Fibulin Is An Extracellular Matrix and Plasma Glycoprotein with Repeated Domain Structure

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Abstract. We have studied the expression of fibulin in cultured fibroblasts and determined its primary structure by cDNA cloning. Our results show that fibulin is a secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix when expressed by cultured cells or added exogenously to cell monolayers. In addition, we find that fibulin is present in plasma at a level of 33 ± 3 μg/ml. Sequencing of multiple fibulin cDNAs indicates that a process of alternative splicing results in the expression of three fibulin transcripts. The transcripts encode overlapping polypeptides differing only in carboxy-terminal segments. Common to the three predicted forms of fibulin is a unique 537–amino acid–long cysteine-rich polypeptide and a 29-residue signal peptide. The amino-terminal portion of fibulin contains a repeated element with potential disulfide loop structure resembling that of the complement component anaphylatoxins C3a, C4a, and C5a as well as proteins of the albumin gene family. The bulk of the remaining portion of the molecule is a series of nine EGF-like repeats.

FIBULIN is a recently described calcium-binding protein which has been shown to interact with a synthetic peptide representing the cytoplasmic domain of the integrin β_1 subunit as well as native $\alpha_5\beta_1$ fibronectin receptor (Argraves et al., 1989). Indirect immunofluorescent staining of cultured fibroblasts revealed that fibulin colocalized with the integrin β_1 subunit, in vivo, at sites of cellular interaction with underlying fibronectin substratum. It was therefore suspected that fibulin might be an intracellular protein involved with mediating cytoplasmic connections of the β_1 integrins. Herein we report the results of characterization of fibulin expression and structure. The results indicate that fibulin is glycosylated and secreted by cultured fibroblasts and becomes incorporated into an extracellular matrix in a fashion similar to fibronectin. Salient structural features deduced from cDNA clones reveal that fibulin is a multidomain protein with two types of repeat motifs, one of which is homologous to the anaphylatoxins C3a, C4a, and C5a, as well as elements of proteins of the albumin gene family, and the other which is homologous to EGF.

Materials and Methods

Antibodies

The following antisera were used for the immunoprecipitation and immunofluorescent staining experiments described herein: mouse monoclonal anti–human fibronectin (not cross-reactive with the bovine fibronectin used in slide coating) was purchased from Telios Pharmaceuticals, La Jolla, CA;

mouse monoclonal anti–human integrin β_1 subunit was provided by Dr. E. Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA; and rabbit anti–human fibulin serum was prepared in this laboratory and has been described previously (Argraves et al., 1989). As a precaution, the antifibulin serum used in the immunofluorescent staining experiments was absorbed on columns of human fibronectin and fibronectin receptor coupled to Sepharose.

For immunoadsorption of fibulin and ELISA, the mouse mAb 5D12/H7 was used. This hybridoma cell line was produced by fusion of immune mouse spleen cells with myeloma X63Ag8.653 cells according to published methods (Ruoslahti et al., 1982). 5D12/H7 reacts specifically with fibulin in ELISA, immunoprecipitation, and in immunoblotting under both reducing and nonreducing conditions (Dickerson, K., and W. S. Argraves, unpublished observations).

Indirect Immunofluorescent Microscopy

Human gingival fibroblasts (primary fibroblast line obtained from Dr. M. Somerman, University of Maryland, Baltimore, MD) were seeded at a density of 1.5×10^4 cells/ml onto Lab-Tek chamber slides (Nunc Inc., Naperville, IL) coated with bovine fibronectin (10 μg/ml, Telios Pharmaceuticals). Cells were fixed for 30 min with 3.7% paraformaldehyde (Fluka AG, Buchs, Switzerland), 0.1% Triton X-100 in PBS, pH 7.2. In indicated experiments, the detergent was omitted from the fixing solution. The slides were washed with PBS and then incubated in 3% normal goat serum–PBS (PBS-serum) for 1 h at room temperature. The primary antisera were diluted in PBS-serum and incubated with the fixed cells for 2 h at 37°C. The slides were then washed with PBS three times for 5 min. The fluorochrome-conjugated antisera, either fluorescein-conjugated sheep anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), were diluted 1:40 in PBS-serum, and incubated with the slides for 20 min at room temperature. The slides were again washed with PBS. A solution of 50% glycerol in PBS was applied to the surface of the slides and a glass coverslip overlaid and fixed to the surface with clear nail polish.

Stained cells were examined and photographed using an Olympus BHS microscope equipped for fluorescent microscopy and having additional exciter filters so as to narrow wavelength bands and restrain crossover excita-

tion during double-fluorochrome label experiments. Photographs were taken using Fujichrome 1600 reversal film with exposure settings controlled with an Olympus (model PM-10ADS) exposure control unit.

Biotinylation of Fibulin

The fibulin used for biotinylation was purified by immunoadsorption from extracts of human placenta. Ground placental tissue was extracted with 4 M KSCN. Extracts were then clarified by centrifugation, dialyzed against TBS, 10 mM EDTA, and passed over a column of plain Sepharose CL-4B. The flow-through was then applied to an affinity matrix of monoclonal 5D12/H7 IgG coupled to Sepharose. The column was washed with 0.5 M NaCl, 50 mM Tris, pH 7.4, and bound fibulin eluted with a solution of 4 M KSCN. The eluted fibulin was dialyzed against TBS and affinity selected on wheat germ agglutinin (WGA)[1]–agarose (see below). Purified fibulin was incubated with sulfo-N-hyroxysuccinimide-biotin (S-NHS-biotin; Pierce Chemical Co., Rockford, IL) in 0.1 M sodium carbonate, pH 8.5 (at a 1:200 molar ratio of protein to S-NHS biotin) for 3 h at 4°C. After the reaction, the samples were dialyzed against serum-free DME supplemented with penicillin, streptomycin, glutamine, sodium bicarbonate, and sodium pyruvate.

Gingival cells were grown for 24 h in Lab-Tek chamber slides coated with 25 μg/ml bovine fibronectin. Medium was removed and the cell monolayers were washed three times with serum-free DME. Biotinylated fibulin, human fibronectin, and human IgG, each diluted to 0.5 mg/ml in DME-ITS (insulin, transferrin, selenous acid, BSA, and linoleic acid; Collaborative Research Inc., Waltham, MA) were added separately to the cells and allowed to incubate for 12 h at 37°C. The media were removed and the cell layers washed three times with PBS. The fluorochrome conjugate, FITC-avidin (Pierce Chemical Co.), was diluted to 30 μg/ml in PBS, added, and incubated for 30 min at room temperature. The cell layers were washed three times with PBS, mounted, and examined by immunofluorescent microscopy.

Immunoprecipitation Analysis

Nearly confluent human gingival fibroblasts, in 100-mm-diam culture dishes (Becton Dickinson, Lincoln Park, NJ), were radiolabeled for 18 h with 250 μCi of [^{35}S]cysteine (New England Nuclear, Boston, MA) in DME (Mediatech, Herdon, VA) containing 10% bovine calf serum supplemented with iron (HyClone Laboratories, Logan, UT). The media was removed and centrifuged at 5,000 g for 15 min. The media supernatant was then dialyzed against 0.5 M NaCl, 2 mM PMSF, 0.1% Triton X-100, 0.1% Tween-20, 50 mM Tris-HCl, pH 7.4 (wash buffer) for 18 h at 4°C. The dialyzed media was pre-cleared with 0.2 vol of protein A-Sepharose (Sigma Chemical Co., St. Louis, MO, mixed 1:1 vol/vol in wash buffer). After a 1-h incubation, the protein A-Sepharose was removed by centrifugation at 2,500 g for 5 min. Antiserum (2 μl) was added to 2-ml aliquots of media and incubated for 18 h at 4°C. Immune complexes were precipitated with protein A-Sepharose and washed repeatedly in wash buffer. After a final wash in TBS, pH 7.4, bound protein was released by addition of SDS electrophoresis sample buffer and analyzed by SDS-PAGE on 7.5% gels.

