Fibrillin-1 and -2 contain heparin-binding sites important for matrix deposition and that support cell attachment

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Fibrillin-1 and -2 are large modular extracellular matrix glycoproteins found in many vertebrate organ systems and are known to be key components of the elastic fibre. In the present study, we identify a new heparin-binding region in fibrillin-2 between exons 18 and 24. Additionally, we have narrowed the location of heparin-binding activity previously identified in fibrillin-1 to the last 17 residues of the mature proteolytically processed protein. This domain demonstrated higher activity as a multimer than as a monomer. The fibrillin-1 C-terminal site supported cell attachment in each of nine cell types tested. Attachment was shown to be mediated by cell-surface heparan sulphate proteoglycans. Fibrillin-1 has been shown previously to have heparin-binding activity that is important for matrix deposition of the molecule by fibroblasts. This function in deposition was confirmed in two additional fibrillin-producing cell types (osteosarcoma and epithelial cells) for the deposition of both fibrillin-1 and -2 into the extracellular matrix.

Key words: extracellular matrix, fibrillin, heparin/heparan sulphate binding.

INTRODUCTION

Fibrillin occurs in early multicellular organisms, such as jellyfish, and although highly conserved throughout evolution, two versions are known to exist in chordata. Its retention through time may have allowed it to develop the multiple roles it is thought to support in more recent organisms. The fibrillin protein is expressed as early as pre-gastrulation stage in a developing chick embryo, is extensively expressed in subsequent midline structures [1,2], and eventually plays a role in several organ systems throughout the mammalian body. It is likely that fibrillin has many functions that vary according to the developmental stage and tissue in which it is expressed. These functions may range from an adhesive substrate for migrating cells in a developing embryo, to providing part of the matrix-based signals for induction of differentiation or to a structural template for the deposition of elastin in the lung and blood vessels. Despite fibrillin’s apparent range of molecular function, little is known about how cells interact with the molecule during and after its deposition into the extracellular matrix (ECM).

Along with integrin–ligand interactions, another major mode of cell–matrix interaction employs cell-surface HSPGs (heparan sulphated proteoglycans), such as members of the syndecan or glycan families. Syndecans are found on the surface of almost all adherent cell types and are known to bind a variety of extracellular ligands, including ECM molecules, such as fibronectin, laminins, interstitial collagens, tenasin, thrombospondin and vitronectin. Cell-surface HSPG are vital to a cell’s perception of its environment, as syndecans are able to induce cytoskeletal reorganization in response to extracellular ligands (see [6,7] for reviews). The presence of heparin/HS binding sites in matrix molecules provides an important mechanistic alternative to integrin-mediated cellular interactions with the ECM.

Information about the functional roles of heparin-binding sites in ECM proteins is rapidly emerging and it is already clear that they play important roles in cell migration, cell adhesion and signalling, and protein–protein interactions. The present study identifies a heparin/HS-binding site within the C-terminal domain of fibrillin-1 and a region of heparin/HS-binding activity in fibrillin-2. In epithelial and osteosarcoma cells, soluble heparin competitively inhibits an unknown interaction that is needed for ECM deposition of both fibrillin-1 and -2. The heparin-binding site in the C-terminal domain of fibrillin-1 can support cell attachment through a cell-surface HS-mediated interaction. Thus cell attachment to fibrillins can potentially produce simultaneous signalling from members of two ECM receptor families: integrins and cell-surface HSPGs.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co., unless stated otherwise. Purified collagen was purchased from Collagen Corp., Palo Alto, CA, U.S.A.

Abbreviations used: CHO, Chinese-hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; HS, heparan sulphate; HSPG, heparan sulphated proteoglycan; ITS, insulin-transferrin-selenium; LTBP, latent transforming-growth-factor-β-binding protein; MAGP, microfibril-associated glycoprotein; PE, pigmented epithelium.

