CMLS, Cell. Mol. Life Sci. 54 (1998) 606–613
1420-682X/98/060606-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Ultrastructural localization of microfibrillar fibulin-1 and fibulin-2 during heart development indicates a switch in molecular associations

N. Miosge[a,*], T. Sasaki[b], M.-L. Chu[c], R. Herken[a] and R. Timpl[b]

[a]Zentrum Anatomie, Abteilung Histologie, Universität Göttingen, Kreuzbergring 36, D-37075 Göttingen (Germany), Fax +49 551 397067, e-mail: nmiosge@gwdg.de
[b]Max-Planck-Institut für Biochemie, D-82152 Martinsried (Germany)
[c]Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia (Pennsylvania 1907-5541, USA)

Received 3 April 1998; accepted 8 April 1998

Abstract. The microfibrillar proteins fibulin-1 and fibulin-2 were previously identified as prominent components of the endocardial cushion tissue (ECT) during heart development and shown to persist in adult valves and septa. Immunogold staining has now been used to compare their localization in embryonic (days 9–11) and adult mouse heart with that of fibronectin and the chondroitin sulphate proteoglycan versican. All four proteins were deposited in the ECT, which consists of a hyaluronan-rich, mainly unstructured matrix, but were barely detectable in myocardial basement membranes or within endocardial cells. Digestion with hyaluronate lyase selectively released the fibulins and versican but not fibronectin from the ECT. Yet neither of the two fibulins bound to hyluronan in solid-phase assays, in contrast to versican. In the adult heart valve, all four proteins could be detected close to cross-striated collagen fibrils or microfibrils, but only versican was lost upon exposure to hyaluronate lyase. The data indicate that fibulins are associated with the hyaluronan-matrix of ECT through a bridge of versican, but that this association changes upon valve development to another supramolecular, presumably microfibrillar organization based on fibronectin and/or fibrillins.

Key words. Heart development; extracellular matrix; heart valves; hyaluronan; immunogold staining; microfibrils.

The extracellular matrix contains many different microfibrillar systems that are based either on some collagen types, or on fibronectin or fibrillins, as well as some related proteins which are still incompletely characterized. A novel protein family consisting of fibulin-1 [1, 2] and fibulin-2 [3] was shown, like fibrillins, to contain long tandem arrays of epidermal growth factor (EGF) modules with a consensus sequence for calcium-binding, indicating that these proteins should have a rodlike shape [4]. This was in fact demonstrated for recombinant fibulin-1, which was obtained as a 90-kDa monomer [5], and recombinant fibulin-2, which forms a disulphide-bonded 350-kDa dimer with a more variable shape [6]. In vitro binding studies demonstrated that fibulin-1 associates with fibronectin, several basement membrane components (laminins, collagen IV, nidogen) and fibrinogen [5, 7–12]. A similar binding repertoire was also found for fibulin-2 [13], but also included

* Corresponding author.

fibrillin [14] and laminin-5 [15]. This was in accordance with light microscopic tissue studies which localized fibulins to several basement membrane zones, vessel walls and various other interstitial spaces in adult tissues [3, 7, 16]. Ultrastructural analyses demonstrated a close association of fibulin-2 with fibronectin microfibrils in fibroblast cultures [17] and with some but not all fibrillin-containing microfibrils surrounding elastic plaques [14]. Fibulin-1 may also associate with fibronectin [18, 19], and additionally with the amorphous core of elastin fibres [16]. Together the data indicate that fibulins are versatile proteins, able to associate with different extracellular supramolecular structures.

Both fibulins were also shown by immunohistology and messenger RNA (mRNA) detection to be expressed in early stages of embryonic organogenesis, particularly in regions of epithelial-mesenchymal interactions involving skeletal development, tooth, neuronal structures and several basement membranes [20–22]. A high but restricted expression of fibulin-2, but not fibulin-1, was also observed during wound healing in skin [23]. The most striking synthesis, however, occurred in early heart development at the stage where the myocard separates from the endocard in a tubelike structure with the endocardial cushion tissue (ECT) in between, as shown for avian [20, 24], mouse [25, 26], human [22] and zebrafish [27]. The ECT eventually gives rise to most of the valve and septal structures of the adult heart [28] which still maintain a high production of fibulin-1 and fibulin-2 [26]. Electron microscopy of the ECT reveals an amorphous structure with some granular deposits, but distinct assembly patterns such as collagen fibrils, microfibrils or basement membranes have not been identified. Yet immunohistology demonstrated that, besides the fibulins, laminin, fibronectin, tenascin-C, basement membrane collagen IV and fibrillar collagens are also present [25, 29, 30]. Their supramolecular structures may, however, become obscured by the presence of large amounts of hyaluronan and sulphated proteoglycans [31, 32].

