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The Proteolytic Activity of the Recombinant Cryptic Human Fibronectin Type IV Collagenase from *E. coli* Expression

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Human plasma fibronectin (pFN) contains a cryptic metalloprotease present in the collagen-binding domain. The enzyme could be generated and activated in the presence of Ca^{2+} from the purified 70-kDa pFN fragment produced by cathepsin D digestion. In this work we cloned and expressed the metalloprotease, designated FN type IV collagenase (FnColA), and a truncated variant (FnColB) in *E. coli*. The recombinant pFN protein fragment was isolated from inclusion bodies, and subjected to folding and autocatalytic degradation in the presence of Ca^{2+}, and yielded an active enzyme capable of digesting gelatin, helical type II and type IV collagen, α- and β-casein, insulin b-chain, and a synthetic Mca-peptide. In contrast, isolated plasma fibronectin, type I collagen, and the DNP-peptide were no substrates. Both catalytically active recombinant pFN fragments were efficiently inhibited by EDTA, and batimastat, and, in contrast to the glycosylated enzyme isolated from plasma fibronectin, were also inhibited by TIMP-2.

KEY WORDS: Fibronectin; type IV collagenase; metalloprotease; TIMP-2.

1. INTRODUCTION

Fibronectin (FN)[3] is a multifunctional plasma and extracellular matrix glycoprotein which is secreted as a dimer of two similar but not identical polypeptide chains of approximately 220–250 kDa connected by two disulfide bonds near the C-terminus of the protein. The molecule is composed of blocks of repeating homologous sequences: type I and type II repeats (45 and 60 amino acids, respectively) are disulfide-bonded loops, and type III repeats (90 amino acids) do not contain disulfide bonds, but some of them contain two free sulfhydryl groups. The structure of the FN polypeptide varies due to alternative splicing of the mRNA. FN is found in soluble form in plasma and most body fluids (plasma fibronectin, pFN), and in insoluble form (cellular fibronectin, cFN) as a component of the extracellular matrix and basement membranes. Fibronectin isolated from plasma consists of polypeptide chains that differ from one another in molecular weight and are distinctly smaller than those produced by most types of cultured cells. FN is encoded by a single gene, and diversity is generated by alternative splicing of the primary transcript. Three alternatively spliced regions have been identified: EDA, EDB, and IIICS (Potts and Campbell, 1994). Functional binding sites of fibronectin include those for fibrin, heparin, and *Staphylococcus aureus* within one domain, for collagen and gelatin in another, and cells, heparin, and fibrin(ogen) in separate domains. FN also has two sites for transglutaminase-catalyzed cross-linking to other proteins. In addition, it binds a number of other molecules, including IgG and C3 complement, and plasminogen. Finally, fibronectin also

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[3] Abbreviations: BCIP, 5-Bromo-4-chloro-3-indolyl; BSA, bovine serum albumin; cFN, cellular fibronectin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FN, fibronectin; FnCol, fibronectin type IV collagenase; GSSG, oxidized glutathione; IPTG, isopropyl-β-D-thiogalactopyranoside; MMP, matrix metalloproteinase; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction; pFN, plasma fibronectin; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline Tween; TIMP, tissue inhibitor of metalloproteinases.

contains binding sites for self-association (Mosher, 1989; Hynes, 1990) (Fig. 1).

These binding properties indicate that FN is implicated in a variety of cellular properties including cell adhesion, wound healing, cytoskeletal organization, migration, differentiation, phagocytosis, hemostasis, thrombosis, and pathogenesis of diseases including cancer and bacterial infection (Clark and Colvin, 1985; Mosher, 1989; Hynes, 1990; Humphries, 1993; Akiyama *et al.*, 1995). In contrast to these properties, several studies demonstrated that FN fragments may perform quite different biological functions than the intact molecule. For example, for some well-defined FN fragments transformation-promoting activity and potent inhibition of endothelial cell growth were found (De Petro *et al.*, 1983; Homandberg *et al.*, 1986). Other fragments have been shown to elevate the expression of collagenase, stromelysin, TIMP-1, tumor necrosis factor-α, and urokinase-type plasminogen activator (Beezhold and Personius, 1992; Tremble *et al.*, 1992; Xie *et al.*, 1994; Bewsey *et al.*, 1996; Homandberg *et al.*, 1998). Finally, several studies described various proteolytic activities of pFN fragments. There are four latent proteinases in human pFN, designated fibronectinase (Ser-enzyme), FN-gelatinase (Asp-enzyme), FN-lamininase (Asp-enzyme), and Fn type IV collagenase (metalloenzyme) (Emod *et al.*, 1990; Planchenault *et al.*, 1990; Lambert Vidmar *et al.*, 1991*a, b;* Unger and Tschesche, 1999).