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Cell culture and cell types
Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) bovine calf serum (HyClone, Logan, UT, U.S.A.) was used for the growth and maintenance of all cell lines. The following cell lines were used: CHO-K1, Chinese-hamster ovary; COS7, green monkey kidney; FBC, fetal bovine calf auricular chondrocytes; FCL, fetal bovine calf nuchal ligament; HEK-293, human embryonal kidney; HSF, human skin fibroblasts; Jurkat, human lymphoma; MG63, human osteosarcoma; NIH 3T3, Swiss mouse embryo; PE, bovine pigmented epithelium. FBC, FCL and HSF were from primary cultures established as described previously [3]. PE cells were a gift from Dr Martin Wax, Department of Ophthalmology, Washington University, St. Louis, MO, U.S.A. All other cells were obtained from American Type Culture Collection (Rockville, MA, U.S.A.).

Peptides and recombinantly expressed proteins
Bacterially expressed proteins
The pQE series of vectors (Qiagen, Valencia, CA, U.S.A.) were used for production for the following domains of human fibrillins 1 and 2 with N-terminal His6-tags. The additional amino acids shown encoded by vector sequence are indicated (using one-letter symbols) for each clone. Fibrillin-1 C-terminal domain (FBN1-CT): MRGSHHHHHHHT plus fibrillin-1 Asp-1493 to His-2871 terminus. Fibrillin-1 C-terminal-pro region (FBN1-CT PRO): MRGSHHHHHHHTDL plus fibrillin-1 Glu-2735 to His-2871 terminus. In full-length wild-type fibrillin, this region is lost to a proteolytic processing post-translational modification and is not present in the mature molecule. Fibrillin-1 exons 36–44 (FBN1 × 36–44): MRGSHHHHHHHT plus fibrillin-1 Asp-1493 to Cys-1824 plus SQA. Fibrillin-2 exon 24 (FBN2 × 24) is an LTBP (latent transforming-growth-factor-β-binding protein)-like 8-Cys RGD-containing domain: MRGSHHHHHHGSACELTPGRAAASVMDCIRPCL plus fibrillin-2 Asp-996 to Lys-1071 plus LARANSSTLAAVTSGSGLTKLN. Fibrillin-2 exons 37/38 (FBN2 × 37/38) is an LTBP-like 8-Cys RGD-containing domain: MRGSHHHHHHGSACELTPGRAAASVMDCIRPCL plus fibrillin-2 Asp-1572 to Glu-1648 plus LARANSSTLAAVTSGSGLTKLN. The proteins were produced and purified on Ni2+-resin columns as described previously [8] and the amino acid sequence was confirmed by N-terminal sequencing. The folding of the bacterially expressed products was not evaluated. To determine whether the three arginine residues C-terminal to the His6-tag in FBN1-CT contribute to heparin binding, purified protein was treated with CNBr overnight, which, by cleaving at methionine residues, removes the His6-tag, the arginine-containing linker region and the first 17 and last 7 amino acids of the fibrillin portion of the peptide. The putative heparin-binding sequence within FBN1-CT remains intact. The protein mixture was then analysed by heparin chromatography as described below.

Synthetic peptides
Peptides were synthesized using an ABI 431A protein synthesizer using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and purified by FPLC using Superdex 30 in an XK16 column (Pharmacia, Uppsala, Sweden) in 25 mM NH4CO3 buffer at a flow rate of 2.0 ml/min. MS was performed to confirm correct synthesis. The peptide sequences were as follows: RGD (GRGDSP); RGE (GRGESP) is the inactive analogue of RGD; FIB15D (YLDIRPAGDNGDTAC) is the inactive analogue of FIB15D; CT-29 (CVSGMGMGGRPEPPVSGEMDDNLSPEA) corresponds to amino acids 2686–2714 of human fibrillin-1; CT-17 (CYECKINGPKR) corresponds to amino acids 2715–2731 of human fibrillin-1. Multimeric CT-17 was produced by incubation under oxidizing conditions and checked by HPLC.

CHO cell and baculovirally expressed proteins
FBN1 exons 23–44 was produced as described previously [8]. FBN2 exons 1–25, exons 10–17 and exons 18–25 were produced as described previously [9].

