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Fibronectin-binding 36 kDa protein in human fibroblasts

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A 36 kDa fibronectin-binding protein was identified from electrophoretically separated proteins of the deoxycholate-soluble fraction of cultured fibroblasts by blotting with fibronectin and using poly- or monoclonal antibodies and immunoperoxidase staining to detect the bound fibronectin. The 36 kDa protein was purified by preparative electrophoresis and used to raise specific antibodies. Solid-phase 36 kDa protein bound plasma and fibroblast fibronectins equally well. The 36 kDa protein is an amphipathic protein with p*I* 5.9. It is monomeric with a tendency to dimerize and appears to be distinct from the cell surface fibronectin receptors which interact with the Arg-Gly-Asp recognition site in the fibronectin molecule.

Fibroblast; Fibronectin-binding protein; Protein isolation; 36 kDa protein

1. INTRODUCTION

Fibronectin is a high-molecular-mass glycoprotein composed of two similar non-identical subunits of 240 kDa. A characteristic feature of fibronectin is its wide distribution; it is found in soluble form in plasma and other body fluids and in insoluble form in tissues and the pericellular matrix of cultured cells [1]. Fibronectin is also well known for its many interactions, including those with fibrin [2], collagen [3], heparan sulfate proteoglycan [4] and cell surfaces [5]. It can mediate the interaction between cells and extracellular matrices. The binding sites for these and many other interactions have been located to the different domains of the fibronectin molecule; the best characterized is the Arg-Gly-Asp (RGD) cell recognition sequence [6] in the COOH-terminal half within one of the 'type III' internal repeats in fibronectin. Derivatives of this peptide inhibit the adhesion of cells to fibronectin and, if used as an insoluble substrate, can simulate the action of the whole molecule.

In human osteosarcoma cells [7], several fibroblasts [8–11], and murine erythroleukemia cells [12] glycoproteins composed of two different 120–160 kDa subunits interact specifically with fibronectin and have been proposed to represent fibronectin receptors on the cell surface. In human platelets the membrane glycoprotein IIb/IIIa (subunits of 142 and 95 kDa) interacts specifically with fibronectin [13,14]. All these interactions are inhibited by the RGD cell recognition peptides. This sequence may not, however, be the only region in the fibronectin molecule that binds to cell surfaces; the two heparin-binding domains in the NH_2- and COOH-terminal regions, respectively, possibly carry a similar function. Interaction of fibronectin with plasma membrane heparin sulfate proteoglycan may be involved in cell spreading and cytoskeletal stabilization [15]. We now report the isolation of a 36 kDa fibronectin-binding protein present in normal human fibroblasts. This protein

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Abbreviations: DOC, deoxycholate; EIA, enzyme immunoassay; PBS, 0.01 M phosphate-buffered, 0.15 M saline, pH 7.4; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Tween, polyoxyethylene sorbitane monolaureate 20

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appears to be distinct from the previously identified fibronectin-binding proteins.

2. MATERIALS AND METHODS

2.1. *Cell cultures, fibronectins and synthetic peptide*

Fibronectins were purified from human plasma and serum-free medium of cultured human embryonic fibroblasts using gelatin- and arginine-Sepharose, as described [16]. In SDS-PAGE under reducing conditions purified fibronectins (5 μg), gave single bands at 250 kDa. The human fibrosarcoma cell line HT-1080 was from the American Type Culture Collection (CCL 121; Rockville, MD). The synthetic peptide GRGDSP was provided by Dr M.-L. Huhtala (Labsystems, Helsinki) and was used at 1 mg/ml.

2.2. *SDS-PAGE and blotting experiments*

SDS-PAGE was performed on 5–16% acrylamide slab gels according to Laemmli [19]. Immunoblotting of proteins was performed using immunoperoxidase staining [20] and primary antibodies as follows: polyclonal anti-fibronectin [16] at 1:1000, peroxidase-labeled anti-fibronectin at 1:400, monoclonal f-33 [21] and f-42 [18] anti-fibronectin IgG_1 at 10 μg/ml, and polyclonal anti-36 kDa protein at 1:250. Proteins transferred to nitrocellulose filters were incubated for 16 h with purified human plasma fibronectin (50 μg/ml) in Tween-containing buffer. The filters were then washed and immunoblotted.