In the present study we cloned and expressed two variants of the FN type IV collagenase (FnColA and FnColB) (Lambert Vidmar *et al.*, 1991*b*). This metalloprotease is located in the collagen-binding domain of human pFN. We tested various physiological and synthetic substrates and also studied the inhibition of the enzyme with inhibitions of metalloproteases.

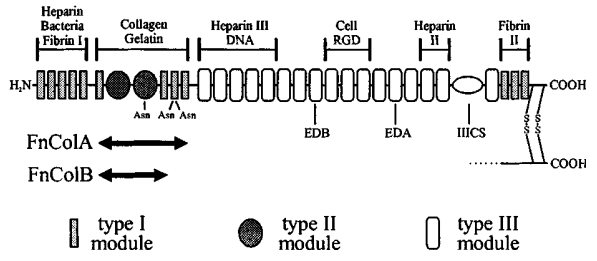

Fig. 1. Schematic domain structure of fibronectin. Fibronectin is composed of three types of internal repeating modules designated types I, II, and III. The EDA and EDB modules are absent in plasma fibronectin as a result of alternative splicing. The binding domains of fibronectin are indicated at the top. The localization of FN type IV collagenases A and B is indicated by arrows.

2. MATERIALS AND METHODS

2.1. Reagents

The *Escherichia coli* strain [JM83] was used as the host for plasmid amplification and *E. coli* [BL21(DE3)] for expression of the FN type IV collagenase. Primers for polymerase chain reaction were obtained from MWG-Biotech. The restriction enzymes *Nde*I, *Sal*I, and *BamH*I were purchased from New England Biolabs. Tth polymerase, T4 DNA ligase, and alkaline phosphatase were obtained from Promega. Chromatography media Q-Sepharose and Sephacryl S-100 were from Pharmacia. Monoclonal mouse anti-human fibronectin IgG (clone IST-10) against the collagen-binding domain was obtained from Chemicon. All other chemicals were of p.a. quality and purchased from Sigma, Serva, Merck, Bachem, Invitrogen, and Roth.

2.2. Cloning and Expression of FnColA and FnColB cDNA

Template for polymerase chain reaction (PCR) amplification of the FN type IV collagenases FnColA and FnColB was the cDNA plasmid pFH134/pAT53, a gift of Dr. F. E. Baralle, Trieste, Italy. Primers were designed based on the known cDNA sequence of fibronectin (FnCol-F 5′-GGTCGTCATATGGTTTACCAACC-3′; FnColA-R 5′-TTCGACGGATCCTC ATGAGCTTG-3′; FnColB-R 5′-GTCATCGTCGACTCACTGATCTCG-3′). *Nde*I and *BamH*I or *Sal*I restriction sites were added to the 5′ and 3′ primers, respectively, to enable insertion of the cDNA into the expression vectors pET11a and pET12b. PCR was performed for 39 cycles (95°C, 30 sec; 54°C + 0.2°C/cycle; 72°C, 60 sec) followed by a final extension at 72°C for 3 min. PCR products were ligated into the expression vectors and the expected sequences were confirmed by restriction analysis and DNA sequencing.

For expression, the *E. coli* strain [BL21(DE3)] was transformed with the plasmids FnColA/pET11a and FnColB/pET12b, respectively. The cells were grown in LB medium at 37°C up to an optical density of 0.8 before inducing the expression with 2 mM isopropyl-β-D-thiogalactopyranosid (IPTG). After 4 hr, cells were lysed and purified, and the obtained inclusion bodies were solved in i.b. buffer (8 M urea, 100 mM 2-mercaptoethanol, 20 mM Tris/HCl, pH 8.5).