Heparin affinity chromatography
HiTrap heparin (Pharmacia) columns were used in an FPLC system to assess the heparin-binding ability of several regions of fibrillin-1 and -2. Samples were loaded on to the column in low-salt conditions and then washed until the flow-through absorbance at 280 nm returned to baseline. Elution was carried out in a stepwise gradient from 25 mM to 750 mM NaCl in 25 mM Tris, pH 7.2. Conductivity was continuously monitored to determine the salt concentration of each fraction. After precipitation with acetone to concentrate and desalt, one quarter of each fraction was assessed by Western blot using standard techniques.

Immunofluorescence of fibrillin assembly in heparin co-cultures
PE and MG63 cells were plated at 1.0 × 10⁶ cells per 35-mm dish and cultured at confluence on glass coverslips for 6 days in the presence of 25 µg/ml (PE) or 100 µg/ml (MG63) soluble heparin (porcine intestinal mucosa; Sigma Chemical Co.). The culture medium was changed every 24 h. Cells were fixed with 2.5% paraformaldehyde in PBS, quenched, and then stained with rabbit polyclonal antibodies FBN1-CT, FBN2 exons 1–24, MAGP (microfibril-associated glycoprotein) or fibronectin, at a dilution of 1:500. Secondary antibody was goat anti-rabbit Alexa Fluor® 594 or 488 conjugate (Molecular Probes, Eugene, OR, U.S.A.) at 1:1500. Photography was performed using a Zeiss Axioshot microscope with an AxioCam 3CCD digital camera.

Radiolabelling of newly synthesized fibrillin
PE cells were cultured as above for 5 days in the presence of 25 µg/ml heparin and the medium was changed daily. On the fifth day, regular culture medium with or without heparin was replaced with 500 µl of a 1:9 mixture of regular DMEM with serum and methionine-free medium containing 125 µCi [15S]methionine and heparin. Control wells were treated identically except no heparin was added. Conditioned medium was harvested after 24 h and evaluated by SDS/5% PAGE and autoradiography.

Relative attachment of nine cell types on seven substrates
All assays that involve cell attachment to protein-coated plastic followed the general protocol described below and any modifications are noted in subsequent sections. Non-adherent polystyrene suspension culture 24-well (1 cm diameter) plates (Costar, VWR, USA) were coated overnight with 100 µg/ml (PE) or 1000 µg/ml (MG63) of the following substrates: collagen I, type I, porcine tail, 500 µg/ml; fibronectin, 10 µg/ml; Type V fibronectin, 5 µg/ml; vitronectin, 10 µg/ml; laminin, 10 µg/ml; collagen type IV, 50 µg/ml; Fibrillin-1 multimeric CT-17, 25 µg/ml; and heparin, 10 µg/ml (Sigma Chemicals Co.). After washing, the plates were incubated for 2 h at 37°C in 5% CO₂/95% air. After washing again, cells were detached with MACS buffer and resuspended in 0.05% trypsin-EDTA in MEM. Cells were counted and 1.5 × 10⁵ cells were plated on each well and cultured for 24 h.

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Cambridge, MA, U.S.A.) were coated overnight at 4 °C with 200 μl of the indicated proteins at saturating concentrations (25 μg/ml F2 × 24, F2 × 37/38; F1CT; 100 μg/ml F15D, F1CT-Pro). Before use, unbound protein was removed and PBS containing 1 mg/ml BSA was added to the wells at room temperature for 1 h to block non-specific binding. Control wells with no substrate were blocked with BSA in all runs.

Cells were washed with PBS and incubated with 0.02 % EDTA at 37 °C until detachment, after which the cells were vortexed to insure a suspension of single cells. Detached cells were washed twice with 15 ml of DMEM without serum and with 1 mg/ml BSA using 4 °C centrifugation at 800 g. Cells were resuspended at 1 × 10^6 cells/ml in DMEM containing 1 mg/ml BSA, and 200 μl (2 × 10^5 cells) was dispensed into each well. The cells were allowed to attach for 45 min at 37 °C in a CO₂ incubator. After the attachment period, the wells were washed three times with PBS containing 1 mg/ml BSA and attached cells were quantified by a hexosaminidase assay [10]. Each experimental condition was performed in triplicate and an average and standard deviation was calculated.

