

Monoclonal Antibodies in the Analysis of Fibronectin Isoforms Generated by Alternative Splicing of mRNA Precursors in Normal and Transformed Human Cells

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Abstract. Recent results showing that a single fibronectin gene can give rise to several different mRNAs by alternative splicing have offered an explanation for fibronectin polymorphism. Here we report on monoclonal antibodies that show specificity for a fibronectin segment (ED) that can be included or omitted from the molecule depending on the pattern of splicing of the mRNA precursors. Using these monoclonals, we have quantitatively analyzed the expression of the ED sequence in human fibronectin

from different sources. The results demonstrated that, at the protein level, the ED segment is not expressed in plasma fibronectin and that, in fibronectin from the tissue culture medium of tumor-derived or simian virus-40-transformed human cells, the percentage of fibronectin molecules containing the ED segment is about 10 times higher than in fibronectin from normal human fibroblasts. These results suggest that in malignant cells the mechanisms that regulate the splicing of mRNA precursors are altered.

FIBRONECTINS (FNs)¹ are high-molecular-mass, adhesive glycoproteins present in the soluble form in plasma and other body fluids and in insoluble form in the extracellular matrices and basement membranes. FN molecules act as bridges between the cell surface and extracellular material. In fact, the FN molecules contain a cell-binding site and binding sites for collagen, heparin, gangliosides, and fibrin. Because of their multiple interactions, FNs play an important role in diverse biological phenomena, including cell adhesion, cell migration, hemostasis and thrombosis, wound healing and the ability to induce a more normal phenotype in transformed cells (for reviews on distribution, structure, and biological functions, see references 1, 7, 8, 18, 24).

It has been demonstrated that FN polymorphism may be at least partially due to alternative splicing schemes in two regions (ED and IIICS) because as many as 10 different mRNAs may originate from the primary transcript of a single gene (9, 12–15, 25, 26, 29) localized on chromosome 2 (11, 34). In fact, Schwarzbauer et al. (25) have shown that an antiserum specific for the rat fibronectin IIICS sequence recognizes the larger subunit of rat plasma FN (pFN), but not the smaller one.

Here we report the characterization of mAbs for the ED

fragment of fibronectin. Using these mAbs in a quantitative assay, we have demonstrated that this sequence is not present in pFN and that tumor-derived and SV-40-transformed human cells release a population of FN molecules in which the percentage of subunits containing the ED sequence is about 10 times higher than in the FN released by normal human fibroblasts.

Materials and Methods

Materials

Eagle's minimum essential medium (MEM) was obtained from Flow Laboratories Inc. (Irvine, Scotland). FCS was obtained from Sera-Lab (Sussex, England). Thermolysin (protease type X), cathepsin D, peroxidase, concanavalin A, pepstatin A, BSA, phenylmethylsulfonyl fluoride, and 3-(cyclohexylamino) propane sulfonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose, cyanogen bromide-activated-Sepharose 4B, and heparin-Sepharose CL-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite (DNA-grade), acrylamide, bisacrylamide, SDS, and low- and high-molecular-mass calibration kits were obtained from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose was from Schleicher & Schüll (Kassel, Federal Republic of Germany). Polyvinyl microwell plates were obtained from Cooke Labs (Alexandria, VA). Peroxidase-conjugated wheat germ agglutinin, peanut agglutinin, and *Ricinus communis* agglutinin were from Miles-Yeda (Rehovot, Israel). Peroxidase-conjugated antibodies were from Dako (Copenhagen, Denmark). Aprotinin was obtained from Lepetit (Milan, Italy). Other chemicals were reagent-grade reagents from Merck (Darmstadt, Federal Republic of Germany).

1. *Abbreviations used in this paper:* cFN and pFN, fibronectin from cultured cell media and plasma, respectively.

Cell Lines

Cultured normal human fibroblast lines (LZ and GM-3651-IC from adult human skin, GM-5386 from embryonic human skin, and WI-38 from embryonic human lung) and transformed cell lines (HT-1080 from a human fibrosarcoma, RD from an embryonic human rhabdomyosarcoma, IgR3 from a human melanoma, WI-38VA13, and SV-40-transformed WI-38 cells) were grown in MEM supplemented with 10% FCS, which had been depleted of bovine FN by passage through a large-capacity gelatin-Sepharose column. The rR5 is an SV-40-transformed human mammary epithelial cell line (4).