In the present study, we have used embryonic and adult mouse heart to examine the ultrastructural localization of fibulin-1 and fibulin-2 in comparison to their potential ligands, fibronectin and versican. All of these proteins were present in the ECT and heart valves but did not reveal any particular assembly pattern. Yet they showed different dependencies on the presence of hyaluronan, which, with the binding data, clearly indicated a switch in the associations of fibulins to different structures during heart development.

Materials and methods

Source of tissues and tissue preparation. NMRI (New Mexico Research Institute) mice were kept on a normal day/night cycle and received Altromin commercial food and water ad libitum. Day 0 of gestation was defined as starting at 11:00 AM on the day on which a vaginal plug was detected after a mating period of 3 h. On days 9–11 of gestation the pregnant mice were anaesthetized with ether, killed by cervical dislocation, and the embryos removed from the uterus. The mitral valves from two 20-day-old NMRI mice were also dissected. The specimens were transferred to 0.1 M phosphate-buffered saline (PBS) pH 7.2, at 4 °C.

Relevant tissue sites of the embryos or the mitral valves were dissected under a stereomicroscope and fixed by immersion in 4% paraformaldehyde and 0.5% gluteraldehyde in PBS at 4 °C for 30 min. Specimens were then treated with 10 mM ammonium chloride in PBS for 45 min at 4 °C, and dehydrated in a graded series of ethanol up to 70%. The specimens were then embedded in the acrylic resin LR-Gold (London Resin Company, Reading, UK). The resin was hardened at −25 °C with the addition of 0.8% of the light-sensitive initiator benzil and the light of a halogen lamp [33].

For morphological investigations, specimens were fixed in 3% paraformaldehyde and 3% glutaraldehyde in 0.2 M phosphate buffer for 2 h at 4 °C, postfixed for 1 h in 1% osmium tetroxide and embedded in epon [34]. Semithin sections were cut and stained with toluidine blue for orientation purposes. Ultrathin sections were cut with a Reichert's ultramicrotome and collected on formvar-coated copper grids. After staining for 10 min with uranyl acetate and 5 min with lead citrate, sections were examined with a Zeiss EM 109 electron microscope.

Sources of antibodies. Rabbit antisera were prepared against recombinant mouse fibulin-1C [5] and fibulin-2 [3] and were affinity-purified. The antibodies were specific for each individual fibulin and reacted in immunoblots of nonreduced mouse fibroblast medium selectively with a 90-kDa band (antifibulin-1) or a 450-kDa band (antifibulin-2). Antibodies against fibronectin (rabbit antimouse) were purchased from Biomol (Hamburg, Germany). Affinity-purified rabbit antibodies against a fusion protein containing a central region of human versican [35] were a kind gift of D. R. Zimmermann, Zürich.

Immunogold histochemistry. Nickel grids carrying the sections of tissue embedded in LR-Gold were incubated for 15 min at room temperature (RT) with PBS, 1% bovine serum albumin. The grids were then incubated with the primary antibodies against fibulin-1 or fibulin-2 diluted 1:150 with PBS for 1h at RT. After rinsing with PBS, the secondary gold-labelled goat-antirabbit immunoglobulin G (IgG) antibody (Medac, Hamburg, Germany), diluted 1:200 with PBS, was applied for 30 min at RT. The gold labelling of the antibody was described in detail in a previous investigation [36]. Reactions with antibodies against fibronectin and versican at a concentration of 1:200 were performed for 1 h, the

remaining part of the procedure being identical to that described above. These immunoreactions were also carried out after pretreatment of sections with 0.1% hyaluronate lyase (Sigma, St. Louis, USA) for 10 min at RT. Controls for nonspecific binding included incubations with uncoated colloidal gold probes alone and the replacement of primary antibodies by normal rabbit antiserum. Neither of these two controls showed any reaction.

Binding assay. High molecular weight hyaluronan (Sigma, St. Louis) was used to coat plastic wells, which were then blocked with 1% bovine serum albumin. Coated wells were incubated with various soluble ligands followed by antibody detection as described in previous protocols [13]. Soluble ligands were recombinant mouse fibulin-2 [13] and fibulin-1C [5], collagen VI purified from a pepsin digest of human placenta [37] and rat brain-derived neurocan [38], which was a kind gift of Dr. U. Rauch, Martinsried.

Results

Ultrastructural localization of fibulins, fibronectin and versican in embryonic mouse heart. On embryonic day 9, the mouse heart anlage consists of the endocardial tube and the developing myocardial layer separated from each other by the cell-free, homogeneous cardiac jelly (CJ), which also surrounds individual myocytes. Mesenchymal cells started to invade the CJ on day 10 and to form the endocardial cushion tissue (ECT). At this time the extracellular matrix had a homogeneous appearance with some granular structures, but no typical fibrils could be identified (fig. 1A). On day 11, the extracellular matrix had further diminished at the expense of an increasing cell number in the developing myocardium and within the ECT. These developmental stages were previously shown to be correlated with an abundant expression of fibulin-1 and fibulin-2 in the ECT, as demonstrated by light microscopy and in situ hybridization [20–22, 24–26].

