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# Expression of fibulin-2 by fibroblasts and deposition with fibronectin into a fibrillar matrix

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## SUMMARY

**The extracellular matrix protein fibulin-2 was shown to be a typical product of cultured human and mouse fibroblasts by several immunological assays. It is secreted and deposited in cells and tissues as a disulfide-bonded oligomer identical in size to the previously described recombinant fibulin-2. Most of the fibroblast fibulin-2 is deposited into a dense fibrillar meshwork which requires treatment with EDTA and/or 6 M urea for solubilization. Fibulin-2 and fibronectin are synthesized at equivalent levels and both colocalize in the fibrils as shown by immunofluorescence. Metabolic labelling and pulse-chase studies demonstrated fibulin-2 oligomers in detergent extracts of cells and their rapid translocation to extracellular EDTA-sensitive assembly forms. Unlike for fibronectin and fibulin-1 only a little fibulin-2 was found in the cell culture medium. Immunogold staining of confluent human fibroblasts showed localization of fibulin-2 to a fine meshwork or bundles of amorphous microfibrils in the matrix. This also demonstrated a distinct colocalization of fibulin-2 and fibronectin at the electron microscope level, indicating that the interaction between these two protein shown in in vitro assays may also exist in situ. No distinct colocalization of both proteins could, however, be observed with cross-striated fibrils of collagen I and collagen VI microfibrils.**

Key words: Extracellular matrix, Fibroblast, Microfibril, Biosynthesis

## INTRODUCTION

Fibulin-2 was recently identified as a novel extracellular matrix protein by cDNA cloning and recombinant protein production (Pan et al., 1993a). It belongs to a larger family of extracellular proteins including fibulin-1, fibrillins and latent TGF-β-binding proteins which are characterized by long tandem arrays of EGF-like modules of which many possess a calcium-binding sequence (Campbell and Bork, 1993). As a consequence of this modular arrangement, these proteins mainly consist of large rod-like elements but can in addition possess some small globular structures. Rotary shadowing of recombinant fibulin-2 showed, however, a star-shaped structure due to the formation of disulfide-linked oligomers (Pan et al., 1993a). Yet the closest relative, fibulin-1, is a monomer and has the shape of an elongated dumbbell (Sasaki et al., 1995a). These data also suggested that both fibulins should be constituents of larger extracellular assembly forms which serve structural functions.

Support for such predictions originated mainly from in vitro interaction studies which showed that fibulin-2 (Sasaki et al., 1995b) and fibulin-1 (Balbona et al., 1992; Pan et al., 1993b; Sasaki et al., 1995a) bind to fibronectin, nidogen and several other extracellular ligands. Most of these ligand interactions are dependent on calcium indicating the involvement of fibulin's rod-like structures. Functional impacts of these interactions are not known except for the competition of fibulin-2 and laminin for nidogen binding (Sasaki et al., 1995b) which indicates a regulatory role in basement membrane assembly. The ubiquitous nature and probably versatile functions of the fibulins are also suggested by their broad occurrence during embryonic development and in the adult phenotype including in basement membranes, mesenchymal compartments, elastic, skeletal and neural tissues (Pan et al., 1993a; Roark et al., 1995; Miosge et al., 1996; Zhang et al., 1996). Particularly prominent was their strong expression during cardiac development and their postnatal persistence in heart valves and septa (Spence et al., 1992; Zhang et al., 1993, 1995).

The nature and compartmentalization of fibulin-2 in cell cultures and tissues has so far not been examined at the molecular and ultrastructural level. Fibroblasts seem to be a convenient source for biosynthesis and assembly studies of fibulin-2 as indicated in previous northern blot and radioimmunoassay analyses (Pan et al., 1993a). Here, we show that fibroblasts produce substantial amounts of disulfide-bonded fibulin-2 and deposit it into a delicate meshwork. Such networks also contain fibronectin, consistent with a distinct calcium-dependent binding between these two proteins (Sasaki et al., 1995b). This supported a structural role for fibulin-2 in mesenchymal tissues such as the dermis.

## MATERIALS AND METHODS

### Sources of cells and antibodies

Human embryonic fibroblasts were obtained from the American Type Culture Collection and fibroblasts from adult human skin were kindly provided by Dr B. Adelmann-Grill (Martinsried). Fibroblasts from 14.5 day mouse embryos were obtained by a standard procedure for isolating feeder cells and were a gift from Dr U. Mayer (Martinsried). They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Affinity-purified antibodies were obtained by standard procedures (Timpl, 1982) from rabbit antisera against recombinant mouse fibulin-1 or fibulin-2 (Pan et al., 1993a; Sasaki et al., 1995a). An antiserum against human recombinant fibulin-2 (Sasaki et al., 1995b) was used without purification. Rabbit antibodies against the aminopropeptide of collagen I have been previously described (Nowack et al., 1976). Monoclonal mouse antibody IST-4 against human fibronectin (Carnemolla et al., 1989) was kindly supplied by Dr L. Zardi and rabbit anti-mouse fibronectin was obtained from Gibco BRL (Eggenstein, Germany). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (H+L) (Bio-Rad, Munich) were used for immunoblotting. Cy3-conjugated affinipure goat anti-mouse IgG (H+L), fluoresceine (DTAF)-conjugated affinipure anti-rabbit IgG (H+L), 6 nm colloidal gold-affinipure goat anti-mouse IgG (H+L) and 12 nm colloidal gold-affinipure goat anti-rabbit IgG (H+L) from Jackson Immuno Research Laboratories (West Grove, PA) were used in indirect immunofluorescence and immunogold double staining.

### Cell and tissue extractions

Confluent cells grown on a 10 cm dish were used to obtain serum-free culture medium and subsequently washed with phosphate-buffered saline, pH 7.2 (PBS). The cell layer was then treated on ice (30 minutes) with 1 ml 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS), containing 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM *N*-ethylmaleimide (NEM) and the supernatant (detergent extract) collected. The remaining matrix was then treated successively (each 30 minutes on ice) with 1 ml TBS, 10 mM EDTA, 2 mM PMSF, 5 mM NEM (EDTA extract) and 1 ml TBS, 6 M urea, 2 mM PMSF, 5 mM NEM (urea extract). All extracts were centrifuged in microfuge tubes (13,000 ***g***, 15 minutes) and stored in aliquots at −80°C. In separate experiments successive extracts of cell layers were obtained after 0.5-5 days in culture by 1% deoxycholate/4% SDS (McKeown-Longo and Mosher, 1983).