Pulse–chase Immunoprecipitation Analysis

Human gingival fibroblasts were grown to near confluence in 35-mm-diam culture dishes. Cell layers were washed three times with cysteine-free RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with ITS and 10 mM Hepes, pH 7.0. The cells were then grown for 15 min at 37°C in the cysteine-free RPMI-ITS medium. The cultures were then pulse labeled for 2 min with cysteine-free RPMI-ITS medium containing 0.5 M Ci/ml [^{35}S]cysteine. After the 2-min pulse labeling the medium was removed and the cell layers were washed three times with RPMI-ITS containing 1 mM unlabeled cysteine, 10 mM Hepes, pH 7.0, and then allowed to incubate for various periods of time in the same medium at 37°C. At the appropriate time intervals, medium was isolated, and the cell layer extracted with 1 ml of 1% Triton X-100, 0.5 M NaCl, 0.05% Tween 20, 0.05 M Tris-HCl, pH 7.4, 2 mM PMSF, using a disposable cell scraper. The cell extracts and culture medium fractions were clarified by centrifugation at 100 g in an ultracentrifuge (model TL-100; Beckman Instruments, Inc., Palo Alto, CA). The resulting supernatants were pre-absorbed with protein A-Sepharose, used in immunoprecipitation, and analyzed by SDS-PAGE as

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1. *Abbreviations used in this paper*: PCR, polymerase chain reaction; WGA, wheat germ agglutinin.

described above. After SDS-PAGE, gels were treated with Enlightning (New England Nuclear, Boston, MA), dried, and used to expose x-ray film at −70°C.

ELISA for Determining Fibulin Concentration in Plasma

To determine the amount of fibulin in plasma, a two-antibody sandwich ELISA was developed. Microtiter wells were coated overnight with 3 μg/ml mouse anti-fibulin monoclonal 5D12/H7 IgG in 0.1 M sodium carbonate buffer, pH 9.5. Nonspecific binding sites were quenched by addition of 1 mg/ml BSA in PBS. Human plasma, pooled from five donors, was serially diluted and incubated with the antibody coating for 1 h at room temperature. Rabbit antifibulin serum at a dilution of 1:10,000 was incubated for 1 h at room temperature followed by goat anti-rabbit IgG alkaline phosphatase for an additional hour. The chromogenic substrate p-nitrophenyl phosphate (Sigma Chemical Co.) was used to measure enzymatic activity bound to the wells. Resulting absorbance values of the plasma samples were compared to those of a serially diluted standard of purified placental fibulin. The concentration of the fibulin standard was determined by protein–dye binding assay (Bradford, 1976).

Lectin Affinity Chromatography of Fibulin

Fibulin was purified from placental extracts by affinity chromatography on the synthetic $β_1$ subunit cytoplasmic domain peptide Sepharose as previously described (Argraves et al., 1989). Fibulin, in 25 mM octyl-$β$-D-glucoside, 20 mM EDTA, 2 mM PMSF, TBS was then applied to a column of WGA coupled to agarose (Vector Laboratories, Inc., Burlingame, CA) equilibrated in the same buffer. The column was washed with 10 column volumes of TBS and eluted with 2 column volumes of TBS containing 0.5 M N-acetyl-D-glucosamine (Sigma Chemical Co.). Eluted protein was electrophoresed on SDS–polyacrylamide gels and protein bands stained using Coomassie blue.

Detection of N-linked Oligosaccharides

To determine the presence of N-linked oligosaccharides on fibulin, WGA-agarose-selected fibulin was first boiled for 3 min in 0.5% SDS, 0.1 M $β$-mercaptoethanol and then digested with N-glycosidase F (Genzyme Corp., Boston, MA), according to the manufacturer's protocol, for 18 h at 37°C. After the digestion, samples were analyzed by SDS-PAGE.

Protein Sequence Analysis

Fibulin was purified from placental extracts by affinity chromatography on the synthetic integrin $β_1$ subunit cytoplasmic domain peptide Sepharose. The affinity-selected material was electrophoresed on SDS–polyacrylamide gels and the 100-kD fibulin polypeptide electroeluted from gel slices (Hunkapiller et al., 1983). This material was digested with trypsin in 0.1 M ammonium bicarbonate, pH 8.0, for 18 h at 37°C. The digest was fractionated on an RP300 column (Applied Biosystems, Foster City, CA) using a microbore HPLC (model 130; Applied Biosystems). Protein fragments from individual peaks were then subjected to Edman degradation using a protein sequencer (model 477A; Applied Biosystems).

Isolation and Sequencing of Fibulin cDNAs

A human placental cDNA λgt11 library (Millan, 1986) was immunologically screened (Young and Davis, 1983) using antibodies affinity selected from rabbit antifibulin serum (Argraves et al., 1989) on a column of fibulin coupled to Sepharose. Clones that expressed insert-encoded protein reactive with these antibodies were isolated and through successive screenings cloned to homogeneity. Insert cDNAs were subcloned into the phage vector M13mp19 and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using modified T7 polymerase (United States Biochemical Corp., Cleveland, OH) and synthetic oligonucleotide primers based on derived sequences. All sequences reported are based on the sequencing of both strands of the cDNA inserts. Sequence analysis and protein database searches were performed using PC-Gene (IntelliGenetics, Mountain View, CA) and Seq-it (CompuRight, Newtown, CT).

RNA Hybridization Analysis

Human placental poly(A)$^+$ RNA was electrophoresed in denaturing 0.8%

agarose gels containing 6% formaldehyde (Lehrach et al., 1977), and blot transferred to nitrocellulose (Thomas, 1980). The filters were probed with a 150-bp DNA segment generated by polymerase chain reaction (PCR) (Saiki et al., 1988) using a fibulin cDNA insert at template and upstream and downstream oligonucleotide primers both taken from a region of fibulin cDNA common to the three cDNA types. After hybridization the filters were washed under high stringency and used to expose x-ray film at −70°C.

PCR Analysis

Total human placental RNA (1 μg) was used with random hexanucleotide primer (200 ng, Pharmacia Fine Chemicals, Piscataway, NJ), RNasin (30 U, Promega Biotec, Madison, WI), 1 mM deoxynucleotide triphosphates (dNTPs), and Moloney murine leukemia virus reverse transcriptase (200 U, Bethesda Research Laboratories, Gaithersburg, MD) to synthesize cDNA. Using 1/100 of the cDNA product, Taq DNA polymerase (3 U, Stratagene, La Jolla, CA), upstream and downstream synthetic oligonucleotide primers (800 ng each), and dNTPs (0.25 mM each), polymerase chain amplification was performed. Primer pairs specific for the cDNA of fibulin A, B, or C were taken from the following positions within the respective target DNA sequence: 1657–1674 and 2142–2159 for A, 1657–1674 and 2248–2265 for B, and 1442–1459 and 1966–1983 for C. The following temperature parameters were cycled 35 times: 1 min at 94°C, 2 min at the $T_m − 4°C$ of the primer with the lower T_m of the given pair, and 3 min at 72°C. Aliquots of the reactions were analyzed by agarose gel electrophoresis and the separated DNA was stained with ethidium bromide.

