Biol. Chem., Vol. 382, pp. 1707 – 1714, December 2001

Recombinant Cryptic Human Fibronectinase Cleaves Actin and Myosin: Substrate Specificity and Possible Role in Muscular Dystrophy

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The N-terminal heparin/fibrin binding domain of human plasma fibronectin (pFN) contains a cryptic proteinase. 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 of pFN. In this work we cloned and expressed the serine proteinase, designated fibronectinase (Fnase), in *E. coli*. The recombinant pFN protein fragment was isolated from inclusion bodies, subjected to folding and autocatalytic degradation in the presence of Ca^{2+}, and yielded an active enzyme capable of digesting fibronectin. Cleavage of pFN and the synthetic peptides Ac-I-E-G-K-pNA and Bz-I-E-G-R-pNA demonstrated identical specificity of the recombinant and the isolated fibronectinase. Further investigations of the substrate specificity revealed for the first time the muscle proteins actin and myosin as being substrates of fibronectinase. The enzyme can be inhibited by α_1-proteinase inhibitor. In the context of induced cathepsin D release, *e. g.* from granulocytes under inflammatory conditions, these results indicate an increase in specific proteolytic potential against muscular proteins in dystrophic diseases by the release of cryptic fibronectinase.

Key words: Actin / Fibronectinase / Muscular dystrophy / Myosin / Serine proteinase.

Introduction

Fibronectin (FN) is a multifunctional glycoprotein, which occurs in blood plasma (pFN, plasma fibronectin) and also on cell surfaces (cFN, cell surface fibronectin). It consists of two almost identical units subdivided into different characteristic binding domains (Figure 1). The variety of binding affinities to extracellular macromolecules, cells, DNA, and bacteria is important for the participation of fibronectin in a multitude of biological processes like cell adhesion, migration, wound healing, metastasis, embryonic development, thrombosis and bacterial infection (Clark and Colvin, 1985; Akiyama *et al.*, 1987; Hynes, 1989; Brown *et al.*, 1993; Humphries, 1993). In contrast to these characteristics, various studies showed that fragments of fibronectin may perform completely different functions compared to the intact protein. Some fragments were found to be responsible for inhibition of endothelial cell growth or promotion of tissue reconstruction. Other fragments stimulate the expression of matrix metalloproteinases, tumor necrosis factor-α, TIMP-1 and urokinase-type plasminogen activator (De Petro *et al.*, 1983; Homandberg *et al.*, 1986; Beezhold *et al.*, 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 from four cryptic proteinases in human pFN designated as fibronectinase (Ser-enzyme), FN-gelatinase (Asp-enzyme), FN-lamininase (Asp-enzyme) and FN-type IV-collagenase (metallo-enzyme) (Emod *et al.*, 1990; Planchenault *et al.*, 1990; Lambert Vidmar *et al.*, 1991a, b; Unger and Tschesche, 1999).

Fibronectinase was first described by Lambert Vidmar *et al.* in 1991. A 70 kDa amino-terminal fragment of isolated plasma fibronectin obtained from cathepsin D digestion underwent autodigestion in the presence of Ca^{2+} and led to 45 kDa and 27 kDa fragments. Further incubation of the the 27 kDa fragment, which contains the N-terminal heparin/fibrin binding domain of pFN, resulted in the appearence of a 23 kDa fragment. The activation step contains the cleavage of the bond K^{86}-D^{87} combined with the opening of the second type I-module 'finger-loop' and the formation of the catalytically active 22 kDa form (Lambert Vidmar *et al.*, 1991a) (Figure 2).

The active 22 kDa fragment consists of an 8 kDa chain, which corresponds to the N-terminus of the pFN, and a 14 kDa chain with the N-terminal sequence DSMIW (D^{87}-W^{91}). The opening of the 'finger-loop' in the second type I-module causes a flexibility of the tertiary structure, which enables the formation of the catalytic active site. The catalytic triad is probably formed by amino acids D^{24}, H^{128} and S^{164}.

In the present study we describe the cloning and expression of human cryptic fibronectinase. Further aims are the activation and the characterization of both the recombinant and the isolated serine proteinase to define the substrate specificity and consequently possible roles in physiological or pathological processes.