Tissues were obtained from 5- to 7-week-old female Balb/c mice and homogenized (1 ml/100-200 mg) either in TBS, 10 mM EDTA, 2 mM PMSF, 5 mM NEM (EDTA extract) or in the same buffer containing in addition 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (detergent extract) and, after incubation for 1 hour on ice, supernatants were collected and stored as described above. Protein contents of the extracts were determined with the BCA assay (Pierce, Rockford, IL).

### Metabolic labelling and immunoprecipitation

Fibroblasts ($2.5\times10^6$ cells) were cultured at 37°C on 10 cm dishes in DMEM/FCS (6-14 hours), preincubated with cysteine-free DMEM (2 hours) and then labelled with [$^{35}$S]cysteine (>1,000 Ci/mmol, Amersham) at 10 μCi/ml for 16 hours. Medium was harvested and the cell layer extracted as described above with buffers containing in addition aprotinin (2 μg/ml). In pulse-chase experiments with $10^6$ cells/6 cm dish, preincubations were followed by 1 hour labelling with 150 μCi/ml of [$^{35}$S]cysteine. The medium was then removed, the cell layer washed once with medium and incubated in unlabelled medium. Medium and cell layer extracts were then obtained at different times (0.5-6 hours) after the pulse.

Samples for immunoprecipitation were centrifuged (13,000 ***g***, 10 minutes) and precleared with Protein A-Sepharose (Pharmacia, Uppsala) overnight at 4°C with gentle shaking. Supernatants were then divided into three aliquots and incubated with affinity-purified anti-mouse fibulin-1, anti-mouse fibulin-2 or monoclonal anti-human fibronectin at 4°C for 5 hours. Immune complexes were precipitated with Protein A-Sepharose (3 hours, 4°C) and washed five times with TBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA. Pellets were subsequently solubilized in SDS-PAGE sample buffer (95°C, 5 minutes), centrifuged and used either directly or after reduction with 50 mM dithiothreitol for SDS-PAGE on 3-12% gels. Radioactive bands were then visualized by fluorography following established protocols.

### Radioimmunoassays

Concentrations of individual proteins were determined by radioimmuno-inhibition assays specific for mouse or human fibulin-2 (Pan et al., 1993a; Sasaki et al., 1995b), mouse fibulin-1 (Sasaki et al., 1995a) and human fibronectin following established protocols (Timpl, 1982). Biological samples were used at 3-4 different dilutions to obtain average values with a standard deviation of less than 20%.

### Immunoblotting

Cell extracts (0.1-2 μg protein) or tissue extracts (5-100 μg protein) were separated by SDS-PAGE on 3-12% gels and electroblotted onto Immobilon-P (Milipore, Bedford, MA). Filters were blocked by incubation (2 hours, room temperature) with TBS either containing 5% bovine serum albumin (BSA) (w/v) or 5% normal goat serum (v/v) followed by specific antibodies (0.1-0.2 μg/ml) diluted in TBS with either 2% BSA or goat serum. After washing with TBS, 0.1% Tween-20, bound antibodies were detected with ECL western blotting reagents (Amersham, Buckinghamshire, UK).

### Immunofluorescence microscopy

Fibroblasts ($5\times10^4$ cells/chamber) were cultured in Lab-Tek 4-chamber slides (Nunc Inc, Napperville, IL) for 4 days, the slides were washed three times with PBS and then fixed with cold acetone or 3% paraformaldehyde. After further washings with PBS, cells were incubated with the first antibodies in PBS, 1% BSA (1.5 hours, room temperature) followed by three washings with PBS. Further incubation with optimal dilutions of fluorescence-tagged secondary antibodies (1 hour) and washings followed the same protocol. Cells were then mounted with PBS/glycerin (1:9) containing β-phenylenediamine (1 mg/ml) and examined with an Axiophot Fluorescence microscope (Zeiss, Oberkochen, FRG).

### Immunogold staining

Human skin fibroblasts were plated onto Thermanox coverslips (Miles Laboratories, Naperville, IL) at a density of $2.5\times10^4$ cells/cm$^2$ in DMEM/FCS containing 25 μg/ml L-ascorbic acid and cultured for 5 days. Cells were washed three times with PBS and fixed with 3% paraformaldehyde on ice for 1 hour. After further washing with PBS cells were incubated (4°C, overnight) with a mixture of monoclonal mouse antibody against fibronectin (culture medium diluted 1:50) and rabbit antiserum against procollagen I amino propeptide (dilution 1:50) or affinity-purified rabbit anti-fibulin-2 (20 μg/ml). After three washes with PBS, cells were incubated with a mixture of colloidal gold-labelled antibodies (see above) for 1.5 hours at room temperature followed by extensive washing with PBS. Negative controls for fibulin-2 were done with normal rabbit IgG (20 μg/ml). Cells were then fixed with 2% glutaraldehyde, 1% $OsO_4$, stained with 1% uranyl acetate in 70% ethanol during dehydration, and embedded in epoxy resin and thin sections were stained with uranyl acetate and lead citrate following a previous procedure (Bruns et al., 1986). Sections were then examined with a CM10 electron microscope (Philips).

## RESULTS

### Characterization of fibulin-2 in fibroblast cultures and tissues

Immunofluorescence staining of confluent human fibroblast cultures demonstrated a dense meshwork of fibulin-2 containing fibrils mainly deposited on top of the cells. Double staining with a monoclonal antibody to fibronectin demonstrated a strict colocalization of the two proteins (Fig. 1). Such meshworks could also be detected on subconfluent human fibroblasts and on mouse fibroblasts, but appeared less dense. Similar networks were also detected with antibodies against fibulin-1, fibrillin-1 and collagen VI demonstrating that they were intermingled at the level of light microscopic resolution. Exposure of the cell layer of human fibroblasts to detergents removed most of the cells but did not change fibulin-2 deposits. A weak decrease in staining was observed after subsequent extraction with EDTA-containing buffer (Fig. 1). Further extraction with 6 M urea solubilized most residual material leaving no recognizable structures. This demonstrated that the extracellular deposits of fibulin-2 are mainly dependent on non-covalent interactions.