Results

Fibulin Is Incorporated into an Extracellular Matrix

We have previously reported that fibulin expressed by cultured fibroblasts accumulates at sites of expected cellular interaction with underlying fibronectin substratum (Argraves et al., 1989). The observed pattern of fibulin staining is coincident with that of the integrin β_1 subunit. These results were based on experiments confined to the period of 4–6 h after plating of the fibroblasts onto fibronectin-coated surfaces. At this early time period, fibulin and the integrin β_1 subunit each appear in immunofluorescent staining as numerous colocalizing streaklike accumulations (Fig. 1, C and D). In the absence of permeabilizing agent, such staining patterns are not evident (Fig. 1, A and B). When we extended immunofluorescent staining studies to periods beyond 6 h we found fibulin accumulated into extensive fibrillar patterns (Fig. 1 K). Furthermore, the fibrillar staining pattern was apparent in the absence of permeabilizing agent (Fig. 1 I) indicating that the immunologically detected fibulin was extracellular.

It was also apparent that the meshwork staining pattern of fibulin was similar to that of fibronectin (Mautner and Hynes, 1977). Indeed, when double-label immunofluorescent staining was done using antibodies to both fibulin and fibronectin, very similar staining patterns were seen both at the early and late periods of culture (Fig. 1, G–L). The staining patterns obtained using fibulin antibodies could be completely blocked by preincubation of the antibodies with 25 μg/ml fibulin (results not shown). In addition, preincubation of fibulin antibodies with human fibronectin at 25 μg/ml failed to block antibody staining of fibulin (data not shown). A further indicator of fibulin antibody specificity is demonstrated by immunoprecipitation experiments shown in Fig. 2. The results indicate that fibulin, like fibronectin, accumulates extracellularly, forming dense networks of fibrils.

It has been shown that exogenously added fibronectin binds to cultured cell monolayers, and becomes assembled into a matrix (Hayman and Ruoslahti, 1979; McKeown-Longo and Mosher, 1983). To similarly evaluate the ability of exogenously added fibulin to bind to cell monolayers, and become incorporated into a matrix, biotinylated fibulin was incubated with fibroblast monolayers. In parallel experiments, biotinylated fibronectin and human IgG were also incubated with fibroblast monolayers as control proteins. After 12 h of incubation, the exogenously added biotinylated fibulin was found bound to the cell monolayer, accumulating in elaborate fibrillar networks (Fig. 1 M). A similar pattern of incorporation was obtained with biotinylated fibronectin (Fig. 1 N), but not with biotinylated IgG (Fig. 1 O). The patterns of incorporation of exogenously added fibulin and fibronectin closely resembled the patterns of endogenous matrix accumulation for each protein as described above (Fig. 1, I–L).

Fibulin Is Secreted by Cultured Fibroblasts

The fact that the immunofluorescent staining results indicated that fibulin was accumulating extracellularly prompted us to verify whether cultured cells secreted fibulin into their medium. Culture medium from fibroblasts metabolically radiolabeled with [^{35}S]cysteine was analyzed for the presence of fibulin. As shown in Fig. 2, fibulin antibodies immunoprecipitate a single polypeptide with an apparent reduced molecular mass of 100 kD, which corresponds to that of previously characterized placental fibulin. In the absence of reducing agent, the immunoprecipitated polypeptide exhibits the increased electrophoretic mobility characteristic of fibulin. The results indicate that fibulin is secreted by the cultured fibroblasts.

To examine the temporal biosynthesis of fibulin, we performed pulse–chase immunoprecipitation analyses. Within the first minutes of chase two immunoreactive polypeptides of ∼80 and 100 kD were present in the cell layer extract (Fig. 3 A). After 5 min of chase the level of 80-kD polypeptide diminished. Between 30 and 60 min of chase, 100-kD fibulin polypeptide appeared in the medium (Fig. 3 B), with a subsequent decrease in the 100-kD polypeptide in the cell extracts. The results suggest a precursor–product relationship between the 80- and 100-kD polypeptides. The 80-kD band may then correspond to the nascent fibulin polypeptide which is subsequently processed to the 100-kD molecule that is secreted.

Fibulin Is a Blood Protein

The presence of fibulin in blood was investigated using immunoadsorption chromatography and ELISA. Plasma, preabsorbed on a column of plain Sepharose, was passed over a column of monoclonal antifibulin IgG coupled to Sepharose and eluted with a solution of 4 M KSCN. SDS-PAGE analysis showed that the immunologically selected polypeptide displayed electrophoretic properties indistinguishable from those of placental fibulin (results not shown). Based on the results of a two-antibody sandwich ELISA we determined the amount of fibulin in plasma to be 33 ± 3 (mean ± SD) μg/ml.

