## Cryptic activities of fibronectin fragments, particularly cryptic proteases

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## 1. ABSTRACT

Fibronectin (FN) is a modular glycoprotein encoded by a single gene. A soluble form of this protein is found in the plasma of several animals. Alternative splicing of pre-mRNA at three sites produces cellular and plasma FNs. The plasma form contributes to blood clotting and thrombosis. Many extracellular matrices (ECM) contain an FN network associated with a variety of cell activities through binding to cell surface integrin receptors. Fragments of FN can have cryptic activities that are specific to these fragments rather than to the intact protein. The metalloprotease activity present in the basement membrane and plasma fibronectins has been intensively studied in humans, bovine and rats. Organic inhibitors that are selective for the human cryptic enzyme have been produced.

## 2. INTRODUCTION

Fibronectin (FN) is a modular dimeric disulfide-bonded glycoprotein. The molecular mass of each subunit is about 230-270 kDa. Each subunit has three types of repeating modules, type I, II, and III (Figure 1). Types I and II modules each have two intrachain disulfide bonds, but type III modules have none (1). A single gene encodes FN. Alternative splicing of pre-mRNA at three sites produces the cellular and plasma FNs. This complex splicing mechanism generates 20 FN variants in human (2). Many mouse models with targeted mutations have been constructed and used to identify the possible roles of these variants (3). The assembly of the ECM FN network depends on the binding of FN to cellular integrin receptors and the activation of intracellular signaling pathways. These stepwise mechanisms involve numerous
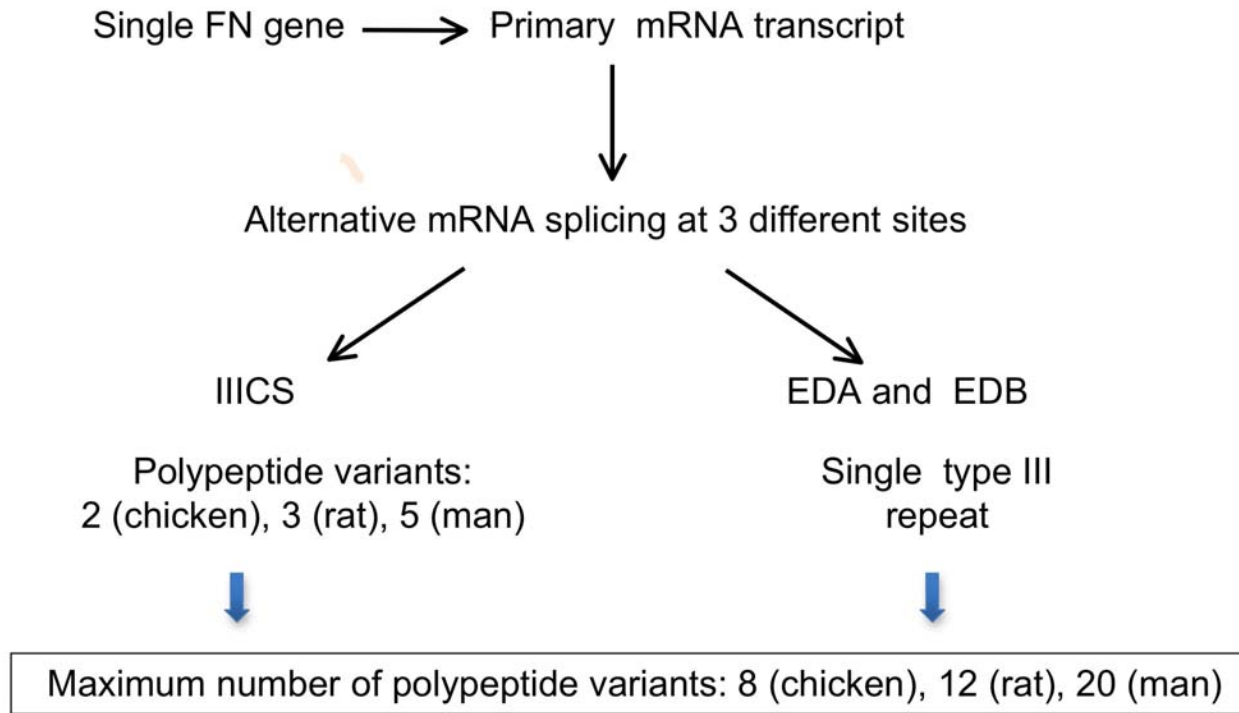
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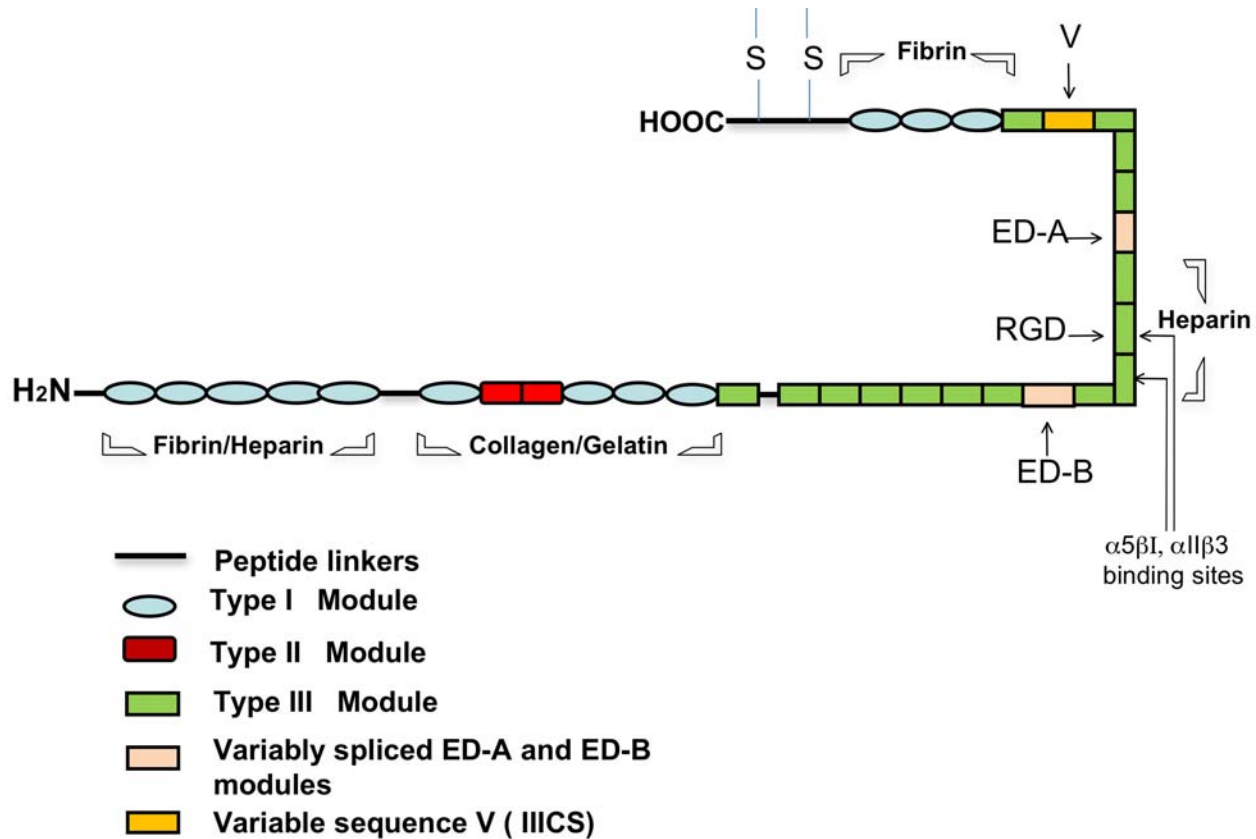