The quantitative distribution of fibulin-2 between the culture medium and the three cell layer extracts of embryonic and adult human fibroblasts was determined by specific radioimmunoassays (Table 1). This demonstrated low levels in the medium and detergent extract which is assumed to contain mainly the intracellular pool. More than 80% of fibulin-2 was found in the EDTA and urea extracts. Higher amounts were typically observed in the EDTA extract, demonstrating calcium requirement for association. A similar distribution, apart from a slight relative increase in the medium and detergent extract, was also observed for mouse fibroblasts. The same analysis for fibronectin showed approximately the same total amount as fibulin-2 in human fibroblasts, indicating synthesis in stoichiometric proportions. Yet almost all of the fibronectin was found in the culture medium and the urea extract. If the content of the EDTA and urea extract could be equated with the fibrillar structures detected by immunofluoresence, these structures should contain a 2- to 4-fold molar excess of fibulin-2 (molecular mass about 420 kDa) compared to fibronectin (molecular mass about 450 kDa). Fibulin-1 (molecular mass about 90 kDa) could only be quantitated in mouse fibroblasts (Table 1). This demonstrated that, as for fibronectin, the major proportion was in the culture medium and urea extract. The molar content was about 2-fold lower than fibulin-2.

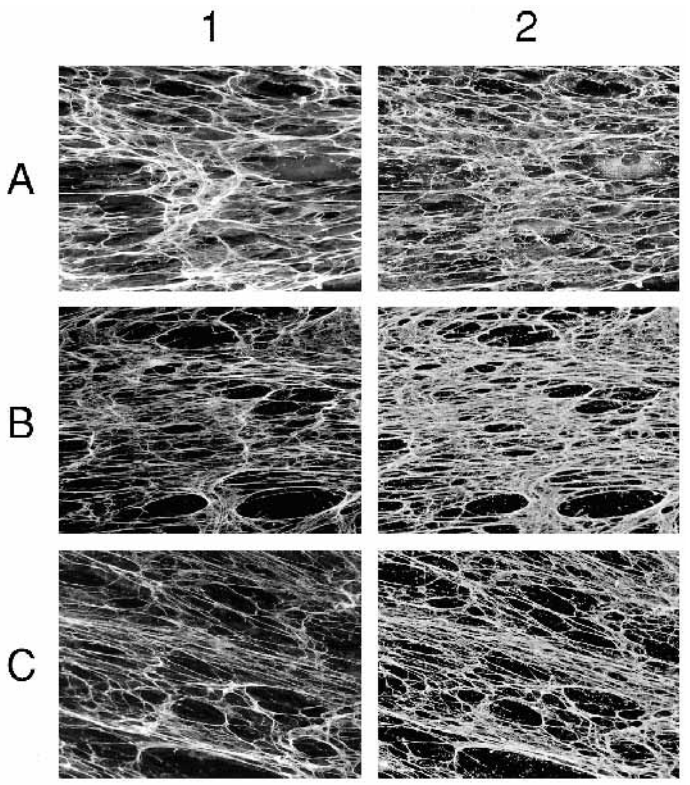


**Fig. 1.** Double immunofluorescence staining of confluent human skin fibroblasts for fibulin-2 and fibronectin. Cells were used without extraction (A) and after detergent extraction (B) which was followed in (C) by EDTA extraction. Staining was with rabbit antibody against fibulin-2 (1) and mouse monoclonal antibody against fibronectin (2).

Immunoblotting was used to determine the size of the proteins analyzed and to confirm the radioimmunoassay data at a qualitative level (Fig. 2). For human fibroblasts, the relative intensities of the bands in medium and the three cell layer extracts corresponded to the quantities of fibulin-2 and fibronectin determined by radioimmuno assay (Table 1). The major fibulin-2 band comigrated with non-reduced recombinant fibulin-2 and approximately with non-reduced fibronectin (molecular mass about 450 kDa), demonstrating that fibroblasts produce a disulfide-bonded form of the fibulin-2 chain. This was confirmed by disulphide bond reduction which shifted the band to a 195 kDa position consistent with similar

**Table 1. Amounts of total protein (TP), fibulin-2 (F-2), fibulin-1 (F-1) and fibronectin (FN) synthesized by fibroblasts and their distribution in different compartments**

| | Human embryonic fibroblasts | | | Human adult fibroblasts | | | Mouse fibroblasts | | |
|---|---|---|---|---|---|---|---|---|---|
| Medium or cell extract | TP(μg) | F-2(μg) | FN(μg) | TP(μg) | F-2(μg) | FN(μg) | TP(μg) | F-2(μg) | F-1(μg) |
| Medium | nd | 0.11 | 1.31 | nd | 0.11 | 2.12 | nd | 0.50 | 0.153 |
| Detergent extract | 252 | 0.14 | 0.26 | 333 | 0.57 | 0.23 | 1,050 | 0.38 | 0.013 |
| EDTA extract | 103 | 1.57 | 0.05 | 81 | 2.34 | 0.04 | 390 | 1.37 | <0.004 |
| Urea extract | 95 | 0.35 | 0.87 | 91 | 1.36 | 0.86 | 260 | 0.61 | 0.070* |
| Total | 450 | 2.17 | 2.49 | 505 | 4.38 | 3.25 | 1,700 | 2.86 | 0.236 |

Confluent fibroblast cultures were used to obtain serum-free culture medium and three successive extracts of the cell layer. Total protein was determined by a colorimetric assay and individual proteins by radioimmunoassays. Values are total amounts per 10 cm culture dish. nd, not determined because of interference by residual serum proteins.

*Lower amounts are present in human fibroblasts (see Figs 2 and 4).

