

FIBROBLAST CELLULAR AND PLASMA FIBRONECTINS ARE SIMILAR BUT NOT IDENTICAL

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ABSTRACT

Fibronectins are multimeric, adhesive glycoproteins present on cell surfaces and circulating in blood. Cellular fibronectin produced by fibroblasts *in vitro* and fibronectin isolated from plasma are known to be very similar immunologically and biochemically. We investigated whether or not they are identical.

Purified chicken and human cell-surface fibronectins are 150-fold more active in hemagglutination of fixed erythrocytes than plasma fibronectins. Cell-surface fibronectin is also 50-fold more active in restoring a more normal morphology to transformed cells originally missing the protein. However, in two other assays that measure cell attachment to collagen and cell spreading, cell-surface and plasma fibronectins have identical specific activities.

In sodium dodecyl sulfate polyacrylamide gels, the subunits of human and chicken plasma fibronectins have significantly smaller apparent subunit molecular weights than cellular fibronectins present on cell surfaces or secreted into culture media. These differences are also present in a characteristic large subfragment of both forms of fibronectin after limited proteolysis by trypsin. We conclude that by both biological and biochemical criteria, cellular and plasma fibronectins are similar but not identical.

KEY WORDS cell surface · cell adhesion · hemagglutination · transformation · collagen · proteolytic fragments

A major cell surface glycoprotein of fibroblasts *in vitro* is usually decreased after malignant transformation. This protein is known as cellular fibronectin, large external transformation-sensitive (LETS) protein, cell surface protein (CSP), or galactoprotein a and is thought to be an adhesive protein (reviewed in references 12, 28, and 30). Cellular fibronectin is present on cell surfaces as well as in a form secreted or sloughed into culture media. An immunologically indistinguishable protein of similar size called plasma fibronectin or cold insoluble globulin is present in plasma and serum (5, 21, 26, 28, 30). Plasma fibronectin also has adhesive properties, and helps to mediate the attachment of cells to collagen or to plastic substrata *in vitro* (9, 10, 18).

These proteins also have similar electrophoretic mobilities, subunit organizations, amino acid and carbohydrate compositions, and peptide maps (3, 13, 28–32). Some investigators consider these proteins to be indistinguishable or identical (6, 16, 20, 29). However, other researchers have reported subtle differences in electrophoretic mobility between cell-surface and plasma fibronectins (7, 17, 22), while others find no size differences between cellular fibronectins (cell surface or secreted) and plasma fibronectin, (2, 6, 15, 20) or even co-migration of only one band of a doublet of plasma fibronectin with cellular fibronectin (13). Partially purified cellular and plasma fibronectins can both mediate attachment of cells to collagen (6, 18, 24), and both are equally effective in promoting baby hamster kidney (BHK)-cell spreading on plastic substrata (25). However, plasma fibronectins are reportedly twofold less

effective than cellular fibronectins in promoting attachment of a transformed hamster cell line to a plastic substratum (13).

This confusing controversy as to the identity or nonidentity of these proteins can probably be resolved only by detailed, direct comparisons of the structures and biological specific activities of plasma and cellular fibronectins. In this study, we have directly compared and quantitated several biological activities of plasma and cell-surface fibronectins from chicken and man, and have also compared the subunit sizes of plasma, cell-surface, and secreted fibronectins from both of these species.

We find that, although plasma and cell-surface fibronectins have identical specific activities in two biological assays, they do not share two other activities. By the use of an electrophoretic system with high resolution for large proteins, we also find that both cell-surface and secreted fibronectins have subunit and subfragment polypeptide sizes that are significantly larger than those of the two subunits of plasma fibronectin.

MATERIALS AND METHODS

Chicken cell-surface fibronectin was isolated from tertiary chick embryo fibroblasts cultured in roller bottles as described previously, utilizing extraction with 1 M urea and ammonium sulfate fractionation (31, 32). Plasma fibronectin was isolated from fresh chicken and human blood anticoagulated with acid citrate dextrose (ACD, National Institutes of Health formula A) or occasionally with heparin (5 U/ml). Plasma was applied to columns of immobilized gelatin (collagen) and eluted with either 8 M urea as described by Engvall and Ruoslahti (8), or with pH 11 buffer B (100 mM NaCl, 1 mM CaCl₂, 50 mM cyclohexylaminopropane sulfonic acid, pH 11.0). The urea elution procedure was utilized unless otherwise indicated, although there were no detectable differences in biological activity of plasma fibronectins prepared by either method. Variable amounts of fibrinogen in many plasma fibronectin preparations were removed by chromatography on a 1.5- × 100-cm Sepharose CL 4B column as described previously for cellular fibronectin (32).

