroblast migration stimulated by IGD peptides is dependent on both αβ3 functionality and tyrosine phosphorylation of focal adhesion kinase. The reduced migration at high concentration may thus arise from effects on integrin clustering and/or subsequent downstream signaling.

Although integrin αβ3 cannot be confirmed as the prime candidate receptor for the IGD motif, it is clear that the presence of αβ3 and its downstream signaling components (phosphatidylinositol 3 kinase and focal adhesion kinase) are required for IGD motogenic activity (8). αβ3 integrin also appears to cooperate with activated growth factor receptors, such as epidermal growth factor receptor, in mediating their respective motogenic signaling in response to ligand binding (24). The RGD loop has been established as a key recognition sequence for αβ3 (10), but changing IGD to RGD in Fn1 domains results in loss, not gain, of motogenic activity. This apparent discrepancy may be due to the inherent inflexibility of the RGD in this context.

A recent report suggested that Asn can spontaneously deamidate to isoD to create a DGR sequence (25) that can interact with αβ3. There is a potential isoDGR in 7Fn1 that could constitute a binding ligand for the αβ3. However, because the 8Fn1**-9Fn1 lacks the NGR sequence that converts to isoDGA, and 7Fn1-8Fn1** and 8Fn1**-9Fn1 both exhibit the same potency in our assay, it is unlikely that this receptor activation mechanism is part of the IGD response. Of course, integrins could play a critical mechanistic role in mediating motogenic signaling by IGD ligation to some unknown cell surface receptor.
The four IGD motifs at the N terminus of FN are invariant in human, rat, and mouse (see Fig. 1). Three are also invariant in frogs, with a conservative mutation in the fourth. From the current work and knowledge of Fn1 module structure it seems likely that all these IGD motifs will display bioactivity as long as the Fn1 modules are exposed. Only two of the four IGD motifs of MSF (7Fn1 and 9Fn1) appear to be active regarding stimulation of fibroblast migration (5). Consistent with this conclusion, a proteolytically derived gelatin-binding domain of FN (Sigma) that contains both 7Fn1 and 9Fn1 displays motogenic activity, whereas the proteolytically derived 5Fn1-9Fn1 (Sigma) was inactive (5). This suggests that 3Fn1 and 5Fn1 may not be exposed in the IGD motifs studied here could also contribute to nature of the response. The number of cryptic sites revealed at any one time could define the size and change and molecular rearrangement. The number of cryptic tension has also been shown to unravel FN (30), and exposed simply by selective sequence deletion (29). Mechanism not seen in full-length FN (27, 28), and cryptic sites can be bioactive in smaller fragments. Fibronectin when exposed; for example, as shown here and previously observation that MSF displays proteolytic activity (26). Of further interest is the containing Fn1 modules that have high collagen/gelatin binding and distinguish them from the N-terminal domain, containing Fn1 modules that have high collagen/gelatin binding to matrix, followed by receptor encounter and intracellular signaling. It is additionally possible that the IGD-containing Fn1 modules might partially denature the collagen triple helix, exposing RGD triplets to which the \( \alpha_v \beta_3 \) integrin could then bind. This would explain the high potency of those IGD-containing Fn1 modules that have high collagen/gelatin binding activity and distinguish them from the N-terminal domain, which has no motogenic activity. Of further interest is the observation that MSF displays proteolytic activity (26).

Another intriguing observation is that full-length FN does not show bioactivity (5). The usual explanation is that IGD motifs are hidden in assembled, intact FN but are bioactive when exposed; for example, as shown here and previously (6), bioactivity appears in smaller fragments. Fibronectin fragments created by proteolysis exhibit many properties not seen in full-length FN (27, 28), and cryptic sites can be exposed simply by selective sequence deletion (29). Mechanical tension has also been shown to unravel FN (30), and potential new sites can be revealed through conformational change and molecular rearrangement. The number of cryptic sites revealed at any one time could define the size and nature of the response.

Previous studies have suggested that biological activities of fibronectin, such as fibrillogenesis, can be controlled by concealing active motifs within the tertiary structure (31). The 70-kDa FN fragment has been shown to be critical in fibril assembly (32), and much work has gone into establishing the matrix assembly site. A possible explanation is that the IGD motifs studied here could also contribute to fibrillogenesis.

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