Localization of the chemotactic domain in fibronectin

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Fragments derived from human plasma fibronectin by enzymatic degradation were tested in the Boyden chamber for chemotactic activity towards various fibroblast strains. The results provide clear evidence that the chemotactic activity is restricted to a defined region of the fibronectin molecule which is the same for various fibroblast strains. The active domain is localized between the collagen binding site and the major heparin binding site, about 170 kDa apart from the N-terminal and about 70 kDa from the C-terminal ends of the two subunit peptide chains.

Fibroblast chemotaxis *Boyden chamber* *Fibronectin*

1. INTRODUCTION

Fibronectins (FN) are high-M_r, multifunctional glycoproteins, present in the extracellular matrix, in most basement membranes and, in a soluble form, in the blood plasma. They exhibit affinity to cell surfaces and bacterial cell walls, to heparin, fibrin, gelatin and actin. Fibronectin is capable of mediating cell attachment to collagen as well as cell spreading on matrix substances (reviews [1–3]). Another property of FN is the induction of fibroblast chemotaxis, a process which may play a role in wound healing [4]. Both plasma and cellular fibronectin are equally active in the induction of fibroblast chemotaxis [5].

Human plasma fibronectin is composed of two similar but not identical polypeptides (A and B chains) with slightly different M_r-values of ~250000 [6–8]. These subunits are linked via disulfide bridges close to their carboxy-terminal ends [9,10].

The FN polypeptide chains consist of highly structured domains separated by regions of flexible segments. Proteolytic digestion renders fragments of FN which can be isolated by affinity chromatography and subsequently tested for their biological activities [1–3,19]. Here, we used this approach to identify the chemotactically active region towards fibroblasts by the use of the Boyden chamber assay.

2. MATERIALS AND METHODS

2.1. *Cell culture technique*

Human embryo fibroblasts were derived from a skin biopsy of a 16-week-old fetus after legal abortion and subcultivated in Dulbecco's MEM supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), glutamine (300 μg/ml), ascorbic acid (50 μg/ml) and 10% fetal calf serum (FCS). For chemotaxis experiments cells were used in passages 8–12 and not later than 3 days after last trypsinisation.

Cells derived from fibrosarcoma, HT-1080 (strain ATCC-CCL 121) and from rhabdomyosarcoma RD (strain ATCC-CCL 136) were obtained from the American Type Culture Collection and grown in Dulbecco's MEM supplemented as above.

2.2. *Chemotaxis*

Polycarbonate filters (8 μm pore; Nucleapore

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Corp., Pleasanton CA) were coated with gelatin (5 μg/ml; Sigma) as in [11]. Cells were harvested by short trypsinisation (0.25% trypsin, 0.1% ethylenediamine tetraacetate), suspended in MEM containing 10% FCS to inactivate the enzyme, centrifuged and resuspended in MEM without FCS. Cells ($1.5–2.5 \times 10^5$/ml) were placed in the upper compartment of the Boyden chamber (0.8 ml). The lower compartment (0.2 ml) contained the peptide to be tested for chemoattractivity (protein was 1–200 nM) in MEM without FCS. The chambers were incubated at 37°C in 5% CO_2, 95% air for 4 h. Non-migrated cells on the upper side of the filter were removed mechanically. Migrated cells on the lower side were fixed on the filter with ethanol and stained with toluidine blue. Migrated cells were counted at a 400-fold magnification with a Zeiss microscope (C. Zeiss, Oberkochen). Each sample was assayed in triplicate and cells in 5 unit fields were counted.

2.3. *Purification of fibronectin and its fragments*

Human plasma fibronectin purified as in [12] was used as chemoattractant and for the production of proteolytic fragments. A chymotryptic digest [12] was dialysed against 0.05 M Tris–HCl (pH 7.4), 1 mM EDTA and fractionated on heparin–Sepharose (column 2.5 × 17 cm) using stepwise elution with 0.05, 0.1 and 0.5 M NaCl. The chymotryptic peptide with 125 kDa (designated: Ch-125) was purified from the 0.05 M eluate fraction by removal of contaminating peptides on gelatin–Sepharose followed by molecular sieve chromatography. The gelatin non-binding Ch-160,180 material was present in the 0.5 M NaCl eluate and was purified as described for the Ch-125 peptide.