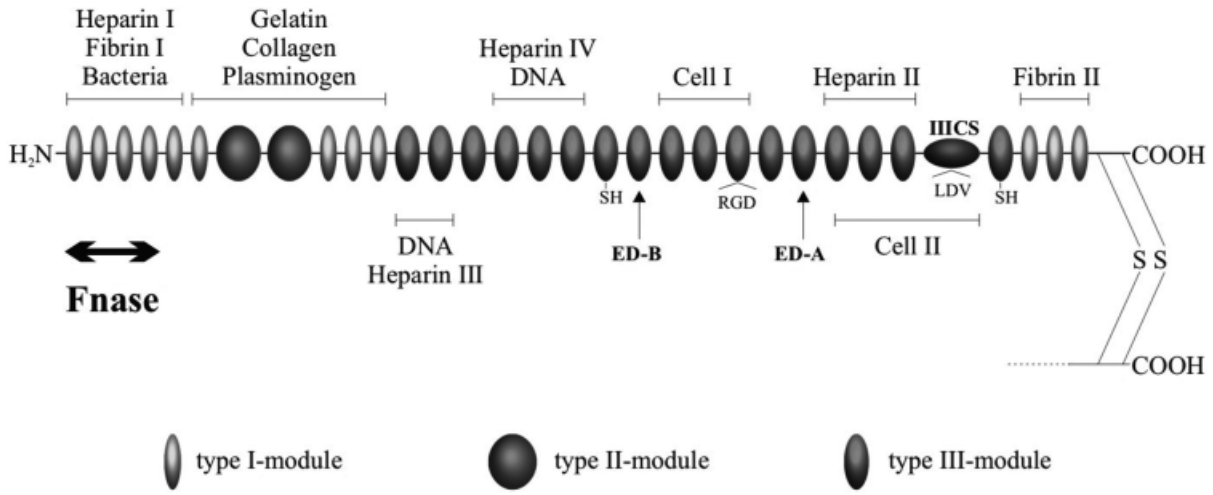

Fig. 1 Schematic Domain Structure of Fibronectin.
Fibronectin is composed of three types of internal repeating modules designated type I, II, and III. The EDA and EDB (extra domains A and B) modules are absent in plasma fibronectin as a result of alternative splicing. Various binding domains of fibronectin are indicated at the top. The localization of fibronectinase (Fnase) is indicated by a bold arrow.