Fibulin Is a Glycoprotein

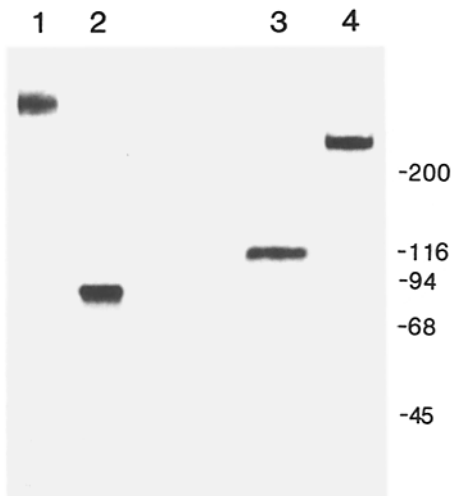Generally, secreted proteins are glycosylated and can be shown to interact with various lectins. We therefore inves-
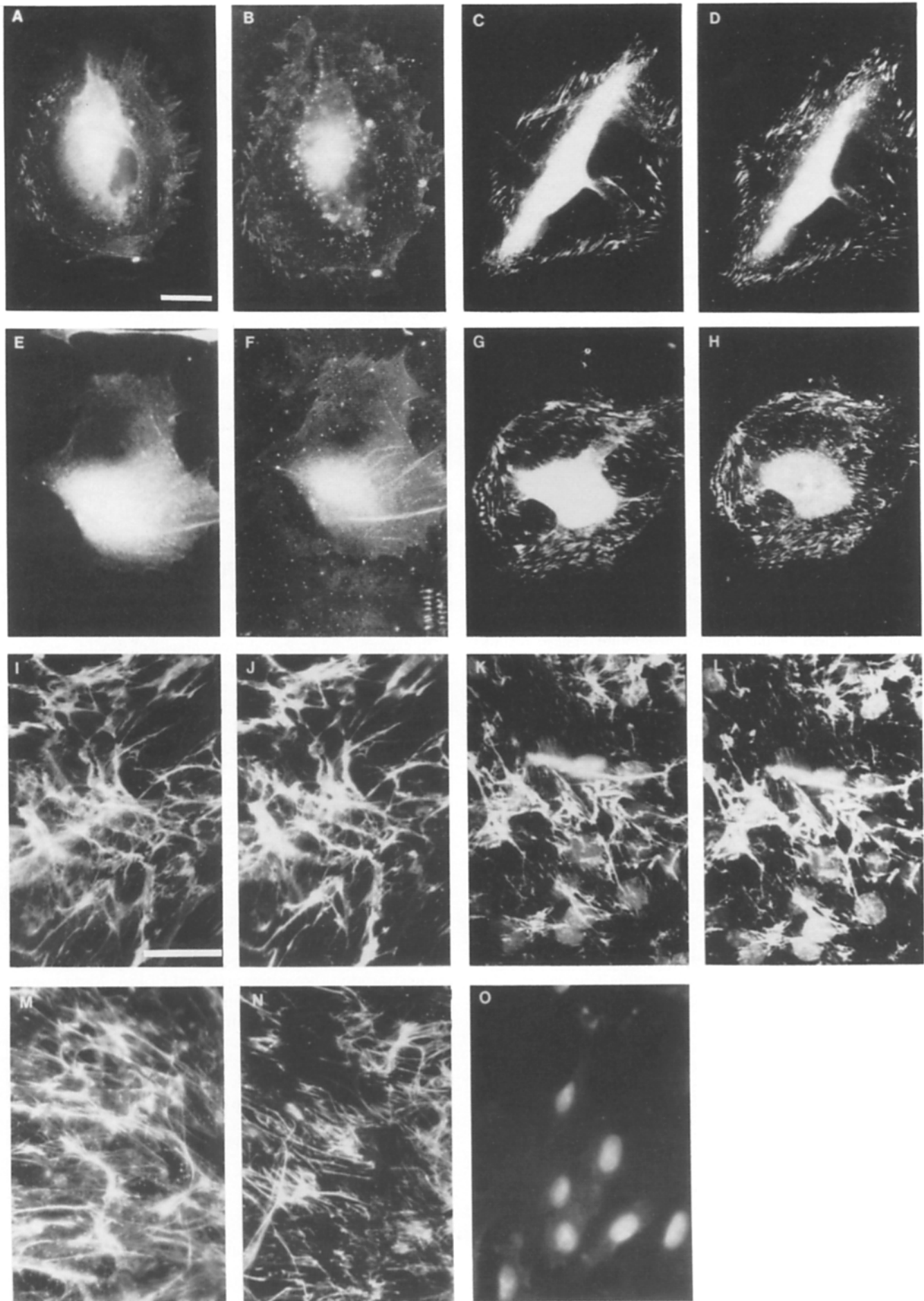
Figure 2. Immunoprecipitation of fibulin from fibroblast culture medium. Antifibronectin serum (lanes *1* and *4*) and antifibulin serum (lanes *2* and *3*) were used along with protein A–Sepharose to immunoprecipitate reactive species from culture media of human gingival fibroblasts metabolically labeled with [³⁵S]cysteine. Samples were electrophoresed in SDS-7.5% acrylamide gels under non-reducing (lanes *1* and *2*) and reducing (lanes *3* and *4*) conditions. After electrophoresis the gels were used to expose x-ray film. The sizes of protein molecular mass markers are indicated on the right in kilodalton.

Figure 3. Pulse–chase labeling and immunoprecipitation analysis of fibulin. Fibroblasts were pulse labeled with cysteine-free growth medium containing [³⁵S]cysteine and chased with medium containing unlabeled cysteine. At designated chase time intervals, culture medium and cell layer extracts were subjected to immunoprecipitation with fibulin antibodies. Immunoprecipitates were electrophoretically separated by SDS-PAGE and the gels analyzed by fluorography. Shown are immunoprecipitates from indicated chase times (in minutes) of cell layer extracts (*A*) and culture medium (*B*).

tigated the interaction of fibulin with WGA. Chromatography of placental fibulin preparations on columns of WGA coupled to agarose and subsequent SDS-PAGE analysis revealed that fibulin bound to the lectin and could be eluted using a solution of the sugar *N*-acetyl-glucosamine (Fig. 4). No 100-kD polypeptide was found in the material that passed through the lectin column (Fig. 4, lane 2) indicating that virtually all the fibulin bound. The results indicate that fibulin is a glycoprotein containing *N*-acetyl-glucosaminyl carbohydrate constituents.

Preparations of fibulin were subjected to digestion with *N*-glycosidase in an effort to estimate the amount of asparagine (N)-linked oligosaccharide on the polypeptide. As shown in Fig. 5, the electrophoretic mobility of fibulin increased after digestion with the enzyme. The mobility of the digested material corresponded to a molecular mass of 95 kD. Controls in which fibulin preparations were incubated under similar conditions, without the enzyme, showed no change in electrophoretic mobility. Assuming a molecular mass of 1,500 D for an average N-linked carbohydrate side chain, native fibulin may then have three N-linked oligosaccharide chains.
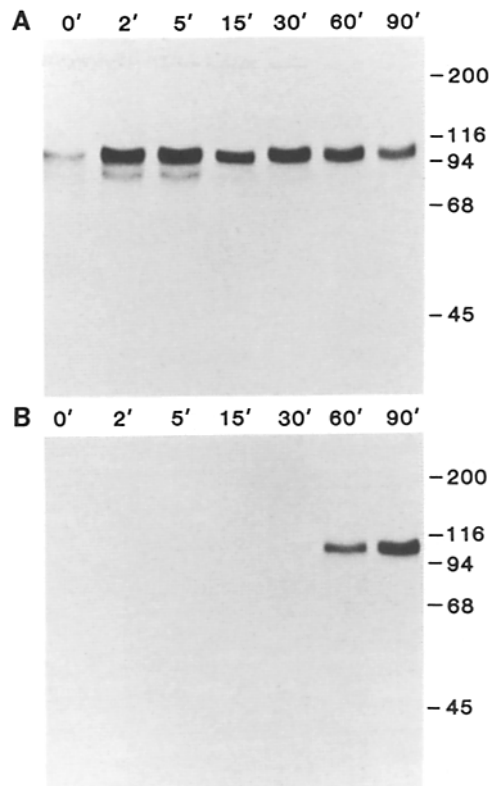
Fibulin Is Encoded by Multiple Transcripts

Immunological screening of a placental cDNA library resulted in the isolation of seven related clones. As individual cDNAs were sequenced it was found that they could be categorized into three types (A, B, and C). The nucleotide sequence of all three types of cDNAs were identical from their 5′ ends to a divergence point at position 1707, after which they were distinct through to the poly(A) tail. The categorization was therefore based on the sequence following the divergence point. Shown in Fig. 6 are the nucleotide sequences determined from the three types of cDNAs isolated.