The cathepsin D-derived fragment Ca-140 was prepared by mild digestion (enzyme:substrate = 1:300; 4 h) of human FN and purified as in [8]. Fragments Ca-95 and Ca-23 were prepared from a heparin non-binding fraction of a more processed digest (fraction I in [13]) by chromatography on Ultrogel AcA44. Ca-18 was obtained from the gelatin binding chymotryptic fragment Ch-60 by further digestion with cathepsin D and purified on heparin–Sepharose [12]. Material of same pI and similar amino acid composition was obtained from a cathepsin D digest (enzyme:substrate = 1:30; 24 h) of purified Ca-140 peptide (unpublished). Ca-15 is present in the above digest of Ca-140 as well as in a similar digest of Ch-125 from which it was isolated by chromatography on Ultrogel AcA54.

The plasmic fragments Pl-85 (gelatin binding) and Pl-90 (gelatin non-binding) resulted from a prolonged digest (Forschungsplasmin KABI, enzyme:substrate = 1:20; 24 h; 37°C) of isolated Ca-200 peptide [8] and were isolated by successive chromatography on gelatin–Sepharose, heparin–Sepharose and Ultrogel AcA44 as will be described elsewhere. M_r-Values of the peptides were determined by SDS gel electrophoresis as in [14]; globular proteins of M_r reference kits from Bio-Rad and Pharmacia were used as standards. [Protein] was estimated spectrophotometrically using $A^{1\%}_{280} = 12.8$ [6].

3. RESULTS AND DISCUSSION

The chemotactic response to fibronectin and to proteolytic fragments of this glycoprotein was in-

Table 1

Number of migrated cells/unit field using FN peptides as chemoattractants in the Boyden chamber (protein was 100 nM)

Lower compartment	Upper compartment			Migrated cells (%)
	HT 1080	RD	HE-F	
FN	165 ± 15	125 ± 15	70 ± 10	100%
Ca-200	n.d.	n.d.	70 ± 10	100%
Ca-70	3 ± 2	n.d.	2 ± 1	<5%
Ca-140	170 ± 10	130 ± 12	75 ± 10	100%
Ca-75-SS-65	8 ± 3	6 ± 2	4 ± 1	5%
Ca-18	n.d.	n.d.	2 ± 1	<5%
Ca-95	5 ± 2	4 ± 2	3 ± 1	<5%
Ca-23	20 ± 4	15 ± 3	8 ± 2	12%
Ca-15	7 ± 3	5 ± 2	4 ± 2	5%
Pl-30	n.d.	n.d.	3 ± 1	<5%
Pl-85	3 ± 1	n.d.	3 ± 2	<5%
Pl-90	155 ± 15	115 ± 10	60 ± 8	95%
Ch-160,180	165 ± 10	120 ± 15	65 ± 10	100%
Ch-125	n.d.	n.d.	68 ± 12	100%
Cathepsin D digest of Ch-125	8 ± 3	6 ± 2	3 ± 2	5%
MEM control	3 ± 2	3 ± 1	2 ± 1	<5%

