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DNA-BINDING DOMAINS OF HUMAN FIBRONECTIN

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SUMMARY. Here we report the use of the technique of transferring proteins from polyacrylamide gels to nitrocellulose sheets to identify the DNA - binding domains of human plasma fibronectin. After separation of fibro - nectin and its tryptic digest on a sodium dodecylsulfate-polyacrylamide gel , the polypeptides were transferred to nitrocellulose sheets and the DNA - binding polypeptides were identified by incubation of the nitrocellulo se sheets with ^{32}P-labeled human DNA followed by autoradiography.

The following results were obtained: a) only two fibronectin trypsin resistant peptides (50 and 60 kd) showed DNA-binding capacity; b) in competition experiments a 300 fold excess of E. coli DNA did not inhibit the human DNA-human fibronectin binding, indicating the specificity of the interaction.

INTRODUCTION. Fibronectins are a family of immunologically related high molecular weight glycoproteins that are present in vertebrates in both soluble and insoluble forms. The soluble form is present in plasma and other body fluids while the insoluble form is found in the pericellular matrix and basement membranes together with collagen, glycosaminoglycans, laminin and other components.

The biological functions of fibronectin are still a matter of speculation, but in in vitro systems, fibronectin exerts many different effects (1-8 and references therein). In cell cultures fibronectin is secreted into the medium or deposited in the pericellular fibrillar matrix. In malignantly transformed cells the amount of fibronectin deposited in the pericellular matrix is generally greatly reduced. Fibronectin promotes cellular aggregration, cell substratum adhesivness, partially reverts trans-

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formed fibroblasts to a more normal phenotype, promotes locomotion of certain types of cells and facilitates reticuloendothelial system clearance of particles and is a potent chemoattractant for cultured fibroblasts (9).

Fibronectin has been shown to interact with cells and other molecules such as collagen, glycosaminoglycans, polyamines and actin. Some of the domains responsible for these interactions have been located on the fibronectin molecule. Several lines of evidence indicate that fibronectin is composed of several rigid domains which are connected by flexible protease-sensitive polypeptide segments (1-8). Much effort has been made to separate structural domains without losing their biological activities. In all these studies, fibronectin fragments were purified by affinity chromatography prior to their analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (1-8, 10-15 and references therein).

We have recently demonstrated that human plasma fibronectin binds to DNA with an equilibrium constant of 4.6×10^{-6} M (16). In this paper we have studied whether fibronectin contains specific DNA-binding domains. We have employed the bidirectional "protein blotting" technique (17) to determine which tryptic peptides contain the DNA-binding domains. This new approach has the great advantage that minute quantities of peptides can be directly analysed for their interaction with macromolecules after blotting on nitrocellulose filters, thus eliminating purification of the various peptides by affinity chromatography as required by the more traditional approach.

MATERIAL AND METHODS

Purification of fibronectin and digestion with trypsin. Human plasma fibronectin was isolated by affinity chromatography on gelatin-Sepharose 4B as previously described (18-19). Purified fibronectin (1mg/ml) was digested at 37°C for 20 minutes with increasing amounts of trypsin (chymotrypsin free; Millipore, Freehold, N.J.). After digestion, soybean trypsin inhibitor (Boehringer, Mannheim, West Germany) was added to an equimolar concentration (see legend Fig. 1A).

Protein blotting and binding of fibronectin to ^{32}P-labeled human DNA. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (20) using vertical slab gels. Polypeptides were transferred onto Schleicher-Schuell Ba 85 nitrocellulose filters (Schleicher-Schuell, Keene,New Hampshire) by the bidirectional diffusion method previously described by Bowen et al. (17). After transfer of the proteins, nitrocellulose filters were washed in prebinding buffer (50 mM NaCl, 0.2% Ficoll, 0.2% polyvinyl pyrollidone, 10 mM Tris, 1 mM EDTA pH 7.5) for 30 minutes at room temperature. The prebinding buffer was removed and 15 ml of binding buffer (50 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM Tris,

1mM EDTA pH 7.5 and, when appropriate, 15 μg/ml of sonicated unlabeled E. coli DNA 1 kb in length) was added. The binding buffer also contained 50 ng/ml ^{32}P-"nick-translated" (21) human DNA ($1x10^7$ cpm/μg) with an average length of 1 kb either as native (double-stranded) or heat-denatured (single-stranded) DNA. The filters were incubated on an orbital shaker for 5 hr at room temperature, whereafter they were washed 4 times 15 min. each in 100 ml of 0.25 M NaCl, 10 mM Tris, 1 mM EDTA pH 7.5 and then left overnight to wash in an additional 100 ml of the same buffer. The filters were dried at room temperature and exposed at -70°C using XAR-5 film (Kodak, Rochester, New York) and an intensifying screen (Dupont-lighting plus).