Human cell-surface fibronectin was routinely prepared by extraction of confluent WI38 fibroblasts with 2 M urea in serum-free Dulbecco-Vogt modified Eagle's medium (31) followed by affinity purification on immobilized gelatin and elution with 8 M urea by a protocol identical to that utilized for plasma fibronectin (8). As a control, two preparations of chick cell-surface fibronectin were diluted to 50 μg/ml in phosphate-buffered saline, then affinity-purified and eluted with 8 M urea as described above.

Preparations of cell-surface and plasma fibronectins

were dialyzed against two to three changes of buffer A (0.15 M NaCl, 1 mM CaCl₂, 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0) for 18–24 h at 4°C, and stored frozen in buffer A at ~1 mg/ml at -60°C.

To rule out proteolytic degradation as a possible source of artifactual electrophoretic migration of plasma fibronectin, we collected blood from a rooster directly into the following final concentrations of protease inhibitors: 5 mM *N*-ethylmaleimide, 10 mM EDTA, 5 μM pepstatin, and 2 mM phenylmethanesulfonyl fluoride. After centrifugation for 2 min in a Beckman microcentrifuge at 10,000 *g* (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), an aliquot of the plasma was brought to 2% in sodium dodecyl sulfate (SDS), 10 mM sodium phosphate, pH 7, and heated to 100°C for 3 min. A parallel aliquot of plasma in protease inhibitors without SDS was applied to a gelatin-Sepharose column to remove plasma fibronectin, and the eluate was also homogenized in SDS and electrophoresed.

Chicken cell-surface and plasma fibronectins were subjected to limited proteolysis with trypsin as described previously (31). 0.2 mg/ml of each protein was incubated with a 1:400 (wt/wt) ratio of crystalline trypsin in 1 mM CaCl₂ and 10 mM sodium phosphate, pH 7, at 23°C. The reaction was stopped at specified intervals by the addition of soybean trypsin inhibitor to 5 μg/ml, then SDS was added to 2% and the samples were heated to 100°C for 2 min and electrophoresed as described previously in a continuous 5% polyacrylamide gel system (33).

Tertiary chick embryo fibroblasts or WI38 human embryonic lung fibroblasts were cultured as previously described (36). To label secreted fibronectin, confluent cultures were incubated in 5 μCi/ml [¹⁴C]leucine for 24 h in regular medium, then the conditioned medium was aspirated and trichloroacetic acid was added to 5%. The acid-insoluble precipitate was collected by centrifugation, washed with 5% trichloroacetic acid, and then neutralized with 1 N NaOH and homogenized in 2% SDS (33). The ¹⁴C-labeled fibronectin band is a major band in SDS-polyacrylamide gels of conditioned medium from chick and human fibroblasts, and was identified by immunoprecipitation with affinity-purified antifibronectin and by electrophoretic criteria (1, 23, 28–31).

Cell-surface fibronectin was labeled with ¹²⁵I by lactoperoxidase-mediated iodination and homogenized in SDS as previously described (11, 33), and samples were analyzed utilizing a high-resolution system with 3% stacking and 4% polyacrylamide-resolving gels (27) containing an additional 3% prepolymerized linear polyacrylamide to decrease protein diffusion without altering gel cross-linking.

Hemagglutination (34), effects of fibronectin on cell flattening and alignment (31, 35), attachment of CHO cells to type I collagen (19), attachment of SV40-transformed 3T3 cells to rat tail collagen (18), and spreading of BHK cells on tissue culture dishes (10) were quantitated exactly as described in the references cited. The Kleinman collagen attachment assay (19) differs from

the original Klebe assay (18) primarily in its use of a much thinner layer of collagen as the attachment substratum.

Materials

N-Ethylmaleimide, EDTA, and pepstatin A were purchased from Sigma Chemical Co., St. Louis, Mo. Trypsin (3 × crystallized, 206 U/mg) was obtained from Worthington Biochemical Corp., Freehold, N. J.; soybean trypsin inhibitor (1 mg inhibited 1.5 mg of trypsin) from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; polyacrylamide (mol wt > 5,000,000) from B.D.H. Chemicals, Ltd., Poole, England; and pooled human serum from Flow Laboratories, Inc., Rockville, Md. Carrier-free Na ¹²⁵I and L-[U-¹⁴C]leucine (287 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. Type I lathyritic rat skin collagen was a generous gift of Dr. Hynda Kleinman, National Institute of Dental Research. All other materials were obtained from the sources described previously (32, 36).

