Transformed Human Cells Release Different Fibronectin Variants Than Do Normal Cells

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Abstract. Fibronectin molecules are dimers composed of subunits whose primary structures may differ. This is due to alternative splicing in at least two regions (ED and IIICS) of the pre-mRNA.

Using two monoclonal antibodies specific for two different epitopes of domain 5 (high affinity for heparin), we have quantitatively analyzed the expression of the IIICS sequence in human fibronectins from different sources. The results demonstrated that the percentage of fibronectin subunits containing the IIICS is higher in fibronectins from tumor-derived or simian virus 40–transformed human cells than in fibronectins from human plasma or normal human fibroblasts.

Furthermore, we observed that 45–65% of fibronectin subunits from transformed cells or normal embryonic fibroblasts are sialylated on the heparin-binding domain 5, whereas this occurs in only 24–28% of fibronectin subunits from normal adult fibroblasts. On the contrary, no sialylation was observed on domain 5 in fibronectin from human plasma.

FIBRONECTINS (FNs)[1] are high molecular weight adhesive glycoproteins present in 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, ganglioside, and fibrin. Due to 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, 3, 4, 13, and 16).

Recently it has been demonstrated that the FN polymorphism may be due to alternative splicing schemes since many different mRNAs may originate from the primary transcript of a single gene (5, 7–10, 17, 18, 22) localized on chromosome two (6, 27).

Here, using the domain-specific monoclonal antibodies IST-7 and IST-2 we have studied the presence of the IIICS sequence in FN from plasma and from the conditioned media of normal and tumor-derived human cells.

Materials and Methods

Cell Lines and Monoclonal Antibodies

Cultured normal human fibroblast cell lines (LZ, from adult human skin; GM-3651-C, from adult human skin; GM-5386, from embryonic human skin; 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; and WI-38VA13, simian virus 40 [SV-40]–transformed WI-38 cells) were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) that had been depleted of bovine FN by passage through a large capacity gelatin-Sepharose column.

Monoclonal antibodies to human plasma FN were prepared as previously described (25). Partial characterization of the monoclonal antibody IST-2 has been previously reported (19, 21). The IST-2 monoclonal antibody is available from Sera Lab (Crawley Down, England).

Purification and Thermolysin Digestion of FN

FNs were purified from human plasma and from the conditioned media of the various cell lines as previously reported (26). Thermolysin digestion of FNs was performed as described by Sekiguchi and Hakomori (20). SDS PAGE and immunoblotting were carried out as described (11, 23).

Purification of Domain 5 and Neuraminidase Treatment

Domain 5 was purified as previously described using a hydroxyapatite chromatography column (28). Neuraminidase (Neuraminidase-Test, Behringwerke, Marburg, FRG) treatment of purified domain 5 was carried out by incubating the polypeptides (0.2 mg/ml final concentration) in 0.05 M sodium acetate buffer, 0.154 M NaCl, 9 mM $CaCl_2$ (pH 5.5) with 0.5 U/ml of neuraminidase for 1 h at 37°C.

Affinity Chromatography on Heparin-Sepharose

5 mg of FN thermolysin digest (5 μg of thermolysin/mg of fibronectin in 25 mM Tris-HCl, pH 7.6, containing 0.5 mM EDTA, 50 mM NaCl) were loaded on a heparin-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography column (1.6 × 4 cm) previously equilibrated in the above-mentioned buffer.

After washing with 40 ml of the same buffer (heparin-unbound fraction),

1. *Abbreviations used in this paper*: FN, fibronectin; RIA, radioimmunoassay; SV-40, simian virus 40.