Mini-filter assays. Either 1 μg fibronectin or 0.1 μg calf-thymus histones were spotted on 5 mm diameter Schleicher-Schuell Ba 85 filters. Prebinding and binding of ^{32}P-DNA were as described above. For more details see Figure legends.

RESULTS

Interaction of double and single stranded ^{32}P-DNA with fibronectin and its tryptic digest.

Fig. 1A shows SDS-PAGE patterns of fibronectin digested with increasing amounts of trypsin. The polypeptides range from full length fibronectin (220 kd) down to 20 kd fragments at the dye front (Fig. 1A). The polypeptides were transferred from the SDS-PAGE to a nitrocellulose filter by diffusion using the procedure described by Bowen et al.(17) with a yield ranging from 50 to 75%. The bidirectional diffusion ("sandwich") technique gives two faithful images of the gel with equal amounts of protein on each filter. Fig. 1B shows two of these filters obtained by blotting proteins from the other half of the gel shown in Fig. 1A and stained with amido black.

The nitrocellulose filter containing transferred polypeptides were incubated with ^{32}P-labeled double-stranded human DNA and washed as described (see Materials and Methods). The bound labeled DNA was detected by autoradiography. Only a few polypeptides of the tryptic digest interacted with DNA. The major DNA binding fragments have molecular weights of: 150, 125, 110, 85, 60 and 50 kd; only two faint bands of 40 and 42 kd were observed (Fig. 2A). Note that a basic protein such as lysozyme (Lane6 and 7,Fig 2) did not bind the radioactive DNA in presence of excess E. coli DNA.

The 50 kd and 60 kd polypeptides which are not predominant bands as visualized by amido black or Coomassie blue staining bind much more labeled human DNA than other polypeptides which are present in larger amounts. For example, the two heavily stained 35 kd and 55 kd bands indicated by arrows in Fig. 1A do not bind DNA.