RESULTS

We first compared several biological activities of purified cell-surface and plasma fibronectins. Cell-surface fibronectins from both chick and human cells are 150-fold more active in agglutinating formalin-fixed sheep erythrocytes than purified plasma fibronectins (Table I). The same differences in agglutination activity between cell-surface and plasma fibronectins are found whether the fibronectins are from human or chicken sources, whether the plasma fibronectins are eluted by 8 M urea or alkaline elution from the immobilized collagen purification columns, or whether the plasma fibronectin is isolated from chicken or human blood anticoagulated with heparin or ACD, or from human serum. In addition, fibro-

nectin from human plasma that was applied to an immobilized collagen column within 30 min after venupuncture, eluted with pH 11 buffer, and assayed immediately without further processing also had very low hemagglutinating activity (requiring 250 μg/ml for half-maximal agglutination).

We evaluated the possibility that an inhibitor of hemagglutination was present in the plasma fibronectin preparations by mixing equal volumes of plasma and cell-surface fibronectins immediately before assay. In four experiments, the final activities were always the average of the two activities; e.g., a chicken cell-surface fibronectin preparation with an endpoint of 1/512 mixed with a plasma fibronectin preparation with endpoint of 1/2 had a net endpoint of 1/256. There is consequently no evidence for an inhibitor of hemagglutination in the plasma fibronectins.

A second biological activity of cellular fibronectin can be identified after it is reconstituted on the surface of transformed cells originally lacking this protein (reviewed in references 13, 30, and 31). Reconstituted cellular fibronectins can partially restore a more normal fibroblastic morphology and adhesiveness to many transformed cells, as well as restoring the capacity of some cell types to align in parallel arrays. In contrast, chicken and human fibronectins display only weak activity in cell flattening and elongation (Fig. 1). In dilution experiments, determinations of the lowest concentration at which fibronectin-treated cells can be distinguished from controls in "blind" evaluations of photographs of random fields indicate that cell-surface fibronectin is 50-fold more effective than plasma fibronectin (1 μg/ml for cell-surface fibro-

TABLE I
Concentrations of Fibronectins Required for Half-Maximal Hemagglutination or Cell Spreading

| Fibronectin | Species | Hemagglutination | Cell spreading |
|--------------|---------|----------------------------------|----------------|
| | | μg/ml | μg/ml |
| Cell surface | Chicken | 1.7 ± 0.5 (3) [1.3 ± 0.3] (7) | 1.3 ± 0.3 (3) |
| | Human | 1.2 ± 0.3 (3) | ND |
| Plasma | Chicken | 248 ± 55 (7) | 1.5 ± 0.5 (2) |
| | Human | 238 ± 42 (6) | 1.0 ± 0.0 (2) |

Agglutination of formalin-fixed sheep erythrocytes and spreading of BHK cells were assayed as described in Materials and Methods. Values in brackets were obtained with preparations assayed before affinity chromatography on immobilized collagen and elution with 8 M urea. ND, not determined. Values indicate mean ± SE. Numbers in parentheses indicate the number of independent experiments, each performed with a different preparation of cellular or plasma fibronectin, usually with duplicate determinations in each.

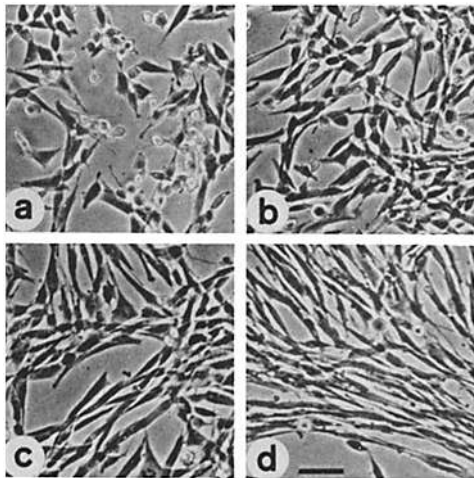


FIGURE 1 Comparison of effects of purified chicken plasma and cell-surface fibronectins on morphology of subconfluent, SV40-transformed 3T3 fibroblasts. (a) Control. (b) Plus 50 $\mu\text{g/ml}$ plasma fibronectin. (c) Plus 1 $\mu\text{g/ml}$ cell-surface fibronectin, the minimum concentration at which an effect was detectable. (d) Plus 50 $\mu\text{g/ml}$ cell-surface fibronectin. Phase microscopy 24 h after the addition of fibronectin. Bar, 50 μm .

nectin vs. 50 $\mu\text{g/ml}$ for plasma fibronectin in three experiments).