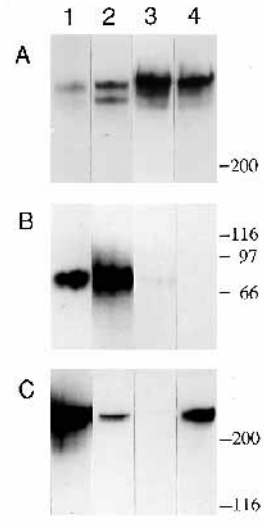


**Fig. 2.** Immunoblot analysis of fibulin-2 (A), fibulin-1 (B) and fibronectin (C) in various human fibroblast compartments. Samples used were serum-free culture medium (1, 20 µl) and successive cell extracts obtained with detergent (2, 8 µl), EDTA (3, 1.5 µl) and 6 M urea (4,1.5 µl). An 8-fold increase in sample load demonstrated also the presence of fibulin-1 in lanes 3 and 4 (not shown). Fibulins were analyzed in non-reduced form and fibronectin after reduction. Positions of molecular mass markers are indicated in kDa.

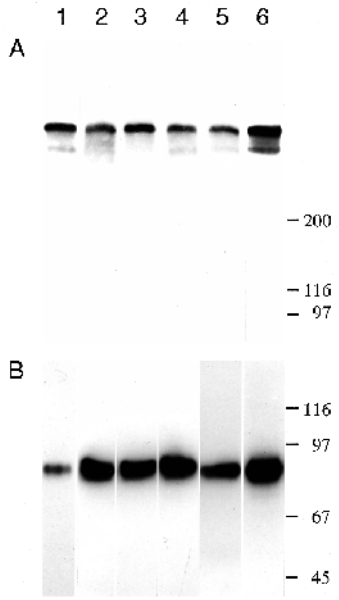


**Fig. 3.** Detection of fibulin-2 (A) and fibulin-1 (B) in EDTA extracts of mouse tissues by immunoblotting. Samples containing 0.5-1 ng fibulins according to radioimmunoassays were obtained from kidney (lane 2), heart (lane 3), placenta (lane 4), skeletal muscle (lane 5) and skin (lane 6). Lanes 1 were loaded with recombinant mouse fibulin-2 (A) or fibulin-1 (B). All samples were non-reduced and molecular mass markers are indicated in kDa.

observations for recombinant fibulin-2 (Pan et al., 1993a; Sasaki et al., 1995b). In non-reduced cell detergent extracts, the fibulin-2 antibodies also detected a distinct band of faster mobility which could represent a precursor with incomplete posttranslational modifications. Immunoblotting also demonstrated a strong 90 kDa fibulin-1 band in medium and cell detergent extracts of human fibroblasts while much less material was detected in the EDTA and urea extracts (Fig. 2). This is consistent with radioimmunoassay data determined for mouse fibroblasts (Table 1).

Solubilization by deoxycholate followed by SDS was previously used to demonstrate time-dependent immobilization of fibronectin exogenously added to human fibroblast cell layers (McKeown-Longo and Mosher, 1983). The same method was now applied to endogenous fibronectin produced by human fibroblasts and showed by immunoblotting about equal staining over a period of 0.5-5 days for both deoxycholate-soluble and -insoluble fibronectin. Yet a distinct increase in deoxycholate-insoluble fibulin-2 was observed over the same period indicating a delayed association with presumably more stable extracellular structures (data not shown). Various mouse tissue extracts prepared either with EDTA-containing (Fig. 3) or SDS-containing buffers (not shown) were in addition used for an immunoblot analysis of the fibulins. This demonstrated mainly a disulfide-bonded form for fibulin-2 and a monomeric form for fibulin-1. The amounts of fibulin in the EDTA extracts (ng/mg wet tissue) were in the range 0.4-1.2 ng/mg for fibulin-2 and 0.6-7 ng/mg for fibulin-1 as determined by radioimmunoassay (Table 2). This indicated that fibulin-1 exists in tissues in an EDTA-sensitive form while only small amounts of this form could be detected in mouse fibroblast culture (Table 1).

**Table 2. Content of fibulins in EDTA extracts of mouse tissues**

| Tissue | Fibulin-2 | Fibulin-1 |
|---|---|---|
| Kidney | 0.40 | 2.21 |
| Heart | 1.02 | 1.21 |
| Placenta | 1.19 | 6.82 |
| Muscle | 0.51 | 0.62 |
| Skin | 0.51 | 0.90 |

All determinations were carried out by radio-immunoassays and are expressed as ng/mg wet weight tissue.

## Pathways of newly synthesized fibulins and fibronectin

Metabolic labelling with [$^{35}$S]cysteine was used to study the distribution of the proteins in the medium and cell layer extracts of human fibroblasts. After a long pulse, immunoprecipitation with specific antibodies for fibulin-2 demonstrated a characteristic 195 kDa band after reduction, primarily in the EDTA and urea extracts (Fig. 4). This band showed a much slower migration when analysed without reduction (data not

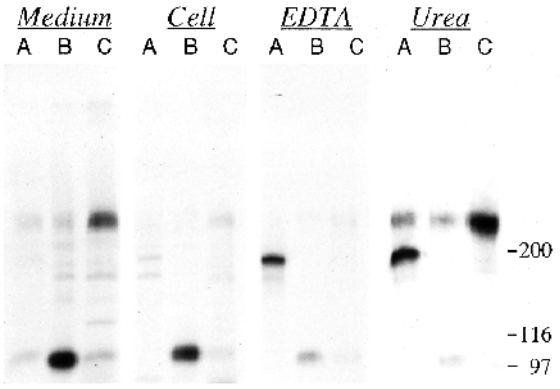


**Fig. 4.** Immunoprecipitation of fibulin-2 (A), fibulin-1(B) and fibronectin (C) from metabolically labelled human fibroblasts. Cells were labelled with [$^{35}$S]cysteine for 16 hours and precipitates analysed by SDS-PAGE under reducing conditions and fluorography. The samples used were serum-free culture medium, cell detergent extracts and subsequent extracts obtained with EDTA and 6 M urea. Some immunoprecipitation of fibronectin was obtained with antisera to fibulins in the medium and urea extract. Since this was also observed with non-immune sera (not shown) it indicates unspecific precipitation. A few molecular mass markers are indicated in kDa.

