A large cathepsin D-derived fragment from the central part of the fibronectin subunit chains

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A mild cathepsin D digest of fibronectin only contained single-chain peptides of 200, 140 and 70 kDa and double-chain fragments of about 300 and 140 kDa containing the C-terminal disulfide link. Among the single-chain fragments the 200 kDa peptide was a precursor of the 140 kDa and 70 kDa peptides. The latter was correlated to the N-terminal and the former to the central region of the fibronectin subunit chains.

Fibronectin *Cathepsin D* *Gelatin–Sepharose* *Heparin–Sepharose*

1. INTRODUCTION

Fibronectin is a large protein present in the pericellular matrix of various adherent cells. A soluble form closely related to the cell-bound protein is found in plasma. As reviewed in [1–3] the molecule expresses affinity to numerous substrates including collagen, fibrin, heparin, bacterial cell walls and surface structures of cells capable of binding this component. It mediates attachment and spreading of the cells on matrix substances as well as binding of suitable substrates to phagocytes.

The more intensively investigated soluble fibronectin consists of two very similar long chain subunits connected by disulfide bonds close to their C-terminal ends [4–6]. So far, the knowledge of the subunit structure is restricted to their terminal regions. The N-terminal one is represented by a cathepsin D derived 70 kDa fragment [5,7], comprising a N-terminal fibrin- and heparin-binding 30 kDa domain and a subsequent gelatin binding one of 40 kDa [8,9]. From the C-terminal region the same protease liberated a strong heparin-binding 140 kDa peptide which, after reduction, dissociated into two peptides of 65 kDa and 75 kDa showing a high degree of homology within the major part of their length [10]. Each peptide being derived from the longer and shorter subunit, respectively, consisted of a heparin-binding basic domain and a disulfide-rich acidic one, those in the longer fragment being well-separable by various proteases [11]. Most of the amino acid sequences of the N-terminal and C-terminal regions of bovine fibronectin have been elaborated [12,13].

Little is known about the central part of the fibronectin subunit chains covering the stretch between the N-terminal 70 kDa fragment and the C-terminal peptides of 65 kDa or 75 kDa, respectively. This region should contain the cell-binding [14] and chemotactic-active [15] sites of fibronectin. This paper describes a cathepsin D-derived fragment spanning this gap and characterizes other peptides as fragments from this region.

2. MATERIALS AND METHODS

Fibronectin was adsorbed from citrated human plasma by gelatin–Sepharose [16], eluted with 1 M KBr (pH 5.3) and purified by chromatography on DEAE-cellulose [17]: 150 mg was digested with 0.5 mg cathepsin D (Sigma; EC 3.4.23.5) in 150 ml 50 mM Na-acetate (pH 3.5), 0.2 mM phenylmethane–sulfonyl fluoride at 30°C and stopped after 4 h with 0.1 mg pepstatin (Sigma) as

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in [7]; 50 ml were dialyzed against 0.1 M NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 7.4) and fractionated on a column (1.6 × 15 cm) of heparin–Sepharose [18] using stepwise elution with 0.1, 0.25 and 0.5 M NaCl containing application buffer. The 140 kDa fragment in the 0.1 M NaCl eluate was depleted from 70 kDa peptide by gelatin–Sepharose and purified on Ultrogel AcA44 [11]. The 200 kDa peptide was isolated from the 0.25 M NaCl eluate by chromatography on Ultrogel AcA34 and digested with cathepsin D (enzyme:substrate, 1:30) as described.

SDS gel electrophoresis was performed in 5–20% logarithmic-gradient polyacrylamide according to [19]. Standards were taken from the high-M_r reference kit of Bio-Rad (Munich) containing samples of 200, 116, 93, 67 and 45 kDa and the low-M_r reference kit of Pharmacia (Uppsala) with proteins of 93, 67, 45, 30, 20 and 14 kDa. Protein concentration was estimated spectrophotometrically using $A^{1\%}_{280} = 12.8$ [20].

3. RESULTS

After 4 h digestion of fibronectin with cathepsin D at an enzyme:substrate ratio of 1:300 a rather simple fragmentation pattern was obtained with peptides of about 300, 200, 140 and 70 kDa as revealed by SDS gel electrophoresis (fig.1). They were partially resolved by chromatography on heparin–Sepharose. Stepwise elution with 0.1, 0.25 and 0.5 M NaCl containing buffer yielded 3 fractions. Fraction I contained mainly a 140 and 70 kDa fragment, fraction II the 200 kDa fragment and some additional 70 kDa one and fraction III a major fragment of 140 kDa diffusely migrating in gel electrophoresis and components of about 300 kDa. All the fragments of fraction III dissociated after reduction yielding single peptide chains of 75 and 65 kDa as well as some undegraded fibronectin subunits. (In our hands these subunits migrated with a rate according to 250–260 kDa rather than 220 kDa as generally reported.) These results indicate that the strong heparin binding fraction III contained the already described C-terminal fragment and very early degradation products consisting of a complete fibronectin subunit connected to a C-terminal remnant of the other chain. All these peptides of fraction III had been characterized previously [10,11].