If, instead of blotting with native fibronectin, ^{125}I-fibronectin or biotinylated fibronectin combined with peroxidase-avidin was used, a great variety of 'fibronectin-binding' proteins were seen.

2.3. *Binding experiments*

Proteins were immobilized onto polystyrene microtiter wells from 4 μg/ml (fibronectin or albumin) or 400 ng/ml (36 kDa protein) using previously described conditions [21,22]. The binding to immobilized protein was studied by adding a volume of 75 μl of different concentrations of purified protein. Tween (0.02%) was included to prevent nonspecific binding and to minimize hydrophobic interactions. The antibody-binding activity of solid-phase-bound ligand protein was assayed using diluted antibodies (polyclonal anti-fibronectin, 1:1000; polyclonal anti-36 kDa protein, 1:250; monoclonal f-33 anti-fibronectin IgG_1, 10 μg/ml) in EIA [21,22]. In all experiments, either the soluble or solid-phase proteins were replaced, as a control, with bovine serum albumin or buffer control. When the specific antibodies were omitted or replaced with the respective preimmune rabbit sera or a 10 μg/ml concentration of the anti-trinitrophenyl monoclonal antibody [22] no reaction occurred.

2.4. *Isolation of the 36 kDa protein and immunization*

Confluent cultures of fibroblasts were rinsed twice with ice-cold PBS, and 1 ml of 10 mM Tris-HCl (pH 8.0) buffer containing 0.5% (w/v) DOC and 1 mM phenylmethylsulfonyl fluoride was added per 175 cm^2 culture flask tilted gently for 10 min at ± 0°C. The DOC-soluble material was collected and clarified by low-speed centrifugation in the cold.

Preparative SDS-PAGE under nonreducing conditions was used to prepare the antigen for immunization. After SDS-PAGE the proteins were transferred electrophoretically onto a nitrocellulose sheet. A track was cut off to locate the 36 kDa protein using blotting with fibronectin. A slice of the sheet containing the 36 kDa protein was dissolved in dimethyl sulfoxide and used to immunize rabbits. IgG of the anti-36 kDa protein rabbit serum was isolated by conventional methods.

For the binding experiments the 36 kDa protein was purified by a modification of the above procedure. ^{14}C-labeled molecular mass marker proteins (Amersham International) were included in the sample. A wet preparative SDS-PAGE slab gel was dried on a cellophane dialysis membrane (LKB, Bromma, Sweden), autoradiographed to locate the proteins and the relevant area cut out and dissolved in 10 mM NH_4HCO_3 containing 0.01% SDS. Electroelution was carried out using a model 1750 electrophoretic sample concentrator (ISCO, Lincoln, NE) at 1 W (7 mA) for 3 h at 4°C.

2.5. *Two-dimensional electrophoresis*

The procedure described by Pollard [23], involving isoelectric focusing in the first dimension and

SDS-PAGE (8% polyacrylamide) in the second, was followed using LKB ampholines (pH 6–8.5).

3. RESULTS AND DISCUSSION

Normal human fibroblasts can be expected to contain not only the putative plasma membrane receptors but also fibronectin-binding proteins in the extracellular matrix in which fibronectin is a major component. To separate these two classes of molecules, we used a modification of the deoxycholate procedure [24] developed to isolate the matrix of cultured fibroblasts.