Monoclonal Antibodies

Six mAbs to different human FN epitopes were used. The mAb 3E3 (23), directed to the cell-binding region of FN, was a gift from Drs. M. D. Pierschbacher and E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). The characterization of the mAbs IST-2, IST-7, and IST-4 has been previously reported (3, 28, 33). They are specific both for pFN and cell media-cultured FN (cFN). In particular, the epitope recognized by IST-2 is localized within the first three type III homology repeats of the heparin-binding domain (see Fig. 3) (3, 28); the epitope recognized by IST-7 within the last type III homology repeat of the FN molecule (3) (see Fig. 3), and the epitope of IST-4 within the first four type III homology repeats of the FN molecule (28). All three of these mAbs were elicited using pFN as antigen.

The mAbs FN-3 (10) and IST-9, both specific for cFN only, were obtained using as antigens the SV-40-transformed human mammary epithelial rR5 cell line (4), and cFN from WI-38VA13 cells, respectively.

Purification of Antibodies and Preparation of Immunoabsorbents

Unique monoclonal hybridomas were expanded as ascites tumors in syngenic mice. Antibodies were purified from these ascites with protein A-Sepharose equilibrated with borate-buffered saline at pH 8.3. These antibodies were then coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. This resulted in a coupling of an average of 2 mg IgG/ml of gel.

Purification and Proteolytic Digestion of FN

FNs were purified from human plasma and from the conditioned media of the various cell lines as previously reported (35). Thermolysin digestion of FN was performed as described by Sekiguchi and Hakomori (27). Cathepsin D digestion of FN was performed according to Balian et al. (2). SDS PAGE and immunoblotting were carried out as described (16, 31).

Purification and Thermolysin Digestion of FN Fragments Containing the Immunological Determinant Recognized by the mAb IST-9

About 200 mg of cFN from WI-38VA13 cells in 250 ml of 50 mM sodium acetate buffer, pH 3.5, 1.8 mM 3-(cyclohexylamino) propane sulfonic acid, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 10 kallidinogenase inhibitor units/ml of aprotinin was digested with 2.5 μ g/ml of cathepsin D for 4 h at 30°C. Cathepsin D was chosen because preliminary experiments indicated that the immunological determinant recognized by IST-9 was quite resistant to this enzyme. This first step was required to avoid FN precipitation (owing to the low solubility of intact cFN) during the second step, adsorption of FN on IST-9-Sepharose. Cathepsin D digestion was stopped by the addition of a 10-fold molar excess of pepstatin A, and the sample was neutralized with 2.5 M Tris, pH 8.8. After dialysis against PBS containing 0.1% Na₂S₂O₅ and 20 kallidinogenase inhibitor units/ml of aprotinin, the FN digest was added to 20 ml of IST-9-Sepharose previously equilibrated in the same buffer. After slow rotation in an end-over mixer for 4 h at room temperature, the IST-9-Sepharose was transferred to a chromatography column and washed with PBS. When all the unbound polypeptides were washed out, the immunoabsorbent was washed with 25 mM Tris-HCl buffer, pH 7.6, 0.5 mM EDTA, 50 mM NaCl, and 2.5 mM CaCl₂. The 20 ml of immunoabsorbent were then resuspended in 20 ml of the same buffer, and thermolysin digestion was carried out directly in the chromatography column, adding 100 μ g of thermolysin (final concentration 2.5 μ g/ml). After slow rotation of the chromatography column in an end-over mixer for 3 h at room temperature, the reaction was stopped by adding EDTA (5 mM

final concentration) and the released polypeptides were washed out by PBS. The bound polypeptides were then eluted by 0.2 M glycine buffer, pH 2.7. The eluting fractions were immediately neutralized by 2.5 M Tris, pH 8.8. Analysis in SDS PAGE of the eluting material showed two polypeptides of 44 and 47 kD plus a small amount of a 52-kD fragment. Purification of each of these fragments was obtained by fractionation on a hydroxyapatite chromatography column (the detailed procedure will be described elsewhere, Borsi, L., and L. Zardi, manuscript in preparation). These purified fragments were then redigested by thermolysin (0.5 μ g/ml) for 1 h, and the polypeptides obtained were purified on a hydroxyapatite chromatography column (32).