**Figure 1.** The modular structure of an FN monomer, showing the regions involved in adhesion and assembly and the variably spliced ED-A and ED-B modules and variable sequence V (IIICS). The C-terminal cysteine implicated in disulfide bridges leading to FN dimer formation are also shown.

conformational changes in the structure of the FN dimer leading to the formation of FN fibrils. This supramolecular structure takes part in cell adhesion and movement (4). FN also has cryptic activities that are specific to FN fragments and not the intact protein. These activities have been associated with cartilage metabolism (5), proliferative diabetic retinopathy (6) adipocyte differentiation (7) and the initiation of fibrillogenesis (8). Some fragments have cryptic protease activities. The first report did not clearly characterize these activities of human plasma FN (9) but a second has provided more information on a collagenase located inside the gelatin binding domain (GBD) of this type of FN (10). We have described a similar gelatinase activity located in the same domain of both plasma and basement membrane FNs (11). This activity is related structurally and functionally to the matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) and has be found in humans, bovine and rats (10-12).

**3.    FIBRONECTINS:    STRUCTURE,    GENE EXPRESSION AND POLYMORPHISM**

The modular structure of FN is dictated by the modular structure of its gene (2). Alternative mRNA splicing of the primary transcript of a single gene gives rise to a complex pattern of FN variants. The processing of this primary transcript involves three cleavage sites: IIICS, EDI

and EDII, the so-called V, EDA and EDB sites. IIICS processing gives several variants because of exon subdivisions, while EDA and EDB are single exons each coding for a single type III repeat (2). The results of this complex gene expression mechanism in some species are summarized in Figure 2. The inactivation of the FN gene is lethal in the mouse embryo, indicating the essential nature of FN (13). The functions of these polypeptides variants *in vivo* are still not understood, but cells involved in skin wound healing contain increased quantities of FN with EDA and EDB domains, i.e cellular FN (cFN) (14). Similar patterns are found in other regenerating tissues, such as regenerating rat liver and lung fibrosis (15-16). A tissue injury leads to plasma FN (pFN) rapidly binding to fibrin and fibrinogen to form hemostatic clots (17). More recent *in vivo* experiments using a model of ferric chloride arterial injury indicate that plasma FN (pFN) takes part in the formation of thrombi in the arterial wall (18-20). Hepatocytes synthesize pFN and secrete it as a heterodimer, one of whose subunits contains the IIICS variant (21; figure 2). FN also binds to the cellular heterodimeric transmembrane integrin receptors. It binds to the $\alpha 5\beta 1$ integrin via its RGD tripeptide, located in an accessible loop of FN (22). Numerous integrin receptors can react with the RGD site (23, 24).   The interaction between FN and the integrin receptors initiates the formation of a multimeric FN structure; this also activates

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Single FN gene ⟶ Primary mRNA transcript

Alternative mRNA splicing at 3 different sites

IIICS

Polypeptide variants:
2 (chicken), 3 (rat), 5 (man)

EDA and EDB

Single type III
repeat

Maximum number of polypeptide variants: 8 (chicken), 12 (rat), 20 (man)

**Figure 2.** Complex gene expression of FN in chickens, rats and humans.

intracellular signaling pathways (4). The three-dimensional structure of the GBD fragment of FN has been reported recently (25).
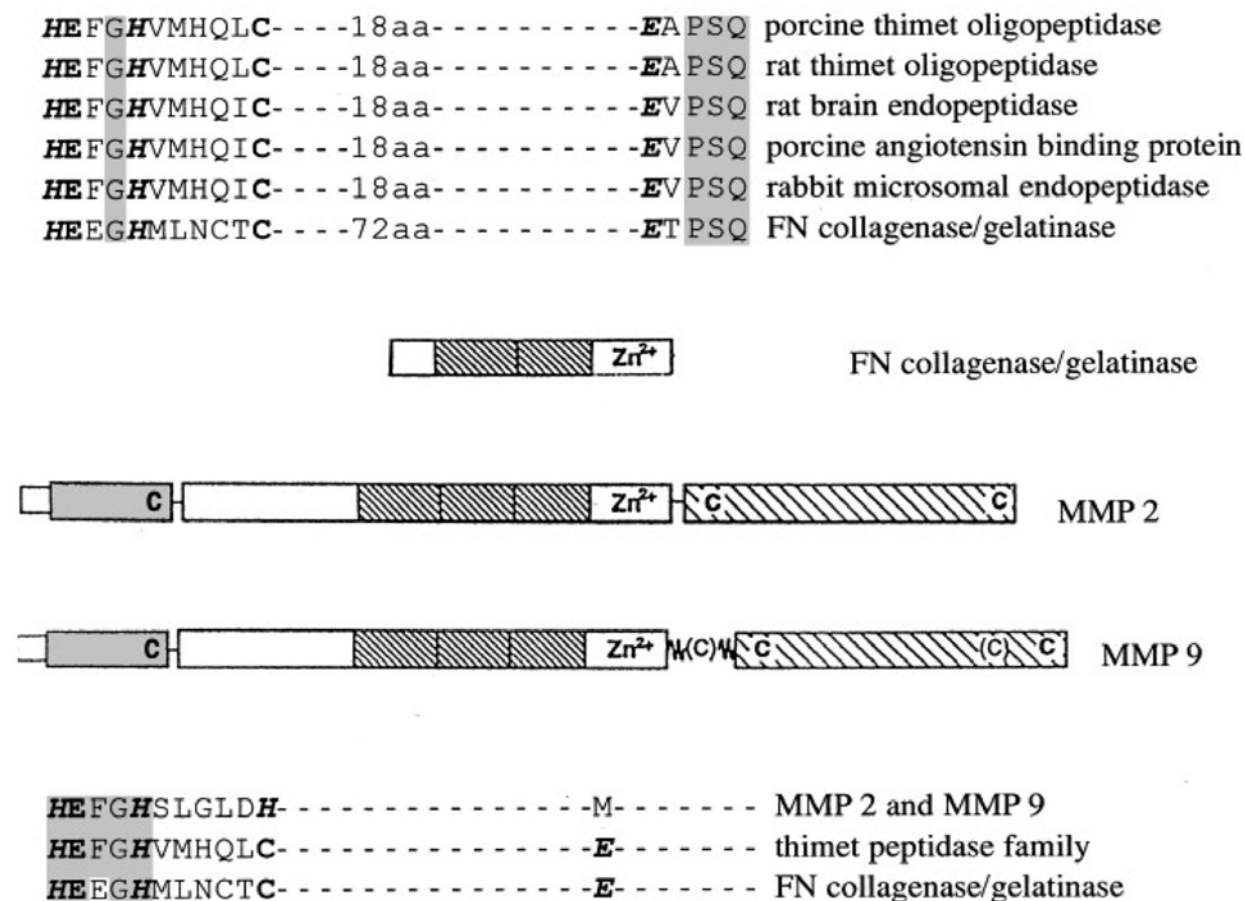
## 4 . THE CRYPTIC ACTIVITIES OF FIBRONECTINS