**Inhibition of cell attachment to ECM protein substrates**

MG63 or PE cell suspensions were prepared as described above. Cells were pre-incubated with the RGD, RGE, FIB15D or RtoA peptides at 250 μM for 10 min at 37 °C and then plated into 24-well dishes coated with the indicated proteins as described above. EDTA (10 mM) was added to the indicated cells just before plating and was present during attachment, but was not present in the cultures containing soluble peptides. Cells were allowed to attach for 45 min before quantification.

**Soluble heparin inhibition of adhesion**

Cells were prepared for plating as described above. Polystyrene 24-well plates were coated with FBN1-CT (25 μg/ml) and blocked as described above, except that the indicated concentrations of soluble heparin from porcine intestinal mucosa were included in the blocking solutions for a minimum of 1 h at room temperature. Cells were plated in the presence of the indicated concentrations of heparin and allowed to attach at 37 °C for 45 min before quantification.

**Inhibition of adhesion through heparitinase II digestion**

COS7 cells were incubated for 2 h with DMEM containing 1 mg/ml BSA and 25 μg/ml cycloheximide, which was also included in all subsequent steps. One dish was incubated with 0.1 units/ml heparitinase II (Seikagaku, Tokyo, Japan) and another dish with 0.2 units/ml chondroitinase ABC for 1 h at 37 °C just prior to detachment. Control cells received no enzymic treatment. All cells were then detached with 0.02 % EDTA and washed twice in the DMEM/BSA/cycloheximide mixture. Cells were plated in 24-well dishes coated with 25 μg/ml FBN1-CT, allowed to attach, and quantified as before.

**RESULTS AND DISCUSSION**

**Heparin/HS-binding activity of fibrillin-1 and -2 sequences**

Heparin-binding activity has been previously described in full-length [3] and large fragments of fibrillin-1 [11]. To further localize fibrillin-1 heparin-binding properties and to assess the heparin/HS-binding activity of fibrillin-2, full-length fibrillin, recombinant proteins and synthetic peptides were evaluated by heparin-Sepharose affinity chromatography (Figure 1, Table 1). Full-length fibrillin-1 from MG63 cell-conditioned medium bound to the column and eluted between 259 mM and 341 mM NaCl. Fibrillin-1 binds heparin with high affinity, as demonstrated by the high concentrations of salt required for elution and by the comparative elution of fibronectin at only equal or lower salt fractions as determined by Western blot (fibronectin results not shown). Indeed, heparin affinity chromatography has been employed previously as a fibrillin purification step, although the site of interaction was unknown [3]. Some samples eluted at, or just above, physiological salt conditions (e.g. FBN1 exons 10–25). It is likely that these were retained on the heparin column by low-affinity non-specific charge interactions that are not physiologically relevant. Two regions of fibrillin-1 and one region of fibrillin-2 bound and eluted only under high salt conditions, indicating a specific high-affinity interaction within these regions. Two fibrillin-2 fragments, exons 1–25 and exons 18–25, exhibited heparin-binding activity. As this binding was not seen for exons 10–17, activity in the N-terminal region appears to be localized to exons 18–25, although additional conditions were not used.

![Figure 1 Map of recombinant proteins](image)

This representation of the protein domain organization of fibrillin-1 and -2 shows the exons expressed as recombinant proteins. These regions were evaluated for heparin-binding affinity by chromatography. CT-29 and CT-17 are synthetic peptides. TB domain, transforming-growth-factor-β-binding-domain; EGF, epidermal growth factor.
activity is also possible in the first ten exons of fibrillin-2. Heparin-binding activity was reported for the first nine exons of fibrillin-1; however, fibrillin-1 exons 18–25 were inactive [11].

The heparin-binding activity observed in fibrillin-1 exons 23–44 may be the same site described previously in recombinant fragment ‘rF18’ [11], as both fragments span similar regions of the molecule. Similar to ‘rF18’, exons 23–44 displayed greatly reduced heparin-binding activity if heparin affinity chromatography was performed in the presence of 10 mM EDTA (results not shown). Fibrillin-1 fragment exon 36–44 did not bind, either because the activity is not within this region or because this bacterially produced protein may not be in a native conformation, and as such is unable to bind heparin.

