Undulin, an Extracellular Matrix Glycoprotein Associated with Collagen Fibrils*

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Undulin, a novel noncollagenous extracellular matrix protein, was isolated from skin and placenta. In polyacrylamide gels most of the unreduced protein migrates with M, above 1,000,000 yielding bands A (M, 270,000), B1 (M, 190,000), and B2 (M, 180,000) after reduction. Undulin is biochemically and immunochemically distinct from other previously characterized large matrix glycoproteins. Immunoblotting using monoclonal antibodies suggests that bands A and B are closely related. Electron microscopy reveals undulin as structures consisting of an approximately 80-nm-long tail with a nodule on one end and with one or two shorter arms on the other. Ultrastructurally immunolabeled undulin is found mainly between densely packed mature collagen fibrils. Indirect immunofluorescence shows bundles of uniform wavy fibers in dense connective tissues superimposable on a subpopulation of type I collagen structures. This suggests that undulin serves a specific yet unknown function in the supramolecular organization of collagen fibrils in soft tissues.

As a major determinator of cellular gene expression and differentiation the extracellular matrix (ECM) is of fundamental importance for the coordinate growth and maintenance of multicellular organisms (Hay, 1984; Thiery et al., 1985). With the advent of refined biochemical and ultrastructural methods a perceiving variety of matrix molecules equipped with unforeseen biological activities has been disclosed (Schuppan and Hahn, 1987). Rapid progress has been made with the characterization of at least 12 types of collagen (Miller and Gay, 1987; Gordon et al., 1987), various proteoglycans (Heinegard and Paulisson, 1984; Poole, 1986), and glycoproteins (Hakamori et al., 1984). Each of these molecular species bears specific domains evidently capable of performing multiple structural or signal-inducing functions.

In view of their high potential for interaction, much interest has been focused on the large ECM glycoproteins fibronectin, laminin, thrombospondin, and tenascin. Fibronectin (M, 540,000) is a constituent of plasma as well as the interstitial ECM. It is involved in blood coagulation, wound healing, and chemotaxis. It mediates the interaction of cells with other matrix components such as fibrillar collagen and heparin (Hynes, 1985; Yamada et al., 1985). cDNA cloning of fibronectin mRNA demonstrated three types of internal homologies (Kornblitt et al., 1986) and a tripeptide sequence (Arg-Gly-Asp), which is recognized by the cellular fibronectin receptor and has later been found to be a common feature of many ECM molecules (Ruoslalhti et al., 1987).

Laminin (M, 900,000) is a cross-shaped molecule unique to basement membranes. The glycoprotein, which links cells to the scaffold of type IV collagen and interacts with heparan sulfate proteoglycan and entactin/nidogen, has received broad attention as a promoter of epithelial/endothelial differentiation, neurite outgrowth, and as a target to which metastasizing cells preferentially attach (Martin and Timpl, 1987). Recently, the complete amino acid sequence of its three constituent chains has become available (Sasaki et al., 1988). Lammin harbors at least two domains which mediate binding to cell surface receptors (Aumailley et al., 1987). One of these activities resides in a pentapeptide sequence close to the central part of the molecule (Graf et al., 1987).

Thrombospondin (M, 420,000) composed of three identical subunits, is produced by various cell types and interacts with fibronectin, laminin, type V collagen, and fibrinogen (Lawler, 1986). Its sequence shows only limited homology with that of the other large ECM glycoproteins (Lawler and Hynes, 1986).

Tenascin (Chiquet-Ehrismann et al., 1986), which has a six-armed structure of M, 1,000,000, is identical or similar to glioma mesenchymal extracellular matrix antigen (Bourdon et al., 1983), myotendinous antigen (Chiquet and Farmbrough, 1984), hexabrachion (Erickson and Inglesias, 1984), J1 (Kruse et al., 1985), and cytotoxact (Grumet et al., 1985). It is expressed in regions where epithelial/mesenchymal interactions take place during embryogenesis, e.g. around limb buds (Chiquet and Farmbrough, 1984) and budding kidney tubules (Aufderheide et al., 1987), and is reexpressed in the mesenchyme bordering invasive carcinomas (Mackie et al., 1987). A major part of the protein sequence of tenasin/cytotoxact has been recently determined (Guilcher et al., 1989; Jones et al., 1988; Pearson et al., 1989) showing clear homologies to domains in fibronectin and demonstrating that the different-sized bands observed in polyacrylamide gels are derived from differential splicing.

Here we report on the isolation and characterization of a novel ECM glycoprotein which is associated with mature collagen fibrils. Light and electron microscopical studies with the aid of monoclonal antibodies revealed this glycoprotein, which is produced by several mesenchymal cell lines, as a unique and abundant component of certain dense interstitial...
connective tissues. We propose for the novel protein the name “undulin” which is derived from the Latin “undula” (little wave) and describes its highly characteristic light microscopical pattern of uniform wavy fibers often organized into regular fiber bundles.