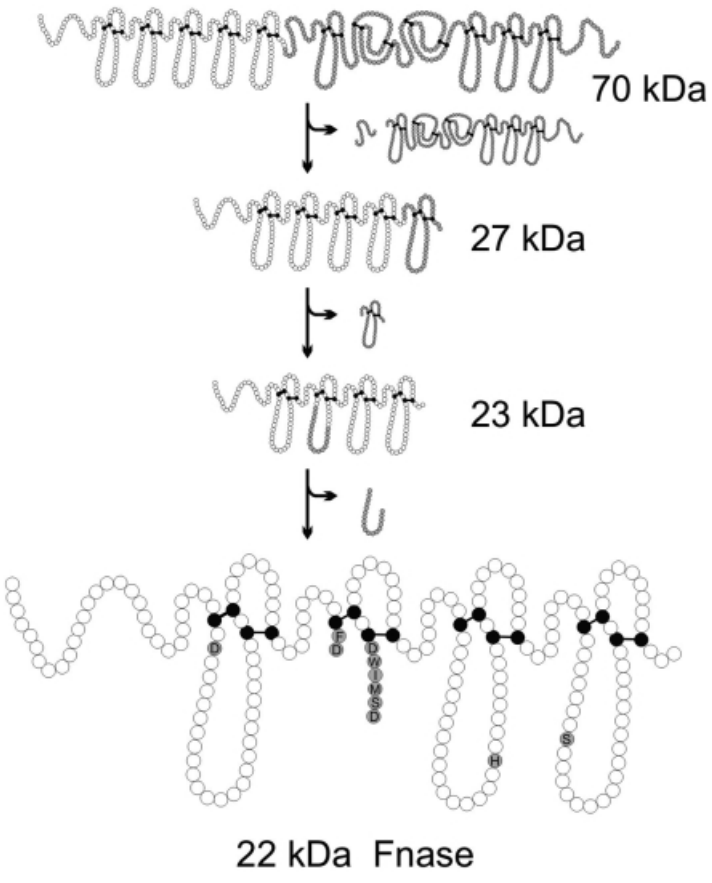

Fig. 2 Model of Processing and Activation of Fibronectinase.
Cleavage of the N-terminal 70 kDa fibronectin fragment is followed by degradation of the 27 kDa fragment resulting in an inactive 23 kDa form. The generation of proteolytic activity is a result of cleavage of the bond K^{86}-D^{87} and a decrease in mass of 1 kDa.

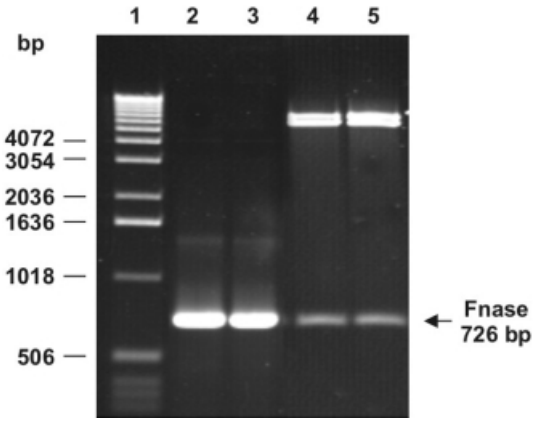

Fig. 3 Agarose Gels (1%) of PCR Amplification and Cloning of Fnase cDNA Stained with Ethidium Bromide.
Lane 1: 1 kb marker; lanes 2 and 3: PCR amplification of Fnase (726 bp); lanes 4 and 5: restriction digest analysis (*Nde*I/*Bam*HI) of the plasmid Fnase/pET-11a.

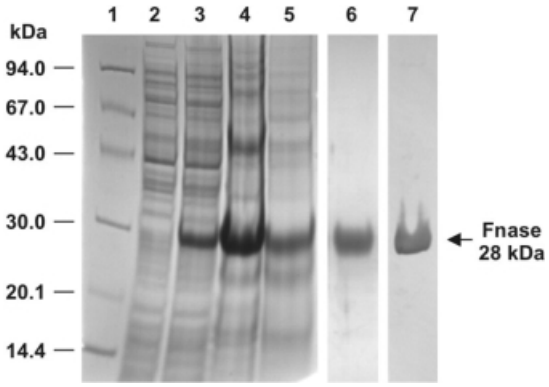

Fig. 4 SDS-PAGE Analysis of the Expression and Purification of Recombinant Fibronectinase.
The homogeneity of the final preparation is documented after silver staining. Lane 1: LMW protein marker; lane 2: expression of the recombinant protein before induction with IPTG; lane 3: expression 3 h after induction with IPTG; lane 4: solution of inclusion bodies; lane 5: Fnase eluted from DEAE-Sepharose; lane 6: homogeneous Fnase after gelfiltration on Sephacryl S-100; lane 7: immunoblot analysis of purified fibronectinase with a monoclonal antibody against the N-terminal domain of fibronectin (Chemicon).

Results

Cloning

Fibronectinase cDNA was amplified by PCR using primers corresponding to the coding sequences of Fnase (R^1-R^{242}). The amplified cDNA was purified and ligated into the *E. coli* vector pET-11a to create a Fnase/pET-11a expression plasmid. The results were verified by restriction analysis with *Nde*I/*Bam*HI (Figure 3). Furthermore, the constructs were confirmed by DNA sequencing.