Both cellular and plasma fibronectins are thought to be capable of mediating attachment of cells to collagen in the absence of serum (18, 24). Utilizing Kleinman's new, sensitive assay for the attachment of CHO (Chinese Hamster Ovary) cells to rat skin type I collagen (19), we find that chicken cell-surface and plasma fibronectins have equal specific activities for attachment; human plasma fibronectin also has the same activity (Fig. 2). The concentration of each of these proteins required for half-maximal attachment was 1–2 $\mu\text{g/ml}$ in this and three other experiments. Chicken cell-surface and plasma fibronectins also had equal specific activities in the Klebe assay (18) for attachment to crude rat tail collagen (unpublished results). We emphasize that these experiments were performed with the identical preparations analyzed above for hemagglutinating and morphological activities.

Finally, cell-surface and plasma fibronectins were compared as to their ability to promote spreading of BHK cells on plastic tissue culture substrata by the assay of Grinnell et al. (10). As recently reported for fibronectins from other species (25), chicken plasma and cell-surface fibro-

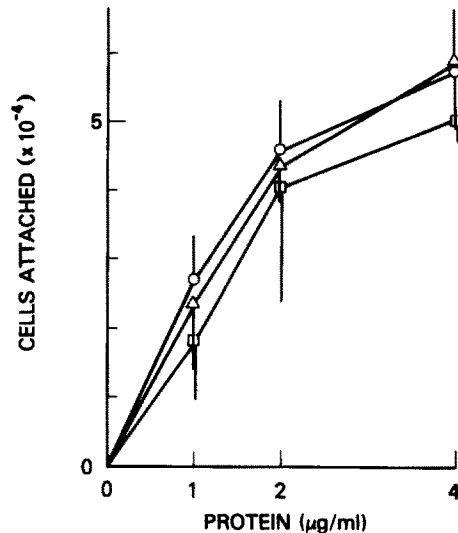


FIGURE 2 Attachment of CHO cells to dishes coated with type I collagen. Ordinate indicates number of cells attached per dish at each concentration of fibronectin after 1 h at 37°C. \circ , Chicken cell-surface fibronectin. \square , Chicken plasma fibronectin. \triangle , Human plasma fibronectin. Points are the means of triplicate dishes; bars indicate SE for each point.

nectins are equally active in permitting cell spreading (Table I).

Since cell-surface and plasma fibronectins have identical specific activities in two biological assays and widely divergent activities in two others, they were examined by several different SDS polyacrylamide gel electrophoretic systems for structural differences. Human cell-surface, secreted, and plasma fibronectins are compared in Fig. 3, and chicken fibronectins in Fig. 4, by utilizing an electrophoretic system found to have excellent resolution for proteins of high molecular weight. The purified cell-surface fibronectins have an apparent subunit mol wt $\geq 10,000$ daltons larger than that of the plasma fibronectins. In addition, the cell-surface fibronectins migrate as a single band, whereas the plasma fibronectins were doublets separated by 5,000 daltons (Figs. 3 and 4). The two bands of human plasma fibronectin appear to be present in equal amounts, whereas the higher molecular weight band of chicken plasma fibronectin appears to be present in slightly decreased amounts compared to the lower band (Fig. 4c and i). The migration of plasma fibronectin as a doublet with a faster mobility than cellular fibronectin is less obvious in a different, continuous gel system (Fig. 5).