In the present study, we have used immunogold staining for the ultrastructural localization of the fibulins in comparison to two of their potential protein ligands, fibronectin and versican. This was done for days 9, 10 and 11, with most of the data only illustrated for day 10 (fig. 1B–H). Fibulin-1 showed a modest random distribution in the CJ and the matrix of ECT, but could not be detected in the basement membranes of developing myocytes or within cells (fig. 1B). The developing epicardium and endocardium, as well as their basement membranes, showed no staining for fibulin-1. Labelling was stronger for fibulin-2, but was again restricted to the CJ and the matrix of ECT (fig. 1C). On day 11, fibulin-2 was mainly detected in areas where the future heart valves form and also in cytoplasmic vacuoles of ECT cells on the side facing the myocardium. Fibronectin showed the most widespread distribution, which, as for the fibulins, included the CJ and the matrix of ECT (fig. 1E), but also the cells and matrix of the developing endocardium and epicardium and their basement membranes and intracellular deposits in myocytes. The proteoglycan versican was also found in the CJ and the ECT matrix (fig. 1G).

Ultrastructural localization in the adult heart valve. The ECT is known to develop into heart valves and septa [28] which show a completely different ultrastructure compared with the primordial ECT. Adult heart valves consist of a duplication of the endocardium lined with endothelial cells, which sit on a distinct basement membrane. Underneath the basement membrane, numerous fibroblasts can be identified, embedded in a matrix rich in cross-striated collagen fibres and some microfibrillar structures (fig. 2A). In the latter region, the strongest immunogold staining was observed for fibulin-2 (fig. 2C). A distinct yet more modest staining could also be demonstrated for fibulin-1 (fig. 2B), fibronectin (fig. 2E) and versican (fig. 2G). The labelling was found either close to the collagen fibres or in more amorphous parts of the matrix that contained structures reminiscent of microfibrils (figs. 2B–E).

Involvement of hyaluronan in binding to the matrix. The amorphous appearance of the ECT matrix is apparently caused by large amounts of hyaluronan and sulphated glycosaminoglycans [31, 32, 39]. To examine the role of hyaluronan in this assembly, we selectively degraded it using a brief digestion with hyaluronate lyase. Subsequent immunogold labelling demonstrated a complete loss of staining for fibulin-1 (not shown), fibulin-2 (fig. 1D) and versican (fig. 1H) but not for fibronectin (fig. 1F). The same treatment of adult valve sections, however, revealed a different pattern. Here versican staining was almost completely lost after exposure to hyaluronate lyase (fig. 2H), whereas staining for fibronectin (fig. 2F), fibulin-2 (fig. 2D) and fibulin-1 was not diminished.

Versican has previously been shown to bind hyaluronan with high affinity [40], as do several related proteoglycans, which explains its loss after digestion. It has not previously been determined whether fibulins show a similar binding, and this was now analysed by a solid-phase assay with immobilized hyaluronan (fig. 3). Collagen VI and the proteoglycan neurocan were used as positive controls and showed a distinct binding above background with half-maximal binding achieved at 10 to 30-nM concentrations. Fibulin-1 and fibulin-2, however, did not bind to hyaluronan up to a concentration of 700 nM.

Discussion

The extracellular matrix seems to play a crucial role in the ECT of embryonic hearts and persists in a different