Expression and Purification

For expression of the recombinant protein the *E. coli* strain [BL21(DE3)] was transformed with the described plasmid. After induction with IPTG the expression reached a maximum after 3 h. A highly expressed 28 kDa protein was detected by SDS-PAGE. After dissolving the obtained inclusion bodies in a buffer containing 8 M urea the recombinant fibronectinase was purified using DEAE-Sepharose as an anion exchanger and Sephacryl S-100 as a matrix for gelfiltration chromatography. The N-terminus of the protein was blocked, but after cleavage with cyanogen bromide a sequence near the N-terminus was identified by automated Edman sequencing (V^7QPQSP-VAVSQSKP^{20}). Furthermore, Western blot analysis with the monoclonal antibody against the N-terminal domain of pFN (Chemicon) was used for identification (Figure 4).

Proteolytic Characteristics

Folding of denatured fibronectinase was performed with the described oxido-shuffling system. After activation of the folded enzyme by incubation in presence of Ca^{2+} (48 h, 37 °C) catalytic activity was tested by zymography. As shown in Figure 5, human plasma fibronectin is a substrate of recombinant fibronectinase. The activation leads to a 22 kDa active fragment as shown previously with isolated plasma fibronectin by Lambert Vidmar *et al.* (1991b).

Determination of the cleavage specificity was also performed with isolated plasma fibronectin as substrate. SDS-PAGE analysis in Figure 6 shows fragmented fibronectin (lanes 2 and 3) after incubation with active recombinant fibronectinase for 16 h. Three fragments (FN1, FN2 and FN3) could be identified by automated Edman sequencing, and thus cleavage occurs at the bonds R^{420}-G^{421}, R^{1244}-F^{1245} and R^{2119}-V^{2120}. Accordingly, fibronectinase cleaves bonds with an arginine residue at the P_1-position flanked by hydrophobic amino acids at P_2, and hydrophilic residues at P_3 (Table 1).

The cleavage of fibronectin with the isolated fibronectinase showed identical results; the same cleavage sites were detected within the protein sequence. In addition

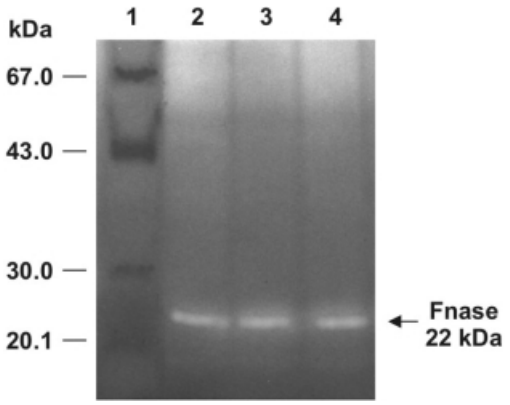

Fig. 5 Fibronectin Zymogram of Fibronectinase Stained with Coomassie Blue.
Lane 1: LMW protein marker; lanes 2–4: folding reactions of Fnase after activation in the presence of 10 nM Ca^{2+} (37 °C, 48 h) yielded an active 22 kDa fragment.

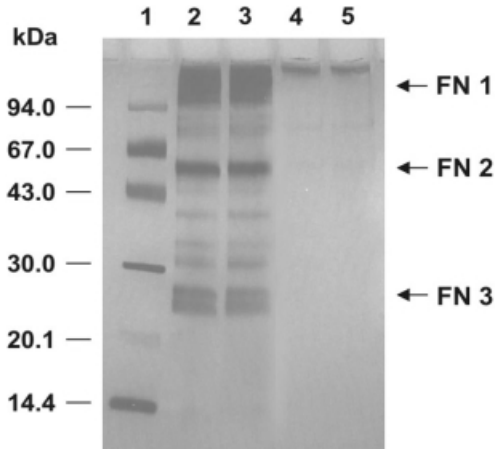

Fig. 6 SDS-PAGE Analysis of the Digestion of Human Plasma Fibronectin.
pFN was incubated with activated Fnase for 16 h at 37 °C and then subjected to SDS-PAGE (10% acrylamid; silver stained). Lane 1: LMW protein marker; lanes 2 and 3: incubation of pFN with Fnase for 16 h; lanes 4 and 5: control incubation of pFN for 16 h. Identified fibronectin fragments (FN1, FN2 and FN3) are indicated.