2.3. Purification and Folding

Purification was performed in two steps: First, the proteins were bound to cation exchanger Q-Sepharose

equilibrated with buffer A (4 M urea, 40 mM 2-mercaptoethanol, 50 mM Tris/HCl, pH 8.5). The column was washed with three bed volumes of buffer A to remove unbound proteins. The recombinant proteins were eluted with a linear gradient from 0 to 700 mM NaCl in buffer A. Protein content of each fraction was determined by measuring the absorbence at 280 nm. The fractions containing the recombinant protein were pooled and concentrated by ultrafiltration. The second step was gel filtration on Sephacryl S-100 to which 3 ml of the sample was applied on a column equilibrated with buffer B (4 M urea, 200 mM NaCl, 40 mM 2-mercaptoethanol, 50 mM Tris/HCl, pH 8.5). Finally, fractions containing FnColA or FnColB, respectively, were pooled and concentrated by ultrafiltration.

The folding procedure was based on an oxido-shuffling system with oxidized gluthathione (GSSG) and dithiothreitol (DTT) (Buchner *et al.,* 1992). This redox system promotes disulfide bond formation. The denatured protein was dialyzed against folding buffer (200 mM NaCl, 0.1 mM $ZnCl_2$, 5 mM $CaCl_2$, 100 mM arginine, 10 mM GSSG, 3 mM DTT, 50 mM Tris/HCl, pH 7.5) and dissolved in 50-fold volume of folding buffer to suppress self-aggregation. After the folding procedure the solution was concentrated by ultrafiltration, dialyzed against buffer C (500 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, 50 mM Tris/HCl, pH 7.5), and used for enzyme assays.

2.4. Immunoblotting

Western blot analysis was carried out using a standard method (Blake *et al.,* 1984). The membrane was blocked for 30 min with 3% BSA in TBST buffer (500 mM NaCl, 0.05% w/v Tween 20, 200 mM Tris/HCl, pH 7.5) and then incubated overnight at room temperature with 0.5 μg/ml monoclonal mouse anti-human fibronectin antibody (IST-10) in 3% BSA in TBST buffer. Finally, the immunoreactive bands were visualized with goat anti-mouse alkaline phosphatase IgG conjugate and BCIP/NBT detection.

2.5. Enzyme Assays

Purified and folded recombinant FnColA and FnColB were used for zymography (Heussen and Dowdle, 1980). Polyacrylamide gels, 10%, containing 1 mg/ml gelatin, α-casein, or β-casein were subjected to electrophoresis under nonreducing conditions at 20 mA. Then the gels were washed twice with 2.5% Triton X-100, three times with destilled water, and once with buffer C (500 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, 50 mM Tris/HCl, pH 7.5). The gels were incubated overnight at 37°C in the same buffer, stained with Coomassie blue, and destained with distilled water. For assays with type II and type IV collagens 15 μl of the substrates (1 mg/ml) was incubated at 37∞C with 5 μl of protease solution and then analyzed by SDS–PAGE (Rabilloud *et al.,* 1994).

Inhibition assays were performed after incubation of the enzymes for 10 min with the respective inhibitors against the Mca peptide ((7-methoxycumarin-4yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2; Calbiochem-Novabiochem) by measuring the residual enzymatic activity using a fluorescence photometer (LS 50 B, Perkin Elmer) at 330 nm excitation and 405 nm emission wavelength. Cleavage of insulin b-chain was performed at 37°C for 2 hr with an enzyme/substrate rate of 1:100.

3. RESULTS

3.1. Cloning

FnColA and FnColB were amplified using primers corresponding to the coding sequences of FnColA (Val^{293}–Ser^{608}) and FnColB (Val^{293}–Gln^{517}). The amplified cDNAs were purified and ligated into pET11a or pET12b, respectively, to create the FnColA/pET11a and FnColB/pET12b plasmids (Fig. 2). The results