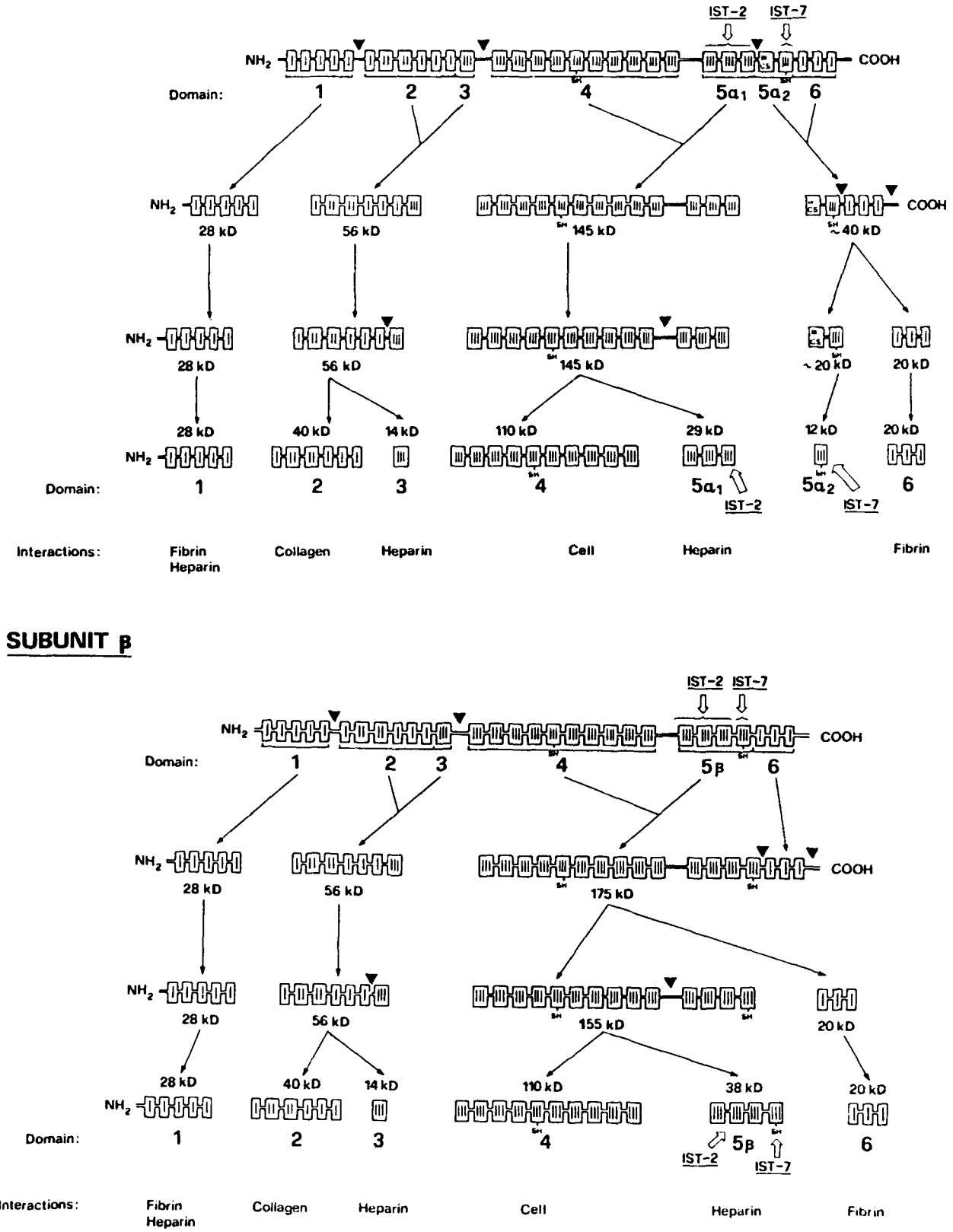

Figure 1. Proposed model for thermolysin fragmentation of the α- and β-subunits of FN. Fragmentation pathways after limited proteolysis with thermolysin of FN subunit α (containing the IIICS sequence) and β (without the IIICS sequence) are schematically presented based on previous reports (19–21, 28), and the results reported are here. White arrows indicate the sites where the two monoclonal antibodies IST-2 and IST-7 react. Arrowheads indicate the thermolysin cleavage sites. The figure also indicates the internal homologies (*I–III*) (15).

