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RESEARCH ARTICLE

INTERACTIONS OF N-TERMINUS OF HUMAN RECOMBINANT FIBRILLIN-3 WITH FIBULIN-2 AND HEPARIN.

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Abstract

Fibrillins are large family of proteins that form a major constituent of microfibrils and subsequently the extracellular matrix. Fibrillin-1 is the most characterized fibrillin member and has been verified to be linked to Marfan syndrome. Fibrillin-2 was linked to congenital contractural arachnodactyly. Fibrillin-3 is expressed during tissues development and it has been linked to Weill Marchesani syndrome and polycystic ovary syndrome. Self-assembly and multiple ligand binding properties of fibrillins are crucial for the proper formation and function of microfibrils. These properties are often compromised in pathological situations. Therefore, we aimed to study the interaction epitopes of the N-terminal of fibrillin-3 with fibulin-2 and heparin in comparison to fibrillin-1, as well as the molecular shapes of the N-terminal region of fibrillin-3. In the present study we compared the binding of fibulin-2 and heparin to the N-terminal polypeptides of both fibrilin-1 and fibrillin-3. Also, we compared folding shapes of their N-termini. Our results indicated the similarity of N-termini of both fibrillin-1 and fibrillin-3 in binding to fibulin-2 and heparin as well as their structures. The N-terminal polypeptides of Fibrillin-3 can interact with Fibulin-2 and heparin in a similar fashion as compared to fibrillin-1, indicating similar functions for both isoforms.

Introduction

Connective tissues serve to support, connect or separate different types of tissues and organs in the body of multicellular organisms (Caplan, 1991). The extracellular matrix (ECM) is the structural backbone of the connective tissues. The ECM is composed of a meshwork of elastic and collagenous fibers, embedded in a hydrophilic ground substance consisting primarily of proteoglycans (Toole, 2000). The Fibrillin family members (fibrillin-1, fibrillin-2 and fibrillin-3 in most mammals) are the major proteins in the structure and function of 10 nm extensible microfibrils within the ECM (Sakai *et al*., 1986; Keene, *et al*., 1991; Zhang *et al*., 1994 and Corson, *et al*., 2004). The microfibrils provide a scaffold for the deposition of elastin. Fibrillins are also thought to aid in sequestering transforming growth factor-β (TGFß) family members either directly or through interactions with the structurally related latent transforming growth factor β-binding proteins (LTBPs).

Fibrillins are collagenase-resistant glycoproteins of about 350 kDa which are secreted into the ECM by fibroblasts (Sakai *et al*., 1986). Fibrillin-1 (FBN1) was first isolated from human fibroblast cultures and identified as the

principal component of 10 nm microfibrils in the ECM (Sakai *et al*., 1986). The FBN1 gene is ubiquitously expressed in mesenchymal cell types (Hume *et al*., 2010 and Summers *et al*., 2010). The Fibrillin-2 (FBN2) gene was identified through its homology with FBN1 (Lee *et al*., 1991) and fibrillin-2 protein was reported to be one of the microfibril components (Zhang *et al*., 1994). FBN2 is expressed in fewer tissues and developmental stages than FBN1. Both FBN1 and FBN2 are expressed at high levels in bone, osteoblasts and chondrocytes. In addition, they regulate the secretion and activation of TGFß family members through the interaction with LTBPs. While, fibrillin-3 (FBN3) was identified in embryonic and fetal tissues, particularly the brain (Corson *et al*., 2004).

Human FBN1 and FBN2 genes have similar structures, with several long introns; FBN3 gene structure is different from FBN1 and FBN2 (Shi *et al*., 2011). On the protein level, fibrillins are large multiple functional domain proteins. Fibrillins consist of 47 epidermal growth factor-like (EGF) domains, 43 of them are calcium-binding EGF (cbEGF) domains. Uniquely, they contain seven domains of the 8-cysteines domain (Charbonneau *et al*., 2004 and Robertson *et al*., 2011). Fibrillin-1 also contains a proline-rich domain in the N-terminus. This domain is a glycine-rich domain in fibrillin-2, and in fibrillin-3 it is a glycine and proline-rich domain. Fibrillin-based microfibrils can have different types, forms and structures. For example, they can form linear fibers like lamina cribrosam of ciliary zonule (Hernandez, 1992). Also, they can form two dimensional networks like in case of the smooth muscle of aortic tunica media; while in skin, fibrillin complexes form three dimensional mesh structure (Ramirez *et al*., 2004).