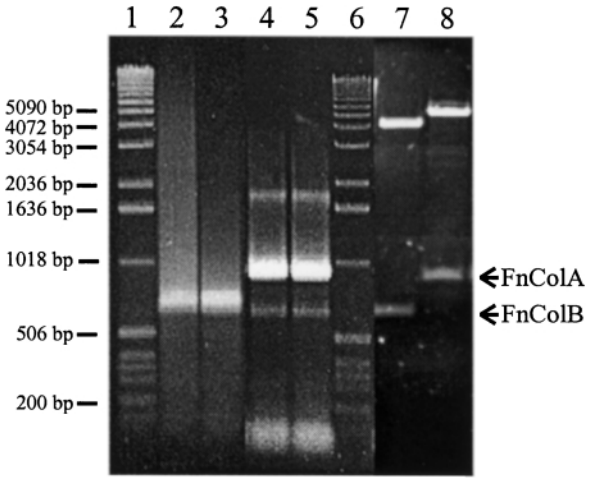

Fig. 2. Agarose gel (1%) PCR amplification and cloning of FnColA and FnColB cDNA stained with ethidium bromide. Lanes 1–6: 1-kb marker; lanes 2 and 3: PCR amplification of FnColB (675 bp); lanes 4 and 5: PCR amplification of FnColA (948 bp); lane 7: restriction digest analysis (*Nde*I/*Sal*I) of the plasmid FnColB/pET12b; lane 8: restriction digest analysis (*Nde*I/*BamH*I) of the plasmid FnColA/pET11a.

were verified by restriction analysis with *Nde*I/*BamH*I and *Nde*I/*Sal*I. The constructs were confirmed by DNA sequencing.

3.2. Expression and Purification

The recombinant proteins were expressed in *E. coli* [BL21(DE3)] transformed with the described cDNA plasmids. After induction with IPTG the expression reached a maximum after 4 hr. Molecular weights of FnColA (35 kDa) and FnColB (28 kDa) were determined by SDS–PAGE. The proteins were purified using Q-Sepharose as an anion exchanger and Sephacryl S-100 as a matrix for gel filtration chromatography. For identification we determined the N-termini by automated Edman sequencing (V^{293}VQPQPHP) as the N-terminus of the collagen-binding domain of plasma fibronectin. Furthermore, Western blot analysis with the monoclonal antibody IST-10 (Chemicon) against the collagen-binding domain was used for identification (Fig. 3).

3.3. Catalytic Characteristics

After folding of the recombinant proteins with the described oxido shuffling system we observed an autocatalytic degradation as shown by Lambert Vidmar *et al.* (1991*b*). Zymography was used to define which of the resulting fragments exhibited catalytic activity against potential substrates. As shown in Fig. 4, gelatin is a substrate for the recombinant FN type IV collagenase. From FnColA and FnColB several catalytic active fragments of 20, 24, 40, 43, and 66 kDa were observed. Fragments with lower molecular weight than the cloned proteins were probably generated by autodigestion, whereas the higher molecular weight forms could be the result of oligomerization. The same results were observed with α- and β-casein as substrates. Further investigations also revealed digestion of type II and type IV collagen (Fig. 5) and the synthetic Mca peptide.

The cleavage specificity against the insulin b-chain was investigated by Edman sequencing of the insulin fragments obtained after digestion with recombinant and with isolated FN type IV collagenase. We found the same cleavage sites for both the recombinant and the isolated form with preference for P_1'=Leu (Tyr). The additional cleavage site at residues Phe^{25}–Tyr^{26} was detected only with the recombinant FN type IV collagenase (Fig. 6). All mentioned substrates were tested with all obtained active fragments and always yielded the same results. No activity of FnColA and FnColB was observed against isolated plasma fibronectin, type I collagen, and the synthetic DNP peptide.