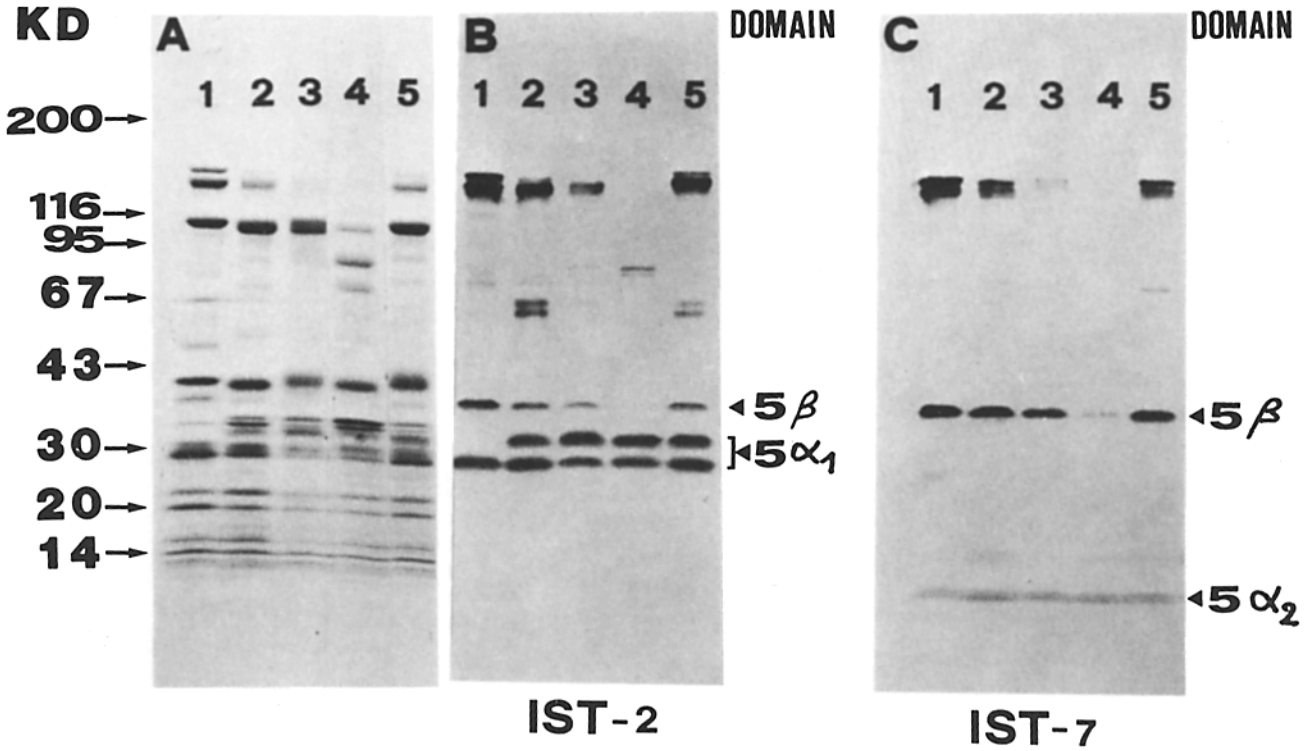

Figure 2. (*A*) 4–18% SDS PAGE gradients of FNs from different sources digested for 4 h at 22°C by thermolysin (50 μg/mg of FN). Lanes: *1*, human plasma FN; *2*, FN from conditioned medium of GM-5386 normal human embryonic fibroblasts; *3*, FN from conditioned medium of HT-1080 human fibrosarcoma cells; *4*, FN from conditioned medium of WI-38VA13 cells SV40-transformed human WI-38 lung fibroblasts; *5*, FN from conditioned medium of IgR3 human melanoma cells. The values on the left are the molecular masses (in kD) of standards. (*B*) Immunoblot analysis of a 4–18% SDS PAGE gradient identical to that shown in *A*, using the monoclonal antibody IST-2. This monoclonal recognizes domains 4+5β (155 kD), 4+5α (145 kD), 5β (38 kD), and domain 5α in the sialylated and nonsialylated forms (33 and 29 kD). For FN domains nomenclature and structure see Fig. 1. (*C*) Immunoblot analysis of a 4–18% SDS PAGE gradient identical to that shown in *A*, using the monoclonal antibody IST-7. This antibody recognizes a 155-kD fragment (domains 4+5β), a 38-kD fragment (domain 5β), and a 12-kD fragment (domain $5\alpha_2$). The 12-kD fragment is barely visible probably because this low molecular mass fragment interacts weakly with the nitrocellulose sheets. In milder digestion conditions this antibody also recognizes a 175-kD fragment (domain 4+5β+6). For FN domains nomenclature and structure see Fig. 1.

bound polypeptides were eluted using 25 mM Tris-HCl, pH 7.6 buffer containing 0.5 mM EDTA, 0.7 M NaCl. All operations were carried out at a flow rate of 20 ml/h. 1.5-ml fractions were collected.

Radioimmunoassay (RIA)

The solid-phase double-antibody RIA method was used for the quantitative competition binding assay (24). Competition reactions were carried out in PBS containing 1 mg/ml of bovine serum albumin (BSA), 5 U/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO).

The heparin-bound and unbound FN fractions (obtained as described above) were, after dialysis, diluted with PBS to identical volumes (10 ml), and BSA and aprotinin were added to give a final concentration of 1% and 10 U/ml, respectively. The competition binding assay was carried out on these fractions using the monoclonal antibody IST-7.

Various dilutions of the heparin-bound and heparin-unbound fractions were incubated with a monoclonal antibody (at a dilution that had given 50% maximum binding in titration experiments) for 1 h at 37°C.