The FBN1-CT recombinant protein demonstrated heparin-binding activity, whereas the FBN1-CT-Pro region did not. Further, the CT-17 peptide bound to the heparin column, whereas the CT-29 peptide did not. These data correspond to the ability of these recombinant proteins and synthetic peptides to support cell adhesion (discussed below). These data localize the heparin/HS-binding site of the fibrillin-1 C-terminal domain to residues Cys-2715–Arg-2731. It should be noted that FBN1-CT has three arginine residues in the linker region C-terminal to the His6- tag that could potentially contribute to heparin binding by this fragment. Removal of a small peptide containing these arginine residues by CNBr cleavage, however, had only a slight affect on the ability of FBN1-CT to heparin.

The C-terminal domain of fibrillin-1 is proteolytically processed as a post-translational modification during the normal maturation of the molecule and before deposition into the matrix [8,12]. Processing results in the removal of the ‘PRO region’, or the last 138 residues of the original molecule. The site of proteolytic processing is a multi-basic furin recognition site and cleavage is thought to occur between Arg-2731 and Ser-2732. The sequence of the CT-17 peptide contains this poly-basic furin recognition sequence and after proteolysis becomes the last 17 residues of the mature fibrillin molecule. It is likely that the basic residues of this sequence mediate heparin/HS binding as the spacing closely matches the consensus derived from other known linear binding sites [13,14]. It is interesting to note that the 17 residue sequence is 100% conserved across mouse, guinea pig, rat, porcine, bovine and human fibrillin-1.

Multimerization increases the heparin-binding affinity of the fibrillin-1 C-terminal site

Fibrillin molecules are incorporated into the ECM as multimers. The net effect of multimerization on the heparin-binding capability of the fibrillin-1 C-terminal heparin-binding site was unknown. To examine this question, monomeric and multimeric CT-17 peptides were evaluated for heparin-binding affinity by FPLC chromatography (Figure 2). Of the monomeric CT-17, 57% bound to the column and of that, 48.6% eluted between physiological salt and 201 mM NaCl and 14.7% eluted above 201 mM NaCl. In contrast, 81.1% of multimeric CT-17 bound the column, and of that, 54.4% eluted above 201 mM NaCl. These data indicate that the fibrillin-1 C-terminal heparin-binding domain has greater affinity as a multimer. A class of lectins, the collectins, are known to increase their carbohydrate affinity through oligomerization (see [15] for review). It is widely accepted that fibrillins are incorporated into the ECM as multimers. As such, fibrillin microfibrils may provide a higher affinity cell–protein interaction than a fibrillin monomer. These

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Table 1

<table>
<thead>
<tr>
<th>Region</th>
<th>Elution range (mM NaCl)</th>
<th>Approximate elution peak (mM NaCl)</th>
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<tr>
<td>Fibrillin 1</td>
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<td></td>
</tr>
<tr>
<td>Full length</td>
<td>184–260</td>
<td>230</td>
</tr>
<tr>
<td>Exons 10–24</td>
<td>140–231</td>
<td>160</td>
</tr>
<tr>
<td>Exons 23–44</td>
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<td>380</td>
</tr>
<tr>
<td>Exons 36–44</td>
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<td></td>
</tr>
<tr>
<td>C-terminal domain</td>
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<td>264</td>
</tr>
<tr>
<td>(full-length)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-terminal (Pro region)</td>
<td>Did not bind</td>
<td></td>
</tr>
<tr>
<td>CT-29 peptide</td>
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</tr>
<tr>
<td>CT-17 peptide monomer</td>
<td>Peak #1 = 104 mM; peak #2 = 187 mM</td>
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<tr>
<td>CT-17 peptide multimer</td>
<td>Wide range, see Figure 2</td>
<td></td>
</tr>
<tr>
<td>Fibrillin 2</td>
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<tr>
<td>Exons 1–25</td>
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<td>279</td>
</tr>
<tr>
<td>Exons 10–17</td>
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<td>Exons 18–25</td>
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<td>329</td>
</tr>
<tr>
<td>Exon 24</td>
<td>Did not bind</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2