Microsequence Analysis

FN fragments were desalted on a Vydac C4 column (4.5 \times 250 mm; The Separations Group, Hesperia, CA) using a linear gradient from solvent A (0.1% aqueous trifluoroacetic acid) to 70% solvent B (0.1:9.9, trifluoroacetic acid/H₂O/CH₃CN) over a 70-min period at a flow rate of 44 ml/h. Detection was performed at 214 nm using equipment previously described (19). Samples (70 pmol) were subjected to automated Edman degradation on a gas-phase instrument built at the City of Hope (5). Amino acid phenylthiohydantoin derivatives were analyzed by reverse-phase HPLC as previously described (6).

Interaction of Fibronectin Fragments with mAbs, Lectins, and Heparin

The reaction of fibronectin proteolytic fragments with mAbs, concanavalin A, wheat germ agglutinin, *Ricinus communis* agglutinin, and peanut agglutinin was determined, after transfer of polypeptides from SDS PAGE to nitrocellulose sheets, as previously described (32). Interaction of the different FN fragments with heparin was studied as reported (32).

Radioimmunoassay

Hybrids were screened for secretion of anti-FN antibodies by the previously described (33) solid-phase double-antibody radioimmunoassay procedure using both purified pFN and cFN as antigens. Only clones showing a negative reaction for pFN and a positive reaction for cFN were selected for further characterization. The solid-phase double-antibody radioimmunoassay method was also used for the quantitative competition binding assay (3, 36). It was carried out using the mAbs IST-9 and FN-3 (both specific for the ED sequence), and IST-4 (specific for a sequence common to all FN variants). Various dilutions of different FNs in PBS, containing 3% BSA and 10 kallidinogenase inhibitor units/ml of aprotinin, were incubated with the mAbs (at a dilution that had given 50% of maximum binding in titration experiments) for 2 h at 37°C. The mixtures were then transferred into the wells of polyvinyl plates previously coated with purified cFN and incubated for 2 h. The mixture was then removed and the wells were washed with PBS. The amount of antibody bound was measured by adding specific ¹²⁵I-labeled rabbit anti-mouse Ig and incubating for 2 h. The entire procedure was carried out at room temperature.

Protein Determination

Protein concentrations of fibronectin and its fragments were estimated spectrophotometrically at 280 nm using an absorption coefficient of $E_{1\%}^{1\text{cm}} = 12.8$ (17).

Results

The mAb IST-9 Is Specific for the ED Sequence

The capacity of the mAb FN-3 to distinguish between human cellular and plasma FN has been previously reported (10). A similar capacity was also established for the mAb IST-9 using both the radioimmunoassay and immunoblotting procedures described in Materials and Methods.

Preliminary attempts to purify a proteolytic fragment containing the immunological determinant recognized by the mAb IST-9 were frustrating because of the high sensitivity of this determinant to proteolytic enzymes. Thus, we have