The mixtures were then transferred into the wells of polyvinyl plates (Cooke Labs, Alexandria, VA) previously coated with purified FN, and incubated for 2 h. Then the mixture was removed and the wells were washed with PBS. The amount of antibody bound was detected by adding specific ^{125}I-labeled rabbit anti-mouse Ig and incubating for 2 h at room temperature. The dilutions of heparin-bound and heparin-unbound fractions that could give a 50% inhibition were used to calculate the ratio between the α- and β-subunits.

Results

Characterization of Monoclonal Antibodies IST-2 and IST-7

The monoclonal antibody IST-2 has previously been partially characterized (19, 21). It reacts with the heparin-binding domain 5 which is comprised of the COOH-terminal four type III homology repeats (21) (see Fig. 1). This domain is present in the different FN molecules with differences in the primary structure since different portions of a 120 amino acid segment (IIICS) may be inserted between the last two type III homology repeats; this is due to alternative patterns of RNA splicing (7–10, 17).

After thermolysin digestion of plasma FN, IST-2 reacts with two fragments of 38 and 29 kD which represent the heparin-binding domain 5 (19, 21, 28). Both the 38 and 29-kD fragments are extremely resistant to thermolysin. Digestion of the purified 38-kD fragment by 50 μg of thermolysin/mg of 38-kD fragment did not generate any 29-kD fragment or other degradation peptides. Identical resistance to thermolysin was shown by the 29-kD fragment. Purification (28) and primary structure studies (14; and Pande, H.,

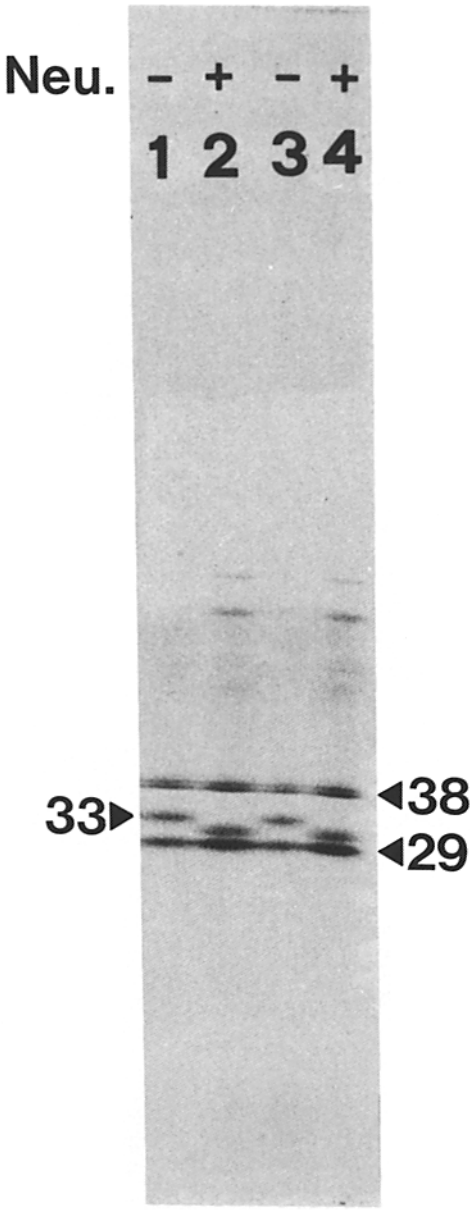

Figure 3. 4–18% SDS PAGE gradients of the purified heparin-binding domains 5β (38 kD) and $5\alpha_1$ (29 and 33 kD) from a thermolysin digest of FN from the tissue culture medium of WI-38 normal human fibroblasts. Lanes *1* and *3*, purified domains 5β and $5\alpha_1$. Lanes *2* and *4*, purified domains 5β and $5\alpha_1$ treated with 0.5 U/ml of neuraminidase (*Neu.*) at 37°C for 1 h and 3 h, respectively (see Materials and Methods). The values on the left and on the right are the molecular masses (in kD).