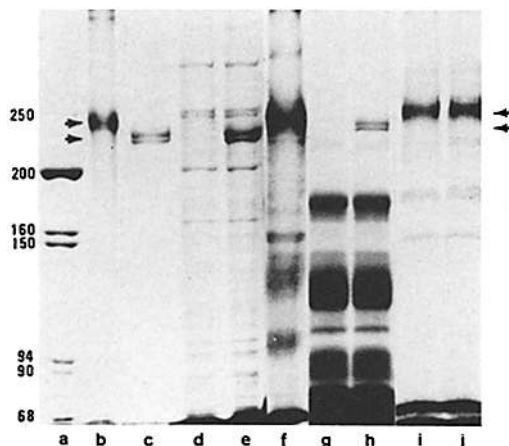


FIGURE 3 SDS gel electrophoresis of human cellular and plasma fibronectins. Samples were analyzed in a 3%/4% polyacrylamide gel containing 3% linear polyacrylamide as described in Materials and Methods. (a) Protein molecular weight standards ($\times 10^{-3}$ daltons). (b) Human cell-surface fibronectin. (c) Human plasma fibronectin. (d) Homogenate of confluent culture of human WI38 cells labeled with ^{125}I . (e) Same as previous lane, plus plasma fibronectin. (f) Autoradiogram of lane e. (g) Conditioned cultured medium collected after labeling WI38 cells for 24 h with ^{14}C leucine. (h) Same as previous lane plus plasma fibronectin marker. (i) Autoradiogram of lane g. (j) Autoradiogram of lane h. Although all lanes are from a single slab gel, the molecular weight markers apply only to lanes b-f. Lanes g-j migrated slightly anomalously because of the high salt content, and comparisons should be relative to the added internal standard of plasma fibronectin in lane h.

Isolated cell-surface fibronectins co-migrate with the cell-surface fibronectin of cells labeled *in situ* by lactoperoxidase-mediated iodination with ^{125}I (Figs. 3 and 4). In mixing experiments, plasma and cell-surface fibronectins do not co-migrate (Figs. 3 and 4d-f). Cellular fibronectin labeled with ^{14}C leucine that is secreted or sloughed into culture media co-migrates with cell-surface fibronectin, but not plasma fibronectin (Figs. 3 and 4g-k). The lower apparent molecular weight of plasma fibronectin is probably not a consequence of proteolytic degradation after collection, because addition of four protease inhibitors during collection (see Materials and Methods) and immediate boiling in SDS yields plasma in which the plasma fibronectin band electrophoretically co-migrates with isolated plasma fibronectin (and which is removed by affinity adsorption to immobilized collagen) (Fig. 4l and m). Both cell-surface and secreted forms of cellular fibronectin are therefore

larger than the two subunits of plasma fibronectin in both chicken and man.

The structural relationships between cellular and plasma fibronectins are clearer after limited proteolysis by trypsin (Fig. 5). It has been reported that chick and hamster cellular fibronectins can be cleaved by trypsin to a large proteolytic fragment that is no longer disulfide-bonded to other subunits; the interchain disulfide bonds are therefore in the portion of the molecule cleaved off by trypsin (13, 31). In other experiments, treatment of human plasma fibronectin with another protease, plasmin, resulted in a very similar pattern of proteolysis (4, 14, 16). We directly compared parallel digestions of chicken cellular and plasma fibronectins using the same protease (trypsin). We find that the plasma fibronectin proteolytic fragments remain a doublet, and that they retain the faster electrophoretic mobility

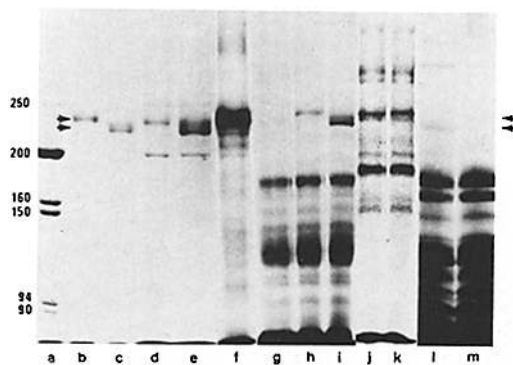


FIGURE 4 SDS gel electrophoresis of chicken cellular and plasma fibronectin. Samples were analyzed in a 3%/4% polyacrylamide gel plus linear polyacrylamide as described in Materials and Methods. (a) Protein standards ($\times 10^{-3}$ daltons). (b) Chicken cell-surface fibronectin. (c) Chicken plasma fibronectin. (d) Homogenate of confluent chick embryo fibroblasts labeled with ^{125}I . (e) Same as previous lane plus chicken plasma fibronectin marker. (f) Autoradiogram of lane e. (g) Conditioned medium from chick embryo fibroblasts after labeling 24 h in ^{14}C leucine as described in Materials and Methods. (h) Conditioned medium plus cell-surface fibronectin marker. (i) Conditioned medium plus plasma fibronectin marker. (j) Autoradiogram of lane h. (k) Autoradiogram of lane i. (l) Chicken plasma collected in protease inhibitors as described in Materials and Methods. (m) Chicken plasma collected in protease inhibitors, then chromatographed on an immobilized gelatin column to remove plasma fibronectin. As in Fig. 3, the protein molecular weight estimates do not apply to lanes g-k, for which the internal standards in lanes h and i should be utilized.