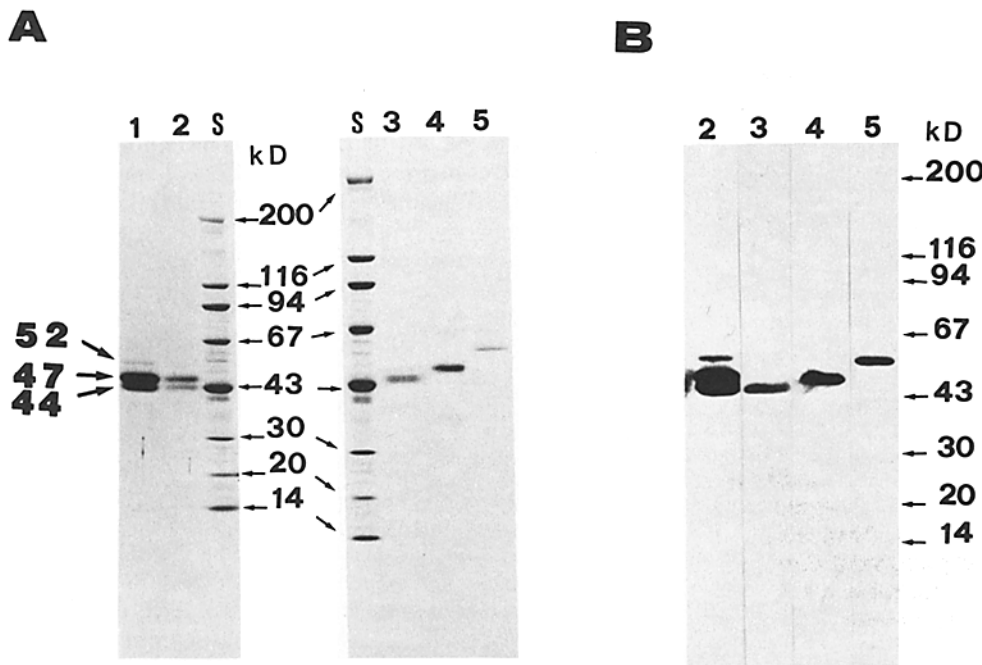


Figure 1. cFN thermolysin fragments obtained from IST-9-Sepharose affinity chromatography column. (A) 4–18% SDS PAGE: lanes 1 and 2, 10 and 2 μ g, respectively, of FN fragments eluted by glycine buffer, pH 2.7, from the IST-9-Sepharose immunoadsorbent after thermolysin digestion; lanes 3, 4, and 5, purified 44-, 47-, and 52-kD fragments. S, molecular mass standards. (B) Immunoblot of a 4–18% SDS PAGE probed with the mAb IST-9. Lanes 2, 3, 4, and 5 as in A. The values on the right are molecular mass.

elaborated the purification protocol described in Materials and Methods.

After incubation of the cFN–cathepsin D digest with IST-9-Sepharose, the bound polypeptides were digested directly on the IST-9-Sepharose immunoadsorbent by thermolysin. In this way we protected the immunological determinant from the proteolytic enzyme. After washing out the released material with PBS, the bound polypeptides were eluted by glycine buffer, pH 2.7. Analysis on a 4–18% SDS PAGE of these polypeptides showed two main fragments having

Table I. NH₂-terminal Sequence Analysis of FN Fragments Eluted from IST-9-Sepharose (52, 47, and 44 kD) and of Those Obtained by Their Subsequent Thermolysin Digestion (38, 33, and 30 kD)

Cycle	M _r of the fragments					
	52	47	44	38	33	30
1	Ile (20)	Ile (30)	Ile (23)	Ile (28)	Leu (41)	Leu (40)
2	Asp (26)	Asp (37)	Asp (29)	Gly (16)	Ile (45)	Ile (25)
3	Arg (12)	Arg (21)	Arg (14)	Thr (10)	Gly (56)	Gly (14)
4	Pro (45)	Pro (53)	Pro (51)	Gln (26)	Thr (41)	Thr (14)
5	Lys (44)	Lys (86)	Lys (49)	Ser (4)	Gln (35)	Gln (33)
6	Gly (20)	Gly (32)	Gly (25)		Ser (13)	Ser (4)
7	Leu (26)	Leu (48)	Leu (31)		Thr (25)	Thr (12)
8	Ala (40)	Ala (37)	Ala (44)		Ala (11)	Ala (25)
9		Phe (27)	Phe (13)		Ile (13)	
10		Thr (NQ)	Thr (NQ)		Pro (14)	
11		Asp (32)	Asp (35)		Ala (21)	
12		Val (51)	Val (22)		Pro (16)	
13		Asp (19)	Asp (32)		Thr (NQ)	
14		Val (27)	Val (21)		Asp (NQ)	
15		Asp (13)	Asp (15)		Leu (13)	
16		Ser (NQ)	Ser (10)			
17			Ile (21)			

Values in parentheses are in picomoles. NQ, residue detected but not quantitated.

molecular masses of 47 and 44 kD, respectively, plus a minor one of 52 kD (Fig. 1 A). All three fragments showed a positive reaction with the mAbs IST-9 and IST-2 in immunoblot analysis (Fig. 1 B), whereas only the 52-kD fragment reacted with IST-7 (see Fig. 3). Each of the three fragments was purified on a hydroxyapatite chromatography column (Fig. 1). All three showed a high affinity for heparin, only the 47-kD fragment reacted with wheat germ agglutinin and *Ricinus communis* agglutinin. Comparison of the NH₂-amino acid sequences of these three polypeptides with the FN sequence (12) demonstrated that all three contain the complete ED sequence at their NH₂-terminal (Table I). In the 44- and 47-kD fragments, the last type III homology repeat is lacking (negative reaction with IST-7) because of the presence of the IIICS sequence, which introduces into the FN molecule, a site extremely sensitive to the proteolytic enzyme thermolysin (3). The difference in mass between the 44- and 47-kD fragments is due to differences in carbohydrate content as indicated by the different reactivity with lectins. In fact, we have previously demonstrated that some FN molecules are sialylated in this region (3). The 52-kD fragment also contains the last type III homology repeat (positive reaction with IST-7); this is due to the fact that this fragment originates from FN subunits in which the IIICS sequence is completely deleted, conferring to this polypeptide resistance to thermolysin (3).