Fibrillin-1 C-terminal heparin/HS binding site has higher affinity as a multimer

The heparin-binding affinity of the CT-17 synthetic peptide was evaluated by heparin chromatography. Chromatograms in (A) and (B) show the elution profiles of equal quantities of monomeric and multimeric heparin/HS binding domains respectively. The percentage of peptide that did not bind the column is shown below the x-axis on the left-hand side. The percentage that eluted between 25 and 200 mM salt is shown in the middle, and the percentage that eluted between 200 mM and 500 mM salt is shown on the right-hand side. Absorbance at 280 nm is shown along the y-axis.
higher-affinity carbohydrate interactions could cluster syndecan receptors along with signalling integrins. This has long been known to be important for cytoskeletal re-organization and stress fibre formation after cell attachment to fibronectin [16], (see [17,18] for reviews). In this way, the secretory pathways of elastogenic cells could be oriented to a developing elastic fibre.

**Soluble heparin competitively disrupts ECM deposition of fibrillin-1 and -2 in epithelial and osteosarcoma lines**

The human osteosarcoma line MG63 produces fibrillin-1 and pigmented epithelial (PE) cells from the eye produce fibrillin-1 and -2. Both lines deposit the fibrillins into the ECM *in vitro*. To determine the physiological importance of the heparin-binding activity of these proteins, the cells were cultured in the presence of soluble heparin and ECM incorporation of fibrillin was assessed by immunocytological staining. Culture in the presence of heparin did not significantly affect cell proliferation or fibrillin production (Figure 4). Co-culture with heparin resulted in an inhibition of ECM deposition of both fibrillin-1 and 2 (Figure 3). In the same cultures, fibronectin deposition was unaffected (MG63) or only slightly inhibited (PE) by the presence of heparin in the medium. A similar disruption of ECM deposition of fibrillin-1 has been reported in fibroblasts [11].

As a control for fibrillin epitope masking by heparin, cultures were stained for MAGP-1, which associates with the fibrillins in the ECM. Under these conditions, an inhibition of MAGP-1 ECM deposition was also observed, presumably due to the lack of fibrillin deposition. This suggests that, of the ECM proteins deposited by MG63 and PE cells, fibrillin is the sole molecule with which MAGP-1 can associate. To determine if non-specific charge interactions were responsible for the effect, the cells were cultured with chondroitin sulphate A (chondroitin 4-sulphate), B (dermatan sulphate) or C (chondroitin 6-sulphate) and no effect was observed (Figure 4A). This indicates that the mechanism of the disruption of fibrillin ECM deposition is specific for heparin sequences.

Previously, our group has shown that the microfibrillar proteins MAGP-1 and fibrillin-1 form a ternary complex with the chondroitin sulphate proteoglycan decorin [19]. In that work, the interaction between MAGP-1 and fibrillin-1 did not appear to be mediated by decorin-associated chondroitin sulphate carbohydrate. In the present work, the use of chondroitin sulphates A, B and C in culture medium would compete carbohydrate-mediated interactions specific for chondroitin sulphates, yet no disruption of either fibrillin deposition or MAGP-fibrillin association was observed. These data confirm and extend our previous work.

When a recombinant construct containing the fibrillin-1 C-terminal domain is expressed in CHO-K1 cells, it is proteolytically processed intracellularly in the early Golgi, probably by a furin family member [8]. If the full-length fibrillin molecule is similarly processed, the intracellular removal of the pro region of the C-terminal domain would allow immediate interaction and retention on the cell surface upon secretion. The C-terminal heparin-binding sequence could play a similar function if the pro region is processed extracellularly. Cell-surface HSPG may bind fibrillin to facilitate subsequent interaction with integrin receptors. Syndecan-2 has been implicated as a co-receptor with integrins in the deposition of fibronectin and laminin [20]. Similarly, the fibrillin-1 heparin/HS-binding site may also be employed during cell-surface-mediated matrix deposition. Indeed, a human fibrillin-1 mutation (Arg2726 → Trp), which prevents proteolytic processing of the C-terminal domain, has been reported to result in impaired deposition of the product of the affected allele [21].

