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Expression of the cell-binding domain of human fibronectin in *E. coli*

Identification of sequences promoting full to minimal adhesive function

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Two cDNA subfragments containing the cell-attachment site of human fibronectin (FN) were expressed as β-galactosidase fusion proteins in *E. coli*. The products were purified to homogeneity by monoclonal antibody affinity chromatography and assayed for activity in a standard cell-adhesion assay. A fusion protein containing an 80 kDa fragment of human FN appeared functionally equivalent to intact FN purified from human plasma, whereas a truncated fusion protein of 33 kDa still containing a previously postulated cell-attachment site was approx. 50-fold less active. Our study establishes a system for analyzing adhesive protein function by DNA manipulation, rules out any major role for eukaryotic post-translational modifications in FN adhesive function, and localizes additional functional activity to a 1.3 kb region.

Fibronectin; cDNA; Cell adhesion; (*E. coli*, Human)

1. INTRODUCTION

The glycoprotein fibronectin (FN) is a multifunctional extracellular protein composed of several functional domains, including sites for binding to fibrin, collagens, heparin, and the cell surface [1–4]. The latter cell-binding or adhesive recognition site is of particular interest, since inhibitors of FN interactions with cells can inhibit crucial steps in embryonic development or metastasis (e.g. see references in [5]). The sequence (Gly)-Arg-Gly-Asp-Ser is a key cell-attachment site [4]. However, this sequence and an 11.5 kDa pepsin fragment containing the putative cell-attachment site show 10–100-fold lower affinities for the FN receptor than intact FN or fragments of ⩾75 kDa [6].

The primary sequence information and post-translational modifications required for FN adhesive function might be analyzed most effectively using an expression system in which protein length and primary sequence could be controlled at will, i.e. by DNA manipulation. We report here the expression of cell-binding domain sequences of human FN in *E. coli* under the control of the right arm promoter of the λgt11 phage vector. One of the affinity-purified products appears to be nearly functionally equivalent to the native human FN molecule in a standard adhesion assay. We also examine for functional roles of the amino-terminal region of this domain and of post-translational modifications.

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2. MATERIALS AND METHODS

2.1. *Preparation of FN cDNA clone pFH60*

An FN cDNA clone spanning 60% of the total FN protein sequence (pFH60) was prepared by ligating two existing cDNA clones, pFH1 and pFH154 [7]. The *Cla*I-*Bam*HI fragment (2 kb) of pFH1 was subcloned into pUC19 as were the *Sac*I-*Bam*HI (1.2 kb) and *Hin*dIII-*Bam*HI (1.3 kb) fragments of pFH154. The pFH1 insert was excised with *Sac*I-*Kpn*I and ligated to the 1.3 kb pFH154 insert, then to the 1.2 kb insert in pUC19.

2.2. *Construction and antibody screening of λgt11 subclones*

The cDNA inserts isolated by double digestion of pFH60 DNA with *Hin*dIII-*Eco*RV or *Bam*HI-*Eco*RV were subcloned at the *Eco*RI site of the λgt11 vector as described by Young and Davis [8] using a mixture of 8-, 10- and 12-mer *Eco*RI linkers (Pharmacia) to obtain a correct reading frame.

Recombinant phages producing β-galactosidase-fused human FN segments in *E. coli* strain Y1090 were identified using rabbit anti-human plasma FN antibody with the ProtoBlot λgt11 immunoscreening system (Promega Biotec).

2.3. *Isolation and purification of β-galactosidase fusion proteins*

Fusion proteins expressed in *E. coli* were analyzed by inducing lysogens in strain Y1089 [8]. The *E. coli* cells were lysed by incubating with lysozyme (2000 U/ml of original culture) and sonication (2 min) in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (STE), adjusted to 8 M urea in STE, incubated for 15 min at 5°C, and clarified by ultracentrifugation. The supernatants were mixed with an equal volume of STE and stored at −85°C.

FN-containing fragments were purified using monoclonal antibody 333 [6] coupled to Sepharose 4B. After incubation for 2 h at 5°C, unadsorbed proteins were eluted with 2 M urea in STE and then with STE. The fusion proteins retained by the affinity matrix were eluted with 0.1 M diethylamine (pH 11.4), and adjusted to pH 8.0 with 0.1 M Tris-HCl. Purity of the purified proteins was analyzed by SDS-polyacrylamide gel electrophoresis [6].

2.4. *Subcloning and DNA sequencing*

Purified recombinant phage DNA was digested with *Eco*RI and subcloned into M13 vectors. DNA sequencing was performed by the method of Sanger et al. [9] using [α-^{35}S]thio-dATP (Amersham).

3. RESULTS AND DISCUSSION

Two human FN cDNA fragments containing the region encoding the (Gly)-Arg-Gly-Asp-Ser cell-binding site and adjacent sequences were subcloned into the *Eco*RI site of the λgt11 phage vector to obtain expression as β-galactosidase fusion proteins (fig.1). Lysogen clones of each recombinant phage in *E. coli* were isolated and cultured in the presence of IPTG to induce the production of fusion proteins.