**EXPERIMENTAL PROCEDURES**

**Isolation of Undulin**—All reagents were obtained from Sigma, Federal Republic of Germany, unless stated otherwise. 2 kg of fresh human placenta or skin of newborn Macaca fascicularis monkeys (a kind gift of Behringwerke, FRG) were homogenized in 2 liters of 100 mM Tris-HCl, 4.5 mM NaCl, 2 mM PhMeSO₂F, 4 mM N-ethylmaleimide, and 10 mM EDTA, pH 7.4 (buffer A). The homogenate was preextracted at 4 °C with 2 x 6 liters of the same buffer to which 1% Triton X-100 had been added. All steps (schematically shown in Fig. 1) were carried out at 4-8 °C and centrifugations were done at 20,000 x g for 30 min. Finally, the material was extracted with 50 mM Tris-HCl, 0.5 M NaCl, 2 mM PhMeSO₂F, 4 mM N-ethylmaleimide, 10 mM EDTA, pH 7.4 (buffer B), 2 x 5 liters, 12-16 h each. The extracts were pooled and ammonium sulfate was added to 30% saturation (168 g/liter). The resulting precipitate was redissolved in 4 liters of buffer A and the cleared solution subjected to fractional salt precipitation at 1.7, 2.6, and 4.5 M NaCl. The fraction precipitating between 1.7 and 2.6 M NaCl was dissolved in 0.5 liters of 50 mM Tris-HCl, 0.3 M NaCl, 2 M urea, 1 mM PhMeSO₂F, 2 mM N-ethylmaleimide, 5 mM EDTA, pH 7.4 (buffer C), and dialyzed against the same buffer. Insoluble material, containing type I and III collagen as judged by SDS-PAGE, was removed by centrifugation. The supernatant was equilibrated with DEAE-cellulose (Whatman, FRG) for 4 h to remove acidic contaminants. Dialysis of the unbound fraction against 50 mM Tris-HCl, 0.02 M NaCl, 2 M urea, 1 mM PhMeSO₂F, 2 mM N-ethylmaleimide, 5 mM EDTA, pH 7.4 (buffer D), caused a precipitate to form. The precipitate consisted of approximately 50% undulin, the remainder being predominantly type I and III collagen and fibronectin (“insoluble fraction”). The material which remained in solution and contained minor quantities of undulin (“soluble fraction”) was stirred with DEAE-cellulose. Unbound material (type I and III collagen) was removed and the proteins bound to the resin were eluted with a gradient of 0.02–0.5 M NaCl. Upon elution undulin, which was about 80% pure, was displaced from the column after type I and III procollagen (Fig. 2). Most of the impurities could be extracted from the insoluble undulin-containing fraction with 50 mM Tris-HCl, 0.5 M NaCl, 4 M urea, 1 mM PhMeSO₂F, 2 mM N-ethylmaleimide, 5 mM EDTA, pH 7.4 (buffer E), which did not dissolve undulin. Finally, undulin was purified by chromatography on a column (1.6 x 110 cm) of Sephacryl S-500 (Pharmacia, FRG) equilibrated in 50 mM Tris-HCl, 6 mM guanidine, pH 8.0. Denatured undulin eluted as a second sharp peak in the region of a mouse tumor laminin standard (M, 900,000). This preparation was judged to be pure by SDS-PAGE and DEAE-cellulose chromatography under the conditions described above.

**Other Antigens**—Human type I, III, IV (7-S long (long form of the tetrameric amino-terminal propeptide of type IV procollagen), triple helical, and NC1 (dimeric carboxyl-terminal propeptide of type IV procollagen) domains), V, and VI collagens as well as laminin fragment P1 (central pepsin-resistant fragment of the cross-shaped laminin molecule) were purified from human placenta and characterized as described previously (Becker et al., 1986a, 1986b; Schuppan et al., 1986a, 1986b). Human plasma fibronectin was obtained by the method of Miekka et al. (1982) and type III procollagen (with the amino-terminal but lacking the carboxyl-terminal propeptide) was isolated from calf or monkey skin (Nowack et al., 1976). Monkey type I procollagen was present in the 2.6 M NaCl precipitate of the neutral salt extract from skin containing the “soluble fraction” of undulin. Type I procollagen was purified by DEAE-cellulose chromatography as described above where it eluted before type III procollagen (partly processed type III procollagen form lacking the carboxyl-terminal propeptide) and undulin (Fig. 2). Judging by SDS-PAGE (Fig. 3) the material isolated consisted of intact type I procollagen and its partly processed precursors and as such was comparable to the forms defined from cell culture media (Burke et al., 1977).

**Antisera, Polyclonal and Monoclonal Antibodies**—Purified undulin was suspended at 0.3 mg/0.5 ml in PBS (50 mM sodium phosphate, pH 7.4), emulsified with an equal volume of complete Freund's adjuvant (Difco), and injected subcutaneously into New Zealand White rabbits. After three booster injections given with incomplete adjuvant at 3-week intervals blood was drained via the ear vein or by antep nickance. For antibody purification undulin was immobilized on CNBr-activated Sepharose 4B (Pharmacia, FRG). The antisera against undulin was pumped over columns of Sepharose-coupled monkey type I procollagen; bovine type III procollagen; human type IV (7-S, triple helical, and NC1 domains), V, and VI collagens; fibronectin, and laminin fragment P1 as described elsewhere (Becker et al., 1986a, 1986b; Schuppan et al., 1985, 1986a, 1986b). The antibodies bound to the undulin column were finally eluted using 3 M KSCN in PBS, 0.05% Tween 20 and 0.05% Tween 20. The production of antisera and affinity purified antibodies against monkey type I and III procollagen; human type IV (7-S long, triple helical, and NC1 domains), V, and VI collagen, and laminin fragment P1 and their characterization as monospecific by sensitive radioimmunoassays have been described before (Becker et al., 1986a, 1986b; Schuppan et al., 1985, 1986a, 1986b). Neutral salt soluble monkey skin type I collagen was purified by an exchange chromatography and used to immunize rabbits according to established procedures (Nowack et al., 1976). Antibodies were affinity isolated by passage over the antigens mentioned above followed by elution from