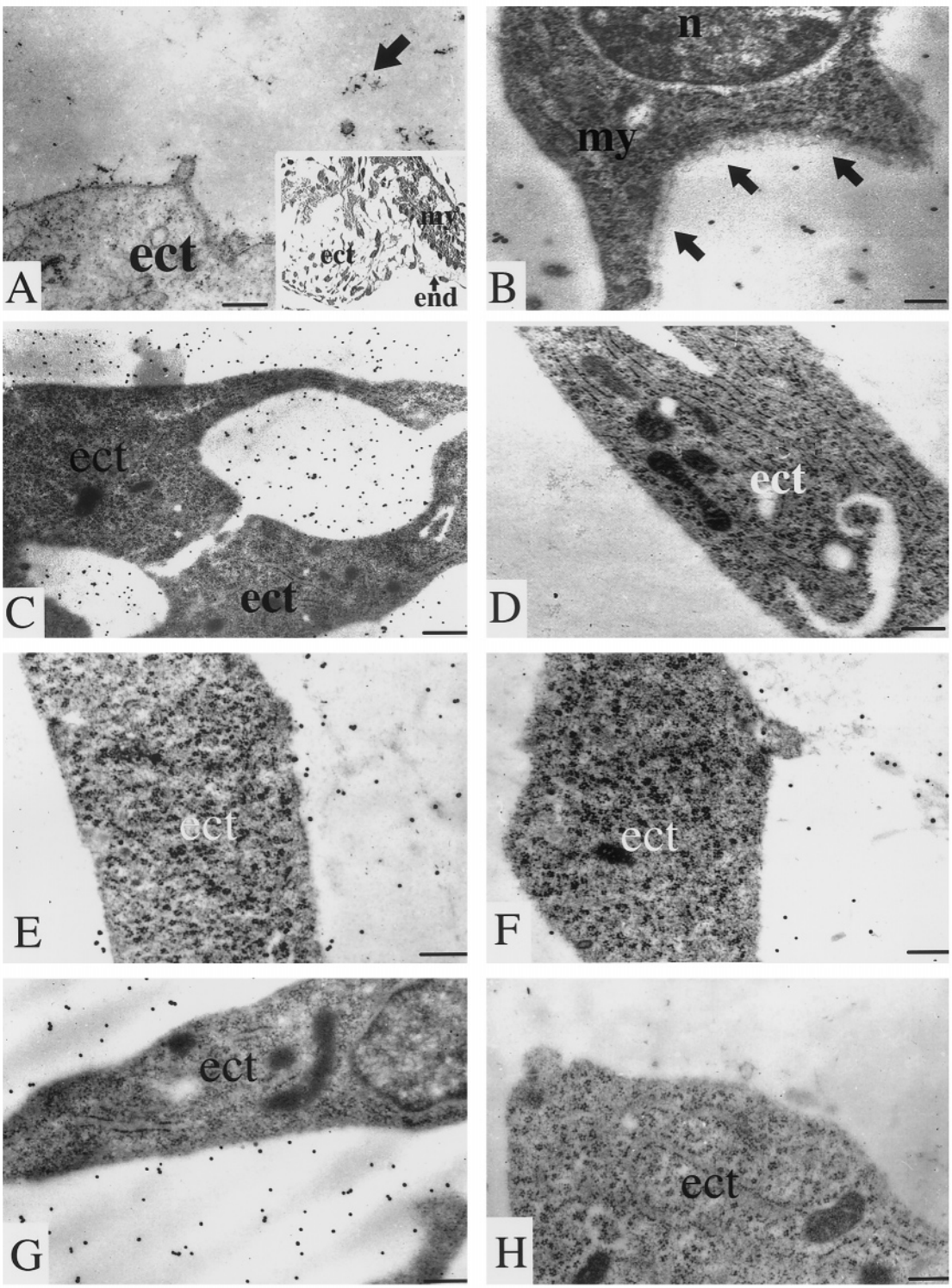

Figure 1. Immunogold labelling of fibulin-1, fibulin-2, fibronectin and versican in embryonic mouse heart (days 9–11). (*A*) Electron micrograph (day 10) of endocardial cushion tissue next to an endocardial cell (ect), showing a homogeneous appearance with sparse filigree structures (arrow); bar, 0.14 μm. The inset shows a lower magnification (bar, 65 μm) of the three basic cellular compartments: endocardial cushion tissue (ect), myocard (my) and endocard (end). (*B*) Staining for fibulin-1 (day 9) of the matrix surrounding a myocyte (my; n, nucleus). Arrows indicate the unstained basement membrane; bar, 0.19 μm. (*C*, *D*) Staining for fibulin-2 on day 10 is shown prior to (*C*) and after (*D*) treatment with hyaluronate lyase. Note the heavy labelling of the ECT matrix in (*C*) but not in (*D*), while the cells (ect) remain unstained; bar, 0.25 μm. (*E*, *F*) Staining of the ECT matrix for fibronectin on day 10 was similar prior to (*E*) and after (*F*) treatment with hyaluronate lyase, bar, 0.19 μm. (*G*, *H*) staining of the ECT matrix for versican on day 10 prior to (*G*) and after (*H*) treatment with hyaluronate lyase. Note the disappearance of labelling in (*H*) bar, 0.19 μm.