Fig.2 shows high production of each fusion protein; for example, the λCBD20 clone produced a product accounting for approx. 10% of total *E. coli* protein. The fusion proteins were purified to apparent homogeneity according to SDS-polyacrylamide gel electrophoresis by affinity chromatography using immobilized monoclonal antibody 333 (fig.2); this rat monoclonal antibody binds near the cell-binding site and is known to inhibit fibroblast adhesion to human FN [6].

These highly purified proteins were assayed for biological activity with BHK cells using a standard adhesion assay measuring the extent of cell spreading [6]. FN purified from human plasma was used as a standard in the assay. As shown in table 1, the λCBD1 fusion protein containing

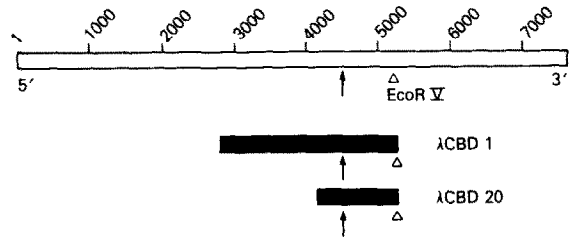

Fig.1. Schematic representation of λgt11 clones containing the cell-binding domain of human FN. The pFH60 cDNA clone covering ~60% of the mature mRNA (▭, see [7]) was subcloned into λgt11 for expression. Subclones (▬) contained bp 2813–5172 (*Eco*RV, Δ) for λCBD1 or bp 4086–5172 for λCBD20, respectively. Arrow indicates cell-attachment site.

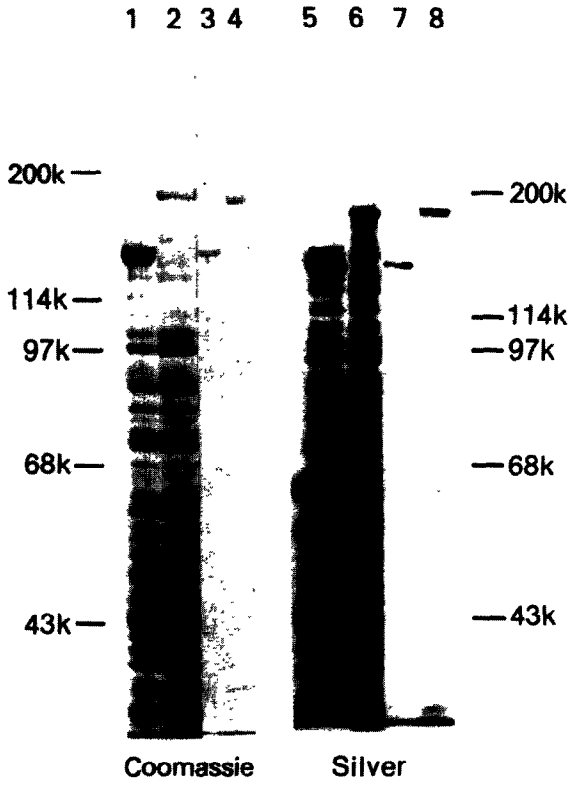

Fig.2. Purification of fusion proteins from two human FN cDNA subclones. Fusion proteins were expressed, purified, and analyzed electrophoretically (see text). Lanes: 1,5, IPTG-induced clone λCBD1 lysate; 2,6, IPTG-induced clone λCBD20 lysate; 3,7, affinity-purified λCBD1 fusion protein; 4,8, affinity-purified λCBD20 fusion protein. Proteins were visualized by silver staining (lanes 1–4; Bio-Rad) or Coomassie blue (lanes 5–8). The blurry 'band' of ~60 kDa seen in lanes 5–8 is a silver-staining artifact also found in blank sample buffer lanes.

~32% of the total FN cDNA sequence was nearly as active on a molar basis as fibronectin itself. In contrast, the λCBD20 fusion protein containing the entire sequence of a previously postulated cell-attachment fragment [10], but missing adjacent N-terminal sequences encoded by bp 2813–4086 in GeneBank file HUMFNMC [7], displayed a 50–60-fold loss of activity. Since ELISA assays of adsorbed proteins confirmed that these proteins attached equally to substrates (not shown), our results appear to provide the most direct evidence to date for an important functional role for this 1.3 kb region. In addition, these results using *E. coli* indicate that all of the eukaryotic post-translational modifications of FN [1–3], including *N*- and *O*-linked glycosylation and phosphorylation, do not appear to be required for expression of virtually full functional activity of FN in this standard assay. The slight reduction of activity we observe may result from absence of *N*-linked carbohydrates [11]. This system should permit further detailed analyses of the molecular basis of FN function.

Table 1

Activities of fusion proteins[a]

Protein	Specific activity (50% cell spreading/pmol)			Relative specific activity
	Expt 1	Expt 2	Average, expts 1,2	
Fibronectin	1.67	1.00	1.34	74
λCBD1	1.18	0.78	0.98	54
λCBD20	0.019	0.017	0.018	1
β-Galactosidase	0.00	0.00	0.00	0

[a] An average of 1000 cells were counted for each protein concentration assayed (~7000 cells/protein)

CBD, cell-binding domain

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