The proteins were separated by SDS-PAGE under non-reducing conditions and transferred to nitrocellulose filter, blotted with purified unlabeled human plasma fibronectin and the bound fibronectin was detected using immunoperoxidase staining. If whole cell layers or matrix material (fig.1A,B) were studied a number of fibronectin-binding proteins were detected: major bands at 480 (comigrating with matrix fibronectin), 160, 140, 90, 47 and 36 kDa. In contrast, only a few fibronectin-binding proteins were seen (fig.1B, lane 2) in the DOC-soluble fraction. These migrated at 160, 140, 47 and 36 kDa. As shown in fig.1C the bands at 160, 140 and 36 kDa could be detected both directly with peroxidase-conjugated polyclonal anti-fibronectin and with two monoclonal anti-fibronectins followed by anti-mouse IgG peroxidase. In different experiments the 36 kDa protein was always present while the others were sometimes weakly stained or absent.

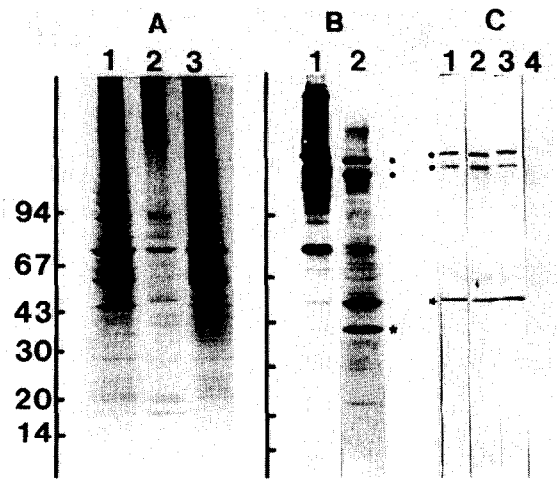

Fig.1. SDS-PAGE of human fibroblast proteins. (A) Protein staining: 1, total proteins; 2, isolated matrix; 3, DOC-soluble material. (B) Fibronectin blotting using polyclonal antibodies: 1, isolated matrix; 2, DOC-soluble material. (C) Fibronectin blotting of DOC-soluble material: 1, peroxidase-labeled polyclonal antibodies; 2, F-33 monoclonal antibodies; 3, F-42 monoclonal antibodies; 4, control without fibronectin. The two dots indicate the positions of 160 and 140 kDa, respectively, and the asterisk 36 kDa. Molecular masses of standards are indicated on the left.

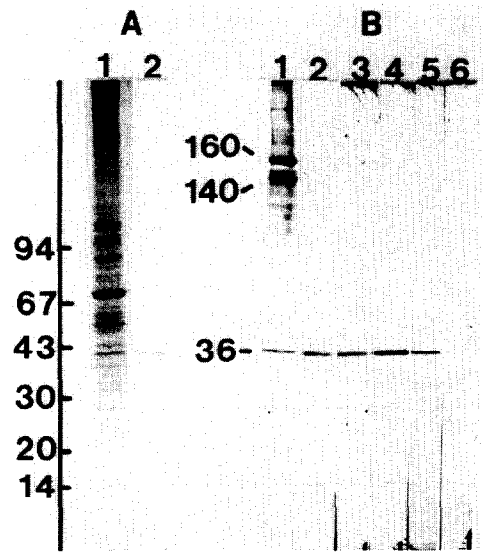

Fig.2. (A) Protein staining of SDS-PAGE: 1, starting material (DOC-soluble fraction); 2, 36 kDa protein purified by preparative SDS-PAGE. (B) Fibronectin blotting: 1, starting material; 2, purified 36 kDa protein. Immunoblotting with anti-36 kDa protein of total proteins from fibroblasts (3) and from HT-1080 sarcoma cells (4), and from fibroblasts with anti-36 kDa protein IgG (5) and with preimmune IgG (6).