Figure 1. Localization of fibulin in cultured human gingival fibroblasts. *A–H* represent double-label immunofluorescent staining images from cells cultured for 4 h on fibronectin-coated surfaces. These cells were either permeabilized (*C*, *D*, *G*, and *H*), or nonpermeabilized (*A*, *B*, *E*, and *F*), and stained with antifibulin (*A*, *C*, *E*, and *G*), mouse monoclonal anti-integrin β₁ subunit sera (*B* and *D*), or mouse monoclonal antifibronectin sera (*F* and *H*). *I–L* represent double immunofluorescent staining images done on cells cultured for 24 h on fibronectin-coated surfaces. These cells were nonpermeabilized (*I* and *J*), or permeabilized (*K* and *L*), and stained with antifibulin (*I* and *K*), and mouse monoclonal antifibronectin sera (*J* and *K*). *M–O* represent fluorescent images made after fibroblast monolayers were cultured in the presence of exogenously added biotinylated fibulin (*M*), biotinylated fibronectin (*N*), and biotinylated IgG (*O*) for 12 h. FITC-avidin was used to detect bound biotinylated probes. Control experiments with each of the fluorochrome conjugates gave negligible background staining. Bars: (*A–H*) 20 μm; (*I–O*) 20 μm.
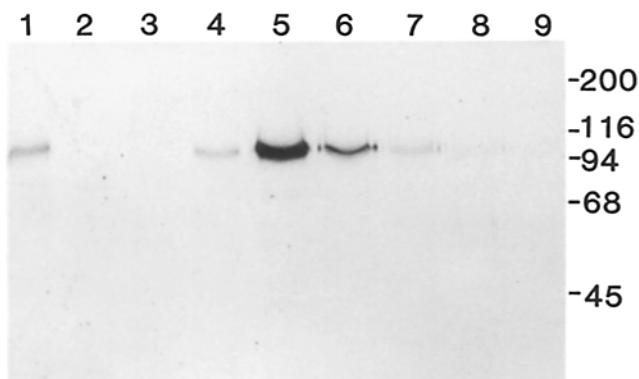
Figure 4. Interaction of fibulin with WGA. SDS-PAGE analysis of placental fibulin before application to the lectin column (lane *1*), the flow-through material (unbound fraction) (lane *2*), and fractions sequentially eluted from the column using a solution of *N*-acetyl glucosamine (lanes *3–9*). After the electrophoresis the gel was stained with Coomassie blue.

RNA hybridization analysis was performed using a fibulin cDNA fragment common to the three types of cDNA (bases 84–234, Fig. 6 *A*) as a probe. As shown in Fig. 7, two transcripts of ~2.4 and 2.7 kb were detected in human placental poly(A)$^+$ RNA. To verify that all the isolated cDNAs corresponded to actual transcripts expressed in placental tissue, a reverse transcriptase PCR analysis was performed (Rappolee et al., 1988). Pairs of synthetic oligonucleotide primers, based on sequence from either side of the divergence point from each cDNA type, were used in PCR to amplify cDNA prepared from placental RNA. The expected sizes for amplified products were 502, 606, and 541 bp for cDNA types A, B, and C, respectively. When the products were analyzed by agarose gel electrophoresis, fragments of the appropriate size were obtained (Fig. 8), thus confirming the presence of each transcript in total placental RNA. The product of PCR using the type A–specific primers was repeatedly the lowest in yield. The results indicate that at least three forms of fibulin transcripts exist, most likely made through a process of alternative splicing of a pre-mRNA transcript.
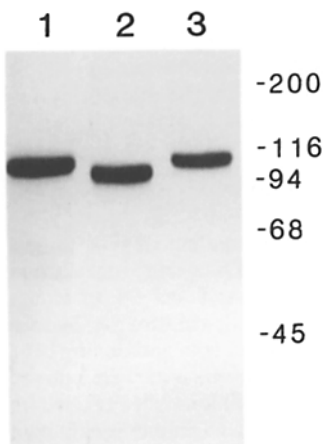

Figure 5. N-glycosidase digestion analysis of fibulin. Coomassie-stained SDS–polyacrylamide gel of undigested placental fibulin (lane *1*), fibulin subjected to digestion with *N*-glycosidase for 18 h at 37°C (lane 2), and fibulin incubated under similar conditions of digestion minus *N*-glycosidase (lane *3*).

Fibulin Is a Modular Protein Containing Two Types of Repeat Motifs

Polypeptides of 566, 601, and 683 amino acid residues are encoded by the type A, B, and C cDNAs, respectively. These polypeptides have in common the first 566 amino acid residues. The alternative B and C cDNA segments encode differing polypeptide elements that add 35 and 117 residues to the 566-residue protein. The amino acid sequence deduced from the cDNAs was found to contain the sequences determined from protein sequencing of fibulin including the amino-terminal sequence (Argraves et al., 1989) and three sequences derived from tryptic fragments of fibulin (Fig. 6). These findings confirmed that the immunologically identified cDNAs indeed corresponded to fibulin. Preceding the amino-terminal sequence in the deduced type A, B, and C sequences is a 29-residue hydrophobic leader sequence that has features consistent with it being a signal peptide (Watson, 1984; von Heijne, 1984). Three potential N-linked glycosylation sites (N-X-S/T) occur in each of the deduced sequences.

The three forms of fibulin are rich in cysteine (~11 mol %), containing 69, 70, and 72 residues for the A, B, and C forms, respectively. Analysis of the sequences with respect to the number and spacing of cysteine residues revealed the presence of two types of repeat motifs (designated type I and II) that each share homology with elements from specific proteins found in the database.

The type I motif has a consensus sequence $CC(X)_{12}C$-$(X)_{9-10}C(X)_6CC$, and is repeated twice (Fig. 9). Separating the two is an imperfect form of this motif that lacks two cysteines. A computer-aided search of the protein database for sequences containing the type I motif or slight variations thereof revealed that $CC(X)_{12}C(X)_{11-12}C(X)_6CC$ is found in complement component anaphylatoxins C3a (de Bruijn and Fey, 1985), C4a (Belt et al., 1984), and C5a (Wetsel et al., 1987). The inverse pattern, $CC(X)_6C(X)_{11-12}C(X)_{12}CC$, is found in the three members of the albumin gene family which include albumin (Brown, 1976), vitamin D–binding protein (Yang et al., 1985; Cooke and David, 1985), and α-fetoprotein (Morinaga et al., 1983). The homology findings suggest that the overall disulfide-stabilized loop structure may be conserved between fibulin and these other proteins even though similarity between residues other than cysteine in the pattern is unremarkable.

The type II motif of fibulin is related to the repeats found in EGF precursor (Scott et al., 1983) as well as a number of extracellular matrix proteins (Engel, 1989). This six-cysteine motif is repeated consecutively nine times in the sequence of fibulin A, B, and C (Fig. 9). Four of the nine type II repeats (2–4 and 9) differ from the typical EGF-like motif in that they have a 4–6-residue insertion between cysteines 4 and 5, instead of the usual single residue separating the two. The ninth type II repeat of fibulin A is imperfect in that it lacks a cysteine in the sixth position of the motif while fibulins B and C both have cysteine residues in the vicinity, but the spacing of these is not conserved relative to the other repeats. Embodied within four of the nine type II repeats (5–8) is a consensus sequence for aspartic acid and asparagine hydroxylation (Stenflo et al., 1988). The seventh type II repeat contains a consensus *O*-glycosylation sequence, CXSXPC, that is found in the EGF-like domains of coagula-

A

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CCCGCCGCCCATGGAGCGCGCCGCCGCGTCGCGCCGGGTCCCGCTTCCGCTGCTGCTGCTCGGCGGCCTTGC  72
                M  E  R  A  A  P  S  R  R  V  P  L  P  L  L  L  L  G  G  L  A   21

GCTGCTGGCGGCCGGAGTGGACGCGGATGTCCTCCTGGAGGCCTGCTGTGCGGACGGACACCGGATGGCCAC  144
L  L  A  A  G  V  D  A  D  V  L  L  E  A  C  C  A  D  G  H  R  M  A  T   45
                         S  X              S  H