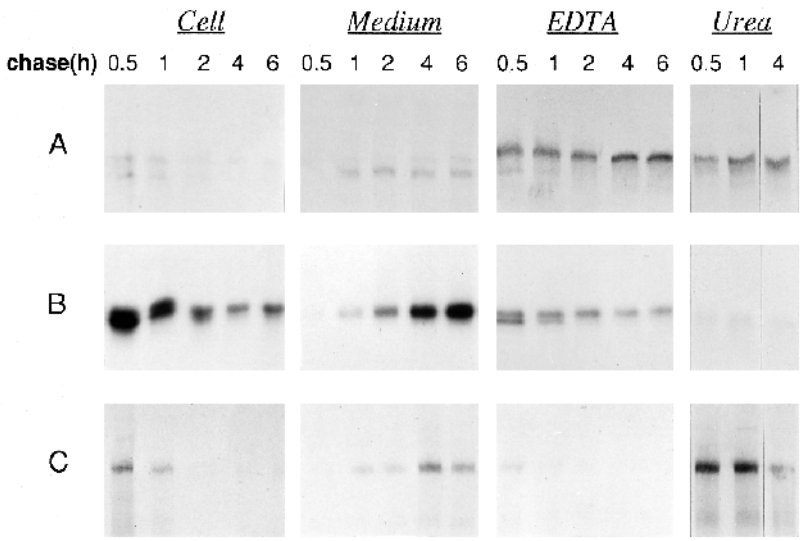


**Fig. 5.** Pulse-chase analysis of newly synthesized fibulin-2 (A), fibulin-1 (B) and fibronectin (C) in human fibroblast cultures. The pulse with [$^{35}$S]cysteine was for 1 hour followed by the chase times in hours indicated on top of the individual gels. The samples analysed by immunoprecipitation and fluorography were cell detergent extracts, serum-free culture medium and EDTA and urea extracts of the cell layer. Only the relevant sections of the gels (see Fig. 4) are shown.

shown). The characteristic 250 kDa chains of reduced fibronectin could be primarily detected in the medium and urea extract. The 90 kDa band of fibulin-1 was mainly detected in the medium and detergent extract (Fig. 4) and showed a slightly faster mobility prior to reduction (not shown). These data were consistent with the distribution determined by radioimmuno assay (Table 1) for cells which had, however, been kept in culture for 4-6 days.

A short labelling (1 hour) followed by a chase period of 0.5 to 6 hours was used to follow the fate of the newly synthesized proteins (Fig. 5). For fibulin-2, a weak doublet band persisted in the cell detergent extract for a short time and subsequently weak bands were detected in the medium. Most of the labelled fibulin-2, however, was found in the EDTA and urea extracts of the cell layer already after a 0.5 hour chase and increased in intensity with time. In contrast, a strong intracellular labelling (detergent extract) of fibulin-1 decreased with time and was paralleled by an increase in the medium. Some labelled fibulin-1 was also detectable in the EDTA extract of the cell layer but showed no change or a moderate decrease in labelling. Fibronectin could be initially detected both in the detergent and urea extracts of the cell layer, while its release into the medium was a later event. This demonstrated a rather rapid deposition of all three proteins into the extracellular meshwork of the fibroblasts and only a late and variable secretion into the medium.

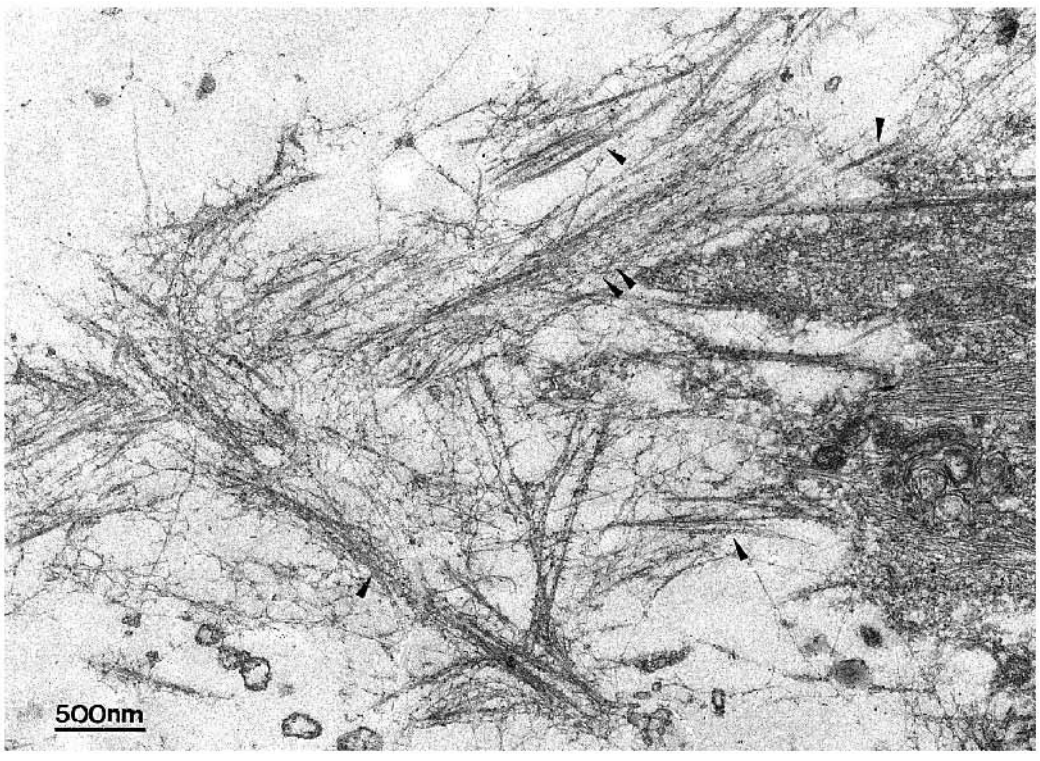


**Fig. 6.** Electronmicroscope visualization of a fibrillar matrix in close vicinity to a cultured human fibroblast (right side). Notice the abundance of thin collagen I fibrils (arrowheads) intermingled with regions of thin filamentous material and the presence of cross-banded structures (double arrowheads) characteristic of aggregated collagen VI microfibrils (Bruns et al., 1986).