The DOC-solubilized fraction of fibroblasts was used to develop a purification procedure for the 36 kDa protein. The two-phase TX-114 detergent separation system [25] was used to define whether this protein is hydrophobic or hydrophilic. The protein was found in both phases. Gel chromatography on Fractogel in the presence of detergent gave several broad peaks and irreproducible results (^{125}I-labeled 36 kDa protein used as marker). In hydrophobic interaction chromatography the protein was not recovered in

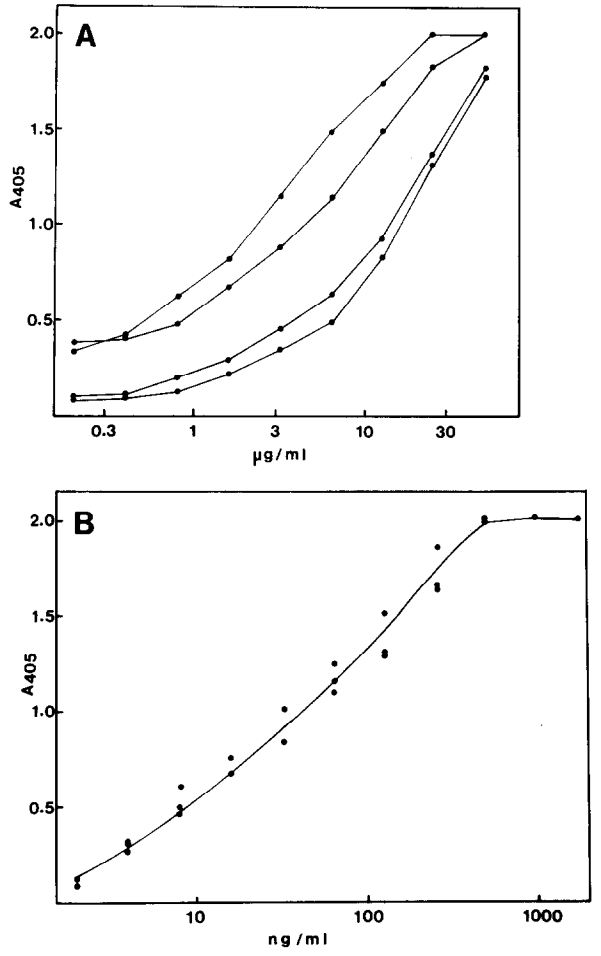

Fig.3. Binding of different concentrations of fibronectin to purified 36 kDa protein immobilized on polystyrene. Bound fibronectin was detected using EIA. (A) Plasma fibronectin and four batches of 36 kDa protein. (B) Cellular fibronectin and purified 36 kDa protein.

the eluants while both the 160 and 140 kDa fibronectin-binding proteins bound to octyl- and phenyl-Sepharose CL-4B. We intended then to use reverse-phase HPLC but the protein lost its fibronectin-binding capacity in acetonitrile. Thus, preparative SDS-PAGE turned out to be the only method applicable (fig.2A). Serum of a rabbit immunized with purified 36 kDa protein reacted specifically with it in immunoblotting of cell extracts (fig.2B). It also reacted with polystyrene wells coated with 36 kDa protein, as judged by EIA.

The purified 36 kDa protein was immobilized onto polystyrene microtiter wells to study binding. As shown in fig.3A, these wells bound fibronectin in a dose-dependent manner. The detection limit for fibronectin varied between 40 and 400 ng/ml for the different 36 kDa protein preparations. Coating of the wells with as little as 4 ng/ml of the 36 kDa protein produced wells able to bind fibronectin specifically as detected in EIA. Wells coated with 4 μg/ml of fibronectin but not uncoated wells or those coated with albumin bound the 36 kDa protein, as detected using anti-36 kDa protein in EIA. The detection limit was 30 ng/ml of 36 kDa protein. Cellular fibronectin, purified from the conditioned culture medium of human fibroblasts, also bound to immobilized 36 kDa