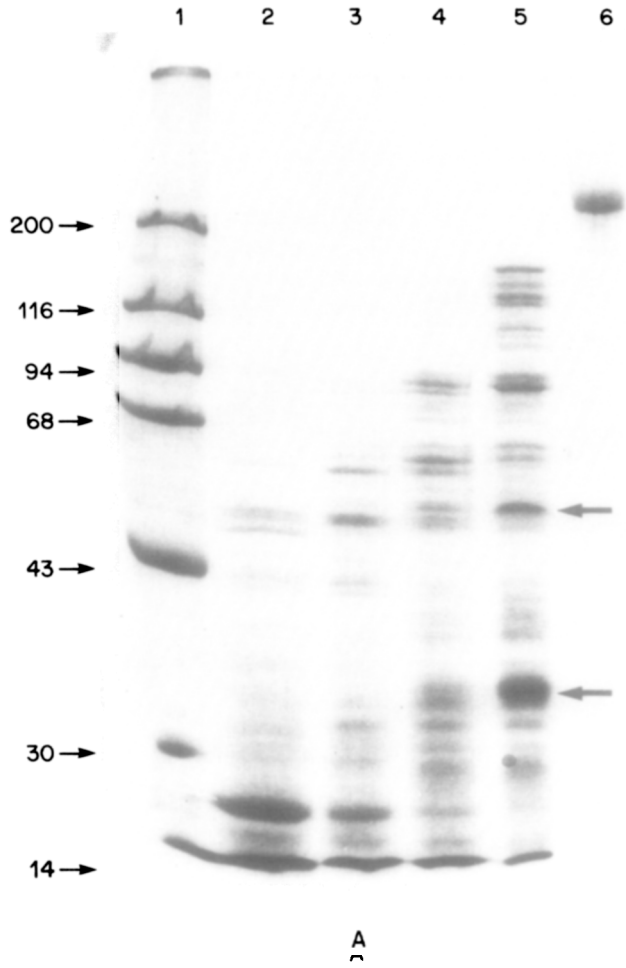

Figure 1 A: SDS-PAGE of fibronectin digested with different amounts of trypsin and stained with Coomassie brilliant blue. Lane 6: undigested fibronectin; lane 2 to 5: 1 mg/ml of human plasma fibronectin in 0.05 M Tris-HCl, 1 mM $CaCl_2$, pH 7.2 digested with 640, 160, 40 and 10 µg/ml of trypsin. After digestion, equimolar amounts of soybean trypsin inhibitor were added. Either 5 µg of undigested or 50 µg of trypsin digested fibronectin were loaded per well onto a 10% SDS-PAGE; lane 1: molecular weight standards, myosin 200 kd, β-galactosidase 116.5 kd, phosphorylase 94 kd, bovine serum albumin 68 kd, ovalbumin 43 kd, carbonic anhydrase 30 kd, lysozyme 14.4 kd. After electrophoresis, one half of the gel was stained in 0.15% Coomassie blue R-250. The other half of the gel was soaked in 4 M Urea buffer for 3 hr and transferred onto nitrocellulose filters by bidirectional diffusion. B: Nitrocellulose filters after transfer of polypeptides from the other half of the 10% SDS-PAGE shown in Fig. 1A. The filters were stained with amido-black. The protein transfer by bidirectional diffusion gives two mirror images with equal amounts of proteins on both filters. Lane 1 and 12: molecular weight markers; lane 2 and 11: undigested fibronectin. Lane 3 to 6 and 10 to 7: fibronectin digested with increasing amounts of trypsin as described for Fig. 1A.

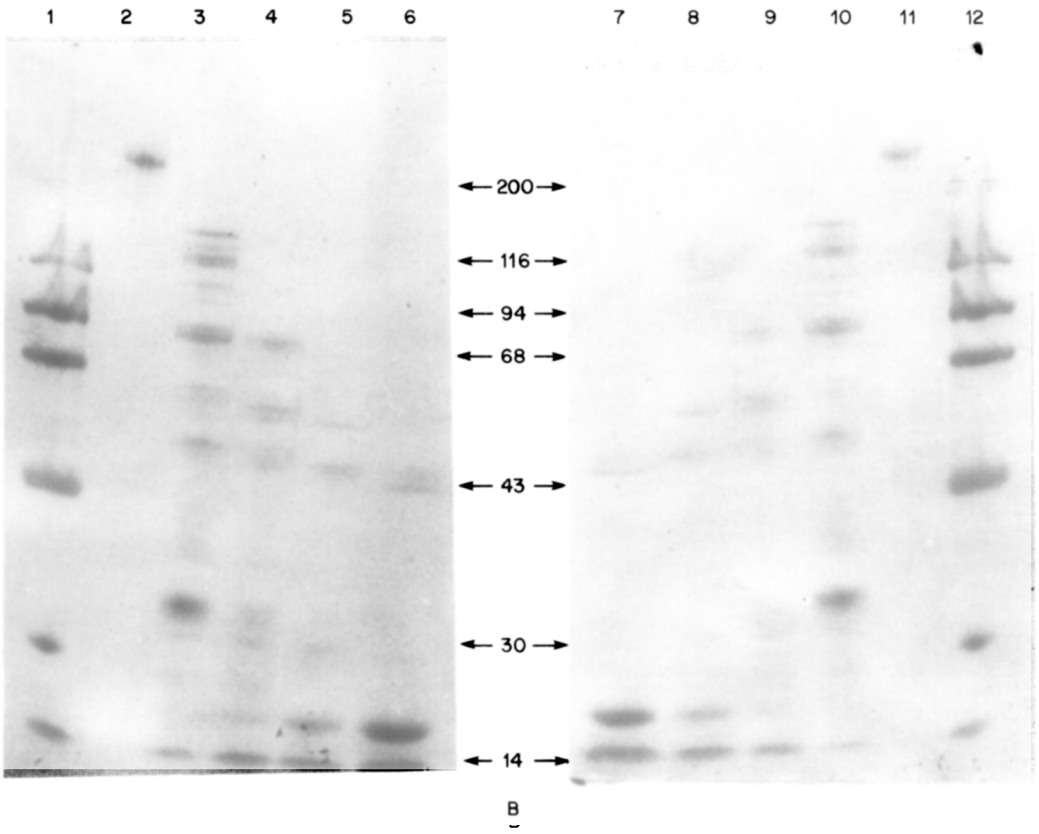

Figure 1 continued.

The binding of single stranded DNA to fibronectin and its tryptic fragments was also tested using the same technique. Figure 2B shows a nitrocellulose filter containing an equal amount of protein as on the filter shown in Fig. 2A (from the same blotting), incubated with ^{32}P-single-stranded DNA having the same specific activity as the double-stranded probe. ^{32}P-single-stranded DNA does not bind to the 50 kd fragment but does bind to the 85 and 60 kd fragments. However, these fragments do not seem to bind as efficiently to the single-stranded DNA as they do to the double-stranded DNA probe.

Specificity of the interaction between DNA and fibronectin.

Fig. 3 shows a comparative study of the binding of native human DNA to fibronectin and histones in the presence and absence of competitor E. coli DNA.