Many diseases have been linked to mutations in fibrillin genes. The most common disease caused by mutations in FBN1 (more than 1000 mutations) is Marfan syndrome (MFS). It is an autosomal dominant connective tissue disorder causes multiple signs and symptoms primarily affecting the ocular, skeletal and cardiovascular systems (Dietz *et al*., 1991; Rock *et al*., 2004; Faivre *et al*., 2007; Loeys *et al*., 2010 and Sengle *et al*., 2012). FBN2 mutations (at least 40-recorded mutations) have been associated with congenital contractural arachnodactyly (CCA) presenting with long limbs, contractures, arachnodactyly and scoliosis (Putnam *et al*., 1995; Beroud *et al*., 2000; Callewaert *et al*., 2009 and Chen *et al*., 2009). Genetic variation at the FBN3 locus was linked to Weill Marchesani syndrome (Corson *et al*., 2004) and polycystic ovary syndrome, PCOS (Urbanek *et al*., 2007).

The fibulins belong to a family of proteins that are associated with basement membranes, elastic fibers, and other entities of the ECM (Campbell and Bork, 1993). One member of this family, fibulin-2, was described to interact with fibrillin-1 and -2 with high affinity (Reinhardt *et al*., 1996b). It is a 195-kDa protein which is able to form disulfide-linked homodimers playing a structural role in larger extracellular assemblies such as basement membranes (Sasaki *et al*., 1997). It was suggested, that fibulin-2 fulfils a role in connecting various ECM proteins and in the formation of elastic fibers (Raghunath *et al*., 1999). Fibulin-2 was shown to co-localize with fibrillin-containing microfibrils in a tissue-specific manner and to interact with the N-terminal region of fibrillin-1 with high affinity, K_d = 56 nM (Reinhardt *et al*., 1996b). In addition, fibulin-2 expression in photo-aged skin is absent from the dermal epidermal junction, whereas it is present in this zone in normal skin (Hunzelmann *et al*., 2001). These data suggest potential pathological consequences if fibulin-2 interactions with microfibrils and basement membranes at the dermal-epidermal junction is disturbed.

It has been demonstrated that heparin as well as heparan sulfate are able to interact with three regions of fibrillin-1, the N-terminal end, the center and the C-terminal end (Tiedemann *et al*., 2001). In addition the heparin/heparin sulfate-binding site in the C-terminal domain was further localized to residues Cys^{2715}-Arg^{2731} of fibrillin-1(Ritty *et al*., 2003). The addition of heparin/heparan sulfate to cell cultures of primary cells inhibited the assembly of fibrillin-1 into higher order structures (Tiedemann *et al*., 2001 and Ritty *et al*., 2003). These data indicated that heparan sulfate proteoglycans (HSPGs) may play a modifying role in microfibril assembly. HSPGs are located in basement membranes and at cell surfaces of various cell types, and are involved in biologic processes such as glomerular filtration, cell adhesion, cell migration, cell proliferation, cell differentiation, and protein-protein interaction (Carey *et al*., 1997; Iozzo *et al*., 1997; Bernfield *et al*., 1999 and Raats *et al*., 2000). These interactions may serve to stabilize the interface between microfibrils and basement membranes.

Here, we describe the expression of the newly constructed N-terminal polypeptides of FBN3, the important region for recruiting and binding other proteins in the microfibrils, Molecular shape and its binding properties to fibulin-2 and heparin in comparison to the same region of FBN1.

Material and Methods

In order to map fibulin and heparin binding sites on fibrillin-3 and to compare them to those in the N-terminal polypeptides fragment offibrillin-1, both N-terminal constructs of fibrillin-1 and fibrillin-3 were generated. Production and characterization of recombinant fibrillin-1 fragment (rF1F, fragment spanning the region between the N-terminus and the second TB/8-Cys domain Ser19–Gly714) have been described in detail previously (Vollbrandt et al., 2004).