### Immunogold staining of fibroblast fibrils

Human skin fibroblasts grown on coverslips in the presence of ascorbic acid produced and deposited an elaborate fibrillar matrix in the close vicinity of the cells as shown by a low power electron micrograph in Fig. 6. This matrix consisted of thin (10-30 nm) cross-striated collagen I fibrils occassionally surrounded by filamentous and globular material (see Furcht et al., 1980), long microfibrils without apparent cross-striation, regions of short amorphous microfibrillar deposits and some cross-banded broad fibrils of 100 nm periodicity which are characteristic of aggregated collagen VI microfibrils (Bruns et al., 1986). These cell layers were used to analyze the colocalization of fibulin-2 (12 nm gold particles) and fibronectin (6 nm gold particles) by electron microscopy at larger magnifications (Fig. 7). This demonstrated a very extensive codistribution of the labels along the extended fibrils lacking cross-striation often over a length of several micrometers. Labelling was, however, scarce or absent along fibrils with a clearly recognizable collagen cross-striation banding and this coincided with a lack of association with filamentous material. The

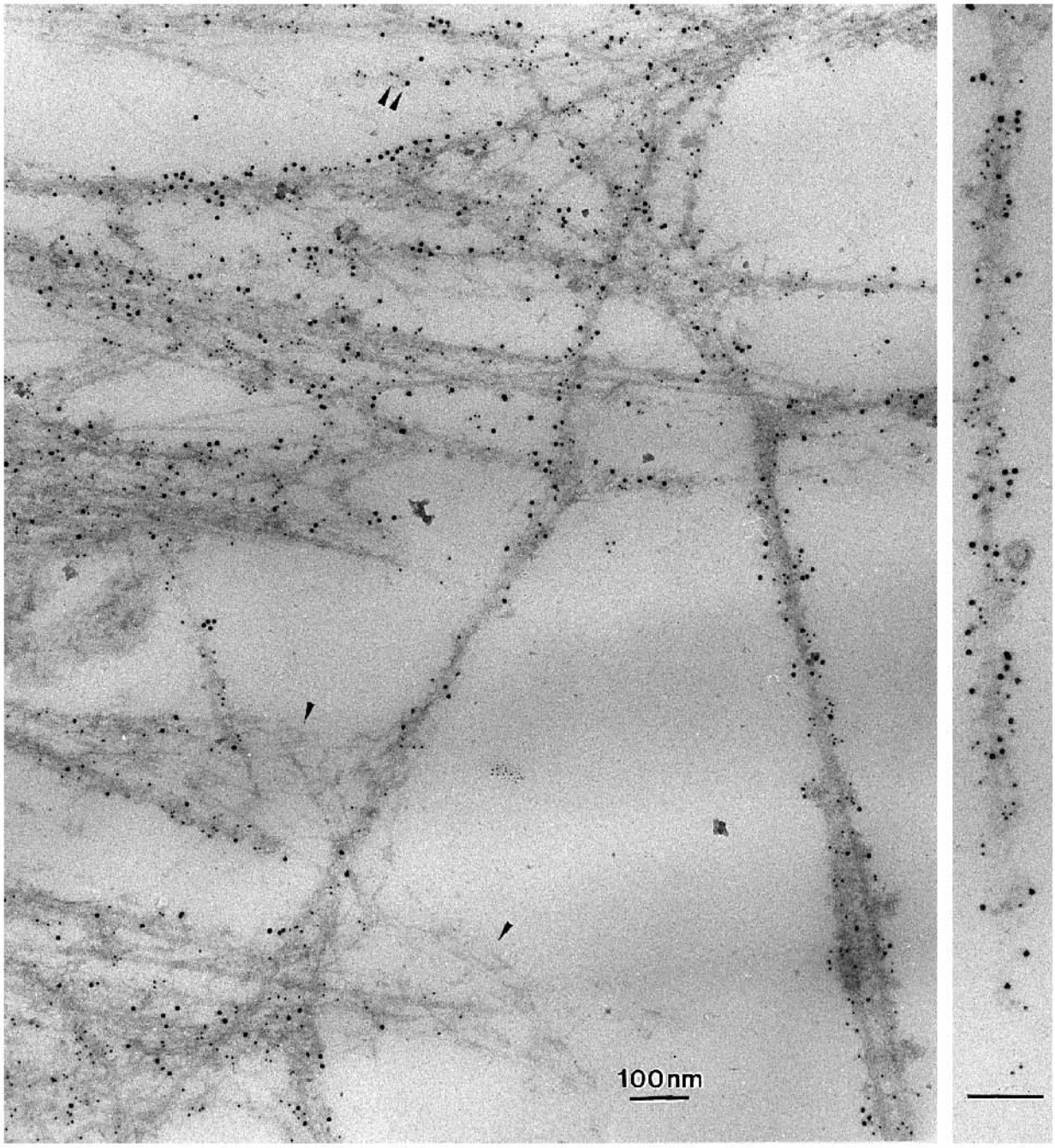


**Fig. 7.** Section of an immunogold-stained human fibroblast matrix demonstrating colocalization of fibulin-2 (12 nm colloidal gold) and fibronectin (6 nm colloidal gold). A particularly well labelled long fibril is shown in the right-hand panel (bar 100 nm). Arrowheads indicate regions of filamentous material apparently not associated with long fibrils which were either labelled for both proteins or where labelling was scarce.

density of fibronectin to fibulin-2 labelling occured approximately in a ratio of 2:1 as illustrated for a 1.5 µm long fibril section (Fig. 7, right side) but this difference could reflect a penetration problem due to the size of the gold particles. There was no apparent periodicity for either label but frequently there seemed to be a segregation of clusters of the two different labels. Yet both different labels could be as close as 10-20 nm. The regions of microfibrillar deposits were either labelled for both proteins or not at all (Fig. 7). Regions where collagen VI fibrils could be identified were also devoid of label.

A potential codistribution of fibronectin and procollagen I in a similar fibroblast matrix was previously suggested by immunoperoxidase and immunoferritin staining (Furcht et al., 1980). Because of our lack of staining of cross-striated collagen fibrils by antibodies against fibulin-2 and fibronectin we used antibodies against fibronectin (6 nm gold particles) and against the aminopropeptide of collagen I (12 nm gold particles) in double staining to clarify such associations (Fig. 8). This demonstrated as expected staining for the aminopropeptide along cross-striated fibrils of about 70 nm periodicity but not in other regions. Fibronectin staining was, however, restricted to non-striated bundles of microfibrils and deposits of small microfibrils. A close colocalization of both proteins was very rare and occured only in regions where both types of fibrils were aligned or crossing each other (Fig. 8). A low codistribution between collagen I and fibronectin could be also observed by double staining immunofluorescence (data not shown).