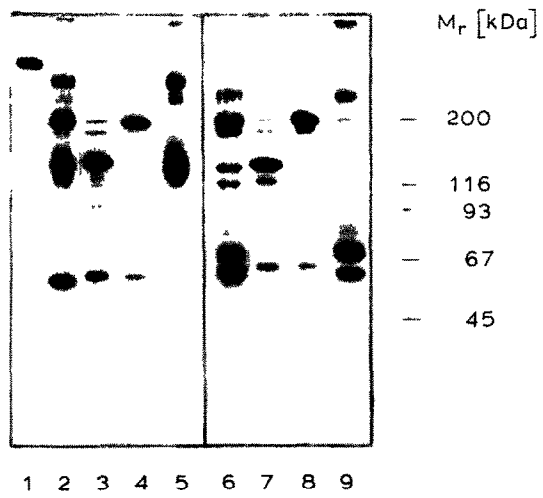

Fig.1. SDS gel electrophoresis of an early cathepsin D digest of fibronectin (lane 2 and 6) and of fractions obtained by chromatography on heparin–Sepharose. Eluates were obtained with 0.1 M (lane 3 and 7), 0.25 M (lane 4 and 8) and 0.5 M NaCl (lane 5 and 9); fibronectin, lane 1; left, non-reduced samples; right, reduced samples.

Fraction I and II consisted of single chain fragments as revealed from their electrophoretic mobility after reduction. The peptides of fraction I were resolved by chromatography on gelatin–Sepharose which retained the 70 kDa piece but showed no affinity to the 140 kDa fragment. In contrast, the two peptides of fraction II were retained by gelatin–Sepharose indicating that both contained the gelatin binding site.

To prove whether the 200 kDa fragment in fraction II was a precursor of the gelatin binding 70 kDa piece, the two peptides were separated by chromatography on Ultrogel AcA34. Subsequently, the isolated 200 kDa fragment was incubated with cathepsin D and the digestion followed by SDS gel electrophoresis (fig.2, left). After 10 min, a 140 kDa and a 70 kDa fragment appeared, the latter giving rise after prolonged digestion to additional fragments predominantly ranging near 120, 95 and 18 kDa. Again the 70 kDa peptide could be adsorbed by gelatin–Sepharose, while the other fragments showed no affinity.

The identity of the 140 kDa peptide derived from the 200 kDa fragment with that found in fraction I was documented by treating the latter with cathepsin D. It yielded the same pattern of

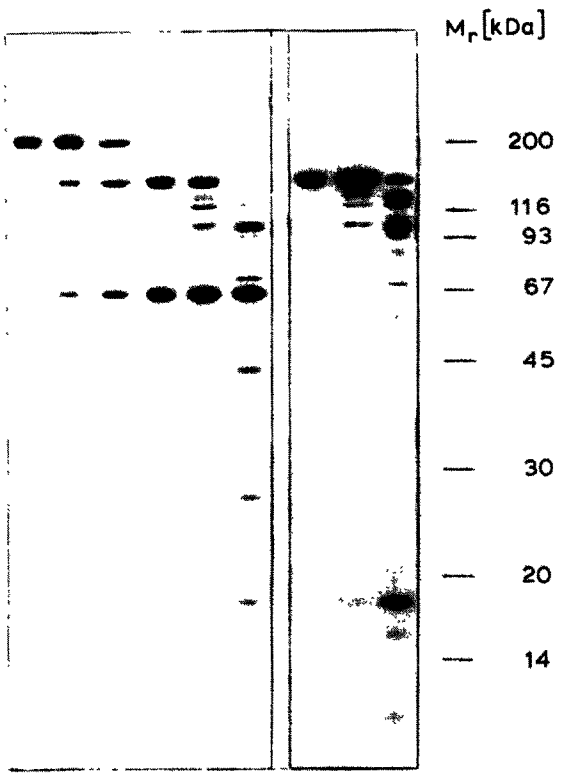

Fig.2. Cathepsin D digests [enzyme:substrate (1:30), 30°C] of 200 kDa (left) and 140 kDa peptides (right) isolated from an early cathepsin D digest as followed by SDS gel electrophoresis. Samples of 200 kDa digest were taken after 0, 0.2, 0.5, 2, 6 and 24 h; 140 kDa peptide was digested for 2 and 6 h. Samples were applied without reduction.

late cleavage products as the digest of the 200 kDa precursor with the exception that the 70 kDa piece and two fragments of lower M_r evidently derived of it were missing (fig.2, right).