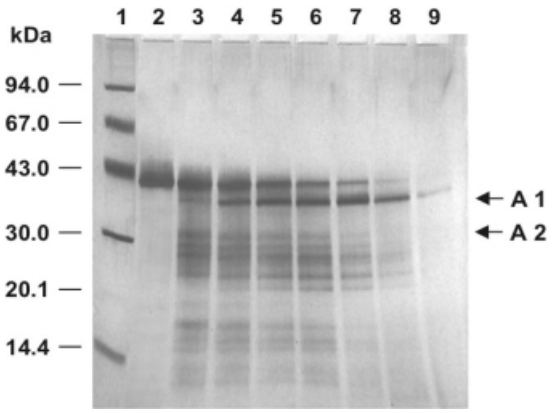

Fig. 7 SDS-PAGE Analysis of the Digestion of Actin.
Samples of actin were incubated with active fibronectinase for various times at 37 °C and then subjected to SDS-PAGE (10% acrylamide; silver stained). Lane 1: LMW protein marker; lane 2: control incubation of actin for 3 h; lanes 3 – 9: incubation of actin with Fnase for 1 min (lane 3), 5 min (lane 4), 15 min (lane 5), 30 min (lane 6), 1 h (lane 7), 2 h (lane 8) and 3 h (lane 9). Identified actin fragments (A1 and A2) are indicated.

several synthetic peptides were used to determine the cleavage specificity (Table 2). Only two of these substrates were cleaved by the fibronectinase. Cleavage took place behind a basic amino acid flanked by a hydrophobic residue in P_2-position and an acidic residue in P_3-position as shown for cleavage of pFN. All exchanges of these three amino acids prevented the substrates from cleavage by fibronectinase. Table 2 also shows the kinetic parameters of fibronectinase for the cleaved peptides.

Further investigations on the substrate specificity of fibronectinase included the cleavage of muscle proteins. Figure 7 shows the analysis by SDS-PAGE of cleavage of human actin with recombinant fibronectinase.

Here, for the first time, both human actin and myosin (data not shown) were identified as substrates of fibronectinase. Cleavage of the proteins was already shown after 1 min of incubation at 37 °C, and after 3 h we observed complete degradation. Two actin fragments (A1 and A2) were characterized by Edman sequencing and revealed cleavages at the bonds V^{47}-G^{48} (A1) and R^{97}-V^{98} (A2). The cleavage at R^{97}-V^{98} corresponded to the specificity observed in the degradation of fibronectin (Table 1) and the pNA-peptides (Table 2). The recombinant enzyme cleaves neither collagens type I, II and IV, nor gelatin. In addition, the isolated proteinase is able to cleave laminin as described by Lambert Vidmar *et al.* (1991).

Inhibition Studies

Human plasma and tissue contains different kinds of proteinase inhibitors. α_1-Proteinase inhibitor (α_1-PI), α_1-an-

Table 1 Cleavage Specificity of Recombinant Fibronectinase.

Substrate	Fragment	N-terminal sequence
Fibronectin	FN1 (120 kDa)	-G-R-W-K-C-D^{455}-
	pFN[a]	-T^{444}-C-F-G-Q-L-R-G-R-W-K-C-D^{455}-
Fibronectin	FN2 (60 kDa)	-V-P-G-T-S-T^{2155}-
	pFN[a]	-E^{2143}-E-P-L-Q-F-R-V-P-G-T-S-T^{2155}-
Fibronectin	FN3 (27 kDa)	-F-T-N-I-G-P-D^{1281}-
	pFN[a]	-P^{1268}-P-P-T-D-L-R-F-T-N-I-G-P-D^{1281}-
Actin	A1 (38 kDa)	-G-M-G-Q-K-D^{53}-
	actin[b]	-R^{41}-H-Q-G-V-M-V-G-M-G-Q-K-D^{53}-
Actin	A2 (31 kDa)	-V-A-P-E-E-H^{103}-
	actin[b]	-S^{91}-F-Y-N-E-L-R-V-A-P-E-E-H^{103}-

pFN and actin (lower line: potential cleaving sites underlined) were subjected to fibronectinase cleavage and the N-terminal amino acid sequences of the obtained fragments (upper line) were determined by automated Edman sequencing. ([a]Swiss Protein Database = Swiss-Prot # P02751; [b]Swiss-Prot # P03996).