**Fig. 2. Elution of undulin from DEAE-cellulose.** Separation of undulin (fraction d) from interstitial collagens (unbound material, not shown) and procollagens (fractions b and c) by DEAE-cellulose chromatography (see “Experimental Procedures”). Protein was eluted from the column with a concave NaCl gradient (0.02–0.5 M NaCl).
5% mercaptoethanol. The positions of γ, β, and α components of type I collagen and of reduced plasma fibronectin (Fn) (M, 300,000, 200,000, 100,000, and 220,000, respectively) used as standards are indicated. Fraction b was composed of type I collagen and procollagen and a mixture of partly processed intermediates (see Burke et al., 1977). Fraction c consisted of type III collagen and procollagen (partly processed type III procollagen form lacking the carboxyl-terminal propeptide). Fraction d contained native undulin, some type I collagen and a mixture of partly processed intermediates (see Burke et al., 1977). Fraction e contained undulin and some type I collagen and of reduced plasma fibronectin (Fn) (M, 300,000, 270,000, 190,000, and 180,000 designated A, B1, and B2, respectively, and low molecular weight material. Nonreduced native undulin did not penetrate the stacking gel. However, after reduction bands of M, 270,000, 190,000, and 180,000 designated A, B1, and B2, respectively, were revealed, considered characteristic for the protein. Human placental undulin purified from material like that shown in Fig. 24 (d). Purification was by chromatography on Sephacryl S-300 under denaturing conditions. Note the blurred B band and the generation of additional bands denoted A' and B'.

immobilized type I collagen. Monospecificity of the type I collagen antibodies was affirmed by radioimmunoassays and immunoblotting (not shown). Monospecific rabbit antisera against chick tenascin has been described by Chiquet-Ehrismann et al. (1986) and monospecific rabbit antisera against human plasma fibronectin was from Dako (FRG).

For hybridoma production female BALB/c mice were immunized subcutaneously with 100 µg of undulin emulsified in complete Freund's adjuvant. The same amount of antigen in incomplete adjuvant was administered after 4 weeks and given daily for the last 4 days before the fusion was carried out. Hybridomas were produced, subcloned, and propagated in ascites fluid as described by Köhler and Miletine (1975). Clone selection was done with the aid of indirect immunofluorescence assay and enzyme-linked immunosorbent assay (Engvall and Perlman, 1971). Antibody clones 11 B1 (IgG1), 15 III IgG2a), and 15 V (IgG2b) were used in the present study.

Radioimmunoassays—Iodine-125 was incorporated into all antigens except for the NC1 domain of type IV collagen by the chloramine-T method. The NC1 domain had to be labeled with the Bolton-Hunter reagent (Schuppau et al., 1985, 1986a, 1986b). Undulin was dissolved in 50 mM Tris-HCl, 2% SDS, pH 8.3, or 50 mM Tris-HCl, 6 M guanidine, pH 8.0, then diluted 1:20 in PBS, 0.05% Tween 20, to a final concentration of 10 µg/ml, and labeled with 1 µCi of iodine-125 for 1 min. Incorporated radioactivity ranged between 4000 and 6000 cpm/pg of protein. For radioassays (Schuppau et al., 1985, 1986a) serial dilutions of antibodies in 0.2 ml of 0.25% carrier rabbit/goat serum in PBS, 0.05% Tween 20 were incubated for 4 h with the radiolabeled antigen (20,000 cpm, 1-5 ng of protein in 0.2 ml), followed by precipitation of immune complexes with a pretested amount of second antisera (usually goat serum against rabbit IgG).

Analytical Methods and Immunoblotting—Proteins were analyzed by SDS-PAGE at concentrations of 2-20 µg/elot (Laemmli, 1971). Antigens run on gels were blotted to nitrocellulose, and, after blocking unspecified binding sites with 25 mM Tris-HCl, 0.05% Tween 20, pH 7.4, containing 5% delipitated milk powder, the blots were developed essentially as outlined by Dziedzic et al. (1985) using primary antibodies at 1-6 µg/ml, goat anti-rabbit IgG, or rabbit anti-goat IgG coupled to peroxidase (Calbiochem, FRG) and a mixture of o-phenyleneborate and o-phenylenediamine as substrates. Alternatively, antigens were detected with alkaline phosphatase-linked secondary antibodies, nitro blue tetrazolium, and o-chloroindolyl phosphate as indicator (Blake et al., 1984).

Peptide mapping of undulin bands A and B was performed according to the method of Cleveland et al. (1977). Briefly, reduced undulin was run on a 6% acrylamide gel and the bands were excised and layered on top of a 13% acrylamide gel with a 4% stacking gel. After addition of Staphylococcus protease V8 (2 µg/10 µl sample buffer, Sigma type XVII) the gel was run at 100 V until the dye front reached the lower third of the stacking gel. After the gel was left to incubate at room temperature for 16 h, the gel was fixed in 70% ethanol/acetic acid (2:1) for 20 min. Tissue sections and cells were fixed in ice-cold acetone for 4 min. Cells cultured on plastic were washed twice with PBS, 0.05% Tween 20, air dried fixed in 70% ethanol/acetic acid (2:1) for 20 min. Tissue sections and cells were incubated with primary antibodies at a concentration of 20-50 µg/ml for 60 min and a secondary fluorochrome-coupled antibody (rhodamine-rabbit anti-mouse IgG, rhodamine-swine anti-rabbit IgG, and fluorescein-rabbit anti-mouse IgG) (Dako, FRG) for 30 min. After each step the specimens were washed three times with PBS, 0.05% Tween 20. In double-labeling immunofluorescence primary rabbit antibodies were visualized first with rhodamine-swine anti-rabbit IgG, followed by the monoclonal antibody detected with fluorescein-rabbit anti-mouse IgG.