TCATCAGAAGGACTGCTCGCTGCCATATGCTACGGAATCCAAAGAATGCAGGATGGTGCAGGAGCAGTGCTG  216
H  Q  K  D  C  S  L  P  Y  A  T  E  S  K  E  C  R  M  V  Q  E  Q  C  C   69
              E

CCACAGCCAGCTGGAGGAGCTGCACTGTGCCACGGGCATCAGCCTGGCCAACGAGCAGGACCGCTGTGCCAC  288
H  S  Q  L  E  E  L  H  C  A  T  G  I  S  L  A  N  E  Q  D  R  C  A  T   93

GCCCCACGGTGACAACGCCAGCCTGGAGGCCACATTTGTGAAGAGGTGCTGCCATTGCTGTCTGCTGGGGAG  360
P  H  G  D  N  A  S  L  E  A  T  F  V  K  R  C  C  H  C  C  L  L  G  R   117

GGCGGCCCAGGCCCAGGGCCAGAGCTGCGAGTACAGCCTCATGGTTGGCTACCAGTGTGGACAGGTCTTCCG  432
A  A  Q  A  Q  G  Q  S  C  E  Y  S  L  M  V  G  Y  Q  C  G  Q  V  F  R   141

GGCATGCTGTGTCAAGAGCCAGGAGACCGGAGATTTGGATGTCGGGGGCCTCCAAGAAACGGATAAGATCAT  504
A  C  C  V  K  S  Q  E  T  G  D  L  D  V  G  G  L  Q  E  T  D  K  I  I   165

TGAGGTTGAGGAGGAACAAGAGGACCCATATCTGAATGACCGCTGCCGAGGAGGCGGGCCCTGCAAGCAGCA  576
E  V  E  E  E  Q  E  D  P  Y  L  N  D  R  C  R  G  G  G  P  C  K  Q  Q   189

GTGCCGAGACACGGGTGACGAGGTGGTCTGCTCCTGCTTCGTGGGCTACCAGCTGCTGTCTGATGGTGTCTC  648
C  R  D  T  G  D  E  V  V  C  S  C  F  V  G  Y  Q  L  L  S  D  G  V  S   213

CTGTGAAGATGTCAATGAATGCATCACGGGCAGCCACAGCTGCCGGCTTGGAGAATCCTGCATCAACACAGT  720
C  E  D  V  N  E  C  I  T  G  S  H  S  C  R  L  G  E  S  C  I  N  T  V   237

GGGCTCTTTCCGCTGCCAGCGGGACAGCAGCTGCGGGACTGGCTATGAGCTCACAGAGGACAATAGCTGCAA  792
G  S  F  R  C  Q  R  D  S  S  C  G  T  G  Y  E  L  T  E  D  N  S  C  K   261

AGATATTGACGAGTGTGAGAGTGGTATTCATAACTGCCTCCCCGATTTTATCTGTCAGAATACTCTGGGATC  864
D  I  D  E  C  E  S  G  I  H  N  C  L  P  D  F  I  C  Q  N  T  L  G  S   285

CTTCCGCTGCCGACCCAAGCTACAGTGCAAGAGTGGCTTTATACAAGATGCTCTAGGCAACTGTATTGATAT  936
F  R  C  R  P  K  L  Q  C  K  S  G  F  I  Q  D  A  L  G  N  C  I  D  I   309

CAATGAGTGTTTGAGTATCAGTGCCCCGTGCCCTATTGGGCATACATGCATCAACACAGAGGGCTCCTACAC  1008
N  E  C  L  S  I  S  A  P  C  P  I  G  H  T  C  I  N  T  E  G  S  Y  T   333

GTGCCAGAAGAACGTGCCCAACTGTGGCCGTGGCTACCATCTCAACGAGGAGGGAACGCGCTGTGTTGATGT  1080
C  Q  K  N  V  P  N  C  G  R  G  Y  H  L  N  E  E  G  T  R  C  V  D  V   357
                                                         S

GGACGAGTGCGCGCCACCTGCTGAGCCCTGTGGGAAGGGACATCGCTGCGTGAACTCTCCCGGCAGTTTCCG  1152
D  E  C  A  P  P  A  E  P  C  G  K  G  H  R  C  V  N  S  P  G  S  F  R   381

CTGCGAATGCAAGACGGGTTACTATTTTGACGGCATCAGCAGGATGTGTGTCGATGTCAACGAGTGCCAGCG  1224
C  E  C  K  T  G  Y  Y  F  D  G  I  S  R  M  C  V  D  V  N  E  C  Q  R   405

CTACCCCGGGCGCCTGTGTGGCCACAAGTGCGAGAACACGCTGGGCTCCTACCTCTGCAGCTGTTCCGTGGG  1296
Y  P  G  R  L  C  G  H  K  C  E  N  T  L  G  S  Y  L  C  S  C  S  V  G   429

CTTCCGGCTCTCTGTGGATGGCAGGTCATGTGAAGACATCAATGAGTGCAGCAGCAGCCCCTGTAGCCAGGA  1368
F  R  L  S  V  D  G  R  S  C  E  D  I  N  E  C  S  S  S  P  C  S  Q  E   453

GTGTGCCAACGTCTACGGCTCCTACCAGTGTTACTGCCGGCGAGGCTACCAGCTCAGCGATGTGGATGGAGT  1440
C  A  N  V  Y  G  S  Y  Q  C  Y  C  R  R  G  Y  Q  L  S  D  V  D  G  V   477

CACCTGTGAAGACATCGACGAGTGCGCCCTGCCCACCGGGGGCCACATCTGCTCCTACCGCTGCATCAACAT  1512
T  C  E  D  I  D  E  C  A  L  P  T  G  G  H  I  C  S  Y  R  C  I  N  I   501

CCCTGGAAGCTTCCAGTGCAGCTGCCCCTCGTCTGGCTACAGGCTGGCCCCCAATGGCCGCAACTGCCAAGA  1584
P  G  S  F  Q  C  S  C  P  S  S  G  Y  R  L  A  P  N  G  R  N  C  Q  D   525

CATTGATGAGTGTGTGACTGGCATCCACAACTGCTCCATCAACGAGACCTGCTTCAACATCCAGGGCGCGTT  1656
I  D  E  C  V  T  G  I  H  N  C  S  I  N  E  T  C  F  N  I  Q  G  A  F   549
F

CCGCTGCCTGGCCTTCGAGTGCCCTGAGAACTACCGCCGCTCCGCAGCCACATGATCGTAGGGAACTCTGCA  1728
R  C  L  A  F  E  C  P  E  N  Y  R  R  S  A  A  T  -   566

TGAGGCCATCGGTGCAGGCTGGAGAAGAGAAGGCAAGTGGCAGGAGTGGAGACCACAGGCATTTGAGCCAC  1800
TTCCTCATGTAACTTAACTTGTGCCTTCAGGACCTGCTCAAGCCCGATCACGTATATACCACTTCCATTTGA  1872
TGATGGAATGCTGCTGTTCATGACCAACTTTATGGCTAGATGGGTCAGAAAGCACCCAGTTCATGATAGGCA  1944
GTTCAGGTCATATGGTGACTTGATGACCCAGAGTCAAACATTCAGTTTCCACCAAAGCCCAGTAACAGGCCA  2016
AGAGCTGTCTCTCAAAAGAAGAGTAGTTATCTGCAGAAGATGGCAGGGCCTTGCTCCGAAAGCCTAGAGACC  2088
GCCACTGTGATTCACCTATGGGGGCCTGCCAAAGCTGCAGCCAGCATCCTTATCTGCCACTGACACCTCAAG  2160
CAACATTGGATCTGCTGGGTCATATGGCCCAAGTGGCAGAGCAACTTGCACAACAGCCTGGACCTGTCATAG  2232
AGCTTTCTCCTGTTCTGGACCCCACTCAAAACTGGCAGCCTTTCAGGTCACTCAATAATGTGCTGGAGTAA  2304
CACACTCAAACGAGGAATGTGTTGCCTCCAAAATCCAATAGGCCCAAAAAA  2355
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B