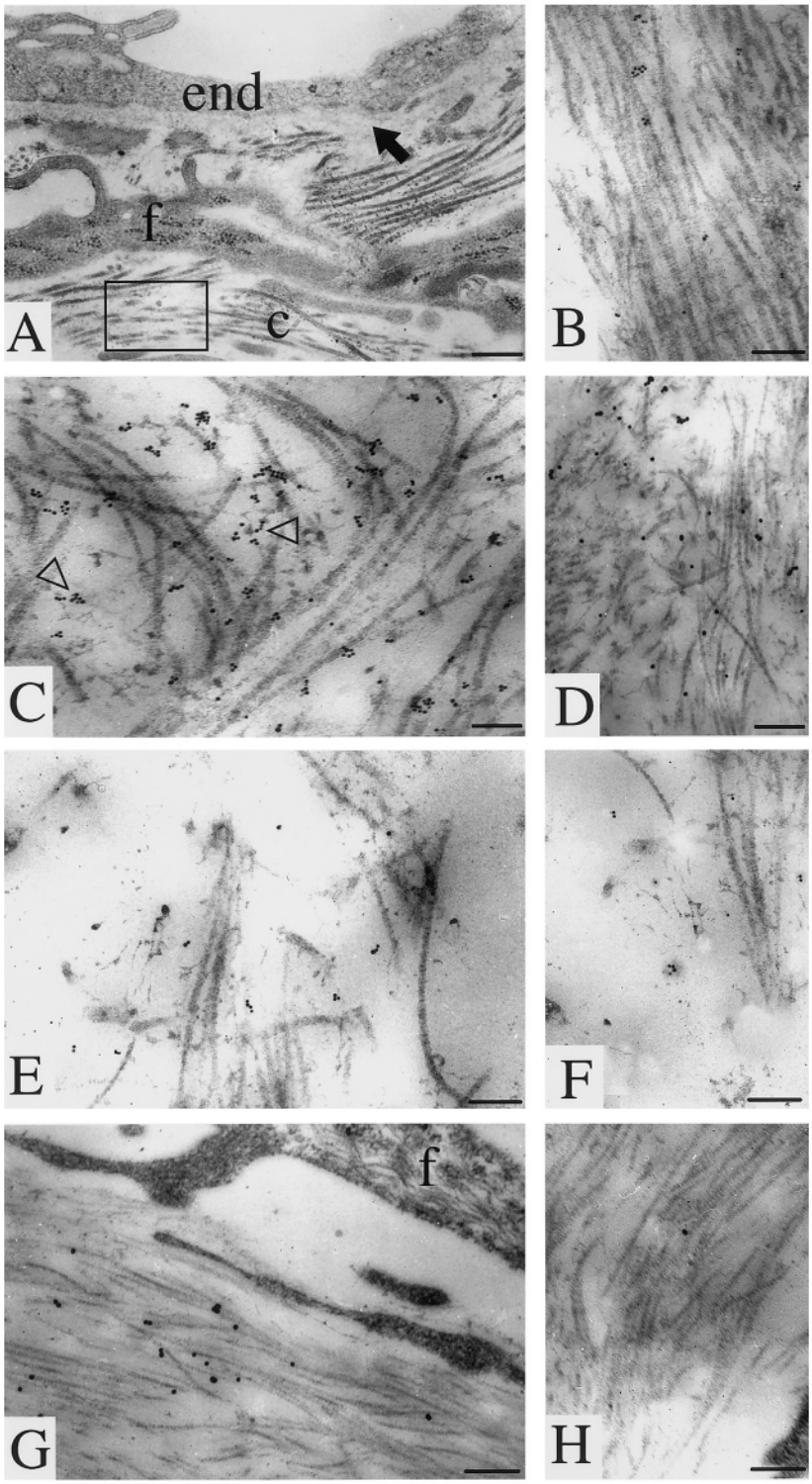

Figure 2. Immunogold labelling of fibulin-1, fibulin-2, fibronectin and versican in the adult (day 20) mouse heart. (*A*) Electron micrograph of a valve section showing an endothelial cell (end) on its basement membrane (arrow) lining the underlying extracellular matrix with fibroblasts (f) and abundant cross-striated collagen fibres (c). The frame denotes the area shown for immunogold staining. (*B*) Staining for fibulin-1; 24 bar, 0.14 μm. (*C*, *D*) Staining for fibulin-2 prior to (*C*) and after (*D*) treatment with hyaluronate lyase reveals no change in labelling close to collagen fibres and microfibrillar structures (arrowheads), bars, 0.21 μm. (*E*, *F*) staining of fibronectin showed only sparse labelling (*E*), mainly remote from collagen fibres, which resisted treatment with hyaluronate lyase (*F*); bars, 0.25 μm. (*G*, *H*) Staining of versican prior to (*G*) and after (*H*) treatment with hyaluronate lyase reveals almost complete disappearance of labelling from the collagen fibres, bars, 0.19 μm.

form in adult heart valves and septa [28]. Studies at the light microscopy level demonstrated a complex composition of this matrix, which contains hyaluronan, proteoglycans, collagens, fibronectin and several microfibrillar and basement membrane proteins [30]. Of particular interest was a high expression of fibulin-1 [20–22, 24, 25, 27] and fibulin-2 [21, 22, 26] in the ECT, persisting in adult heart valves. Besides being basement membrane proteins, both fibulins are also known to associate with fibronectin [17–19] and fibrillin [14] into microfibrillar structures, and fibulin-1 has also been detected within the core of elastin fibres [16]. This broad binding repertoire is reflected in the polymorphic shape of fibulin-2 [6], which enables it to participate in various forms of extracellular organization.