### 4.1. General cryptic activities

The group directed by Homandberg has studied the cryptic activity of FN that is involved in cartilage breakdown. They used cultured bovine cartilage explants to demonstrate large increases in gelatinase and collagenase activities and the release of proteoglycans. These properties are associated with the 29 kDa amino-terminal fragment and the 50 kDa gelatin-binding fragments, whereas intact FN has no enzymatic activity (5). The mechanism involved has recently been elucidated. FN fragments act via the MAP-kinases activation pathway to upregulate the synthesis of MMP3 and MMP13. The most potent fragment is the 29 kDa amino-terminal fragment (26). Another group obtained similar results with the 45 kDa FN fragment, the so-called GBD (26). They found that an aggrecanase activity was also liberated by the action of the FN-GBD fragment. These cryptic fragments could be associated with the cartilage damage that occurs in osteoarthisis (5, 26, 27). Fukai and collaborators described three FN fragments involved in adipocyte differentiation. These properties are linked to conformational changes or limited proteolysis by MMP2 (8). The most potent activity was located inside the heparin 2 C-terminal binding domain. This domain was exposed by urea treatment, which gave an unfolded fragment, or by limited proteolysis with MMP-2 (28). FN fragments may also be associated with angiogenesis, the proliferation and migration of vascular cells in a model of retinal diabetic retinopathy (7). The $III_1$ domain has a cryptic activity that is important for the formation of fibrils. This $III_1$ domain binds essentially to the $III_5$ and $III_7$ modules (29). Tomasini-Johansson *et al* have shown that binding of the N-terminal 70-kDa (70K) fragment of FN to fibroblasts blocks assembly of intact FN and is an accurate indicator of the ability of various agents to enhance or inhibit the assembly of FN (30).

### 4.2. Cryptic protease activity

We digested an intact basement membrane isolated from bovine lens capsule with the lysosomal cysteine proteases, cathepsins B, H and L in order to monitor the fragmentation of collagen IV, laminin and FN. These enzymes became bound to this ECM model in a saturable fashion, with $K_d$ values of 1 to 6 $10^{-7}$ M and 4-22.5 $10^{12}$ binding sites per capsule (31). This digestion led to the production of gelatinase activities that were detected by gelatin zymography. We obtained similar results when the active fragments were isolated on heparin agarose and gelatin agarose (32). These observations agree well with the work of Keil–Doulha and colleagues on plasma FN (33). The gelatin zymography revealed several activities (32, 33). We are now convinced that the multiple bands observed on gelatin gels are best explained by a precursor-product relationship, as reported for the gelatinases MMP2 and MMP9 (34, 35). The well-characterized cryptic protease is located inside the gelatin binding domain (GBD) (11), whose structure was solved recently by X-ray crystallography (25). This zinc-dependent

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HEFGHVMHQLC----18aa----------EAPSQ   porcine thimet oligopeptidase
HEFGHVMHQLC----18aa----------EAPSQ   rat thimet oligopeptidase
HEFGHVMHQIC----18aa----------EVPSQ   rat brain endopeptidase
HEFGHVMHQIC----18aa----------EVPSQ   porcine angiotensin binding protein
HEFGHVMHQIC----18aa----------EVPSQ   rabbit microsomal endopeptidase
HEEGHMLNCTC----72aa----------ETPSQ   FN collagenase/gelatinase
```



FN collagenase/gelatinase



MMP 2



MMP 9

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HEFGHSLGLDH---------------M--------   MMP 2 and MMP 9
HEFGHVMHQLC--------------E--------   thimet peptidase family
HEEGHMLNCTC--------------E--------   FN collagenase/gelatinase
```