Because the determinant recognized by IST-9 is very sensitive to thermolysin while on the contrary, the heparin-binding domain is extremely resistant (3), we mildly digested the three purified fragments with thermolysin. We obtained a 38-kD fragment from the 52-kD, a 33-kD fragment from the 47-kD, and a 30-kD fragment from the 44-kD (Figs. 2 and 3). All fragments (30, 33, and 38 kD) lost their ability to interact with IST-9, but not with IST-2. The 38-kD fragment was still able to react with the mAb IST-7 and the 33-kD with wheat germ agglutinin and *Ricinus communis* agglutinin. The amino acid sequence of the NH₂-terminal of these

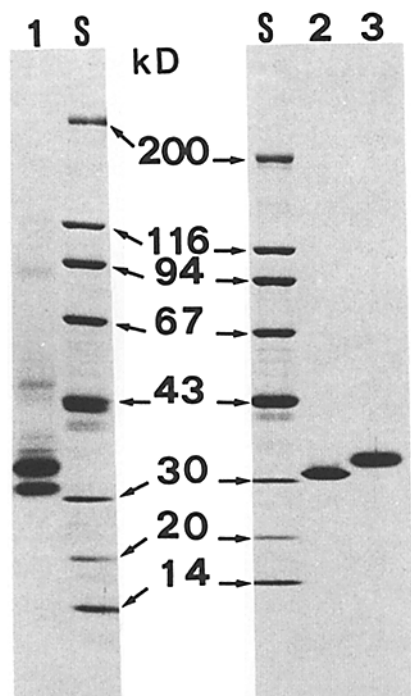


Figure 2. Polypeptides, obtained by thermolysin digestion of the purified FN fragments containing the ED sequence. Lane 1: 4–18% SDS PAGE of a thermolysin digest of polypeptides eluted from the IST-9-Sepharose (shown in Fig. 1 A, lane 1). Lanes 2 and 3: the two purified main fragments obtained by thermolysin digestion of the 47- (lane 2) and 44- (lane 3) kD fragments, respectively. S, molecular mass standards.

three polypeptides showed that the ED sequence was cleaved (Table I). These three fragments have characteristics identical to the heparin-binding fragments usually obtained by thermolysin digestion of cFN (3). Identical results were obtained using the mAb FN-3.

Quantitative Determination of the ED Sequence in FN from Different Sources

To estimate the presence of the ED sequence in FN from different sources, we used the mAbs IST-9 (which recognizes the ED sequence) and IST-4 (which recognizes a determinant common to all FN types) in a competition radioimmunoassay system (for procedure see Materials and Methods).

The results, summarized in Table II, demonstrated that the ED sequence is undetectable in plasma FN, and that the amount of FN molecules containing the ED sequence is about 10 times higher in FN from SV-40-transformed or tumor-derived human cells than from normal cells.

Discussion

Here we report on mAbs (IST-9 and FN-3) specific for the ED sequence of human FN. The localization within the ED sequence of the epitopes recognized by these mAbs is based on the following observations: (a) these mAbs react only with cFN and not with pFN. This result is consistent with experiments showing that the ED segment is absent in hepatocyte mRNAs that are the source of pFN (14, 30). (b) The ED sequence is easily destroyed by very mild thermolysin

digestion. However, this sequence becomes very resistant to thermolysin when FN is bound to the mAbs IST-9 or FN-3. (c) All of the thermolysin fragments that bind to an immunoabsorbent prepared with the mAbs IST-9 or FN-3 contain the complete ED sequence at the NH₂-terminal (see Table I). The fact that the 44- and 47-kD FN fragments are positive for IST-9 and FN-3 even though they lack the last type III homology repeat (negative reaction with IST-7) demonstrates that the epitope recognized by IST-9 and FN-3 is localized in an NH₂-terminal position with respect to the last type III homology repeat (see Fig. 3). Furthermore, when the ED sequence is removed from these three polypeptides (44, 47, and 52 kD) by subsequent thermolysin digestion (Table I, Fig. 3), all of them lose the ability to react with the mAbs IST-9 and FN-3. However, the 38-kD fragment (originating from the 52-kD fragment) still includes the last type III homology repeat. Thus, the epitopes recognized by IST-9 and FN-3 are localized within the NH₂ part of the fragment lost in the thermolysin digestion: the ED sequence.