In the present study, we used the heart as a model to study the organization of fibulin-1 and fibulin-2 at the ultrastructural level. Immunogold staining demonstrated the presence of both proteins in the amorphous matrix of the ECT but not in various basement membranes. A similar localization was also found for versican and fibronectin, the latter also being present within basement membrane and cells. The ECT matrix showed only sparse filigree structures (fig. 1A), even after standard fixation and embedding in epon, and thus we were unable to draw any conclusions about the association of the fibulins with distinct supramolecular structures. All four proteins were also detectable in the adult heart valve, where they were mainly present in a fibroblast-rich extracellular matrix with abundant cross-striated collagen fibres. The fibulins were either in the close vicinity of these fibers or more remote, the latter being consistent with the possibility [17] that they are close to matrix microfibrils. This would suggest molecular interactions with fibronectin and/or fibrillin, as indicated from solid-phase assays with fibulin-1 and fibulin-2 [5, 8, 13, 14]. Similar assays demonstrated lack of binding to the fibril-forming collagens I,III and V [13] and would argue against a molecular association of the fibulins with the cross-striated collagen fibers of the valve matrix.

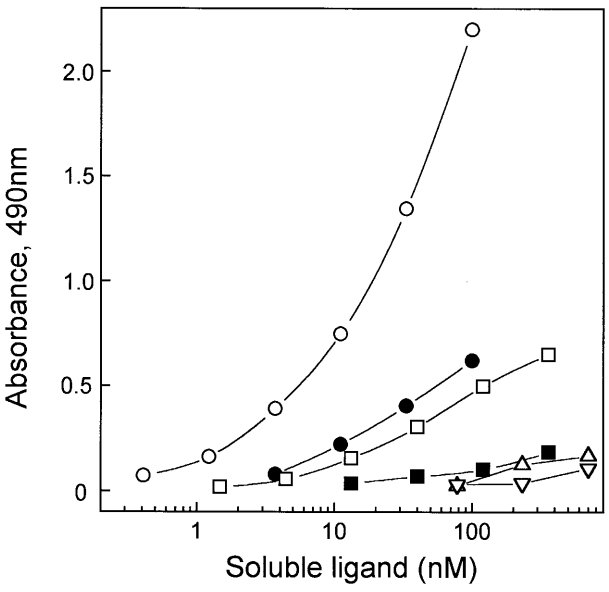

Figure 3. Lack of binding of fibulins to hyaluronan in a solid-phase assay. Hyaluronan was immobilized and incubated with fibulin-1C (▽), fibulin-2 (△), neurocan (□) or collagen VI (○) at the concentrations indicated. Binding was detected by specific antibodies in a colorimetric assay. Background binding to the blocking serum albumin is shown only for neurocan (■) and collagen VI (●).

Treatment of the amorphous ECT matrix with hyaluronate lyase caused a loss of versican, as expected, indicating its binding to hyaluronan through a high-affinity site located in the N-terminal domain [40]. The concomitant loss of both fibulins was, however, a surprising observation, since, as shown here, they lack binding activity for hyaluronan. Fibronectin in the ECT matrix was not released by digestion, which excludes its association with the fibulins. Recent data together with A. Aspberg and D. Heinegard (unpublished) demonstrated high-affinity binding of the C-terminal lectin domain of versican [41] to the rod-like elements of fibulin-1 and fibulin-2. We therefore postulate that the same complexes exist in the ECT and are held in place there through the association of the N-terminal domain of versican to hyluronan. The latter interaction apparently persists in the adult heart valve, as shown by hyaluronate lyase treatment. This digestion no longer released the fibulins, which, as discussed above, may have switched to a microfibrillar association during heart development.

Our ultrastructural analysis failed to demonstrate a basement membrane localization of the fibulins around myocytes and some other places of the embryonic heart, even though they are found in basement membrane zones of adult heart muscle [3]. This suggests a third form of molecular association, which could occur at later developmental stages and would need further examination. A transient appearance of fibulin-1 and fibulin-2 was recently shown for the basement membranes around seminiferous tubules during testis development [42]. This underscores the dynamic and versatile nature of the fibulins, whose biological functions now need to be analysed by genetic strategies.

Acknowledgements. Parts of this work are based on the doctoral thesis of N. Bunzel. The study was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 266, Mi 573/1-1) and by the National Institutes of Health (AR 38923, GM55625).