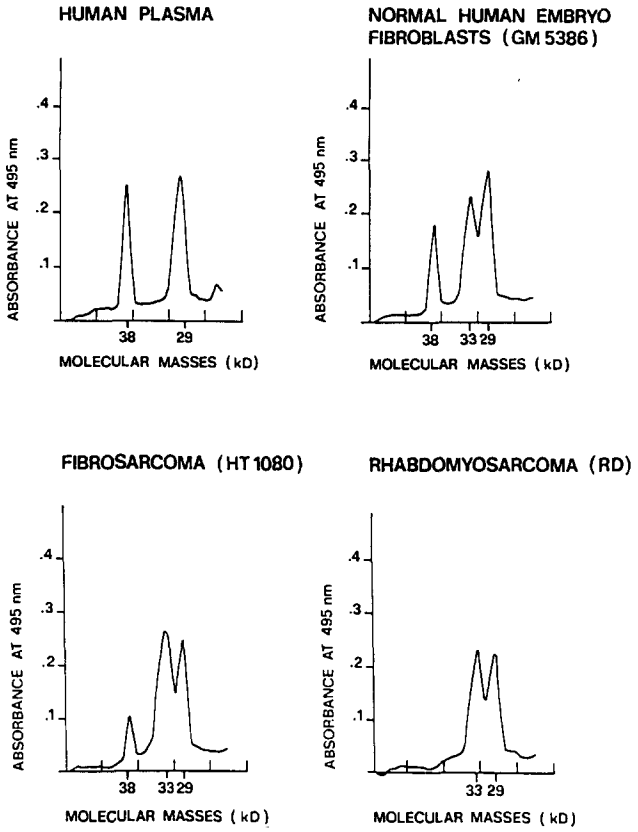

Figure 4. Densitometric scanning of immunoblots, stained using the monoclonal antibody IST-2, of thermolysin digested FNs. 38-, 33-, and 29-kD peaks represent domain 5β, sialylated domain $5\alpha_1$, and domain $5\alpha_1$ respectively.

J. Calaycay, T. Lee, K. Legesse, J. E. Shively, A. Siri, L. Borsi, and L. Zardi, manuscript submitted for publication) of these two fragments demonstrated that the 38-kD fragment (domain 5β) contains the four repeats of type III homology with the deletion of the entire 120 amino acid IIICS sequence, whereas the 29-kD fragment (domain $5\alpha_1$) contains the first three repeats of type III homology plus a small portion of the connecting segment (IIICS) (see Fig. 1). These data indicate that the monoclonal antibody IST-2 reacts with an antigenic determinant present on the first three type III homology repeats (Fig. 1) and that the presence of the IIICS sequence introduces into the FN molecule a site that is very sensitive to the proteolytic enzyme thermolysin.

The monoclonal antibody IST-7 reacts with the 38-kD (domain 5β) but not the 29-kD fragment (domain $5\alpha_1$) (Fig. 2). It also reacts with a smaller fragment of ~12 kD (domain $5\alpha_2$) (Fig. 2), which represents the last type III homology repeat, as also confirmed by the NH_2-terminal amino acid sequence of the purified fragment (data not shown). This 12-kD fragment (domain $5\alpha_2$) does not have heparin-binding activity (data not shown), which is the characteristic of domains $5\alpha_1$ and 5β.

Domain 5 May Be Sialylated

The monoclonal antibody IST-2, which reacts with the 38- and 29-kD fragments of plasma FN, also reacts with FN from the spent media of different human cell lines with an extra band having a molecular mass of ~33 kD (see Fig. 2). This band does not react with the monoclonal IST-7, indicating that this fragment lacks the last type III homology repeat. Furthermore, this fragment shows as great an affinity for heparin as do the 29- and 38-kD fragments (data not shown). All three of these fragments were purified and treated with different amounts of neuraminidase (Fig. 3). The 38- and 29-kD fragments did not show any change in molecular mass, whereas that of the 33-kD fragment decreased to ~30 kD. The fact that neuraminidase treatment does not reduce the 33-kD fragment exactly to 29 kD is very likely due to either incomplete desialylation or the presence of other carbohy-