Equimolar amounts of fibronectin or histones were spotted onto nitrocellulose filters. Five ng of ^{32}P-labeled human DNA (50 ng/ml) were in-

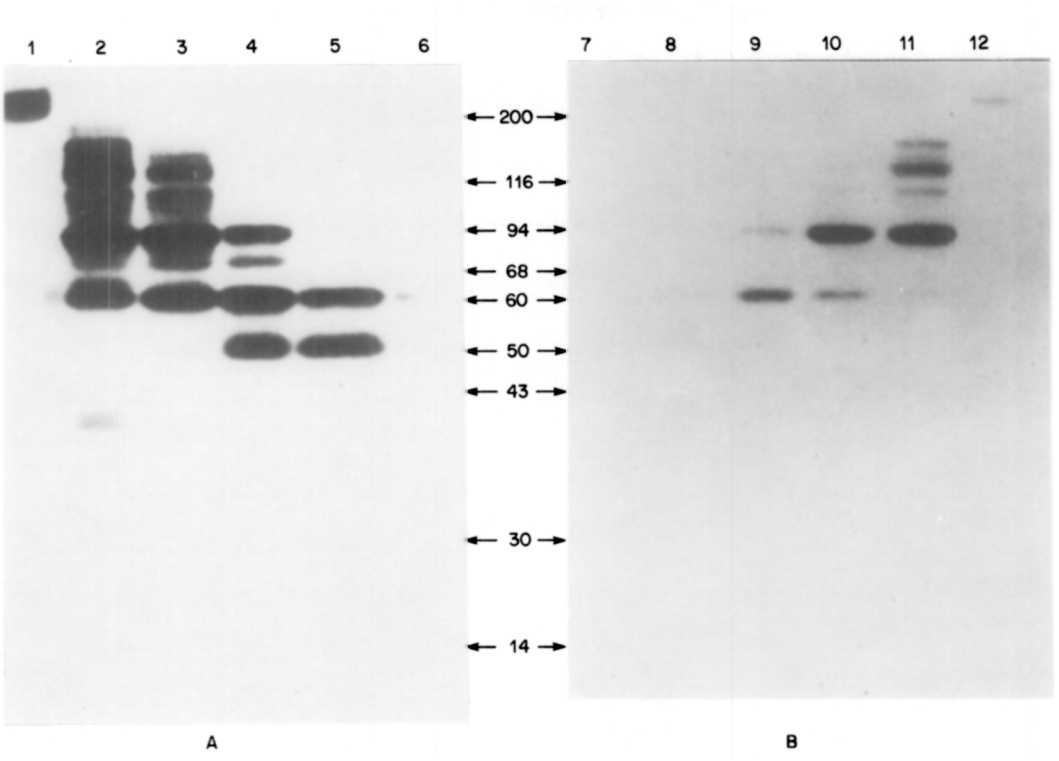

Figure 2: Binding of double and single-stranded ^{32}P-labeled DNA. The nitrocellulose filters were incubated for 5 hr with 50ng/ml ^{32}P-"nick-translated" human DNA (1 x 10^7 cpm/μg) either as native (double-stranded)DNA (A) or heat denatured (single-stranded) DNA (B) in the presence of 15 μg/ml of cold E.coli double or single-stranded DNA, respectively. The filters were washed and dried as described in Materials and Methods and subsequently exposed for 1 hr at - 70°C using XAR-5 film (Kodak) and an intensifying screen (Dupont-Lightning plus). Lane 1 and 12: undigested fibronectin, lane 2 to 5 and 11 to 8: fibronectin digested with increasing amounts of trypsin as described for Fig. 1A. Lane 6 and 7: molecular weight standards.

cubated with each filter for increasing lengths of time either in the presence or absence of 15 μg/ml (300 fold excess) of E coli DNA.

In the absence of E. coli DNA 90% of the radioactive human DNA was bound to histones and 25% to fibronectin after 5 hrs of incubation. In the presence of E. coli DNA, the binding to histones was reduced by about 30 fold, while the binding to fibronectin was lowered by 2-4 times (at 5 hr). When human DNA was added as competitor at the same concentration and average length as the E. coli DNA, the binding of ^{32}P-human DNA to histones and fibronectin was completely inhibited (Fig. 3).

DISCUSSION

Earlier we have reported that antichromatin antibodies from cultured fibroblasts showed specificity for an homologous serum protein (22).