Using these monoclonals (IST-9 and FN-3) in a quantitative assay, we demonstrated, at the protein level, that this sequence is not present in pFN, and that FN from tumor-derived and SV-40-transformed human cells is composed of a population of molecules in which the percentage of subunits containing the ED sequence is about 10 times higher than in FN from normal human fibroblasts. Similarly, FN from transformed cells is composed of a population of molecules in which the IIICS sequence is more expressed than in FN from normal cells (3). This may be due to either a slower catabolism or an increased synthesis of FN molecules containing the ED or IIICS sequences. The fact that FN molecules containing these two sequences are much more susceptible to proteolytic enzymes rules out the first hypothesis. Thus, these results strongly suggest that in transformed cells the mechanisms that regulate RNA processing are altered. Of course, this needs to be confirmed at the mRNA level.

Table II. Competitive Inhibition Binding of Monoclonals IST-9 and IST-4 to Human cFN

FN source	FN required to give 50% inhibition	
	IST-9	IST-4
	μg	
Plasma	>100	0.29
Normal human fibroblasts (LZ)	9.7	0.31
Normal human fibroblasts (GM 5386)	11.2	0.28
Normal human fibroblasts (GM 3651)	10.3	0.37
Normal human fibroblasts (WI38)	8.4	0.25
SV40-transformed fibroblasts (WI38WA13)	0.9	0.25
Rhabdomyosarcoma (RD)	0.85	0.32
Melanoma (IgR3)	1.2	0.33
Fibrosarcoma (HT-1080)	1.0	0.30

The experiments were carried out using the double-antibody radioimmunoassay described in Materials and Methods.

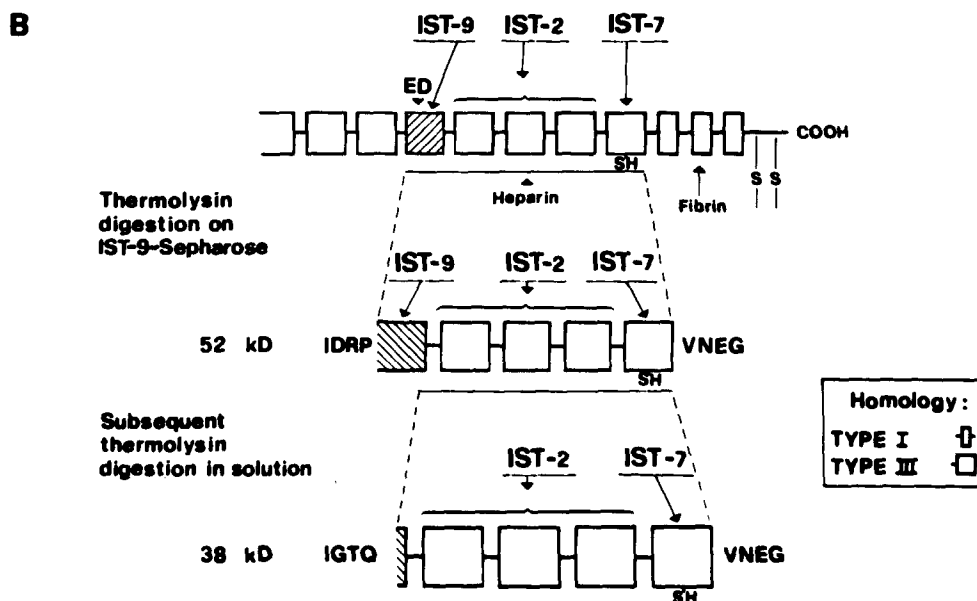
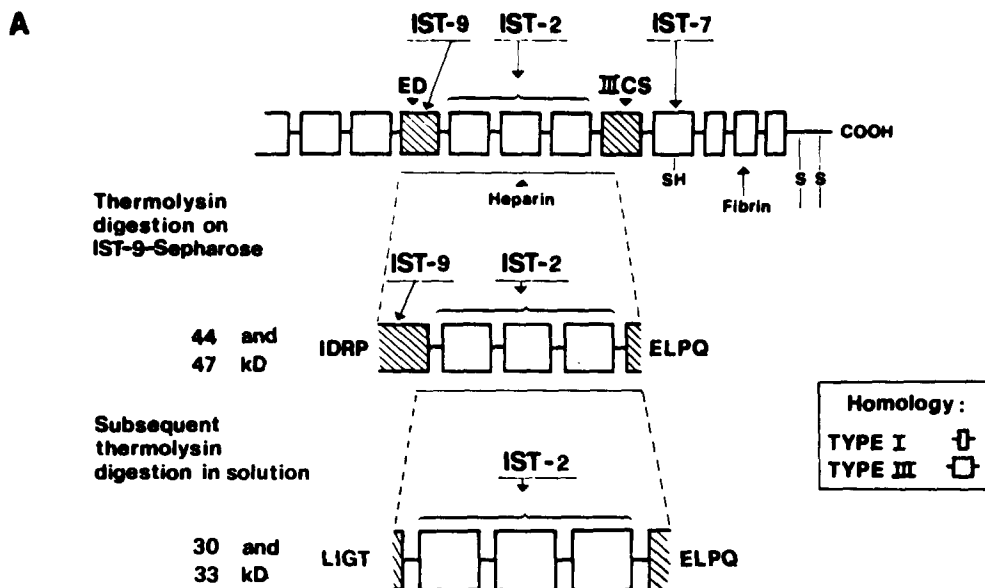