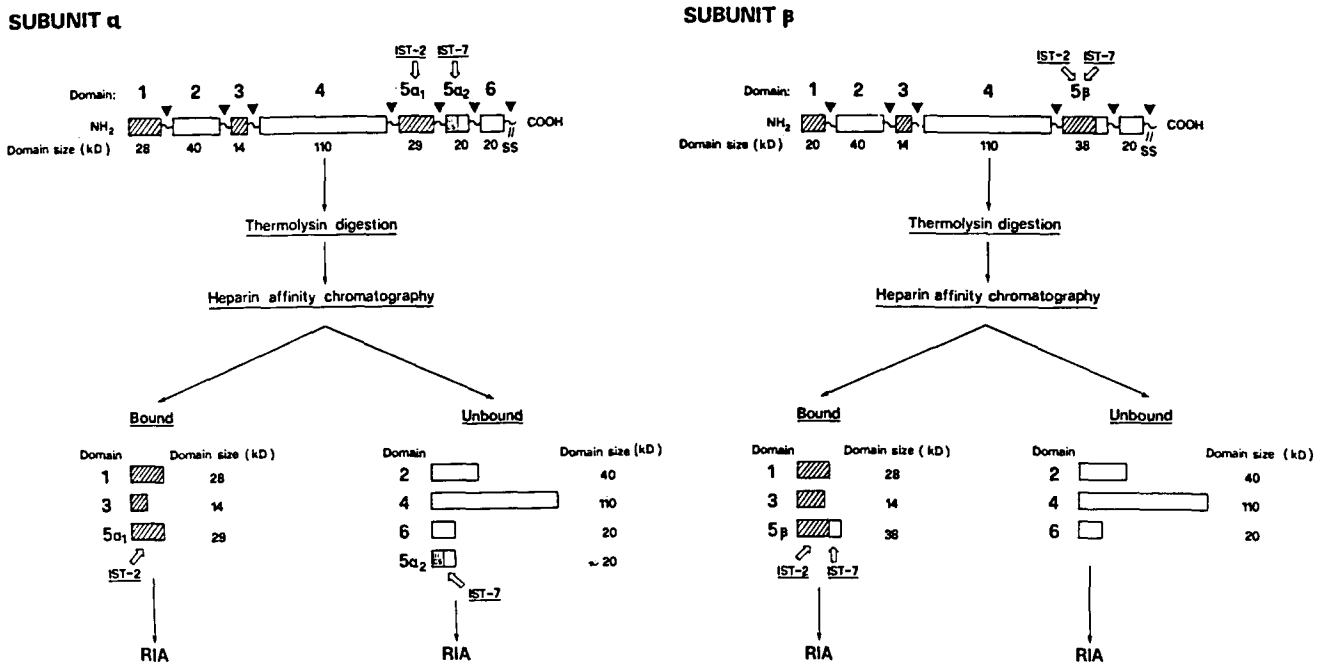

Figure 5. Schematic outline of FN α- and β-subunit determination. Hatched areas indicate heparin-binding fragments; white arrows indicate the reaction sites of the monoclonal antibodies IST-2 and IST-7, respectively. Arrowheads indicate thermolysin cleavage sites. The expression of the IIICS sequence (subunit α) introduces into the FN molecule a new cleavage site and after thermolysin digestion the fragment containing the immunologic determinant recognized by IST-7 is separated from the heparin-binding domain and it elutes in the heparin-unbound fractions. On the contrary, when the IIICS sequence is deleted (β-subunit) thermolysin cannot separate the determinant recognized by IST-7 from the heparin-binding sequence. Using the RIA described in Materials and Methods, the determinant recognized by the monoclonal IST-7 was measured in the heparin-bound and heparin-unbound fractions. The ratio between the amount of determinant present in the heparin-unbound and heparin-bound fractions corresponds to the ratio between the α- and β-subunits.

drates. Neuraminidase treatment also induced a drastic change in the isoelectric point of the 33-kD fragment (from 8.3 to 9.3), whereas it did not induce any change in the charge of the other two fragments. These data indicate that the 33-kD fragment represents a glycosylated form of the 29-kD fragment (domain $5\alpha_1$).

Determination of the Expression of the IIICS Sequence in FN from Different Sources

To estimate the presence of the IIICS sequence in FN from different sources we used two different approaches, using the monoclonal antibodies IST-2 and IST-7, and the different sensibilities of the two FN subunits to thermolysin: (*a*) Since the 38-kD fragment originates from the FN subunit in which the IIICS sequence is completely deleted and the 29- and 33-kD fragments originate from the FN subunit in which the IIICS sequence is at least partially expressed (14; and Pande, H., J. Calaycay, T. Lee, K. Legesse, J. E. Shively, A. Siri, L. Borsi, and L. Zardi, manuscript submitted for publication), the ratio between the 38-kD fragment and the 33 + 29 kD fragments represents the ratio between the β- and α-subunits in the different FNs. To estimate the relative amount of each of these fragments we performed a densitometric scanning of immunoblots stained using the monoclonal antibody IST-2 (Fig. 4). The percentage of the area of each peak is reported in Table I. (*b*) The second procedure involved the use of the monoclonal antibody IST-7 (specific for domains 5β and $5\alpha_2$) and is based on the observation that when the IIICS sequence is deleted, the proteolytic enzyme thermolysin cannot separate the determinant recognized by IST-7 from the sequence with heparin affinity. On the contrary, the expression of the IIICS sequence introduces into the FN molecule a site extremely sensitive to thermolysin and after digestion the fragment containing the determinant recognized by IST-7 cannot bind heparin (see Fig. 5).

Fig. 6 shows competition RIA experiments for the binding of the monoclonal antibody IST-7 using the heparin-bound and heparin-unbound fractions of the thermolysin digests of different FNs. The data are summarized in Table I (for the procedure see Materials and Methods).

The results obtained with the two procedures were nearly identical (Table I) and demonstrated that the percentage of the α-subunit (containing the IIICS sequence) is higher in virus-transformed or tumor-derived cells with respect to normal fibroblasts or plasma.