1 Argraves W. S., Tran H., Burgess W. H. and Dickerson K. (1990) Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structure. J. Cell Biol. **111:** 3155–3164
2 Pan T.-C., Kluge M., Zhang R.-Z., Mayer U., Timpl R. and Chu M.-L. (1993) Sequence of extracellular mouse protein BM-90/fibulin and its calcium-dependent binding to other basement membrane ligands. Eur. J. Biochem. **215:** 733–740
3 Pan T.-C., Sasaki T., Zhang R.-Z., Fässler R., Timpl R. and Chu M.-L. (1993) Structure and expression of fibulin-2, a novel extracellular matrix protein with multiple EGF-like repeats and consensus motifs for calcium-binding. J. Cell Biol. **123:** 1269–1277
4 Campbell I. D. and Bork P. (1993) Epidermal growth factor-like modules. Curr. Opin. Struct. Biol. **3:** 385–392
5 Sasaki T., Kostka G., Göhring W., Wiedemann H., Mann K., Chu M.-L. et al. (1995) Structural characterization of two variants of fibulin-1 that differ in nidogen affinity. J. Mol. Biol. **245:** 241–250
6 Sasaki T., Mann K., Wiedemann H. Göhring W., Lustig A., Engel J. et al. (1997) Dimer model for the microfibrillar protein fibulin-2 and identification of the connecting disulfide bridge. EMBO J. **16:** 3035–3043
7 Kluge M., Mann K., Dziadek M. and Timpl R. (1990) Characterization of a novel calcium-binding 90-kDa glycoprotein (BM-90) shared by basement membranes and serum. Eur. J. Biochem. **192:** 651–659
8 Balbona K., Tran H., Godyna S., Ingham K. C., Strickland D. K. and Argraves W. S. (1992) Fibulin binds to itself and to the carboxyl-terminal heparin-binding region of fibronectin. J. Biol. Chem. **267:** 20120–20125
9 Brown J. C., Wiedemann H. and Timpl R. (1994) Protein binding and cell adhesion properties of two laminin isoforms (AmB1e2e, AmB1sB2e) from human placenta. J. Cell Sci. **107:** 329–338
10 Tran H., Tanaka A., Litvinovich S. V., Medved L. V., Haudenschild C. C. and Argraves W. S. (1995) The interaction of fibulin-1 with fibrinogen. A potential role in hemostasis and thrombosis. J. Biol. Chem. **270:** 19458–19464
11 Adam S., Göhring W., Wiedemann H., Chu M.-L., Timpl R. and Kostka G. (1997) Binding of fibulin-1 to nidogen depends on its C-terminal globular domain and a specific array of calcium-binding epidermal growth factor-like (EG) modules. J. Mol. Biol. **272:** 226–236
12 Tran H., Van Dusen W. J. and Argraves W. S. (1997) The self-association and fibronectin-binding sites of fibulin-1 map to calcium-binding epidermal growth factor-like domains. J. Biol. Chem. **272:** 22600–22606
13 Sasaki T., Göhring W., Pan T.-C., Chu M.-L. and Timpl R. (1995) Binding of mouse and human fibulin-2 to extracellular matrix ligands. J. Mol. Biol. **254:** 892–899
14 Reinhardt D. P., Sasaki T., Dzamba B. J., Keene D. R., Chu M.-L., Göhring W. et al. (1996) Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues. J. Biol. Chem. **271:** 19489–19496
15 Utani A., Nomizu M. and Yamada Y. (1997) Fibulin-2 binds to the short arms of laminin-5 and laminin-1 via conserved amino acid sequences. J. Biol. Chem. **272:** 2814–2820
16 Roark E. F., Keene D. R., Haudenschild C. C., Godyna S., Little C. D. and Argraves W. S. (1995) The association of human fibulin-1 with elastic fibers: an immunohistological, ultrastructural and RNA study. J. Histochem. Cytochem. **43:** 401–411
17 Sasaki T., Wiedemann H., Matzner M., Chu M.-L. and Timpl R. (1996) Expression of fibulin-2 by fibroblasts and deposition with fibronectin into a fibrillar matrix. J. Cell Sci. **109:** 2895–2904
18 Roman J. and McDonald J. A. (1993) Fibulin's organization into the extracellular matrix of fetal lung fibroblasts is dependent on fibronectin matrix assembly. Am. J. Respir. Cell Mol. Biol. **8:** 538–545
19 Godyna S., Mann D. M. and Argraves W. S. (1994) A quantitative analysis of the incorporation of fibulin-1 into the extracellular matrix indicates that fibronectin assembly is required. Matrix Biol. **14:** 467–477
20 Spence S. G., Argraves W. S., Walters L., Hungerford J. E. and Little C. D. (1992) Fibulin is localized at sites of epithelial-mesenchymal transitions in the early avian embryo. Dev. Biol. **151:** 473–484