**Figure 3.** Structure-function relationships between the FN-protease and related enzymes. The most closely related are the matrix metalloproteases MMP-2 and MMP-9. The FN-protease and these MMPs have similar catalytic activities (11). Reproduced by permission of the publisher (Wiley-Blackwell).

metalloproteinase is present in both the extracellular matrix and the plasma FNs (11). Sequence alignments indicate that the minimum consensus sequence HEXXH characteristic of zinc metalloproteinase is located in the $I_8$ domain. It is present in all three of mammalian species that have been studied intensively, humans, bovine and rat (1). Our group has also studied this protease in the three species and demonstrated its general character (11, 12). It is related to the metalloproteinases MMP2 and MMP9, both structurally and functionally, (11) (Figure 3, from 11) despite the putative active site having a non-classical signature (24). The group led by Tschesche have expressed a cDNa coding for the GBD fragment in *E. coli* and used it to characterize a similar protease activity (36). The same group have identified another protease activity located in the central domain of fibronectin. This aspartyl protease was characterized using biochemical methods (37). We have recently demonstrated that a N-terminal proline-rich peptide (14 amino acids) is released from the FN-protease by autoproteolysis (38; Figure 4). The three-dimensional structure of GBD shows that this N-terminal tail is freely accessible (25).

Similar proline-rich peptides have been found in bovine and rats (39-41), (Figure 5). The FN of all these three species may be involved in signaljng as it can be cleaved in a very peculiar manner to give rise to the prolyl-endopeptidase activity that is characteristic of the FN-protease (11, 12). Proline-rich peptides are associated with gene expression and intracellular signaling pathways (42). It has been postulated that FN protease activity arises after an FN becomes bound to integrins on the cell surface. This enzyme could liberate its proline-rich peptide which, in turn, activates intracellular signaling pathways that are linked to cell motility. Schor *et al* (43) showed that the GBD fragment can stimulate the migration of fibroblasts onto a 3D collagen I matrix. These results were obtained with femtomolar concentrations of GBD purified by several methods and apparently devoid of possible contaminants. These findings support the above hypothesis. The same group reported that fetal and cancer fibroblasts produce the migration stimulating factor (MSF). The MSF is a truncated isoform of FN; it is the 70 kDa N-terminal fragment of FN that contains the GBD/FN-protease (Figure 6). This truncated FN has
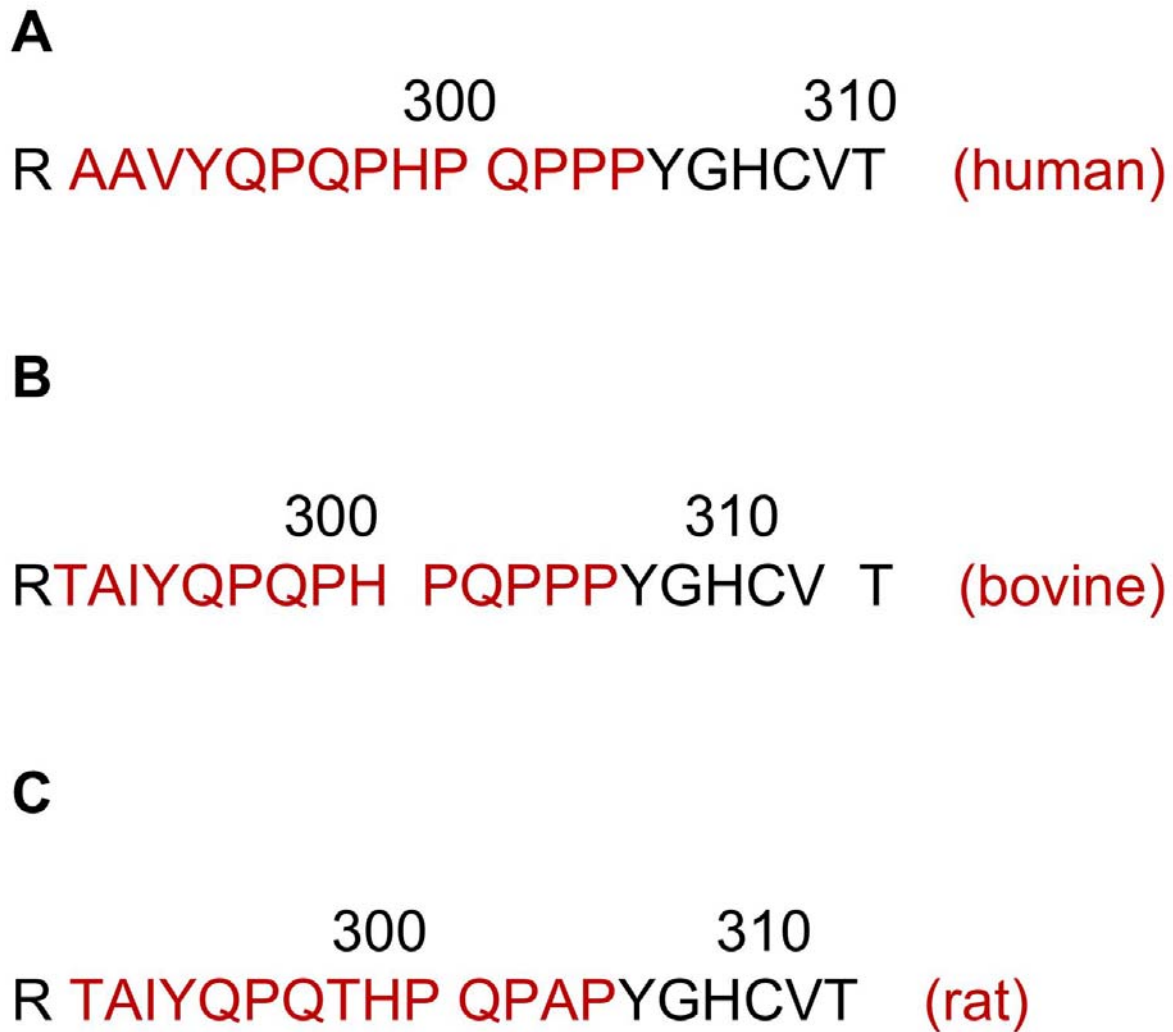
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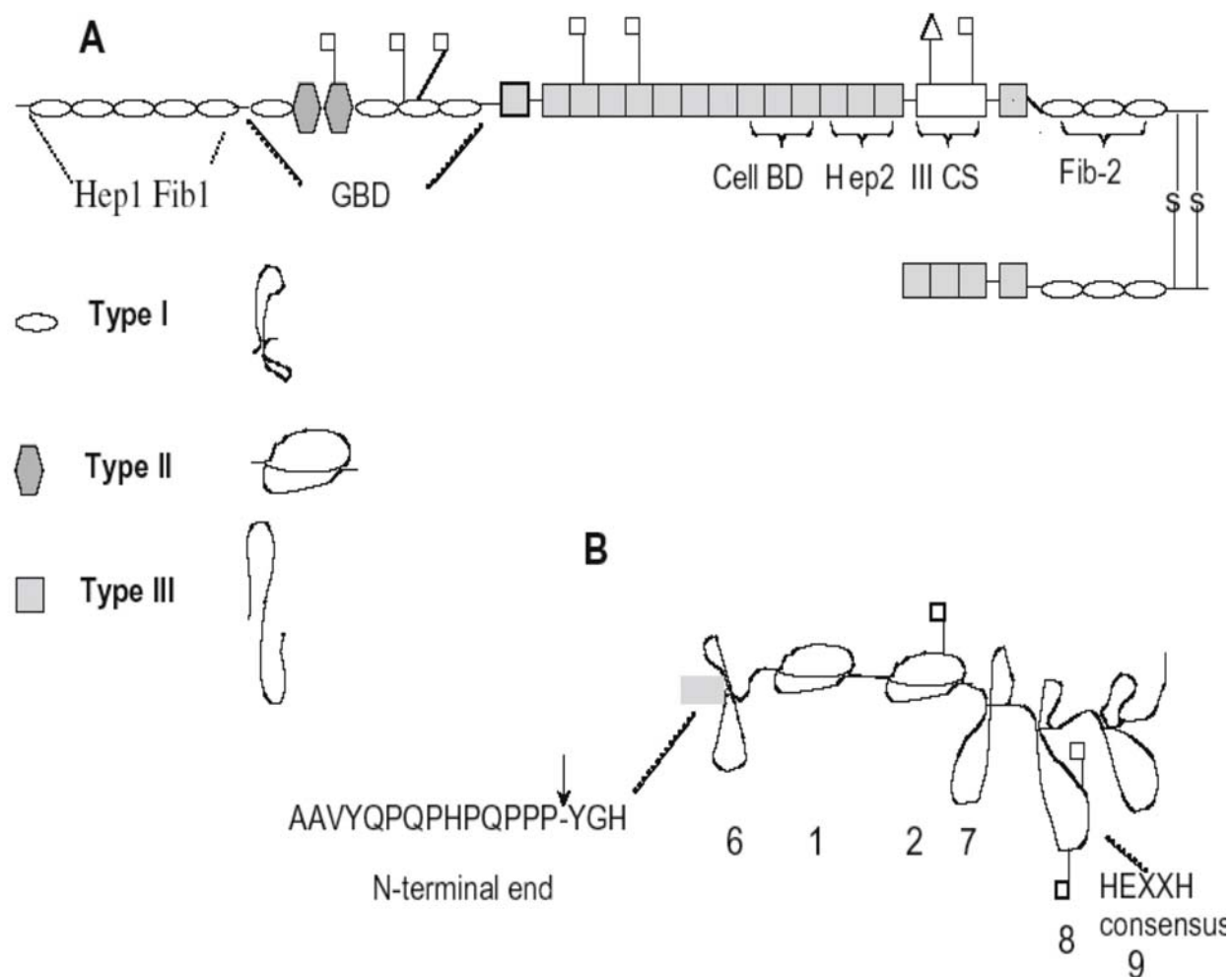