Furthermore, the percentage of the glycosylated domain 5α seems to be higher in FNs from embryonic or transformed cells with respect to normal cells (Table I).

Discussion

In this work we provide evidence that tumor-derived or transformed human cells release into the culture media different FN variants than do normal cells.

It has previously been demonstrated that the FN polymorphism is due to alternative splicing schemes in at least two regions (IIICS and ED) of the pre-mRNA (7-10, 17, 18, 22). Previous studies (8, 9) have suggested that the ED segment seems to be absent in the hepatocyte mRNAs, which are the source of plasma FN, and it is probable that the inclusion of

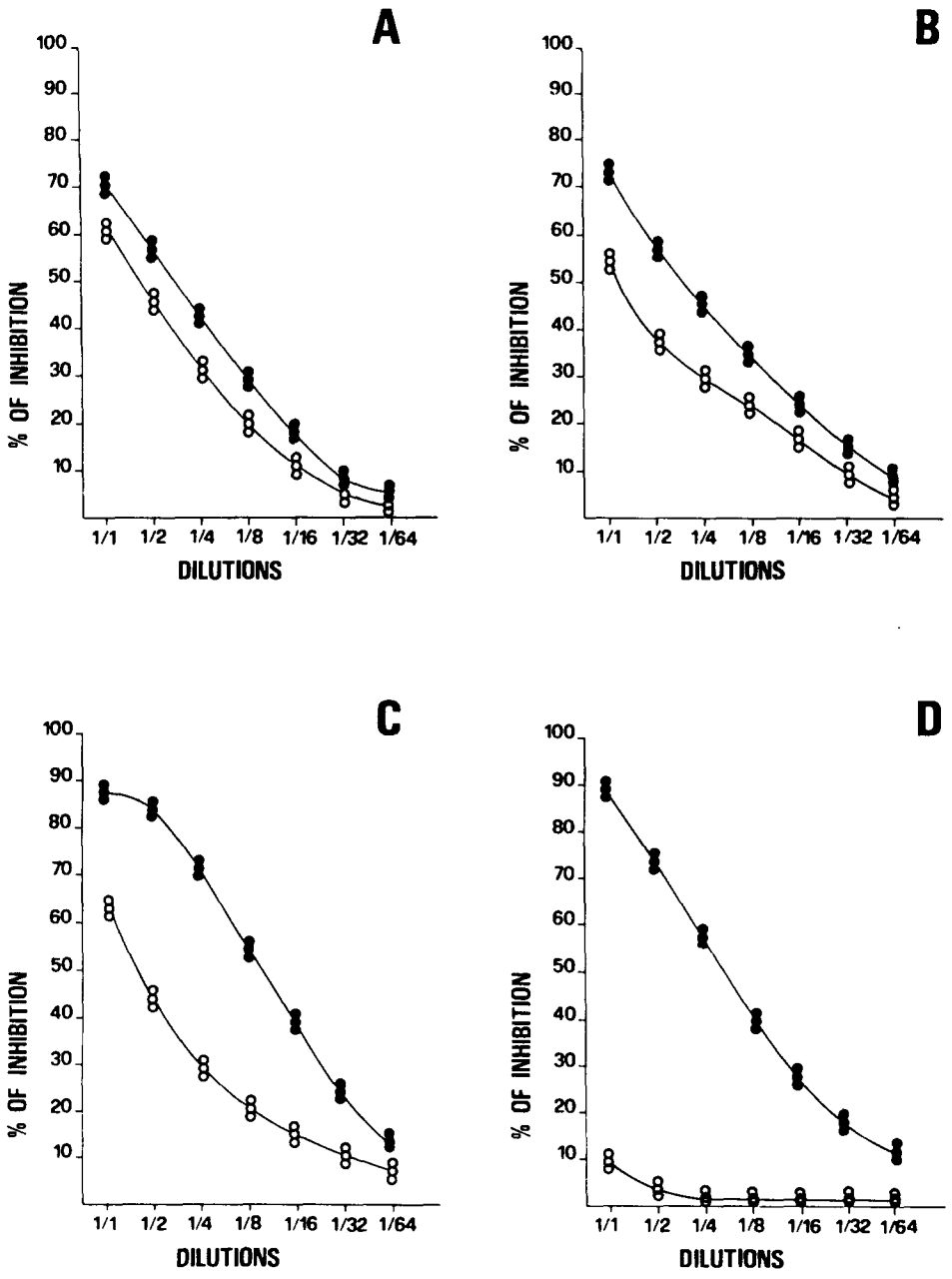

Figure 6. Competitive inhibition of binding of the monoclonal antibody IST-7 to normal human fibroblast FN (GM-3651-C). Aliquots of the monoclonal antibody IST-7 were mixed with different dilutions of the heparin-bound (○) and heparin-unbound (●) peptides from thermolysin digests of various FNs. For the procedure see Materials and Methods. (*A*) Human plasma FN; (*B*) FN from spent medium of normal fibroblasts; (*C*) FN from spent medium of melanoma cells (IgR3); and (*D*) FN from spent medium of SV-40-transformed human fibroblasts (WI-38 VA13).