Rotary Shadowing Electron Microscopy—Undulin dissolved in 50 mM Tris-HCl, 6 M guanidine, pH 8.0, at 50 µg/ml was spread extended against 0.2 M ammonium bicarbonate, pH 7.8, mixed with an equal volume of glycerol, and sprayed onto freshly prepared mica disc. The preparations were shadowed with platinum-carbon at an angle of about 5° and with carbon at 90° in a Balzers BAE 400 coating unit (Furthmayr and Madri, 1982). The carbon replicas were floated onto distilled water, collected on copper grids, and examined in a Philips EM 410 electron microscope.

Indirect Immunofluorescence—Human tissue samples taken for diagnostic reasons or from a deceased kidney donor were mounted and snap-frozen in dry ice. 4-µm cryostat sections were air-dried and fixed in ice-cold acetone for 4 min. Cells cultured on plastic were washed twice with PBS, 0.05% Tween 20, air dried, fixed in 70% ethanol/acetic acid (2:1) for 20 min. Tissue sections and cells were incubated with primary antibodies at a concentration of 20-50 µg/ml for 60 min and a secondary fluorescent-coupled antibody (rhodamine-rabbit anti-mouse IgG, rhodamine-swine anti-rabbit IgG, and fluorescein-rabbit anti-mouse IgG) (Dako, FRG) for 30 min. After each step the specimens were washed three times with PBS, 0.05% Tween 20. In double-labeling immunofluorescence primary rabbit antibodies were visualized first with rhodamine-swine anti-rabbit IgG, followed by the monoclonal antibody detected with fluorescein-rabbit anti-mouse IgG.

Immunoelectron Microscopy—Freshly collected biopsies of human skin and oral mucosa were prepared for immunoelectron microscopy after ultracytometry as described (Gelderblom et al., 1985). Briefly, tissue cubes were fixed in dimethylsuberimidate, pH 9.5, and infiltrated with 70% sucrose. Ultrathin sections were cut in a Reichert-Jung FC-4 cryostat at ~90 °C and transferred to Formvar-coated, 200 mesh copper grids.
carbon-reinforced hexagonal copper grids. Sections were incubated with the desired polyclonal or a mixture of monoclonal antibodies 11 B1, 15 III, and 15 V at 150-250 μg/ml for 10 min and then with goat anti-rabbit or rabbit anti-mouse gold conjugate (5-nm particle size, Janssen, Belgium) diluted 1:60, for 20 min. The specimens were postfixed with 2% glutaraldehyde and stained with 2% uranyl acetate. For control purposes equivalent amounts of rabbit or mouse nonimmune IgG were used in place of the primary antibodies. Immunostaining was observed using a Zeiss 10A electron microscope.

RESULTS

Isolation and Characterization of Undulin—As illustrated in Fig. 1 undulin was isolated from monkey skin and human placenta by extraction with neutral buffer and salt fractionation employing a procedure similar to that used to isolate interstitial collagens and procollagens (Sage and Bornstein, 1982). Material precipitating with type I collagen and procollagen contained the bulk of undulin mainly in a form insoluble in low salt buffers. When chromatographed on DEAE-cellulose, a distinct protein peak was eluted with a salt gradient at 0.2 M NaCl (Fig. 2, fraction d). This peak was only observed when strict protease inhibition was implemented during the initial purification. SDS-gel electrophoresis (Fig. 3A) indicated that the less acidic fractions eluting from DEAE-cellulose (b and c in Fig. 2) contained type I and III procollagen. Electrophoresis of fraction d, however, displayed a hitherto unknown pattern. Most of the unreduced material in fraction d did not penetrate the stacking gel. After reduction, characteristic bands of type III collagen and procollagen were obtained. After reduction high molecular weight material not penetrating the stacking gel or just entering the running gel was seen. Antibodies to human and monkey type I and III collagen and fibronectin failed to react with the bands considered characteristic for undulin. Furthermore, undulin antibodies did not bind to DEAE-cellulose under the conditions used. Separation of collagen, fibronectin, and material of lower molecular weight was achieved by chromatography on a column of Sepharose S-500 in buffer containing 6 M guanidinium. Undulin eluted from the column as a second sharp peak in the region of mouse tumor laminin (Mr 900,000, not shown). Undulin thus purified and subsequently reduced showed the expected pattern with 2 bands less clearly separated than in preparations of native material. In addition, some factor migrating bands designated A' and B' (Fig. 3B), possibly generated due to residual proteolytic activity, appeared. The yield of pure undulin from newborn monkey skin and human placenta was 50–100 mg/kg wet tissue weight.

Amino acid analysis of purified undulin revealed a high proportion of hydrophobic and acidic residues, whereas hydroxyproline and hydroxylsine, which are characteristic for collagens, were absent. The overall composition is distinct from that of fibronectin or laminin (Table I) and of thrombospondin (Coligan and Slayter, 1984, data not shown). Carbohydrate analysis showed that undulin is a glycoprotein with N-Asp (GlcNAc, Man) and Ser/Thr (GlcNAc, Glc)-linked oligosaccharides. As demonstrated in Table II the degree of glycosylation (2% relative to protein) is significantly lower than that reported for tissue fibronectin (~4%) and laminin (~12%).