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                                  GCAGAAATCCAAGAAGGGAAG  1728
                                  Q  K  S  K  K  G  R   573

GCAGAACACCCCAGCGGGATCAAGTAAAGAGGACTGCAGGGTTCTTCCATGGAAGCAGGGGTTGGAGGATAC  1800
Q  N  T  P  A  G  S  S  K  E  D  C  R  V  L  P  W  K  Q  G  L  E  D  T   597

CCACCTTGATGCCTAGTGAGGAAGATGGACCTGGACAGACAGTCAGCTCCACACCTTGCGCTGAGCAGCTGT  1872
H  L  D  A  -  -   601

GATTGTGCCACGGGAGCATGAGCCCTTTTCCCCACGGCCCTTGCCACTGTCTCCTGGCCCTCTCTCTGATCA  1944
TGCCAGGTTTGCACCAGCCTCGAGTCTCCCATGTTGTAGTACATTCTCCAAGATGCAGCCCAGGAGCCTCTC  2016
TGAAGGACCAGTCTGGTTACGATGGTCTGAGCTTCCTTAGAACCTTCCATGGTTGTCTTTCCCAGCAGATG  2088
AAGCATAGCCTCCTTGGAATGGCATGGGAGGCCTGGCCTGATCTGGCCTCTGCCCACCCTTTGAGCTGTACC  2160
TGCCCCACCCCAGCTCATCCATGTGCTTGTACCCTGGCCCCACGGGGAGGCTTGCCCTTCCCTGAATGTGCC  2232
TTCTTGTGGCTTTAGCATGTGCCATGCTGTCCCCCTGCAGACTGCCATTCTCCTTGCAGACTTGGCTCAGAA  2304
GTCACCTCCTCAGTGCAGTTAGCCTGAGCTCCCCTGGCCCCAGGTGCCTCCATCGAGCATTTACCCCATTG  2376
TGTTGTGGCTGTTCCTTAACGTCCCCACTAGCCAGGCTCTTTGAGGGCAGGGATTGTGTCTGGTTAATTTCT  2448
GTATTCTCTGCAACTTTGCAATGTTTGGCTTGAAGAAGGAGCTCAGTAAACATCTGAATAAACATGCAGGTT  2520
GATGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  2555
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C

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                                  CCGCTGTGAGCGCTTGCCTTG  1728
                                  R  C  E  R  L  P  C   573

CCATGAGAATCGGGAGTGCTCCAAGCTGCCTCTGAGAATAACCTACTACCACCTCTCTTTCCCCACCAACAT  1800
H  E  N  R  E  C  S  K  L  P  L  R  I  T  Y  Y  H  L  S  F  P  T  N  I   597

CCAAGCGCCCGCGGTGGTTTTCCGCATGGGCCCCTCCAGTGCTGTCCCCGGGGACAGCATGCAGCTGGCCAT  1872
Q  A  P  A  V  V  F  R  M  G  P  S  S  A  V  P  G  D  S  M  Q  L  A  I   621

CACCGGCGGCAATGAGGAGGGCTTTTTCACCACCCGGAAGGTGAGCCCCCACAGTGGGGTGGTGGCCCTCAC  1944
T  G  G  N  E  E  G  F  F  T  T  R  K  V  S  P  H  S  G  V  V  A  L  T   645

CAAGCCTGTCCCCGAGCCCAGGGACTTGCTCCTGACCGTCAAGATGGATCTCTCTCGCCACGGCACCGTCAG  2016
K  P  V  P  E  P  R  D  L  L  L  T  V  K  M  D  L  S  R  H  G  T  V  S   669

CTCCTTTGTGGCCAAGCTTTTCATCTTTGTGTCTGCAGAGCTCTGAGCACTCGCTTCGCGTCGCGGGGTCTC  2088
S  F  V  A  K  L  F  I  F  V  S  A  E  L  -   683

CCTCCTGTTGCTTTCCTAACCCTGCCCTCCGGGGGTAATAAAGTCTTAGCAAGCGTGGGACACAGTGAAAA  2160
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  2200
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(bases 1–1707) are not shown. Putative polyadenylation signal sequences are boxed. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X53741, X53742, and X53743 for A, B, and C forms, respectively.

Figure 6. Nucleotide sequence of the three types of fibulin cDNAs isolated and the corresponding deduced amino acid sequences for each form. *A* shows the complete nucleotide and predicted amino acid sequence for the A form of fibulin. The putative signal peptide cleavage site is indicated by an arrow pointing upward. Protein sequences of the amino terminus and of three tryptic peptides of fibulin are indicated by solid lines. Amino acid residues beneath the predicted amino acid sequence indicate differences between the cDNA-deduced sequence and those determined from protein sequencing. Potential N-linked carbohydrate attachment sites are indicated by solid squares. The site where the three types of cDNAs diverge is indicated by an arrow pointing downward between 1707 and 1708. *B* and *C* are sequences of the alternatively spliced segments from fibulins B and C, respectively, beginning at nucleotide 1708. Sequences of B and C that overlap with those of form A

-9.5
-7.5

-4.4

-2.4

-1.4

Figure 7. RNA hybridization analysis showing size of fibulin transcripts. Poly(A)$^+$ human placental RNA (3.6 μg) was electrophoresed on a 0.8% agarose, 6% formaldehyde gel, and transferred to nitrocellulose. The filter was incubated with a random primed, ^{32}P-labeled 150-bp fibulin cDNA segment derived from a region common to types A, B, and C fibulin cDNAs. Sizes of standards are indicated on the right in base pairs.
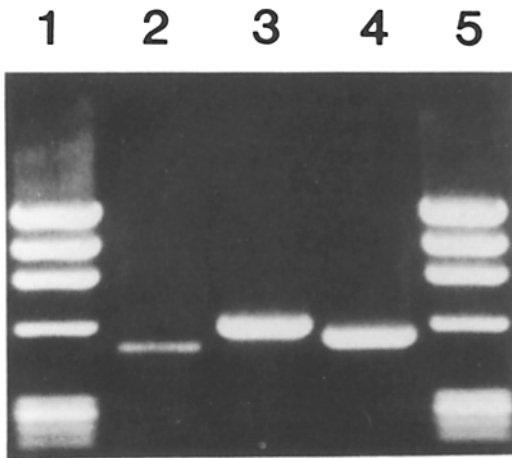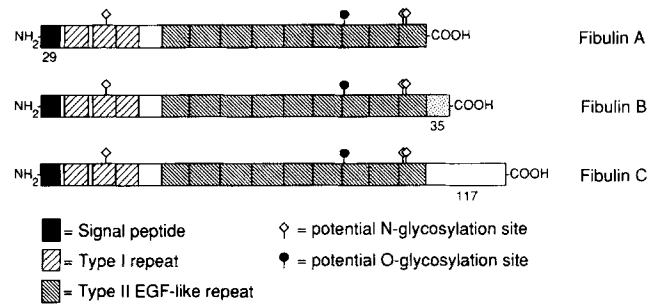
Fibulin A