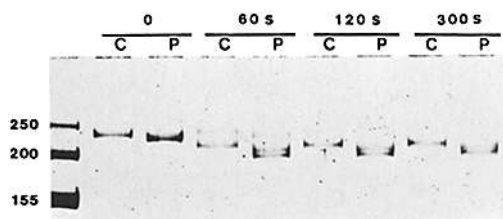


FIGURE 5 Comparison of limited proteolysis products of chicken cell-surface and plasma fibronectins. Samples were incubated in parallel with crystalline trypsin for the times indicated, then electrophoresed as described in Materials and Methods. C, Cellular fibronectin; P, plasma fibronectin. Molecular weights are $\times 10^{-3}$ daltons.

compared to the cellular fibronectin tryptic fragment (Fig. 5). As reported previously, examination of both fibronectins after proteolysis without reduction of disulfide bonds with dithiothreitol showed that the large proteolytic fragments were not disulfide-bonded to other subunits (31; results not shown). These results suggest that plasma and cellular fibronectins have a similar overall structure, but that the differences in polypeptide length found in the intact molecule remain in the large proteolytic fragments. Because this fragment is thought to be at the amino terminus of the molecule (13, 14), the differences in polypeptide length may be localized to the amino rather than the carboxy portion of fibronectin.

DISCUSSION

The major findings of this study are: (a) plasma and cell-surface fibronectins have identical biological activities in mediating both cell attachment to collagen and cell spreading; (b) the same preparations of plasma fibronectin are 50-fold less active than cellular fibronectin in restoring normal fibroblastic morphology and alignment to transformed cells in reconstitution experiments, and they are 150-fold less active in hemagglutination; (c) these differences in biological activity are accompanied by differences in subunit size of plasma and cellular fibronectins; and (d) these size differences are localized to homologous major proteolytic fragments of these molecules. In addition, previous work has reported differences in the solubilities of these proteins; only plasma fibronectins are soluble at physiological pH (see reference 30). Cellular and plasma fibronectins are therefore similar but not identical.

A tentative generalization with respect to biological activities is that both the plasma and cellular forms of fibronectin can mediate cell-to-sub-

stratum interactions, while only the cellular form readily promotes cell-to-cell interactions and strong cell-to-plastic interactions in the presence of serum. It is important to note that the activities attributed to plasma fibronectin are assayed in the absence of serum, and can be mediated by the fibronectin in serum (9, 10, 18, 19). In contrast, the effects of cellular fibronectin on cell shape and alignment are routinely assayed in serum-containing culture media (31); it is therefore not surprising that the addition of more plasma fibronectin has relatively minimal effects. However, it is also noteworthy that the plasma fibronectins are not entirely devoid of these unusual activities; this finding is further evidence for homologies between the proteins.

What accounts for the differences in size and biological activity? Two obvious possibilities are that the two proteins are related but are different gene products, or that plasma fibronectin is derived from cellular fibronectin by processing either of an RNA precursor or of protein. Differences caused by differing amounts of glycosylation are unlikely because their carbohydrate compositions are nearly identical (29, 32). One attractive possibility is that the larger size of cellular fibronectin is because of a short, additional segment at the amino terminus (28) that could promote cell-to-cell interactions via hydrophobic or hydrogen-bonding interactions, as well as low solubility caused by enhanced self-association. This possibility is currently under investigation.

We thank Dr. Hynda Kleinman for providing type I collagen and methods before publication, Dr. Fernando Cabral and Dr. Peter Davies for suggesting the use of linear polyacrylamide, Mr. Raymond Steinberg for photographic work, and Mrs. Wilma Davis for typing the manuscript.

Received for publication 18 September 1978, and in revised form 31 October 1978.

Note Added in Proof: Chicken cellular fibronectin was recently reported to mediate attachment of periosteum cells to collagen with specific activities similar to those we report (Kleinman, H. K., et al. 1978. Proceedings, Mechanisms of Localized Bone Loss. Horton, Tarpley, and Davis, editors. Special Supplement to Calcified Tissue Abstracts. 61-74).

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