n.d. = not determined

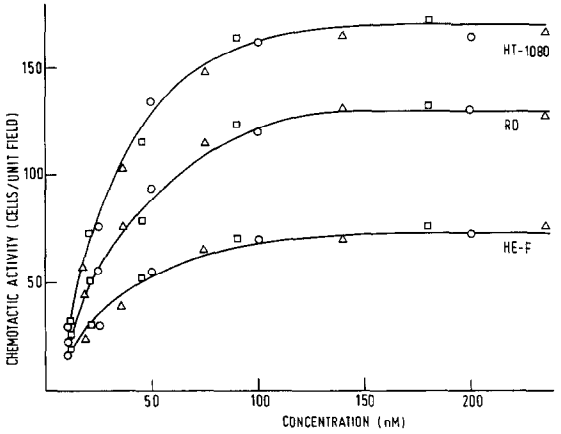

Fig.1. Chemotactic response of human fibrosarcoma (HT-1080), rhabdomyosarcoma (RD) and human embryo fibroblast (HE-F) cells to fibronectin (○), chymotryptic fragments Ch-160,180 (△) and cathepsin D-derived fragment Ca-140 (□). See section 2 for details.

vestigated using 3 different cell strains: human embryo fibroblasts (HE-F), HT 1080 fibrosarcoma cells and RD rhabdomyosarcoma cells. The latter two cell types migrated in a fibronectin gradient with a rather high rate [5,15], in our hands 165 ± 15 and 125 ± 15 cells/unit field, respectively, compared to 70 ± 10 HE-F cells (table 1, fig.1). The number of migrated cells increased up to 100 nM fibronectin (25 μg/ml) in the lower compartment of the Boyden chamber beyond which it remained on a plateau level [5].

The chemotactic region of fibronectin has been localized within a large cell binding chymotryptic 160 kDa peptide [16] and in a gelatin non-binding cathepsin D-derived 140 kDa peptide [17]. To localize the chemotactic active site in the fibronectin subunit chains more exactly, additional purified fragments from proteolytic digests of fibronectin were tested as chemoattractants (table 1). The position of many of those peptides within the fibronectin subunit chains was already known [8,18], while the correlation of others was so far tentative and was corroborated by these results (fig.2).

Among the cathepsin D-derived peptides an early 140 kDa gelatin non-binding fragment (Ca-140) representing the central part of the peptide chains [8] showed full activity (fig.1). This fragment is most probably identical with the above 140 kDa peptide described in [17]. The gelatin binding N-terminal peptide Ca-70 was inactive (<5%) and the double-chain C-terminal fragment Ca-75-SS-65 (present in the heparin binding fraction IV [13]) was very poorly active (5%). Full response was also obtained with the Ca-200 fragment [8] com-