**Figure 3** Immunofluorescent imaging of cells cultured in the presence or absence of heparin

PE or MG63 cells were seeded near confluence on glass coverslips, and 12 h after attachment, the cells were changed into medium containing 20 μg/ml (PE cells) or 100 μg/ml heparin (MG63 cells). The medium was changed daily for 6 days until the culture was harvested. Cells were fixed with paraformaldehyde and were stained with antibodies against human fibrillin-1 C-terminal domain, human MAGP or human fibronectin (FN) as indicated in the figures. Culturing with soluble heparin inhibited fibrillin deposition in both cell types, while fibronectin deposition was unaffected. FBN1, fibrillin-1; FBN2, fibrillin-2.

**FBN1 C-terminal heparin-binding site supports cell attachment**

The ability of cells to bind to the C-terminal domain of fibrillin-1 was investigated using the domain as a solid substrate in cell-adhesion assays (Table 2). Nine cell lines from five species were tested for their ability to adhere to this region of human fibrillin-1 C-terminal domain, human MAGP or human fibronectin (FN) as indicated in the figures. Culturing with soluble heparin inhibited fibrillin deposition in both cell types, while fibronectin deposition was unaffected. FBN1, fibrillin-1; FBN2, fibrillin-2.
In the fibrillin molecule, these exons fold into LTBP-like 8-cysteine domains. Both contain an RGD sequence, although not in analogous locations within the domains, and may be bound by different integrins [5]. The peptide FIB15D, which spans the RGD sequence in exon 37 of fibrillin-1, was used as a third integrin-mediated attachment substrate [3]. Cell culture medium containing 10% bovine calf serum was used to coat wells as a positive control, as it is widely accepted that serum contains several proteins that readily support integrin-mediated cell adhesion. BSA was used as a negative control and blocking agent for non-specific binding.

The human fibrillin-1 C-terminal domain supported attachment in each of the nine cell lines tested (Table 2). The FBN1-CT-Pro region, not present in the mature molecule, did not support attachment of any cell type. In general, for most cell types the fibrillin-1 C-terminal domain supports cell adhesion as well as, or better than, the RGD-containing substrates. The RGD-containing substrate that best supported adhesion varied among the cell types and probably reflects differences in cell-surface receptor expression and species specificity. These data show that in vitro, various cell types from several species readily adhere to the site.
Further, the observation that cells do not adhere to the FBN1-CT-Pro protein indicates that the site of interaction is within the first 45 residues of the domain.

**Cell attachment to the fibrillin-1 C-terminal domain is mediated by a cell-surface HS proteoglycan**

The nature of the adhesive interaction between cells and the fibrillin-1 C-terminal domain was unknown. Although peptide sequences typical of an integrin-binding site were not clearly present, the possibility of this common form of cell–ECM interaction was examined, as some integrins are able to bind a range of RGD- and non-RGD-containing sequences. To satisfy these substrate requirements, fibrillin-2 exon 24 and fibrillin-2 exon 37/38 were used as RGD-containing positive control substrates. As a non-RGD-containing integrin ligand, acid-soluble purified type I collagen was used. PE and MG63 cells were plated on to these three substrates, as well as FBN1-CT with and without inhibitors. It is well established that soluble peptides containing an RGD sequence can inhibit integrin interaction with a matrix ligand by competition. The peptides RGD and FIB15D, along with their respective inactive analogue peptides RGE and RtoA, were used in solution to compete binding to the known integrin substrates. While the RGD and FIB15D peptides readily inhibited adhesion to two RGD containing substrates, FBN2 × 24 and FBN2 × 37/38, they did not affect attachment of PE and MG63 cells to the fibrillin-1 C-terminal domain (Figure 5A). Although type I collagen does not contain an RGD sequence, some inhibition of PE cell attachment to collagen was observed. Several integrin receptors have been shown to bind to collagen and some of these are also able to bind RGD-containing substrates and so the observed disruption is therefore not surprising. Control peptides RGE and RtoA did not inhibit attachment to any substrate and so inhibition by the active peptides is a specific competitive interaction and not due to another mechanism.