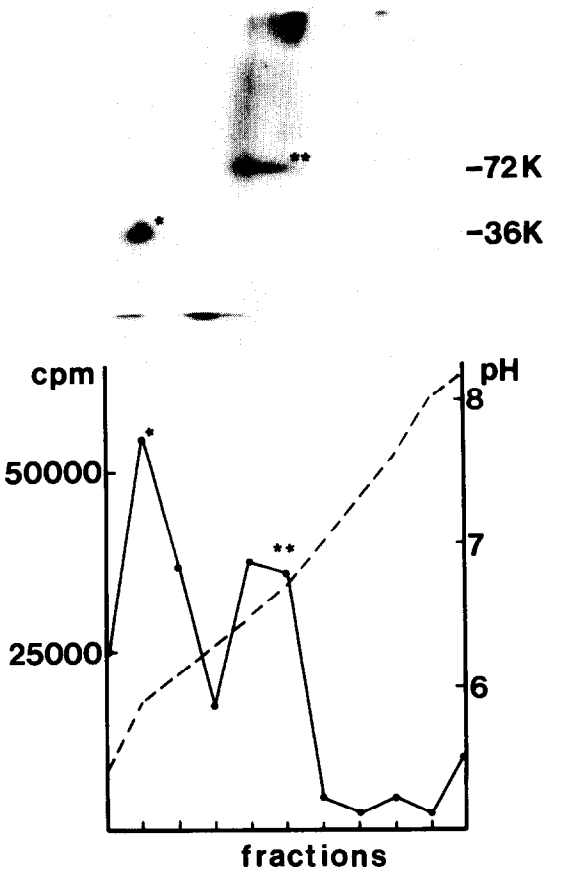

Fig.4. Two-dimensional electrophoresis of purified ^{125}I-labeled 36 kDa protein. The positions of the monomer and dimer are indicated by one and two asterisks, respectively.

protein (fig.3B). Since control experiments with polyclonal anti-fibronectin antibodies showed that they detect immobilized human plasma and cellular fibronectins with equal sensitivity, the binding curves for plasma and cellular fibronectins could be compared. The comparison suggests that cellular fibronectin binds better to the 36 kDa protein.

The synthetic peptide GRGDSP did not affect the binding of fibronectin to immobilized 36 kDa protein. Increasing concentrations of NaCl inhibited the binding, 0.3 M NaCl giving 50% inhibition compared to the standard conditions (0.15 M NaCl). The reaction was maximal at pH 5–6 and was inhibited at pH $\leq$ 4 or pH $>$ 8.

Migration of the 36 kDa protein in SDS-PAGE under non-reducing and reducing conditions suggests that it is a monomeric protein partially dimerized under non-reducing conditions. When stored at 4°C it partly aggregated into a dimeric form. The 36 kDa protein is quite stable, no degradation products having been observed. The protein was regularly stored in the presence of DOC; its behaviour in gel chromatography, among other observations, indicated that it is relatively insoluble. According to the two-phase separation experiments it is amphipathic; it was found to be separated into both the hydrophilic and hydrophobic phases. The isoelectric point (fig.4) of monomeric 36 kDa protein is pH 5.9 and that of its dimeric form (72 kDa) pH 6.8.

Here, we describe a novel fibronectin-binding 36 kDa protein of normal human fibroblasts. When fibroblast proteins were separated by SDS-PAGE and transferred onto nitrocellulose, fibronectin bound to three proteins of 160, 140 and 36 kDa. Purified 36 kDa protein bound both purified plasma and cellular fibronectins. In the approach we have taken, using blotting with fibronectin, we also detected 160 and 140 kDa proteins which may be identical to those described by others (see above). The antibodies raised (anti-36 kDa protein) reacted only with the 36 kDa protein and not with the other two. This indicates that the 36 kDa protein is a distinct protein and not a degradation fragment of the others.

Why was the 36 kDa fibronectin-binding protein not detected previously? One reason could be that this protein is quite adhesive. It could not be purified by chromatographic methods. Detergent was necessary to maintain the protein soluble and prevent its binding to insoluble cellular material. A second reason is that the protein was detected only by native fibronectin. For instance, iodination of fibronectin – previously known to modify fibronectin's interactions [26] – could not be used in the detection of this fibronectin-binding protein.

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