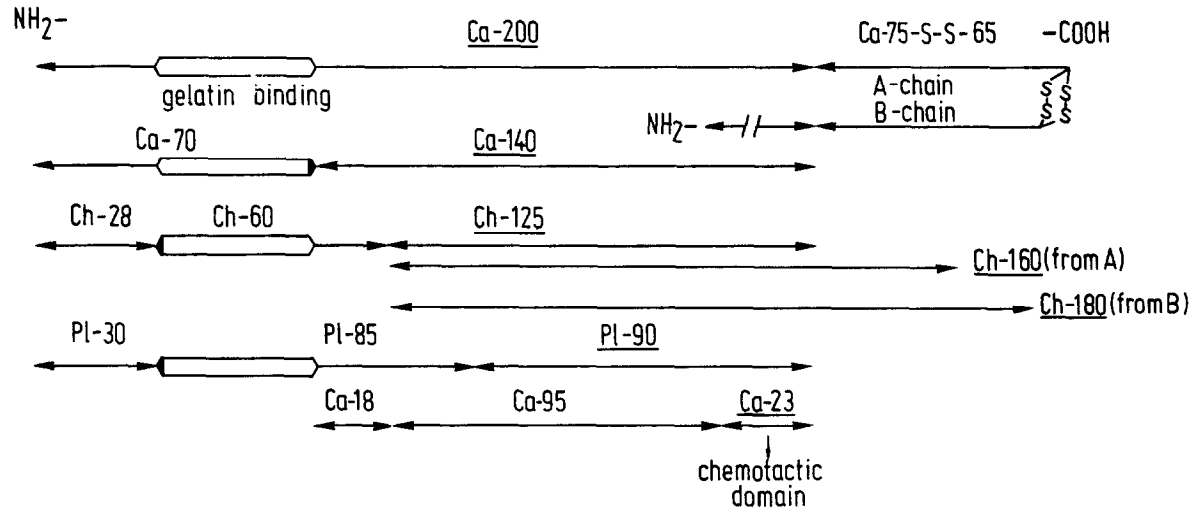

Fig.2. Localization of the chemotactic domain within the fibronectin subunits based on data in table 1. Chemoattractive cathepsin D-derived (Ca), chymotryptic (Ch) and plasmic (Pl) fibronectin fragments are underlined. The white boxes indicate the position of the gelatin binding domain which was useful for affinity purification and localization of respective fragments.

prising the N-terminal 70 kDa and the central 140 kDa region. From a more advanced digest peptides Ca-95, Ca-23, Ca-18 and Ca-15 representing further cathepsin D cleavage products of the central Ca-140 piece (unpublished) were investigated. Ca-23 showed little, but significant activity (12%). It is the precursor of the very poorly active (5%) fragment Ca-15 [18]. The other fragments were inactive. Ca-18 had been localized adjacent to the N-terminal Ca-70 peptide [12] and, therefore, should occupy the N-terminal part of Ca-140.

Further information on the chemoattractive site came from peptides obtained by plasmic digestion of the above Ca-200 fragment ranging from the N-terminus of the fibronectin subunits to the end of the central region. Plasmin clipped off rapidly the N-terminal peptide Pl-30 [9,10] and subsequently generated mainly the two peptides Pl-85 and Pl-90. Pl-85, lacking chemotactic activity, bound to gelatin–Sepharose. This indicates a position adjacent to Pl-30 (fig.2). The gelatin non-binding Pl-90, which is chemotactically active (95%), represents the C-terminal region of Ca-200.

Some chymotryptic peptides were also tested. Large heparin binding fragments of 160 and 180 kDa (designated: Ch-160,180) gave full chemotactic response (fig.1). They lacked gelatin binding capacity and thus were located in a position adjacent to the gelatin binding center up to the highly protease-sensitive site very close to the C-terminal end of the shorter B-chain (Ch-180) or to a chymotrypsin cleavage site about 30 kDa apart from the C-terminus of the longer A-chain (Ch-160), respectively (fig.2). Therefore, the presence of these 2 related peptides reflects the slight difference in the C-terminal part of the 2 fibronectin subunits. Another chemoattractive peptide was Ch-125 (100%) which, as it lacked gelatin and heparin binding capacity, was derived from the central region. Further cathepsin D digestion of the active Ch-125, giving rise to the poorly active Ca-15 isolated, destroys the chemotactic activity nearly completely (5%). This suggests that either a further cleavage of Ca-23 abolishes the chemotactically active center or the peptide might change its original conformation with loss of biological activity.

These data give sufficient information to localize the chemoattractive site within the fibronectin subunits. It is comprised in the central region covered by peptide Ca-140. As peptides Ca-18 and Pl-85 containing sequences from the N-terminal half of Ca-140 failed to give a chemotactic response, the activity must be located within the C-terminal half of Ca-140 covered by peptide Pl-90. Another inactive peptide was Ca-95. Therefore, this peptide only can be correlated to a position adjacent to the C-terminus of Ca-18. Consequently, the chemoattractice site is localized in the utmost C-terminal position of Ca-140 of about 30 kDa. The weakly active peptide Ca-23 has to be derived from this part.

In [19], the cell binding site of fibronectin was attributed to a tryptic 75 kDa fragment, the position of which was shown to be 72 kDa apart from the C-terminal end of the A-chain of 58 kDa from the B-chain C-terminus. According to our data the chemoattractive site should be in the C-terminal half of this fragment.

Our results provide clear evidence that the chemotactic activity is restricted to a defined domain of fibronectin which is the same towards various fibroblast strains.

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