## DISCUSSION

Fibulin-2 has been identified as a novel extracellular matrix

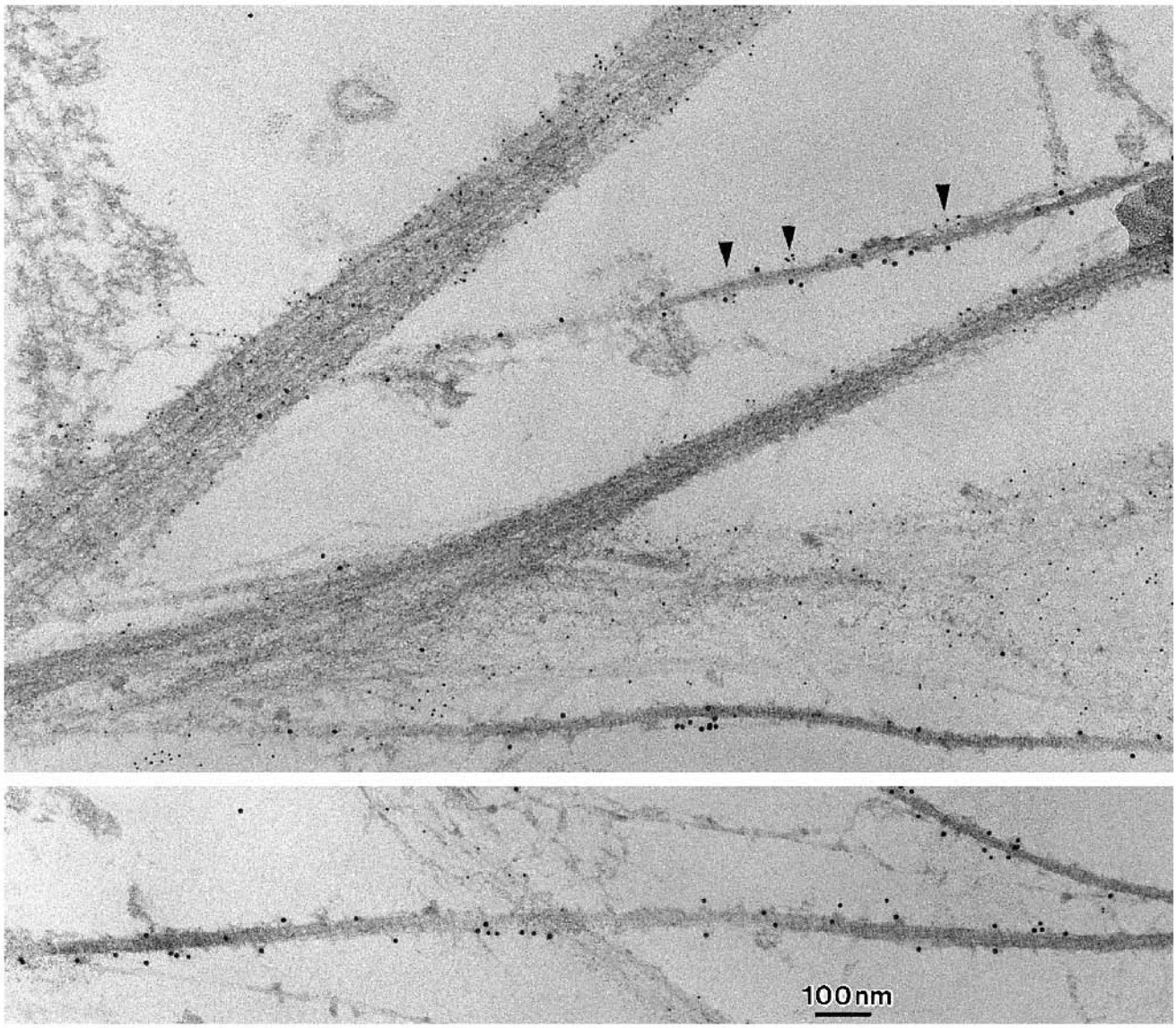


**Fig. 8.** Lack of colocalization of collagen I (12 nm colloidal gold) and of fibronectin (6 nm colloidal gold) by immunogold staining of a human fibroblast matrix. The sections shown include regions of long microfibrillar bundles stained for fibronectin and intermingled with collagen fibers and microfibrillar deposits (top) as well as a section with typical cross-striated collagen fibrils of about 70 nm periodicity (bottom). Note the scarce appearance of a close double labelling (arrowheads).

protein with a ubiquitous distribution in vessel walls, heart valves and many other interstitial localizations (Pan et al., 1993a; Zhang et al., 1995, 1996). Yet the way in which fibulin-2 is incorporated into particular supramolecular assembly forms has so far remained unclear. Here we demonstrate that it associates with fibrillar structures deposited by cultured fibroblasts and shows a distinct colocalization with fibronectin. This identifies fibulin-2 as a microfibrillar component such as the fibrillins and latent TGF-β binding proteins (Rosenbloom et al., 1993; Kielty and Shuttleworth, 1995; Gibson et al., 1995) which all belong to the same superfamily of calcium-binding rod-like proteins.

Three different fibroblast cell lines were shown to produce and secrete high levels of fibulin-2, which confirmed previous predictions from northern blot analyses and the immunofluorescence localization to tissues which are rich in fibroblasts (Pan et al., 1993a) such as the dermis. Radioimmunoassays demonstrated that these levels were as high as those of fibronectin (Table 1) suggesting that such levels are typical for the fibroblastic phenotype. This mesenchymal fibulin-2 was furthermore shown to correspond to a disulfide-bonded oligomer in cell layer and tissue extracts similar to recombinant mouse and human fibulin-2 obtained from transfected kidney cell clones (Pan et al., 1993a; Sasaki et al., 1995b). Fibroblasts were also reported to produce the related isoform fibulin-1 which is monomeric (Argraves et al., 1990). As shown here it is less abundant than fibulin-2 in cell culture but found in equivalent or even higher amounts in EDTA extracts of tissues. This could reflect the different solubilities of the two isoforms, as also indicated from cell culture studies (Table 1), or a higher expression or larger repertoire of fibulin-1 producing cells in the same tissues.