Figure 3. Schematic representation of thermolysin digestion of FN subunits. Thermolysin digestion on IST-9-Sepharose of the FN subunits (A) containing and (B) lacking the IIIICS sequence, and of the subsequent thermolysin digestion in solution of the fragments obtained. Amino acids of the NH₂- and COOH-terminal of different fragments are also indicated. COOH-termini are from references 20 and 21. The model is based on data reported here and in references 3, 20, and 21. Also indicated are the types of internal homology (22).

The results reported here raise three questions: (a) Do tumor cells also express higher levels of the ED sequence *in vivo*? (b) Are the different fibronectin variants produced by malignant cells at least partially responsible for the expression of the transformed phenotype? (c) Are modifications of pre-mRNA splicing in malignant cells limited to FN or is it a more general phenomenon involving other proteins?

Such mAbs specific to sequences, the expression of which is regulated by the alternative splicing of pre-mRNA, may represent tools useful for answering these questions.

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References

1. Alitalo, K., and A. Vaheri. 1982. Pericellular matrix in malignant transformation. *Adv. Cancer Res.* 37:111-158.
2. Balian, G., E. M. Click, E. Crouch, J. M. Davidson, and P. Bornstein. 1979. Isolation of a collagen-binding fragment from fibronectin and cold-insol-

uble globulin. *J. Biol. Chem.* 254:1429-1432.

3. Castellani, P., A. Siri, C. Rosellini, E. Infusini, L. Borsi, and L. Zardi. 1986. Transformed human cells release different fibronectin variants than do normal cells. *J. Cell Biol.* 103:1671-1677.

4. Chang, S. E., J. Keen, E. B. Lane, and J. Taylor-Papadimitriou. 1982. Establishment and characterization of SV40-transformed human breast epithelial cell lines. *Cancer Res.* 42:2040-2053.

5. Hawke, D., D. Harris, and J. E. Shively. 1985. Microsequence analysis of peptides and proteins. V. Design and performance of a novel gas-liquid solid-phase instrument. *Anal. Biochem.* 147:315-330.

6. Hawke, D., P.-M. Yuan, and J. E. Shively. 1982. Microsequence analysis of peptides and proteins. II. Separation of amino acid phenylthiohydantoin derivatives by high-performance liquid chromatography on octadecylsilane supports. *Anal. Biochem.* 120:302-311.

7. Hormann, H. 1982. Fibronectin: mediator between cells and connective tissue. *Klin. Wochenschr.* 60:1265-1277.

8. Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369-377.

9. Hynes, R. O. 1985. Molecular biology of fibronectin. *Annu. Rev. Cell Biol.* 1:67-90.

10. Keen, J., S. E. Chang, and J. Taylor-Papadimitriou. 1984. Monoclonal antibodies that distinguish between human cellular and plasma fibronectin. *Mol. Biol. Med.* 2:15-17.

11. Koch, G. A., R. C. Schoen, R. J. Klebe, and T. B. Shows. 1982. Assignment of a fibronectin gene to human chromosome 2 using monoclonal antibodies. *Exp. Cell Res.* 141:293-302.

12. Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755-1759.

13. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1983. Isolation and characterization of cDNA clones for human and bovine fibronectins. *Proc. Natl. Acad. Sci. USA.* 80:3218-3222.

14. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1984. Human fibronectin: cell specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. *Nucleic Acid Res.* 12:5853-5868.

15. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1984. Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:221-226.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.

17. Mosesson, M. W., and R. A. Unfleet. 1970. The cold insoluble globulin of human plasma part 1 purification primary characterization and relationship to fibrinogen and other cold insoluble fraction components. *J. Biol. Chem.* 245:5728-5736.

18. Mosher, D. F. 1984. Physiology of fibronectin. *Annu. Rev. Med.* 35:561-575.

19. Pande, H., J. Calaycay, D. Hawke, C. M. Ben-Avram, and J. E. Shively. 1985. Primary structure of a glycosylated DNA-binding domain in human fibronectin. *J. Biol. Chem.* 260:2301-2306.

20. Pande, H., J. Calaycay, T. D. Lee, A. Siri, L. Zardi, and J. E. Shively.

Structural analysis of a 29/38 kDa heparin-binding domain of fibronectin: evidence that two different subunits of human plasma fibronectin arise by alternative mRNA splicing. Proceedings of the Symposium of American Protein Chemists 1985. Plenum Press, New York. In press.

21. Pande, H., J. Calaycay, T. D. Lee, K. Legesse, J. E. Shively, A. Siri, L. Borsi, and L. Zardi. Structural differences between the two subunits of human plasma fibronectin in the carboxy terminal heparin-binding domain. *Eur. J. Biochem.* In press.

22. Petersen, T. E., H. C. Thogersen, K. Skorstengaard, K. Vibe-Pedersen, L. Sottrup-Jensen, and S. Magnusson. 1983. Partial primary structure of bovine plasma fibronectin, three types of internal homology. *Proc. Natl. Acad. Sci. USA.* 80:137-141.

23. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259-267.

24. Ruoslahti, E., E. Engvall, and E. G. Hayman. 1981. Fibronectin: current concepts of its structure and functions. *Collagen Relat. Res.* 1:95-128.

25. Schwarzbauer, J. E., J. I. Paul, and R. O. Hynes. 1985. On the origin of species of fibronectin. *Proc. Natl. Acad. Sci. USA.* 82:1424-1428.

26. Schwarzbauer, J. E., J. W. Tamkun, I. R. Lemischka, and R. O. Hynes. 1983. Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell.* 35:421-431.

27. Sekiguchi, K., and S. Hakomori. 1983. Domain structure of human plasma fibronectin. *J. Biol. Chem.* 258:3967-3973.

28. Sekiguchi, K., A. Siri, L. Zardi, and S. Hakomori. 1985. Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. *J. Biol. Chem.* 260:5105-5114.

29. Tamkun, J. K., J. E. Schwarzbauer, and R. O. Hynes. 1984. A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. *Proc. Natl. Acad. Sci. USA.* 81:5140-5144.

30. Tamkun, J. W., J. E. Schwarzbauer, and R. O. Hynes. 1983. Plasma fibronectin is synthesized and secreted by hepatocytes. *J. Biol. Chem.* 258:4641-4647.

31. Towbin, H., T. Stachelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

32. Zardi, L., B. Carnemolla, E. Balza, L. Borsi, P. Castellani, M. Rocco, and A. Siri. 1985. Elution of fibronectin proteolytic fragments from a hydroxyapatite chromatography column: a simple procedure for the purification of fibronectin domains. *Eur. J. Biochem.* 146:571-579.

33. Zardi, L., B. Carnemolla, A. Siri, L. Santi, and R. S. Accolla. 1980. Somatic cell hybrids producing antibodies specific to human fibronectin. *Int. J. Cancer.* 25:325-329.

34. Zardi, L., M. Cianfriglia, E. Balza, B. Carnemolla, A. Siri, and C. M. Croce. 1982. Species-specific monoclonal antibodies in the assignment of the gene for human fibronectin to chromosome two. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:929-933.

35. Zardi, L., A. Siri, B. Carnemolla, E. Cosulich, G. Viale, and L. Santi. 1980. A simplified procedure for the preparation of antibodies to serum fibronectin. *J. Immunol. Methods.* 34:155-165.

36. Zardi, L., A. Siri, B. Carnemolla, L. Santi, W. D. Gardner, and S. O. Hoch. 1979. Fibronectin: a chromatin associated protein? *Cell.* 18:649-657.