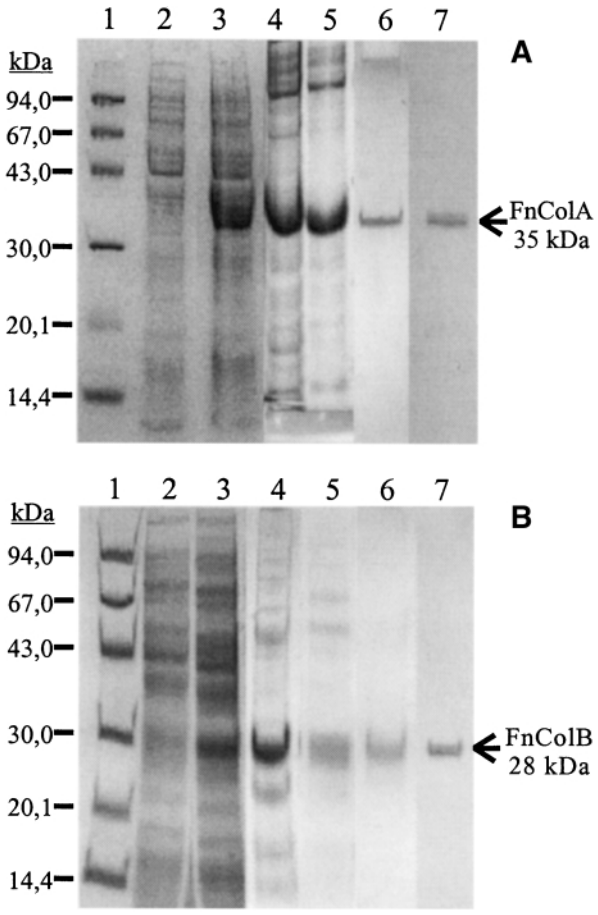

Fig. 3. SDS–PAGE analysis of the expression and purification of recombinant FnColA (A) and FnColB (B). The homogeneity is documented after silver staining. Lane 1: LMW protein marker; lane 2: expression of the recombinant proteins before induction with IPTG; lane 3: expression 4 hr after induction with IPTG; lane 4: solution of inclusion bodies; lane 5: FN collagenases eluted from Q-Sepharose; lane 6: homogenous FN collagenases after gel filtration on Sephacryl S-100; lane 7: immunoblot analysis of purified FnColA (A) and FnColB (B) with monoclonal antibody IST-10 (Chemicon).

Inhibition studies demonstrated that EDTA and batimastat were effective inhibitors of the FN collagenases. In contrast to the protein fragment isolated from the digest of plasma fibronectin (data not shown), bovine TIMP-2 and the N-terminal inhibitory domain of human TIMP-2 (ΔTIMP-2) (Farr *et al.*, 1999) also inhibited the catalytic activity of the recombinant enzymes. Figure 7 shows that TIMP-2 is a better inhibitor for recombinant FN type IV collagenase than batimastat.

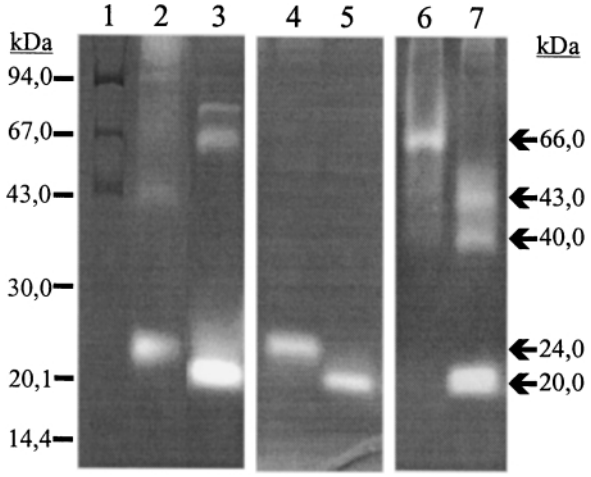

Fig. 4. Gelatin zymogram of FnColA and FnColB folding reactions stained with Coomassie blue. Lane 1: LMW protein marker; lanes 2 and 3: folding reactions of FnColA led to two active low-molecular weight fragments with either M_r 24 or 20 kDa and several high-molecular weight fragments; lanes 4–7: folding reactions of FnColB led to the same active fragments as described for FnCol A. Arrows show most significant active fragments. The same results were observed with α- and β-casein.

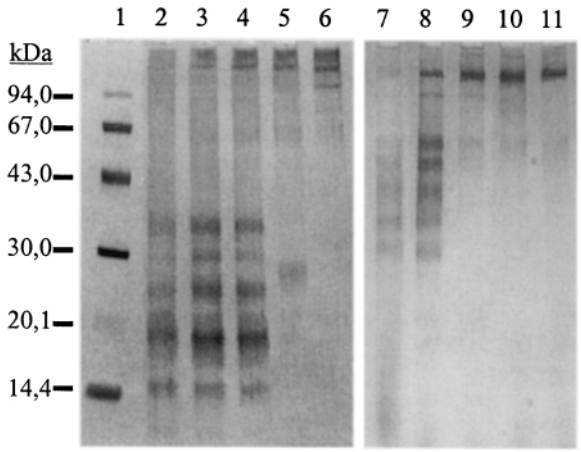

Fig. 5. SDS–PAGE analysis of the digestion of type II and type IV collagen. Samples of type II and type IV collagen were incubated with a 20-kDa active fragment of FN collagenase (Fig. 4; lane 5) for various times at 37°C and then subjected to SDS–PAGE (10% acrylamide; silver-stained). Lane 1: LMW protein marker; lanes 2–4: incubation of type IV collagen with FnColB for 1 hr (lane 4), 6 hr (lane 3), and 15 hr (lane 2); lanes 5 and 6: control incubation of type IV collagen for 15 hr (lane 5) and 0 hr (lane 6). Lanes 7–9: incubation of type II collagen with FnColB for 1 hr (lane 9), 6 hr (lane 8), and 15 hr (lane 7); lanes 10 and 11: control incubation of type II collagen for 15 hr (lane 10) and 0 hr (lane 11).