Undulin Is Unrelated to Other ECM Antigens—In order to test whether undulin is related to other known connective tissue components, a rabbit antiserum was produced against the monkey protein. The antiserum was passed over a wide spectrum of immobilized human/mouse ECM antigens (type I and III collagen and procollagen, type IV (7-S long, triple helical, and NC1 domains), V and VI collagen, fibronectin and laminin fragment P1). Monospecific antibodies were obtained by a final chromatography on an undulin column. There was no significant binding of the undulin antibodies to the radiolabeled ECM antigens mentioned above whereas the antibodies did bind to radiolabeled undulin with high affinity (Fig. 4A). Likewise, radiolabeled undulin did not bind significantly to high titer antibodies against all of the above-mentioned antigens (Fig. 4B). These data suggest that undulin is immunologically unrelated to these collagens and ECM glycoproteins.

Up to this point there was no sound evidence that all of the three bands attributed to undulin were indeed the antigens reactive to the polyclonal undulin antibodies. As illustrated in the immunoblot of a crude monkey skin extract in Fig. 5A, bands A, B1, and B2 were recognized by the antibodies. Without reduction high molecular weight material not penetrating the stacking gel or just entering the running gel was seen. Antibodies to human and monkey type I and III collagen and procollagen; type V (7-S, triple helical, and NC1 domains), V and VI collagen, fibronectin; laminin fragment P1; and chicken tenascin failed to react with the bands considered characteristic for undulin. Furthermore, undulin antibodies

### Table I

| Carbohydrate       | Undulin | Laminin | Fibronectin |
|--------------------|---------|---------|-------------|
| **α** carboxyhydrate/100 g of protein | **g** | **g** | **g** |
| N-Acetylglucosamine | 0.94    | 1.55    | 1.66        |
| N-Acetylgalactosamine | 0.22   | 3.88    | ND*         |
| Mannose            | 0.28    | 2.79    | 1.93        |
| Galactose          | 0.59    | 3.08    | 0.78        |
| Fucose             | 0.05    | 0.63    | ND*         |

* ND, not detected.

### Table II

| Carbohydrate       | Undulin | Laminin | Fibronectin |
|--------------------|---------|---------|-------------|
| **α** carboxyhydrate/100 g of protein | **g** | **g** | **g** |
| N-Acetylglucosamine | 0.94    | 1.55    | 1.66        |
| N-Acetylgalactosamine | 0.22   | 3.88    | ND*         |
| Mannose            | 0.28    | 2.79    | 1.93        |
| Galactose          | 0.59    | 3.08    | 0.78        |
| Fucose             | 0.05    | 0.63    | ND*         |

* ND, not detected.
did not react with the antigens mentioned (not shown). This constitutes further evidence that undulin is unrelated to these ECM proteins and that A, B1, and B2 are related to one parent molecule.

**Bands A, B1, and B2 of Undulin Are Closely Related**—Three monoclonal antibodies were produced using monkey undulin as the immunogen. When a human placental undulin preparation of about 50% purity (prior to molecular exclusion chromatography) was subjected to SDS-PAGE (6% acrylamide) without (−) and after (+) reduction, bands A, Bl, and B2 were detected. Bands considered characteristic for the protein are detected. B, reduced placental undulin probed with monoclonal antibodies 11 B I (lane 1), 15 III (lane 2), and 15 V (lane 3) after transfer from 6% acrylamide gels. With 5 μg/ml the antibody concentration used in lane 3 was 10-fold above that used in lanes 1 and 2, causing detection of presumed degradation products of low molecular weight. Note the different reactivity with material in the region of the A chain. The positions of reduced fibronectin (Fn) and of the α1 and α2 chains of type I collagen with M, 220,000, 100,000, and 55,000, respectively, are indicated.

**Composition of Undulin from Several Tissues**—In order to investigate the composition of undulin from different tissues 20 mg each of human placenta, uterus, liver, and large intestine were extracted directly with nonreducing or reducing SDS sample buffer, run on SDS gels, and transferred to nitrocellulose. As demonstrated in Fig. 7A polyclonal and monoclonal antibodies equally detected material in the position of A, B1/B2, and some higher as well as lower molecular weight material. This implies that 1) undulin is a ubiquitous component of connective tissue and 2) undulins from different tissues are identical or at least similar.

**Cells Producing Undulin in Culture**—The synthesis of undulin by several mesenchymal cell lines was shown with the aid of indirect immunofluorescence. Whereas only less than 10% of preconfluent human foreskin fibroblasts and diploid lung fibroblasts (WI-38, MRC-5) exhibited undulin-positive intracellular granules, cell lines from a rhabdomyosarcoma (RD) and an osteosarcoma (MG-63) consistently displayed strong immunoreactivity in more than 50% of preconfluent cells (Fig. 8). To investigate the composition of undulin produced by cells in vitro, culture medium from RD cells was subjected to immunoprecipitation with monoclonal undulin antibodies followed by SDS-gel electrophoresis and immunoblotting (Fig. 7B) revealing a prominent A and a broad B band. This suggests that 1) the composition of undulin produced by RD cells is quite similar to the form extracted from

**Fig. 4. Undulin lacks cross-reaction with other ECM molecules in radioimmunoassay.** A, binding of 125I-labeled ECM antigens to rabbit antibodies against monkey undulin. Antigens follow: O, monkey undulin (Un); A, human laminin fragment P1 (LamP1); A, human plasma fibronectin (Fn); and D, bovine amino-terminal propeptide (PIIP). Binding to human type I, III, IV (7-S long, triple helical, and NC1 domains) collagen and monkey type I procollagen was less than 5% at an antibody concentration of 10−1, and therefore is not shown. B, binding of 125I-labeled monkey undulin to polyclonal antibodies against ECM molecules. O, antiundulin (α-Un); D, anti-type VI collagen (α-VI); E, anti-monkey type I procollagen (α-PIP). Binding to antibodies against human type I, III, IV (7-S long, triple helical, and NC1 domains), and V collagen, laminin fragment P1, and the bovine amino-terminal type III procollagen propeptide, which was below 5% at an antibody concentration of 10−1, is not shown.