**Figure 4.** (A) showing the structure of the heterodimeric pFN (for details, see the text and Figure 1). (B) The GBD/FN-protease fragment showing the N-terminal proline-rich peptide liberated by autoproteolysis. The limited trypsin proteolysis that generates the GBD indicates that an arginine residue precedes the N-terminal alanine residue in the whole FN molecule. Reproduced by permission of the publisher (Elsevier).

a cryptic motogenic activity on fibroblast migration that is associated with the IGD tripeptide in type I FN motifs (44). NMR studies have shown that a mutation in that part of the gene that encodes the IGD motifs located within the modules I8 and I9 (45; Figure 6) leads to the loss of the motogenic activity but no significant changes in the structure of the FN in solution (45). MSF also has FN protease activity (46). Thus the FN protease could be involved in cell migration in pathological states, such as cancer progression. The evidence supporting this is that the 45 kDa FN protease is enzymatically active in a low zinc ion concentration and when zinc ions are absent (Figure 7). The activity was measured with a fluorogenic peptide whose structure mimics a collagen helix (47). The 45 kDa FN protease also binds to a labeled collagen peptide (25). Ultracentrifugation studies showed that the 45 kDa FN is a monomer in the absence of zinc ions (25). The enzymatic activity is gradually lost as the zinc

ion concentration is increased (Figure 8). This loss of enzyme activity by the 45k Da FN protease is accompanied by the molecules forming dimers that do not bind to labeled collagen peptides and crystallizes as a dimer (25). The observations summarized in Figure 7 indicate that the 45 kDa monomeric FN protease is the active enzyme in the absence of zinc and when its concentration is low (66 M). The dimeric, inactive FN protease is formed in the presence of high zinc ion concentrations, and this form tends to crystallize. We conclude that the monomeric 45 kDa FN protease is a biologically relevant enzyme.

Specific inhibitors of this new enzyme target could be useful for studying the biological role of this protease and may lead to potential drugs. We have developed coumarin-derived compounds in order to obtain specific inhibitors of FN protease (48).

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**A**

300                       310

R AAVYQPQPHP QPPPYGHCVT   (human)

**B**

300                       310

RTAIYQPQPH PQPPPYGHCV T   (bovine)

**C**

300                       310

R TAIYQPQTHP QPAPYGHCVT   (rat)

**Figure 5.** Sequences of the proline rich peptide of human FN-protease (A) and similar sequences in bovine and rat FN-proteases (B and C), the FNs of these species have FN-protease activity at their N-terminus ends. The amino acid numbering (300-310) is that of the corresponding whole FN. They may all have similar signaling pathways.



Crucial
IGDs motifs

Specific
C-terminal decamer
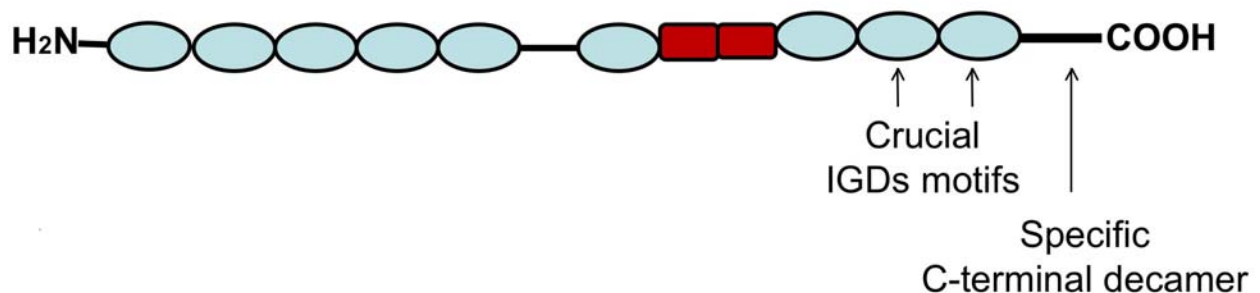
**Figure 6.** Structure of the MSF, a truncated isoform of FN corresponding to the 70 kDa N-terminal fragment of FN. Truncation of the gene generates the specific C-terminal decamer of the protein. The most relevant IGD motifs associated with the motogenic activity are indicated.