Table 2 Cleavage Specificity and Kinetic Parameters of Isolated Fibronectinase.

Peptide	Cleavage	K_m[mM]	k_{cat}[s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
Suc - A - R - pNA	—	—	—	—
Suc - A - A - A - pNA	—	—	—	—
Suc - A - A - F - pNA	—	—	—	—
Suc - F - P - F - pNA	—	—	—	—
Ac - I - E - G - K - pNA	++	1.4±0.05	33.4±0.7	23.86±0.45
Bz - I - E - G - R - pNA	++	1.2±0.05	46.7±0.8	38.92±0.64
Bz - I - F - V - R - pNA	—	—	—	—
Bz - I - P - F - R - pNA	—	—	—	—
Bz - V - L - R - pNA	—	—	—	—
Bz - V - L - K - pNA	—	—	—	—

The peptide substrates were subjected to fibronectinase cleavage. Enzymatic activity was measured by detecting the change of absorbance at 405 nm and the kinetic parameters were calculated by a method of Lineweaver and Burk (1934).

tichymotrypsin and antithrombin III (AT III) belong to the family of serpins ('serine pronteinase inhibitors'). α_2-Macroglobulin (α_2-M) is a glycoprotein that inhibits proteinases of all families. The TIMPs ('tissue inhibitors of matrix metalloproteinase') are responsible for the inhibition of matrix metalloproteinases (MMPs). The effect of these inhibitors on isolated human fibronectinase was tested with the peptide Bz-I-E-G-R-pNA. The catalytic activity of the serine proteinase was effectively inhibited only by α_1-proteinase inhibitor.

Discussion

Fibronectinase (Fnase) is one of four cryptic proteinases inherent in human plasma fibronectin (Emod *et al.*, 1990; Planchenault *et al.*, 1990; Lambert Vidmar *et al.*, 1991a, b). It is a Ca^{2+}-dependent serine proteinase, which can be inhibited by both PMSF and EDTA. Fnase was first described by Lambert Vidmar *et al.* (1991a) and was further characterized by Unger and Tschesche (1999). The proteolytic activity could be demonstrated after digestion of fibronectin (pFN) isolated from blood plasma by a method of Vuento and Vaheri (1979). Cleavage of pFN with cathepsin D results in an N-terminal 70 kDa fragment, from which fibronectinase could be generated by further processing steps. A disadvantage of this method is the time consuming isolation of the enzyme. Moreover, only extremely low yields were obtained and the instability of the proteinase impedes characterization. In order to obtain fibronectinase on a more efficient way an *E. coli*-expression system was developed.

We selected the *E. coli* T7-polymerase-pET-vector system to be able to induce the expression of fibronectinase. Combination with the *E. coli* expression strain BL21(DE3) led to a potential expression system and finally resulted in high level production of recombinant fibronectinase with a yield of 15 mg/l. A disadvantage of the *E. coli*-pET-system was the aggregation of the expression product into inclusion bodies, which are soluble only under denaturing conditions. A two step purification procedure on DEAE-Sepharose as a cation exchanger and Sephacryl S-100 as a matrix for gelfiltration was developed to obtain denatured fibronectinase that was folded into its native conformation. A problem of the folding procedure was the high number of 10 disulfide bonds. Therefore, an oxido-shuffling system with oxidized gluthathione (GSSG) and dithiothreitol (DTT) was used to allow correct cysteine pairing.

SDS-PAGE-analysis of the reaction products after folding and activation revealed only a diffuse band structure of 22 – 27 kDa (not shown). The likely explanation is formation of incorrectly folded proteins due to the high numbers of cysteines with possible occurence of misfolded enzyme.

For identification of catalytically active processed fragments we used pFN-zymography. The only proteolytic fragment was detected with a molecular mass of 22 kDa, which corresponds to the isolated fibronectinase described by Lambert Vidmar *et al.* (1991a). This was evidence for correct folding and activation of parts of the expressed proteinase in a yield of 5 – 10% of the prepurified protein. Thus, for the first time recombinant fibronectinase became available for characterization.