**Fig. 5. Undulin bands A and B are immunologically related to each other.** A, (lanes 1 and 2) a tissue extract from monkey skin with less than 5% undulin was run in SDS-PAGE (6% acrylamide) without (−) and after (+) reduction. (lanes 3 and 4) the same material was run in SDS-PAGE (6% acrylamide) after reduction in the presence of 5 μg/ml antibody against human type I, III, IV (7-S long, triple helical, and NC1 domains) collagen and monkey type I procollagen was less than 5% at an antibody concentration of 10−1, is not shown. B, binding of 125I-labeled monkey undulin to rabbit antibodies against monkey undulin. Antigens follow: O, anti-undulin (α-Un); D, anti-type VI collagen (α-VI); E, anti-monkey type I procollagen (α-PIP). Binding to antibodies against human type I, III, IV (7-S long, triple helical, and NC1 domains), and V collagen, laminin fragment P1, and the bovine amino-terminal type III procollagen propeptide, which was below 5% at an antibody concentration of 10−1, is not shown.
FIG. 6. Peptide mapping shows that band B of undulin is a truncated form of band A. Undulin was run on a 6% polyacrylamide gel, the excised bands A (lanes 1 and 3) and B (bands 2 and 4) digested with Staphylococcus protease V8, rerun on a 13% acrylamide gel and either silver-stained (lanes 1 and 2) or probed with monoclonal anti-undulin antibody 15 III (lanes 3 and 4) as described under "Experimental Procedures." Molecular weight standards: a, bovine serum albumin (M, 67,000); b, ovalbumin (M, 43,000); c, carbonic anhydrase (M, 30,000); d, soybean trypsin inhibitor (M, 20,000); e, lactalbumin (M, 14,400). In the silver-stained gel (lanes 1 and 2) the protease V8 imposes as a prominent doublet between standards c and d. Note identity of peptide maps of undulin bands A and B with the exception of additional higher molecular weight peptides from A migrating slower or slightly faster than standard a. The lines connect the positions of the peptides reactive in the immunoblot (lanes 3 and 4) with the corresponding positions in the silver-stained gel (lanes 1 and 2).

FIG. 7. Immunoblots of tissue extracts and RD cell culture medium reveal a similar pattern of undulin bands. Replicas were obtained from 7.5% acrylamide gels and developed with polyclonal antibody 15 III; polyclonal antibodies gave identical results (not shown); s, front of stacking gel; r, front of running gel; A and B denote positions of undulin bands A and B. For further details see "Experimental Procedures." A, extracts from human placenta (lanes 1 and 5), uterus (lanes 2 and 6), liver (lanes 3 and 7), and large intestine (lanes 4 and 8) without (lanes 1–4) and after (lanes 5–8) reduction of disulfides. 5 μl of extract, equivalent to 0.5 mg of tissue, were applied to each lane. Note the presence of faint bands slightly above and below the A band as well as material of M, 160,000, 140,000, and 120,000 (small arrows) in all preparations. B, immunoprecipitate from RD cell culture medium representing one sixth of the material from preconfluent 75-cm² culture flasks without (−) and after (+) reduction of disulfides.

Undulin Has a Unique Molecular Shape—When examined by rotary-shadowing electron microscopy, putatively monomeric forms of undulin could be visualized (Fig. 9A). Although only preparations of the molecule denatured in 6 M guanidine were studied some distinct features could be observed. The structures consisted of an approximately 80-nm-long thread-like segment, terminating at one end in a nodule with a diameter of ~9 nm and linked at the other end to one or two ~45-nm-long arms. Sometimes the arms appeared to be stud-

FIG. 9. Rotary-shadowing electron microscopy of undulin shows hitherto unknown molecular forms. Selected images of presumed monomers (A) and di/trimers (B). Bar, 100 nm. An approximate 80-nm long threadlike segment is terminated at one end by a nodule with a diameter of approximately 9 nm and linked at the other end to one or two ~45-nm-long arms, which often appear to bear two or three smaller nodules (best visualized in A). For further details refer to "Results" and "Experimental Procedures."
closer inspection: 1) undulin was composed of a long arm and one or two shorter arms compared to the uniform arms in thrombospondin and more importantly 2) the arms in undulin were significantly longer (approximately 45 and 80 nm) than those observed in thrombospondin (approximately 29–30 nm in a contracted state and 38 nm in an extended state; Lawler et al., 1985). Significant amounts of other species were not observed in our preparations. Unfortunately the denaturing treatment of undulin during its purification from tissue invariably led to increased numbers of aggregates as evidenced by SDS-gel electrophoresis even after reduction of disulfides. Thus, visualization of the native protein, e.g. isolated from cell culture, could aid in defining its molecular architecture more conclusively.