**Cryptic activities of fibronectins.**
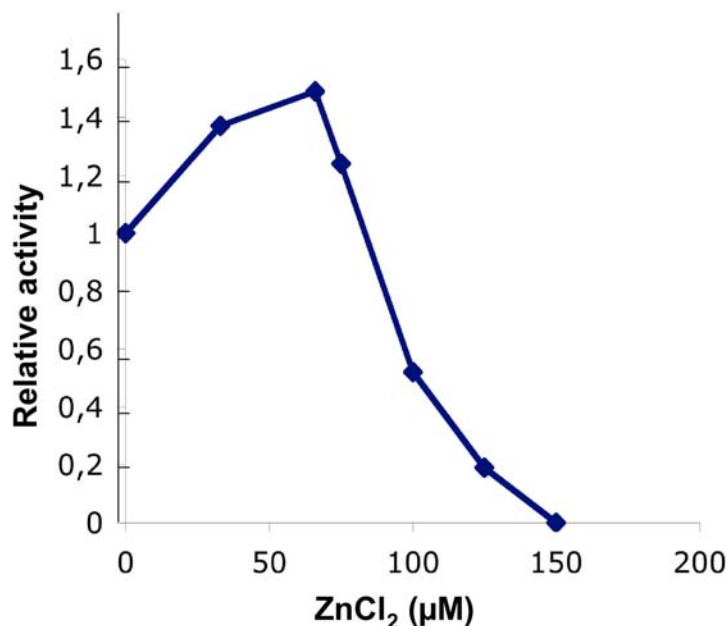


**Figure 7.** Zinc ion dependency of FN-protease activity (activity measured using the fluorogenic substrate wich mimics a collagen helix) pH7.4 Tp 37°C. Initial rates of the enzymatic reaction are measured during 10 min.
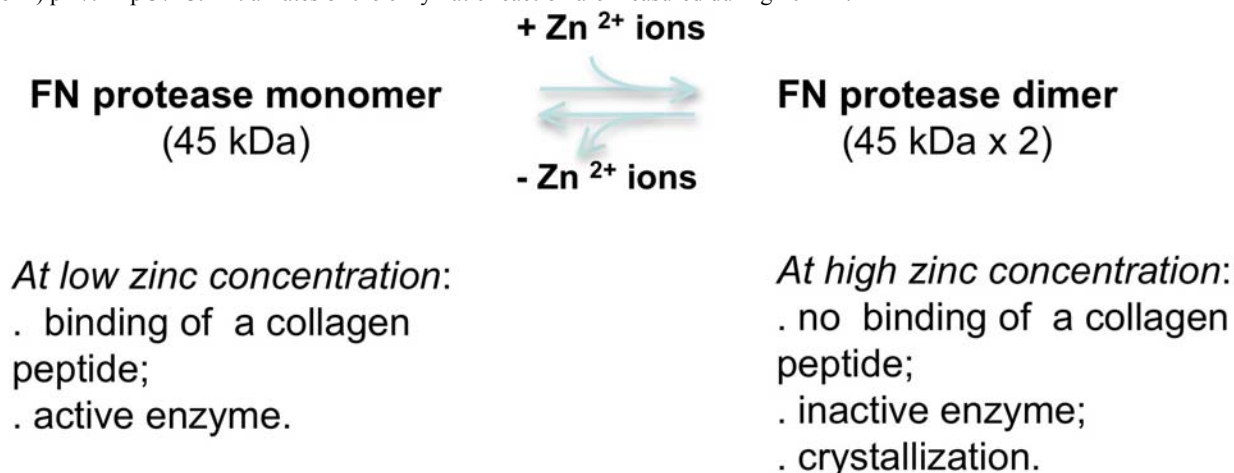


**Figure 8.** The monomer and dimer of the 45 kDa FN protease. The monomer (right) binds to a collagen peptide, is enzymatically active against a fluorogenic substrate that mimics a collagen helix. It has these properties in the absence of added zinc ions and in low zinc concentrations. The dimer (left) is formed in a high zinc concentration, has no enzymatic activity, does not bind to a collagen peptide, and crystallizes.

Fibronectin (FN) [Cleaved into: Anastellin]

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