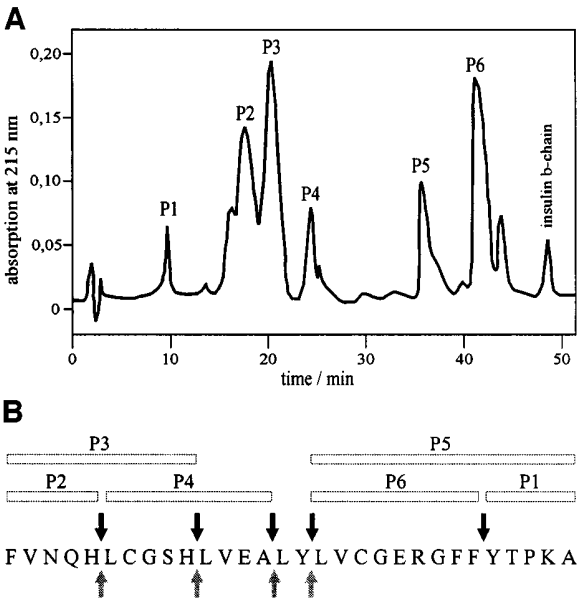

Fig. 6. RP-HPLC pattern of insulin b-chain fragments produced by FN type IV collagenase on an Aquapore RP-300 column. The fragments were eluted with a gradient of acetonitrile (0–50 %) (A). For determination of the cleavage specificity of recombinant FN type IV collagenase (↓) in comparison with the isolated enzyme (↑) we used automated Edman sequencing for identification of the obtained fragments (B).

4. DISCUSSION

Human plasma fibronectin contains four latent proteases (Emod *et al.*, 1990; Planchenault *et al.*, 1990; Lambert Vidmar *et al.*, 1991*a*, *b*). In this study we cloned, expressed, and characterized two variants of the FN type IV collagenase, a metalloprotease, located in the collagen-binding domain of fibronectin. After expression in *E. coli* and naturation catalytic activity was detected against gelatin, type II and type IV collagen, α- and β-casein, and a synthetic Mca peptide. Inhibition studies showed that bovine TIMP-2, the inhibitory domain of human TIMP-2 (ΔTIMP-2), EDTA, and batimastat were efficient inhibitors.

There were two difficulties encountered in the folding of recombinant FN type IV collagenases. First, FnColA and FnColB contained 24 and 16 cysteine residues, respectively. Thus, the probability of formation of incorrectly folded proteins is very high. Only part of the folding reactions were successful and yielded an active enzyme. The high number of cysteine residues also explains the appearance of bands in zymography with higher molecular weights than that of the monomer. Because of partial misfolding outside the catalytic domain,

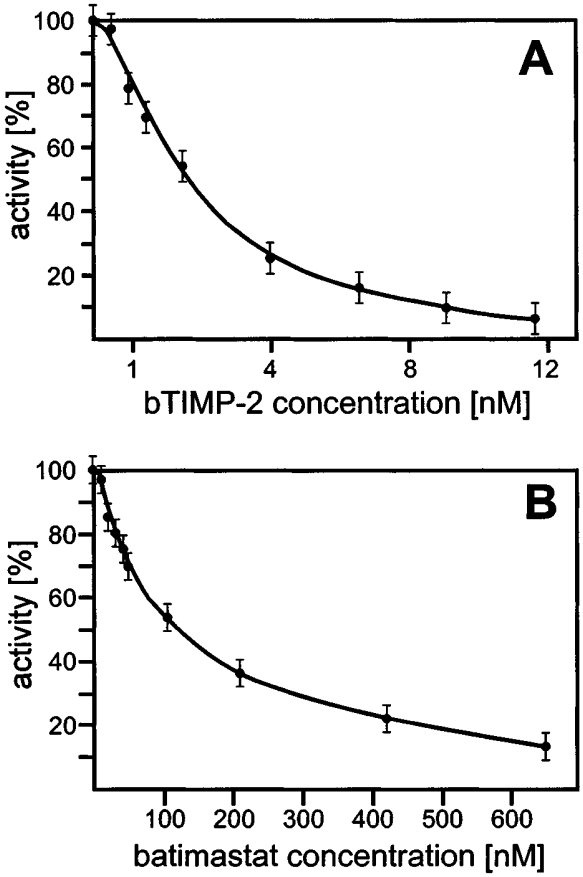

Fig. 7. Inhibition of FN type IV collagenase with TIMP-2 (**A**) and batimastat (**B**). After incubation for 10 min of enzyme and inhibitor inhibition assays were performed against a synthetic Mca peptide by measuring the residual enzyme activity using a fluorescence photometer.