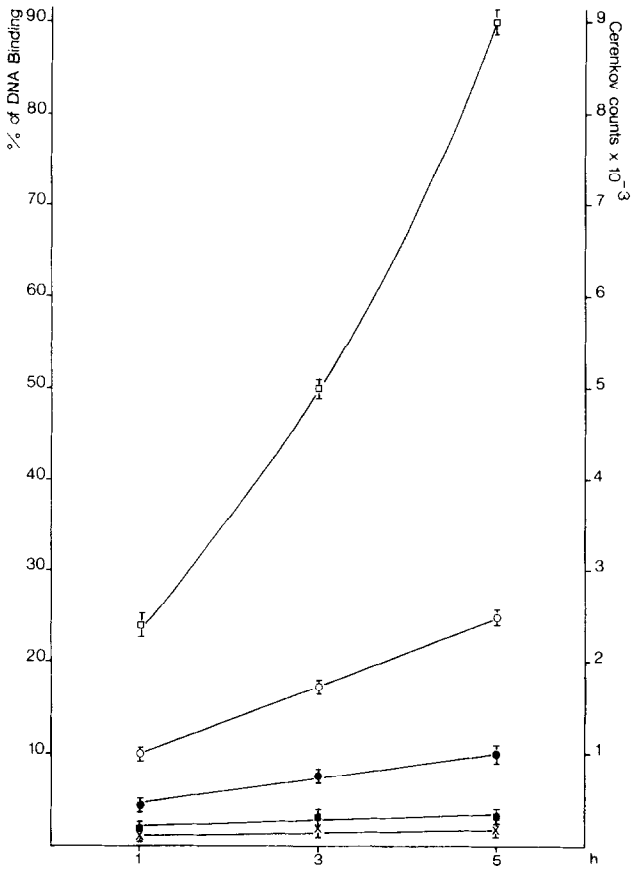

Figure 3: Comparison of binding of native ^{32}P-labeled human DNA to fibronectin and histones in the presence and absence of competing unlabeled E. coli or human DNA. An equal number of Cerenkov counts were incubated with either 1µg of fibronectin or 0.1 µg (equimolar concentration) of calf thymus histones spotted on 5 mm diameter nitrocellulose filters. Binding (1, 3, 5 hr) and washing conditions were as in Fig. 2. The filters were dried before counting. Backgrounds were approximately 25-50 Cerenkov counts and were subtracted from the values shown.

○——○ ^{32}P human DNA bound to fibronectin in absence of competitors.

■——■ ^{32}P human DNA bound to fibronectin in the presence of 15µg/ml of E. coli DNA as competitor.

□——□ ^{32}P human DNA bound to histones without competitors.

■——■ ^{32}P human DNA bound to histones in the presence of 15 µg/ml of E. coli DNA as competitor.

×——× ^{32}P human DNA bound to fibronectin in the presence of 15µg/ml human DNA as competitor.

Subsequently this protein was identified as fibronectin which was found to be a DNA-binding protein in in vitro systems (16).

Now using the technique of blotting proteins from SDS-PAGE to nitrocellulose sheets we have studied the DNA-binding capacity of native fibronectin and its tryptic digest. We found the same DNA-binding pattern

for both reduced and unreduced fibronectin (data not shown). Thus sulfhydryl-groups do not appear to be important for DNA-binding.

A minimum size of the DNA-binding fragments has been observed. Neither mild (10 μg/ml) nor extensive (640 μg/ml) tryptic digestion produced a DNA-binding fragment smaller than 50 kd. Extensive digestion revealed two DNA-binding fragments of 50 kd and 60 kd. We have not yet determined if the 50 kd polypeptide is contained within the 60 kd polypeptide or if they contain independent binding sites. These two trypsin resistent fragments could represent highly glycosilated areas of fibronectin since glycosilation is known to inhibit proteolytic digestion of proteins (23-24). In addition the DNA-binding is not due to an overall basic charge of fibronectin or its tryptic polypeptides, since these have isoelectric points of 6.8 or lower (McMaster, unpublished results). In fact, competition experiments in the presence of excess (300 fold) of E. coli DNA which eliminated basic charge binding to histones, trypsin and lysozyme, did not efficiently inhibit DNA-binding to fibronectin. These data indicate that fibronectin probably interacts with specific DNA sequences.

Recently Hayashi and Yamada have shown the presence of multiple binding domains for actin heparin and DNA in chicken plasma and cellular fibronectin (25).
We have not found inhibition of DNA-binding (Zardi and Destree, unpublished results) to fibronectin either by gelatin or heparin. Similarly Ruoslahti et al. (26) did not detect inhibition of the binding of heparin to fibronectin by DNA. These data suggest that the DNA-binding domains are different from those involved in heparin and gelatin binding.

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