Most remarkable was the strong association of fibulin-2 with the fibroblast cell layer which exceeded that of fibulin-1 and fibronectin. A major fraction of the cell layer-associated protein could be solubilized with EDTA-containing neutral buffer but some required dissociation under denaturing conditions (6 M urea). It was furthermore noticed that translocation of the detergent-soluble, presumably intracellular form of fibulin-2 to the EDTA- and urea-sensitive compartments is a fast process as shown by pulse-chase experiments. These data also indicated that deposition of fibulin-2 to fibrillar structures involved both calcium-dependent and other non-covalent interactions while only low amounts of fibulin-2 escaped this by secretion into the culture medium. This seems to be different for fibronectin, of which more than half is secreted into the culture medium, which, as shown by pulse-chase experiment, occured at a late stage. Fibronectin deposition to the cellular matrix was sensitive to urea but not to EDTA and the final stoichiometric ratio of fibulin-2 to fibronectin was in the range 2-4:1 (Table 1) but some of the fibronectin may not have been extracted due to extensive disulphide or other cross-linking (Hynes, 1990; Choi and Hynes, 1979). It was, however, also obvious that a large fraction of fibulin-2 could be removed by EDTA without disturbing the fibrillar fibronectin matrix. This could indicate that the fibronectin matrix is required for deposition of fibulin-2, even though this was not shown directly. Such dependence was demonstrated for fibulin-1 (Roman and McDonald, 1993; Godyna et al., 1994) which is also produced by fibroblasts but, as shown here, in lower quantities than fibulin-2 and fibronectin. The data were also consistent with a distinct binding of fibulin-1 to fibronectin in solid phase assays (Balbona et al., 1992; Sasaki et al., 1995a) while no significant binding could be detected between fibulin-1 and fibulin-2 (Sasaki et al., 1995b).

The potential interaction between fibulin-2 and fibronectin has been previously demonstrated by solid-phase binding assays and was shown to be blocked by EDTA. These studies also demonstrated by surface plasmon resonance assays a $K_d$ of about 1 μM for this interaction probably followed by the formation of a more stable complex (Sasaki et al., 1995b). All our attempts to produce calcium-dependent aggregates or fibrils from mixtures of fibulin-2 and fibronectin up to concentrations of 0.5 μM have so far failed (T. Sasaki and R. Timpl, unpublished). This could be due to the initial low affinity of both components for each other or to the requirement of preformed fibronectin fibrils for association. The formation of such fibrils from fibronectin in solution is also difficult to achieve (Hynes, 1990) very likely because of the need for integrins α5β1 and/or αvβ3 and some other receptors to provide a cell-bound nucleation site (Peters and Mosher, 1987; Wu et al., 1993; Moon et al., 1994; Wennerberg et al., 1996). In this context, it is of interest that the RGD site of mouse fibulin-2 shows moderate binding to αvβ3 but not to α5β1 integrin (Pfaff et al., 1995). However, since human fibulin-2 and fibulin-1 lack the RGD and a distinct affinity for integrins it appears unlikely that integrin-mediated nucleation could be a general mechanism in the formation of fibrils from fibulins.

Double labelling of the fibroblast-produced matrix by the immunogold technique demonstrated a distinct colocalization of fibronectin and fibulin-2 which could be as close as about 20 nm. The regions of double labelling were mainly restricted to long bundles or a fine meshwork of microfibrils which lacked a distinct morphology and periodicity and were previously equated with supramolecular assembly forms of fibronectin in other studies (Hedman et al., 1978; Furcht et al., 1980; Hynes, 1990). These filamentous structures could be clearly distinguished from fibrillin-containing elastic microfibrils (Kielty and Shuttleworth, 1995) as well as from collagen VI microfibrils (Bruns et al., 1986; Timpl and Chu, 1994). Such microfibrils which could be stained for fibulin-2 and fibronectin were not associated with cross-striated collagen I fibrils which as shown previously with other staining methods (Furcht et al., 1980) could also be identified by immunogold staining for the aminpropeptide of procollagen I. The data of Furcht et al. (1980) suggested also some form of codistribution between fibronectin and procollagen I based on separate staining patterns obtained with single antibodies. This interpretation could not be confirmed in our double staining experiments which rather demonstrated that fibronectin microfibrils and collagen I and VI fibrils form independent supramolecular structures in the matrix deposited by cultured fibroblasts.

The density of double labelling in certain regions of the fibroblast matrix is also consistent with possible molecular contacts between fibronectin and fibulin-2 which both have a length of 90-120 nm in their most extended form (Engel et al., 1981; Pan et al., 1993b). Yet such contacts still need to be verified by cross-linking experiments or by the search for natural intermolecular cross-links. A further approach has become feasible through recent developments which allow fibronectin fibrils to be produced in solution (Morla et al.,

1994). This was achieved by small recombinant fibronectin fragments which apparently disrupt internal contacts in fibronectin molecules followed by partial unfolding and lateral aggregation (Hynes, 1990; Morla and Ruoslahti, 1992; Aguirre et al., 1994; Hocking et al., 1994). This could therefore be a suitable model to study the potential associations of the fibulins as well as other fibronectin-binding proteins in vitro.

The evidence for a localization of fibulin-2 and fibronectin to a microfibrillar compartment of the fibroblast matrix is very likely only one of several ways in which fibulin-2 participates in supramolecular assemblies. Recent studies demonstrated its in vitro binding to fibrillin-1 with a $K_d$=56 nM and its association with certain but not all regions of dermal elastic microfibrils as shown by electron microscopy (Reinhardt et al., 1996). Fibulin-2 may also occur in some basement membranes (Pan et al., 1993a) where it could interact with perlecan and nidogen ($K_d$=0.5 μM) and thus regulate their binding to laminins (Sasaki et al., 1995b). Further association forms of fibulin-2 should be expected in heart valves or developing nerve and cartilage tissues where it is particularly abundant and not codistributed with fibronectin (Zhang et al., 1995, 1996; Miosge et al., 1996). Thus fibulin-2 seems to be a versatile protein of the extracellular space and the molecular as well as biological analysis of its diverse interaction potentials will remain a forthcoming challenge.

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