4. DISCUSSION

Here, an early cathepsin D-derived 140 kDa fragment of fibronectin is described which consisted of a single peptide chain and is degraded by further enzyme treatment to a group of fragments of 90–125 kDa. The peptide is part of a large precursor fragment which is cleaved by cathepsin D to this peptide and a 70 kDa gelatin-binding one. The latter is already well characterized as the N-terminal cathepsin D-derived fragment of the fibronectin subunits [4–8]. Consequently, the single chain 140 kDa fragment should be correlated subsequent to this gelatin binding piece although a gap of small peptides lost during processing of the digest cannot be excluded.

All data indicate that the 140 kDa fragment represents the central region of the fibronectin subunits between the N-terminal 70 kDa region and the C-terminal peptides of 75 kDa or 65 kDa, respectively. Overlapping with the C-terminal region is unlikely as the peptides from this part contain a basic strong heparin binding domain in N-terminal position [11], while the single chain 140 kDa fragment is very little retained by heparin–Sepharose.

Further data are necessary to correlate the later fragments emerging from the 140 kDa peptide after prolonged cathepsin D treatment. A basic 18 kDa domain [21] adjacent to the gelatin binding one on its C-terminal side is most likely part of this central 140 kDa fragment and might be represented by a late cathepsin D-derived peptide of the same size (fig.2). Therefore, the longer fragments of 90–125 kDa should probably be correlated to the central and C-terminal parts of the early 140 kDa piece.

Gel electrophoresis of the 140 kDa fragment sometimes yields a close doublet. It is not clear whether this microheterogeneity is due to a less specific proteolytic cleavage or whether it reflects a difference of the central parts in the longer and shorter subunit peptide chains of fibronectin.

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REFERENCES

[1] Mosher, D.F. (1980) in: Progress in Hemostasis and Thrombosis (Spaet, T.H. ed) vol.5, pp.111–151, Grune and Stratton, New York.
[2] Ruoslahti, E., Engvall, E. and Hayman, E.G. (1981) Collagen Rel. Res. 1, 95–128.
[3] Hörmann, H. (1982) Klin. Wochensch. 60, 1265–1277.
[4] Jilek, F. and Hörmann, H. (1977) Hoppe Seyler's Z. Physiol. Chem. 358, 133–136.

[5] Balian, G., Click, E.M. and Bornstein, P. (1980) J. Biol. Chem. 255, 3234–3236.
[6] Furie, M.B. and Rifkin, D.B. (1980) J. Biol. Chem. 255, 3134–3140.
[7] Balian, G., Click, E.M., Crouch, E., Davidson, J.M. and Bornstein, P. (1979) J. Biol. Chem. 254, 1429–1432.
[8] Hörmann, H. and Seidl, M. (1980) Hoppe Seyler's Z. Physiol. Chem. 361, 1449–1452.
[9] Sekiguchi, K., Fukuda, M. and Hakomori, S. (1981) J. Biol. Chem. 256, 6452–6462.
[10] Richter, H., Seidl, M. and Hörmann, H. (1981) Hoppe Seyler's Z. Physiol. Chem. 362, 399–408.
[11] Richter, H. and Hörmann, H. (1982) Hoppe Seyler's Z. Physiol. Chem. 363, 351–364.
[12] Skorstengaard, K., Thøgersen, H.C., Vibe-Pedersen, K., Petersen, T.E. and Magnusson, St. (1982) Eur. J. Biochem. 128, 605–623.
[13] Petersen, T.E., Thøgersen, H.C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L. and Magnusson, St. (1983) Proc. Natl. Acad. Sci. USA 80, 137–141.
[14] Pierschbacher, M.D., Hayman, E.G. and Ruoslahti, E. (1981) Cell 26, 259–267.
[15] Seppä, H.E.J., Yamada, K.M., Seppä, S.T., Silver, M.H., Kleinman, H.K. and Schiffman, E. (1981) Cell Biol. Int. Rep. 5, 813–819.
[16] Vuento, M. and Vaheri, A. (1979) Biochem. J. 183, 331–337.
[17] Dessau, W., Jilek, F., Adelmann, B.C. and Hörmann, H. (1978) Biochim. Biophys. Acta 533, 227–237.
[18] Miller-Andersson, M., Borg, H. and Andersson, L.O. (1974) Thromb. Res. 5, 439–452.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Mosesson, M.W., Chen, A.B. and Huseby, R.M. (1975) Biochim. Biophys. Acta 386, 509–524.
[21] Seidl, M. and Hörmann, H. (1983) Hoppe Seyler's Z. Physiol. Chem. 364, 83–92.