dimers or oligomers could be generated, nevertheless showing proteolytic activity. The second problem was autodigestion, already described by Lambert Vidmar *et al.* (1191*b*). The native FN type IV collagenases A and B were generated by autodigestion from a 70-kDa amino-terminal fragment of isolated human plasma fibronectin. The obtained enzymes were not stable, underwent further autodigestion, and lost proteolytic activity. We observed the same carboxy-terminal degradation in both recombinant FnColA and FnColB enzymes. Figure 8 shows a possible model for the processing of FN type IV collagenases A and B leading to the active 20- and 24-kDa fragments. The active fragments had lower molecular weights than the initially cloned protease fragments and after storage exhibited a further loss of catalytic activity. These drawbacks made it difficult to obtain reasonable yields of defined protein fragments.

Inhibition by the natural tissue inhibitors of metalloproteases, TIMPs, showed an interesting difference between the isolated FN type IV collagenases and the recombinant enzymes. Bovine TIMP-2 (Fernandez-Catalan *et al.,* 1998) and the inhibitory domain of human TIMP-2 (ΔTIMP-2) (Farr *et al.,* 1999) were efficient inhibitors of recombinant FnColA and FnColB. In contrast, TIMP-2 had no effect on the isolated FN type IV collagenase. A possible explanation for the differences in inhibition might result from the glycosylation of the native proteins. There are three potential glycosylation sites at Asn residues 430, 528, and 542 for carbohydrate binding near the potential active site, perhaps responsible for differences in inhibition due to steric reasons. The differences in molecular weights between the isolated native and the recombinant enzymes are indicative of a glycosylation of the plasma-derived proteases. We also found a difference in cleavage specifity of recombinant and isolated FN type IV collagenase against the insulin b-chain as shown in Fig. 7. Differences in glycosylation may also be responsible for the additional cleavage within the insulin b-chain by the recombinant enzyme.

Similarities in amino acid sequence between FN type IV collagenase and other metalloproteases were found within the family of metzincins, especially with gelatinase A (MMP-2) and gelatinase B (MMP-9) (Fig. 9). The fibronectin-like domain of the gelatinases is similar to the type II repeats of the collagen-binding domain of fibronectin. The sequence of the catalytic site with the zinc-binding motif in the metzincins finds some homology in four residues including the first histidine residue in FN type IV collagenase between type II and type I repeats. The second and the third histidines of the metzincin zinc-binding motif –HExxHxxGxxH– are not present in the corresponding sequence motif of plasma fibronectin. But there are four further histidine residues (H^{421}, H^{429}, H^{488}, H^{492}) near the original zinc-binding motif that may perhaps be involved in zinc binding.

Because of the ubiquitious occurence of fibronectin, the significance of FN type IV collagenase in physiological or pathological processes is not well understood. Most studies on the pathophysiological role of fibronectin fragments have been devoted to the cleavage of articular cartilage in osteoarthritis and rheumatoid arthritis patients (Xie *et al.,* 1992, 1993). Levels of fibronectin fragments are highly elevated in synovial fluids of those patients. They cause the release of the cytokines, TNFα, IL-1α, IL-1β, and IL-6, which appear to be responsible for MMP-3 (stromelysin-1) expression and suppression of proteoglycane synthesis (Homandberg *et al.,* 1998). On the other hand, MMP-3 has been shown to degrade fibronectin into fibronectin fragments and thus could lead to amplification of its own synthesis (Xie *et al.,* 1994;