Fibronectinase was not able to degrade insulin. Therefore, we used the only known substrate, plasma fibronectin, to examine the cleavage specificity of the enzyme. From various cleavage products (25 – 150 kDa), three fragments could be identified by sequence determination of the N-termini. Both isolated and recombinant fibronectinase cleaved bonds with an arginine residue at the P_1-position flanked by hydrophobic amino acids at P_2, and acidic and/or amphoteric hydrophilic amino acids at the P_3-position. The higher k_{kat}/K_m value of 38.92 for Bz-I-E-G-R-pNA in contrast to 23.68 for Ac-I-E-G-K-pNA indicated the peptide with the arginine in P_1-position as the better substrate.

Comparison of the amino acid sequences revealed the largest sequence homologies to the family of prohormone convertases, which belong to the family of subtil-

isin-like proteinases. On the other hand, the cleavage specificity is similar to the trypsin-like proteinases, which also cleave behind basic amino acid residues. On the contrary, pro-hormone convertases require a pair of basic amino acids (Siezen and Leunissen, 1997). A further indication for the similarity between fibronectinase and the trypsin-like proteinases is the pH optimum of 7.2. Most trypsin-like proteinases as well as trypsin, kallikrein or factor Xa have pH optima between 7 and 8, unlike pro-hormone convertases, which develope full activity at acidic pH 6 (Barrett, 1994). Because fibronectin is the only known substrate of fibronectinase, new substrates should be identified in order to determine the role of the enzyme in physiological or pathological processes. A relevance of fibronectinase in the pathological process of muscular dystrophy could result from its release by the action of the aspartatic proteinase cathepsin D. An increased cathepsin D production, *e. g*. from leukocytes under inflammatory conditions (Clausbruch and Tschesche, 1988), was observed in tissue samples of patients with muscular dystrophy combined with a strong decline of muscle tissue (Iodice *et al.*, 1972; Noda *et al.*, 1981; Whitaker *et al.*, 1983; Gopalan *et al.*, 1987). The *in vitro* generation of fibronectinase is initiated by cleavage of pFN with cathepsin D, and thus fibronectinase may contribute to the proteolytic processes in muscular dystrophy. For this reason the capability of the recombinant enzyme to degrade muscle proteins was examined. Both human actin and myosin were degraded after very short incubation times with the proteinase and were identified for the first time as fibronectinase substrates. In addition, two actin fragments could be characterized by Edman sequencing. The cleavage specificities of the recombinant enzyme corresponded to that of the cryptic fibronectinase released from plasma fibronectin.

Identification of these two substrates revealed a possible role of fibronectinase in the pathological process of muscular dystrophy. The development of specific antibodies against the active enzyme is indispensable for further investigations in the occurrence of fibronectinase. Immunohistological detection of both cathepsin D and fibronectinase in dystrophic tissues could reveal information about the spatial and temporal appearance of the proteinases, and thus, could give an indication for the involvement of fibronectinase in muscle degradation.

Materials and Methods

Reagents

Primers for polymerase chain reaction were obtained from MWG-Biotech (Ebersberg, Germany). Tth polymerase, T4 DNA ligase, and alkaline phosphatase were obtained from Promega (Madison, USA). The *E. coli* strain JM83 was used as the host for plasmid amplification and *E. coli* BL21(DE3) for expression of the fibronectinase. The restriction enzymes *Nde*I and *BamH*I were purchased from New England Biolabs (Beverly, USA). Chromatographic media DEAE-Sepharose and Sephacryl S-100 were from Pharmacia (Freiburg, Germany). Monoclonal mouse anti-human fibronectin IgG against the N-terminal heparin/fibrin binding domain was obtained from Chemicon (Temecula, USA). All other chemicals were of p.a. quality and purchased from Bachem (Bubendorf, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma (Munich, Germany).