the ED sequence is characteristic of cellular FN. Recently, Schwarzbauer et al. (18), using a rabbit antiserum to the IIICS sequence, have demonstrated that the great majority of the FN molecules from a rat cell line contains the IIICS sequence while on the contrary, a sizeable proportion of rat plasma FN was lacking the sequence. Furthermore, it has been recently demonstrated that at least part of the IIICS sequence is present in the α-subunit of human plasma FN (2, 14; and Pande, H., J. Calaycay, T. Lee, K. Legesse, J. E. Shively, A. Siri, L. Borsi, and L. Zardi, manuscript submitted for publication).

Here we show that the expression of the IIICS sequence introduces into the FN molecule a region that is susceptible to thermolysin cleavage. This agrees with previous indications of a different susceptibility to proteolytic enzymes in the COOH-terminal region of the two FN subunits (2, 11, 15, 20, 23, 24, 26). Thus, thermolysin digestion of subunits containing the IIICS sequence produces fragments different with respect to those originating from FN molecules in which the IIICS sequence is deleted. Using two monoclonal antibodies (IST-2 and IST-7), the different susceptibilities to thermolysin of the two FN subunits, and the affinity for heparin of the different FN thermolysin fragments, we have set up two procedures for the quantitative determination of the expression of the IIICS sequence in FN from different sources. However, five splicing variants within the IIICS have been demonstrated in human FN (10). In fact, the 120 amino acids of the IIICS segment may be present or deleted, or portions of 95, 89, or 64 amino acids may be expressed in the FN molecules (10). The two procedures described here can only discriminate whether the IIICS sequence is completely deleted or at least partially expressed.

Using these procedures we have observed that: (*a*) the percentage of molecules containing at least part of the IIICS sequence varies depending on the FN source; and (*b*) expression of the IIICS sequence is higher in FN from tumor-

Table I. Percentage of α- and β-Subunits in FN from Different Sources

Source of FN	From RIA		From densitometric scanning of immunoblots	
	α	β	α*	β
Plasma	60	40	60 (0)	40
Normal human embryonic fibroblast (GM5386)	75	25	78 (45)	22
Normal human embryonic fibroblast (WI-38)	75	25	78 (43)	22
Normal human adult fibroblast (GM3651-C)	72	28	73 (28)	27
Normal human adult fibroblast (LZ)	nd	nd	76 (24)	24
Fibrosarcoma (HT-1080)	90	10	88 (62)	12
Rhabdomyosarcoma (RD)	100	0	100 (67)	0
Melanoma (IgR3)	80	20	80 (55)	20
SV-40–transformed human fibroblast (WI-38/VA13)	100	0	100 (65)	0

Percentage of α- and β-FN subunits as estimated using the RIA method with the IST-7 monoclonal antibody and by densitometric scanning of immunoblots with the IST-2 monoclonal antibody. FN was from plasma or from the spent media of the different cell lines.
* The percentages of subunits in which domain 5 is sialylated are indicated in parentheses. nd, not determined.

derived and SV-40–transformed cells with respect to normal fibroblasts and plasma. These results suggest a specifically programmed splicing mechanism in different cell types and that in transformed or tumor-derived cells these mechanisms are altered. Recently Matsuura and Hakomori, using monoclonal antibodies, found a domain that is present in FNs from tumors and fetal tissues but absent in those from adult tissues and plasma (12). We can exclude that the monoclonal antibodies prepared by Matsuura and Hakomori (12) recognize some antigenic determinant on the IIICS domain since this is also present in plasma FN.

Furthermore, we demonstrated that the FN domain $5\alpha_1$ (which contains part of the IIICS sequence) is sialylated in some FN molecules from spent media of culture cells but not in plasma FN. The percentage of the sialylated domain $5\alpha_1$ in FN from the spent media of embryonic fibroblasts is about twice that in FN from the spent media of adult fibroblasts. The percentage of sialylated domain $5\alpha_1$ is even higher in FN from the spent media of transformed cells. We did not observe any sialylation of domain 5β (from which the IIICS sequence is deleted).

Future research should be directed towards establishing the biological consequences of these structural modifications.

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