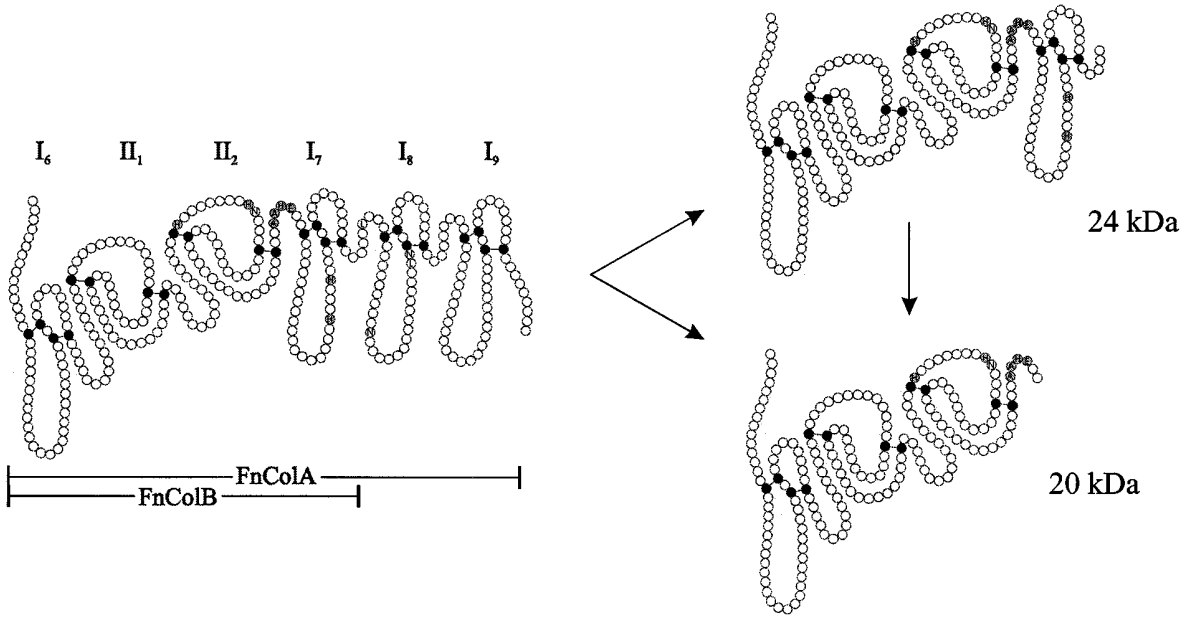

Fig. 8. Possible model of recombinant FN type IV collagenases A and B and their processing leading to active 20- and 24-kDa fragments. Type I and type II modules are indicated by I_x and II_y. Shaded amino acids show the zinc-binding motif –AAHE– and further histidine residues with possibility of zinc binding. The figure also shows three possible Asn residues for glycosylation.

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FnCol     TDCTSEGRRDNMKWCGTTQNYDA
MMP-2     ESCTSAGRSDGKMWCATTANYDD
MMP-9     STCTSEGRGDGRLWCATTSNFDS

FnCol     DQKFGFCP--------MAAHEEI
MMP-2     DRKWGFCPDQGYSLFLVAAHEFG
MMP-9     DKKWGFCPDQGYSLFLVAAHEFG
Metzinc                   AAHExx

FnCol     CTTNEGVMYRIGDQWDKQHDMGH
MMP-2     HAMGLEHSQDPGALMAPIYTYTK
MMP-9     HALGLDHSSVPEALMYPMYRFTE
Metzinc   HxxGxxH       M
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Fig. 9. Alignment of amino acid sequences of FN type IV collagenase (Thr^{432}–His^{492}) with gelatinase A (MMP-2), gelatinase B (MMP-9), and the zinc-binding motif of the metzincin family.

Bewsey *et al.*, 1996; Homandberg *et al.*, 1998). Therefore, fibronectin and fibronectin fragments regulate protease activity in synovial fluid. The question is still unresolved as to what kind of FN fragments are included in this process. Fragments like FnColA or FnColB could not only induce other proteases, but also could contribute to the proteolytic potential of synovial fluid. FN type IV collagenase could perhaps also be involved in malignant processes. Cleavage of type IV collagen has been shown to be a prerequisite for degradation of basement membrane and is thus involved in tumor invasion and metastasis.

In this study cloning and expression of recombinant FN type IV collagenase was shown for the first time. We also demonstrated differences in inhibition of native and recombinant enzyme with TIMP-2. Substrate specificity of active fragments indicates that FN type IV collagenase capable of cleaving matrix components may elevate the proteolytic activity of extracellular areas and thus may contribute to tumor progression, osteoarthritis, and rheumatoid arthritis.

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