Undulin Displays a Distinctive Distribution in the ECM—Since information on the tissue distribution of an assumed novel ECM protein could yield important clues about its potential biological role, we performed indirect immunofluorescence on sections of several human organs. Staining of skin and oral mucosa revealed a highly characteristic, previously unobserved pattern of uniform undulating fibers organized in bundles. The bundles were found extending throughout the dense interstitium, regardless of whether polyclonal or monoclonal antibodies were used to visualize them (Fig. 10, a and b). These fibers were found in areas rich in fibrillar collagen, such as the dermal corium or as an outer sheath around blood vessels, glands, and nerve fibers. Cells, basement membranes, and bone were not stained by the antibodies. In the liver the densely packed fiber bundles of the portal ECM contrasted to the sparsely outlined perisinusoidal space (Fig. 10, c and d). Whereas the medial and intimal layers of small arteries were unstained, the media of larger arteries showed an amorphous fluorescence for undulin bordered by the characteristic undulating fiber bundles in the vascular adventitia (Fig. 10, e and f). This unique restricted pattern of distribution has not been found for other ECM proteins including thrombospondin which contrary to undulin is concentrated at the endothelial aspect of blood vessels and in basement membranes such as that separating epidermis from dermis (Wright et al., 1985).

Double-staining Immunofluorescence—Tissue sections were stained sequentially with antibodies against undulin and other ECM components. As shown in Fig. 11, a and b, tenascin and undulin in placenta, for example, were distributed exclusively of each other, i.e. undulin is expressed where tenasin is absent and vice versa. This exclusive distribution was found in all tissues thus far examined including liver, skin, oral mucosa, and gut. In contrast, undulin was codistributed with a subgroup of type I collagen fibers in the dense fibrillar ECM of the dermis, at the periphery of vascular structures, nerve fibers, glands, and of smooth muscle bordering the interstitium, i.e. structures requiring a pliable sheath of collagen fibers (Fig. 11, e and f). Undulin was only partly codistributed with fibronectin and was absent, e.g. in loose connective tissue and in the walls of blood capillaries (Fig. 11, c and d).

Undulin Is Associated with Collagen Fibers—To explore further the association of undulin with fibrillar collagen, we carried out immunoelectron microscopy with polyclonal or monoclonal antibodies on ultrathin cryostat sections of several collagens/procollagens, fibronectin, laminin, and tenasin/cytotactin; (2) amino acid and carbohydrate analyses showed that this component is not a collagen, but a glycoprotein distinct from the other known ECM glycoproteins; (3) rotary shadowing electron microscopy visualized undulin as a previously unknown molecular species; (4) studies with immunofluorescence microscopy indicated that its distribution in tissues is unique when compared to that of other ECM components; (5) immunoelectron microscopy revealed its close association with collagen fibrils at sites where these fibrils are tightly packed,

![Image](http://www.jbc.org/)

**Fig. 10. Light microscopical distribution of undulin in several human tissues.** Polyclonal antibodies were used in indirect immunofluorescence. Bars, 40 μm. The pattern obtained with monoclonal antibodies was identical (not shown). For details see “Experimental Procedures.” a, weak staining of the upper dermis in oral mucosa. The subepithelial basement membrane (arrowheads) is negative. b, epithelium. c, deeper layers of the oral mucosa. The subepithelial basement membrane (arrowheads) is negative. b, epithelium. c, deeper layers of the oral mucosa. The subepithelial basement membrane (arrowheads) is negative. d, sinusoidal aspect of the liver. Single, branching fibers in the perisinusoidal spaces and a collar of longitudinally oriented fibers ensheathing a central vein (V). e, bronchial arteriole of the lung with a pronounced adventitial sheath of undulin and part of a less well-demarcated venule (V). f, medium-sized artery showing diffuse fluorescence in the media (left half of the micrograph). At the transition to the adventitia (right half of the micrograph) undulin exhibits the characteristic wavy pattern. Adventitial vessels appear as empty spaces (arrows).
Fig. 11. Undulin shows limited codistribution with type I collagen and a complementary distribution to tenasin. Double-staining immunofluorescence on cryosections of human placenta (a and b) and uterus (c-f). Bars, 40 μm. a, rabbit antitenasin shows a distribution completely complementary to that of monoclonal antiundulin in b. Arterioles (arrowheads) and some vascular structures are positive for tenasin. c, monoclonal antifibronectin strongly labels a capillary (arrow) and a vascular structure (arrowhead). d, rabbit antiundulin is partly codistributed with fibronectin, but is absent from the areas marked by arrowheads. e, arteriole. f, rabbit anti-type I collagen stains fibers throughout the uterus and the pattern obtained with monoclonal antiundulin (f) corresponds at specific sites, such as the sheaths surrounding an arteriole (A) and smooth muscle fibers (M), but not at others such as the interstitium between individual muscle cells.

Fig. 12. Undulin is associated with collagen fibrils. Undulin antibodies were applied to ultrathin frozen sections and detected with gold-conjugated secondary antibodies as outlined under “Experimental Procedures.” Bar, 300 nm in a, b, and d, and 200 nm in c. a, dermal layer of human skin incubated with rabbit antiundulin showing irregular deposits along the fibril axis. b, and c, oral mucosa incubated with a mixture of monoclonal antibodies 11Bi, 15III, and 15V. In b label appears to be concentrated at sites of dense packing of collagen fibers (arrowheads), whereas less dense connective tissue in c contains collagen fibrils, which are only lightly decorated with gold particles. The adjacent amorphous extracellular material is completely negative for undulin antigenicity. d, negative control using nonimmune IgG instead of the first antibody.