21 Zhang H.-Y., Timpl R., Sasaki T., Chu M.-L. and Ekblom P. (1996) Fibulin-1 and fibulin-2 expression during organogenesis in the developing mouse embryo. Dev. Dynam. **205:** 348–364
22 Miosge N., Götz W., Sasaki T., Chu M.-L., Timpl R. and Herken R. (1996) The extracellular matrix proteins fibulin-1 and fibulin-2 in the early human embryo. Histochem. J. **28:** 109–116
23 Fässler R., Sasaki T., Timpl R., Chu M.-L. and Werner S. (1996) Differential regulation of fibulin, tenascin and nidogen expression during wound healing of normal and glucocorticoid-treated mice. Exp. Cell Res. **222:** 111–116
24 Bouchey D., Argraves W. S. and Little C. D. (1996) Fibulin-1, vitronectin and fibronectin expression during avian cardiac valve and septa development. Anat. Rec. **244:** 540–551
25 Zhang H.-Y., Kluge M., Timpl R., Chu M.-L. and Ekblom P. (1993) The extracellular matrix glycoproteins BM-90 and tenascin are expressed in the mesenchyme at sites of endothelial-mesenchymal conversion in the embryonic mouse heart. Differentiation **52:** 211–220
26 Zhang H.-Y., Chu M.-L., Pan T.-C., Sasaki T., Timpl R. and Ekblom P. (1995) Extracellular matrix protein fibulin-2 is expressed in the embryonic endocardial cushion tissue and is a prominent component of valves in adult heart. Dev. Biol. **167:** 18–26
27 Zhang H.-Y., Lardelli M. and Ekblom P. (1997) Sequence of zebrafish fibulin-1 and its expression in developing heart and other embryonic organs. Dev. Genes Evol. **207:** 340–351
28 Eisenberg L. M. and Markwald R. R. (1995) Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ. Res. **77:** 1–6
29 Little C. D., Piquet D. M., Davis L. A., Walters L. and Drake C. J. (1989) Distribution of laminin, collagen type IV, collagen type I and fibronectin in chicken cardiac jelly/basement membrane. Anat. Rec. **224:** 417–425
30 Little C. D. and Rongish B. J. (1995) The extracellular matrix during heart development. Experientia **51:** 873–882
31 Markwald R. R. and Smith W. N. A. (1972) Distribution of mucosubstances in the developing rat heart. J. Histochem. Cytochem. **20:** 896–907
32 Manasek F. J., Reid M., Vinson W., Seyer J. and Johnson R. (1973) Glycosaminoglycan synthesis by the early embryonic chick. Dev. Biol. **35:** 332–348
33 Herken R. and Barrach H. J. (1985) Ultrastructural localization of type IV collagen and laminin in the seven-day-old mouse embryo. Anat. Embryol. **171:** 365–371
34 Karnovsky M. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Acta Histochem. **88:** 25–27
35 Zimmermann D. R., Dours-Zimmermann M. T., Schubert M. and Bruckner-Tuderman L. (1994) Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. J. Cell Biol. **124:** 817–825
36 Miosge N., Günther E., Heyder E., Manshausen B. and Herken R. (1995) Light and electron microscopic localization of the α1-chain and E1 and E8 domains of laminin-1 in mouse kidney using monoclonal antibodies to establish the orientation of laminin-1 within basement membranes. J. Histochem. Cytochem. **43:** 675–680
37 Odermatt E., Risteli J., van Delden V. and Timpl R. (1983) Structural diversity and domain composition of a unique

collagenous fragment (intima collagen) obtained from human placenta. Biochem. J. **211:** 295–302
38 Rauch U., Gao P., Janetzko A., Flaccus A., Hilgenberg L., Tekotte H. et al. (1991) Isolation and characterization of developmentally regulated chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of brain identified with monoclonal antibodies. J. Biol. Chem. **266:** 14785–14801
39 Markwald R. R., Fitzharris T. P., Bank H. and Bernanke D. H. (1978) Structural analysis on the matrical organization of glycosaminoglycans in developing endocardial cushions. Dev. Biol. **62:** 292–316
40 LeBaron R. G., Zimmermann D. R. and Ruoslahti E. (1992) Hyaluronate binding properties of versican. J. Biol. Chem. **267:** 10003–10010
41 Zimmermann D. R. and Ruoslahti E. (1989) Multiple domains of the large fibroblast proteoglycan, versican. EMBO J. **8:** 2975–2981
42 Loveland K., Schlatt S., Sasaki T., Chu M.-L., Timpl R. and Dziadek M. (1998) Developmental changes in the basement membrane of the normal and hypothyroid postnatal rat testis: segmental localization of fibulin-2 and fibronectin. Biol. Reprod., in press