Cloning and Expression of Fibronectinase

Amplification of the fibronectinase cDNA with the polymerase chain reaction (PCR) was performed with the cDNA plasmid pFH6/pAT153, a gift of Dr. F.E. Baralle (Trieste, Italy) as template. Primers were designed based on the known cDNA sequence of the N-terminus of fibronectin (Fnase-For 5'-AAG CAT ATG AGG CAG GCT CAG CAA ATG G -3'; Fnase-Rev 5'-CAC GGA TCC CTA CCT CTC ACA CTT CCA CTC -3'). *Nde*I and *Bam*HI restriction sites were added to the 5' and 3' primers, respectively, to enable the insertion of the cDNA into the *E. coli* expression vector pET-11a. PCR was performed for 35 cycles (95 °C, 30 s; 57 °C+0.2 °C/cycle; 72 °C, 60 s) followed by a final extension at 72 °C for 3 min. The PCR product was ligated into the expression vector and the expected sequence was confirmed by restriction analysis and DNA sequencing. For expression of fibronectinase, the *E. coli* strain BL21(DE3) was transformed with the plasmid Fnase/pET-11a. The cells were grown in LB-medium at 37 °C up to an optical density of 0.7, before inducing the expression with 2 mM isopropyl-β-D-thiogalactopyranosid (IPTG). After 3 h cells were lysed and the obtained inclusion bodies were dissolved in buffer (8 M Urea, 100 mM 2-mercaptoethanol, 20 mM Tris/HCl, pH 8.5).

Purification and Folding

First, the recombinant protein was bound to the cation exchanger DEAE-Sepharose equilibrated with buffer A (4 M urea, 40 mM 2-mercaptoethanol, 50 mM Tris/HCl, pH 8.5). After washing the column with 4 bed volumes buffer A to remove unbound proteins, the recombinant fibronectinase was eluted with a linear gradient from 200 to 600 mM NaCl in buffer A. Protein content of each fraction was determined by measuring the absorbance at 280 nm. The fractions containing the recombinant protein were pooled and concentrated by ultrafiltration. The second step of purification was a gel filtration on Sephacryl S-100; 3 ml of the sample were 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, the fractions containing Fnase were pooled and concentrated by ultrafiltration.

The folding procedure was based on an oxido-shuffling system with oxidized glutathione (GSSG) and dithiothreitol (DTT) (Buchner *et al.*, 1992). This redox system promotes disulfide bond formation. The denatured protein was dialyzed against the 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 enzymatic assays.

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 over night at room temperature with 0.5 µg/ml monoclonal mouse anti-human fibronectin antibody (N-terminal) in 3% BSA in TBST buffer. Final-

ly, the immunoreactive bands were visualized with goat anti-mouse alkaline phosphatase IgG conjugate and BCIP/NBT (5-bromo-4-chloro-3-indolyl/nitro blue tetrazolium) detection.

Enzyme Assays

Activation of purified and folded recombinant fibronectinase was performed by incubation at 37 °C for 48 h in the presence of 10 mM Ca^{2+}. Activated Fnase was used for zymography (Heussen and Dowdle, 1980). 10% polyacrylamide gels containing 1 mg/ml fibronectin were subjected to electrophoresis under nonreducing conditions at 20 mA. Then the gels were washed 2 times with 2.5% Triton X-100, 3 times with distilled 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 Brilliant Blue and destained with distilled water. For further assays with fibronectin, actin, and myosin, 10 µl of the substrate (1 mg/ml) were incubated at 37 °C with 5 µl of proteinase solution and then analyzed by SDS-PAGE (Rabilloud *et al.*, 1994). The cleavage specificity against fibronectin and actin was investigated by Edman sequencing of the fragments obtained after digestion with recombinant fibronectinase. Kinetic measurements were performed on a Shimadzu double-beam spectrophotometer with thermostated cell compartiment. The hydrolysis of chromogenic substrates was monitored by measuring the decrease in absorbance at 405 nm as a function of time. For inhibition studies fibronectinase was incubated with the respective inhibitor for 30 min in buffer C and then used for enzyme assays.

Acknowledgements

We are grateful to Dr. F.E. Baralle for preparation of the cDNA plasmid pFH6/pAT153 containing Fnase cDNA. Furthermore, we would like to thank M.O. Luther for amino acid sequencing. This work was supported by the Deutsche Forschungsgesellschaft, special research program SFB 549, project A5.

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Received June 20, 2001; accepted October 25, 2001