Although undulin is clearly a distinct glycoprotein of the ECM, it bears some similarities to other ECM macromolecules, particularly tenasin. Both molecules display a molecular weight above 1,000,000 on SDS gels when unreduced. After reduction of disulfides undulin shows bands of M, 270,000, 190,000, and 180,000, whereas tenasin/tenasin-like molecules show two to three bands between 150,000 and 260,000 depending on the species, its developmental stage, and the tissue of origin (Aufderheide and Ekblom, 1988; Bourdon et al., 1983; Chiquet and Fambrough, 1984; Chiquet-Ehrismann, 1986; Grumet et al., 1985; Hoffman et al., 1988; Kruse et al., 1985). It has recently been shown that these bands are derived from one gene by differential splicing (Gulcher et al., 1989). The three bands observed for undulin isolated from skin and placenta all reacted similarly when probed with one of several individual monoclonal antibodies in immunoblots. Therefore it appears that they are derived from a common gene either by differential splicing of a parent mRNA or by extensive posttranslational modifications of a single nascent polypeptide chain.

Since only one mRNA of approximately 7 kb is found in Northern blots of preparations from both placenta and a rhabdomyosarcoma cell line using various different undulin cDNA clones, the latter possibility seems more plausible. The size of this mRNA is close to that of the human fibronectin message (two differentially spliced forms around 7.9 kb; Vibe-Pedersen et al., 1984), chicken tenasin/cytotactin (messages between 7 and 9 kb; Jones et al., 1988; Pearson et al., 1989) and human hexabrachion (two messages around 6.2 and 7.8 kb; Gulcher et al., 1989).

We recently determined the continuous protein sequence of about 30% of human undulin from cyanogen bromide peptides and cDNA clones locally revealing up to 42% homology with regions in human fibronectin and up to 33% homology with parts of tenasin/cytotactin/hexabrachion, but showing no similarities with human thrombospondin or laminin, further underlining its unique nature as a distinct member of an ECM glycoprotein superfamily.

Augmenting the common features of the subunit composition of tenasin/cytotactin/hexabrachion and undulin, a monoclonal antibody against mouse tenasin has recently been shown to react with all chains of M, 260,000, 210,000, and 200,000 of reduced tenasin in immunoblots of extracts of mouse embryonic and adult intestine (Aufderheide and Ekblom, 1988).

Despite the above-mentioned similarities between undulin and tenasin the data suggest divergent biological roles for the two molecules. In all tissues studied so far their distributions have been found to be mutually exclusive. Tenasin/cytotactin/hexabrachion is expressed in mesenchymal tissues interacting with differentiating or rapidly growing epithelia such as during limb bud morphogenesis (Chiquet and Fambrough, 1984), kidney tubule formation (Aufderheide et al., 1987), early brain cell migration (Crossin et al., 1986), epithelial cell renewal in the gut (Aufderheide and Ekblom, 1988), and cancerous infiltration by mammary tumors (Mackie et al., 1987). Undulin, in contrast, is virtually absent underneath...
epithelia subject to rapid cell renewal and is rapidly degraded in the vicinity of invading tumors such as Kaposi sarcoma and oral squamous cell carcinomas.3

Since undulin is primarily associated with a particular subgroup of collagen fibrils some speculation about its biological function may be made. The fibrils with which undulin is associated appear highly ordered in uniform bundles. These bundles are prominent in tissues like skin and oral mucosa and at the periphery of structures like blood vessels, glands, and glandular ducts where high mechanical flexibility combined with stability are important. In keeping with the ultrastructural data which show undulin preferentially in regions of densely packed collagen fibers, it may be hypothesized that the glycoprotein acts as a flexible anchor allowing limited gliding between individual collagen fibrils. The lack of undulin in bone and its paucity in scar tissue (not shown) are in good agreement with this hypothesis. Furthermore, undulin antibodies label only highly uniform fibers representing bundles of collagen fibrils, thereby yielding a regularity of pattern not seen for any other ECM component. Thus it may be speculated that undulin is somehow involved in the supramolecular organization of collagen fibrils characteristic of differentiated mesenchyme, but lost or reduced, for example, for irreversible fibrosis of the liver and when tumor invasion of the interstitial ECM occur. In this regard Birk and Treelstad (1984) postulated that coalescence of mature collagen fibrils in fibroblast surface folds necessitates the incorporation of another component, possibly a glycoprotein. Whether undulin fulfills such a hypothetical function as well as the nature of the interaction of undulin and collagen fibrils remain to be clarified in the future. The anomalous binding of type I collagen to DEAE-cellulose in the presence of undulin, their coelution during purification, and our recent finding of a specific interaction of mainly the α2 chain of type I collagen with undulin attest to the innate affinity of the two molecules.

Undulin is produced by a variety of cell types in vitro. Low levels are found in cultures of human skin fibroblasts and a high rate of synthesis is found in a mesenchymal tumor cell line, whereas no undulin has been detected in epithelial cell cultures. In these cultures and in extracts from a variety of human tissues a remarkably constant electrophoretic pattern has been observed with band A (M, 270,000) and a broad band B (M, 180,000–190,000) after reduction, which indicates a fairly uniform processing of the presumed parent protein and/or mRNA.

The association of undulin with the surface of mature collagen fibrils and the finding that it appears to be removed more rapidly than any other of the major ECM components during carcinomatous invasion could qualify undulin or its fragments as key molecules for biochemical, histological, and serological studies on tissue differentiation and metastasis.

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