The addition of EDTA to the adhesion assay is expected to disrupt integrin-mediated adhesion as integrin–ligand interactions are cation-dependent. In both cell lines, EDTA thoroughly disrupted adhesion to the substrates known to be integrin ligands. MG63 adhesion to collagen was only partially inhibited, and this suggests that a non-integrin-mediated interaction is responsible. It has been reported that syndecans 1, 2 and 4 can mediate cell signalling and cytoskeletal re-organization. While members of the glypican family are also present on many cells, they do not appear to mediate cellular attachment [22].

Another major mode of cell–ECM interaction occurs through the syndecan family of cell-surface receptors, which are the primary form of HSPG present on most cells (see [6, 7] for reviews). These type I transmembrane proteoglycans bind a variety of extracellular ligands via their heparan sulphated chains, have been localized to focal adhesions and are known to play a role in signalling and cytoskeletal re-organization. While members of the glypican family are also present on many cells, they do not appear to mediate cellular attachment [22].

To determine if cellular interaction with the fibrillin-1 C-terminal domain is mediated by a cell-surface HSPG, various methods were undertaken. Soluble heparin was added to the binding assay in an attempt to competitively inhibit interaction with a cell-surface HSPG. The attachment of five cell types from four out of five cell lines with 10 μg/ml. This is well within the range published for other matrix molecules, such as vitronectin [23], laminin [24a], and fibronectin [25], and demonstrates good specificity. The highest levels of heparin used ablated binding to the domain, yet had no effect on the adhesion of three cell lines to an RGD-containing substrate.

Heparitinase II catalyses the cleavage of α-N-acetyl-D-glucosaminidic linkage in heparin and HS. Pre-treatment of COS7 cells with heparitinase II greatly inhibited attachment to FBN1-CT (Figure 5C). Chondroitinase ABC digestion of the cells had no effect on adhesion and indicates that attachment is not a non-specific charge interaction. The adhesion of all cell types tested
and the small variation of the inhibition curves between cell types is consistent with the nearly ubiquitous expression of syndecans. Taken together, these data indicate that cell attachment to the C-terminal domain of fibrillin-1 is mediated by a cell-surface HSPG.

There are at least two possible mechanisms by which soluble heparin disrupts deposition of fibrillin into the ECM: (i) competition of a cell-surface interaction and/or cell-mediated deposition and (ii) competition of an HS-dependent protein–protein association in the ECM. Interestingly, the dose-dependent inhibition of fibrillin ECM deposition by heparin presented in this work differs from that reported previously for dermal fibroblasts, even though the heparin sources were identical [11]. In the present study, nearly complete inhibition of fibrillin deposition was observed at 5- and 20-fold lower heparin concentrations for MG63 and PE cells respectively, than was needed for complete inhibition in the fibroblastic cultures. Inhibition at lower heparin concentrations indicates interactions of higher specificity in the osteosarcoma and epithelial lines. Differences in carbohydrate composition between the cell types may account for the sensitivity. Alternatively, variation in the insoluble ECM could affect HS-dependent protein–protein interactions necessary for fibrillin deposition.

The data presented here and previous work have shown sequences in exon 37 of fibrillin-1 and exons 24 and 37 of fibrillin-2 to be ligands for several integrins [3,5]. We have also shown that the heparin/HS-binding site at the C-terminus of mature fibrillin-1 supports cell attachment through a cell-surface HSPG. Thus the potential exists for simultaneous signalling from two classes of cell-surface receptors, integrin and HSPG, after attachment to fibrillin. When the potential for more than one integrin to bind the RGIDs in fibrillin-1 and -2 is combined with various cell types expressing different HSPGs, a wide range of signalling possibilities results. These dual interactions may not only be important for fibrillin deposition, but could conceivably impact cell migration, developmental differentiation, wound repair and homeostasis within the mature tissue. Determination of the in vivo function of the fibrillin-1 heparin/HS-binding sites is an important direction for future investigation.

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REFERENCES