Fibulin B

Fibulin C

■ = Signal peptide   ⟨ = potential N-glycosylation site
▨ = Type I repeat   ● = potential O-glycosylation site
▩ = Type II EGF-like repeat

Figure 8. Detection of fibulin A, B, and C mRNAs by reverse transcriptase PCR. Pairs of synthetic oligonucleotide primers taken from regions upstream and downstream of the putative splice site (position 1707) within fibulin A, B, and C cDNAs were used in PCR to amplify cDNA made from human placental RNA. The products of the reactions were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. Products of the primer pairs specific for fibulin A, B, and C are displayed in lanes *2, 3,* and *4,* respectively. Molecular weight size standards (lanes *1* and *5*) are Hae II digest of phiX174 DNA with fragment sizes of 1353, 1078, 872, 603, 310, 281, 234, and 194 bp.

tion factors VII, IX, protein Z, and thrombospondin (Nishimura et al., 1989). Immediately following each type II repeat is a pentapeptide with a consensus sequence XD(I/V)(D/N)E. Separating the third type I repeat and the first type II repeat is a 33-residue segment with 36% (12) of the amino

acids either aspartic or glutamic acid. The structural organization of the three forms of fibulin, including the arrangement of the repeat motifs, are schematically represented in Fig. 10.

Discussion

Our previous results (Argraves et al., 1989) suggested that fibulin might be an intracellular link between the cytoplasmic domain of the integrin β_1 subunit and components of the cytoplasm. This was based on the fact that fibulin could be purified by affinity chromatography on a peptide representing the cytoplasmic domain of the integrin β_1 subunit and that fibulin could be seen by indirect immunofluorescent staining of fibroblasts only after permeabilization. This latter observation was based on immunofluorescent staining studies done on fibroblasts grown for 4–6 h on fibronectin-coated surfaces. As we again show here, little if any fibulin

Figure 9. Complete amino acid sequence of fibulins A, B, and C. The sequences of repeat motifs I and II have been aligned with spaces (−) inserted in the repeats in order to align cysteine residues.

staining can be seen unless such cells are permeabilized (Fig. 1, *A*, *C*, *E*, and *G*). However, when we stained these cells with a mAb to fibronectin or one recognizing a presumed extracellular determinant of integrin β_1 subunit, we also found little or no specific staining unless the cells were permeabilized (Fig. 1, *B* and *D*, *F* and *H*). Evidently, in the absence of permeabilizing agents, the close association between the cell and the substratum prevents access of antibodies to sites where fibronectin and integrin are accumulating. Therefore, the ability to see staining only after permeabilization may be a misleading indicator that the target antigen is an intracellular protein.

When we extended our immunofluorescent staining studies to periods beyond 4–6 h, we began to see staining of fibulin in the absence of permeabilization (Fig. 1 *I*). With progressive culture time, fibulin was found to accumulate extracellularly into extensive fibrillar patterns resembling the pattern of accumulation of fibronectin. Using pulse–chase labeling and immunoprecipitation analyses, we established that fibulin was indeed a secreted protein. Furthermore, we showed by lectin affinity chromatography and *N*-glycosidase digestion that fibulin was a glycoprotein containing N-linked carbohydrate. These findings were supported by the results of cDNA cloning which showed the predicted amino acid sequence of fibulin to have a signal sequence and three potential *N*-glycosylation sites. The presence of the repeated EGF-like motif is yet another feature not found in cytoplasmic proteins but common to a number of extracellular matrix, plasma, and membrane proteins (Engel, 1989). Taken together the findings are consistent with fibulin being an extracellular matrix protein rather than a cytoplasmic protein. The significance of the fact that fibulin can be purified by affinity chromatography on the putative cytoplasmic domain of the β_1 subunit remains to be explained.

In SDS-PAGE analysis, fibulin, purified by both affinity chromatography and immunoprecipitation, migrates as a single band with an apparent molecular mass of 100 kD. Based on the results of cDNA cloning it can be predicted that there exist three forms of fibulin (designated *A*, *B*, and *C*) encoded by three transcripts likely derived from a common pre-mRNA. The fact that our fibulin preparations seem only to have a single polypeptide may indicate that predominantly one form is being isolated. We are attempting to prepare antisera to synthetic peptides unique to the B and C forms to help address this. Another puzzling issue has to do with the disparity between the molecular weight of fibulin estimated from SDS-PAGE and that determined from cDNA. The polypeptides (minus signal peptides) predicted from the nucleotide sequences of the three cDNAs have molecular masses of 58,670, 62,561, and 71,551 D. These values are not in agreement with fibulin's apparent molecular mass of 100 kD obtained from SDS-PAGE. Our results indicate that *N*-linked glycosylation only accounts for ~4–5 kD of the molecular mass of the 100-kD polypeptide. Other types of substitution, such as *O*-glycosylation, may account for the remaining difference. The seventh EGF-like repeat of fibulin does contain a consensus *O*-glycosylation sequence of the kind found in the clotting factors VII, IX, protein Z, and thrombospondin (Nishimura et al., 1989). Overestimation of molecular mass by SDS-PAGE has been reported for a number of proteins rich in negatively charged amino acids and having low isoelectric point values (Takano et al., 1988;

Graceffa et al., 1988; Saunders et al., 1989). Fibulins A, B, and C have an average content of aspartic and glutamic acid residues of 13.5% and average estimated pI of 4.7. It is therefore possible that anomalous electrophoretic behavior of fibulin on SDS-PAGE results in an overestimation of its size.

We demonstrated previously that fibulin is a calcium-binding protein. Analysis of the predicted amino acid sequence indicated no sequence homologous to the consensus divalent cation–binding sequences of proteins such as calmodulin, troponin C, and parvalbumin (Szebenyi et al., 1981) to be present. The analysis did reveal however, the presence of four potential asparagine hydroxylation sites, $CX(D/N)(X)_4(F/Y)XCXC$ (Stenflo et al., 1988), embodied within EGF-like repeats 5–8. EGF domains containing β-hydroxylated residues have been implicated in calcium binding (Sugo et al., 1984) and are found in numerous proteins including the vitamin K–dependent blood coagulation proteins, complement protein C1r, low density lipoprotein receptor, and thrombomodulin. Work of Ohlin et al. (1988) showed that protein C has a high-affinity calcium-binding site residing within an EGF-like element containing β-hydroxylaspartic acid. Whether the EGF-like domains of fibulin contain β-hydroxylated aspartic acid, or asparagine, and bind calcium remains to be determined.

Based on our sequence analysis of the fibulin cDNAs we propose that fibulin is a modular protein containing distinct domains that include two types of repeated cysteine-containing motifs and two alternatively spliced elements. In the first type of motif, the arrangement of cysteines closely resembles that of sequences found in the complement proteins C3a, C4a, and C5a as well as proteins of the albumin gene family. The second repeat is homologous to EGF. Whether these structural homologies are indicative of functional similarities is not known. The availability of cDNA and antibody probes provide the means to learn more about the expression of fibulin in various types of cells and tissues. Future studies will focus on understanding the function of fibulin as an extracellular matrix and blood protein.

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