Constructions of N-terminal of fibrillin-3 and protein expression

In this study, a wild-type recombinant fragment of human fibrillin-3 spanning the region between the N-terminus and the second TB domain (amino acid position Met1-Arg681) was designed, including the multifunctional interaction site. To generate the construct, template pBS-FBN3 (Nagase et al., 2001) containing the full-length cDNA for human FBN-3 was amplified. PCR resulted in a product of 523 bp using oligonucleotides rF3F-S (5'-GCTTCGAGCTCAGCCCTGACGGC-3') and rF3F-AS (5'-GCATGCGGCCGCTATTAGTGATGGTGATGGTG-ATGTCGACCATCCGTGGTAATGC-3'). The sense primer was designed to introduce an additional restriction site for BlpI, while the antisense primer contained the sequence coding for six His residues, two stop codons and restriction site for NotI for subsequent cloning. The PCR product was ligated to the pCR-BluntII-TOPO vector (Invitrogen) resulting in plasmid pCR-BluntII-rF3F (4042 bp). The pCR-BluntII-rF3F was double digested using BlpI and NotI. Then, the digested fragment (504 bp) was sub-cloned into pBS to produce pBS-rF3F (5003 bp). Finally, pBS-rF3F and the expression plasmid pcDNA3.1 (+) were digested with NheI and NotI. The expected fragments of 2092 bp from pBS-rF3F and 5344bp from pcDNA3.1 (+) were isolated and ligated resulting in pCDNA-rF3F (7436 bp). The expression of pCDNA-rF3F produced rF3F protein including the sequence of the authentic fibrillin-3 signal peptide and a 6-histidine tag at the C-terminal (Met1-Arg681-His6).

Cell culture and protein purification

According to Lin *et al*. (2002), expression vectors were stably transfected to the human embryonic kidney 293 cells (HEK 293, American Type Culture Collection) and were grown on complete Dulbecco's modified Eagle's medium (DMEM, Carl Roth) supplemented with 10% (v/v) fetal calf serum (Biochrom AG), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were cultivated at 37°C under 5% CO2 atmosphere. The cell culture medium was typically replaced after 2-3 days. Expressed proteins were purified using ÄktaPurifier (GE Healthcare Life sciences) using HiTrap Chelating column charged with Ni2+ ions (GE Healthcare Life sciences) and detected on the nitrocellulose membrane using monoclonal antibody for the six-Histidine tag (mAb-His6, Invitrogen).

Extraction of human fibulin-2

For extraction of authentic fibulin-2 from cell culture, confluent layers of normal human skin fibroblasts (American Type Culture Collection) were first washed two times with TBS including protease inhibitors (PMSF and NEM). Then, the extracellular layer was extracted with 0.1 ml/cm2 TBS including protease inhibitors and 10 mM EDTA for 10 min at 20°C. The extracts were stored at -80°C (Reinhardt et al., 1996b).

Protein interaction assays

Solid-phase binding experiments were performed as described elsewhere with minor modifications (Lin *et al*., 2002). A total of 10 µg/ml (100 µl per well) of rF1F or rF3F in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) was used to coat 96-well plates previously blocked with 5% nonfat milk in TBS and incubated with serial dilutions (1:2) of ligands. Bound proteins were detected using horseradish peroxidase conjugate secondary antibodies (HRP-Ab) (Pierce) and 5-aminosalicylic acid (Sigma). Developed color measures were implemented at 492 nm using Microplate EL310 Auto reader (Bio-Tek Instruments).

Blot overlay assay

Fibulin-2 extracts were TCA precipitated and loaded on SDS-PAGE. The proteins were transferred onto nitrocellulose membrane. After membrane blocking, ligands and antibodies were diluted in TBSM including 2 mM CaCl2 (binding buffer). Then, membranes were incubated with 100 µg/ml of recombinant fibrillin-1 fragments as soluble ligands for 3h. Incubation with binding buffer alone served as a negative control. After washing, the membranes were incubated with specific polyclonal or monoclonal antibodies against the soluble ligands. The membranes were incubated with horseradish peroxidase conjugated goat-anti-rabbit or goat-anti-mouse antibodies (1:800 diluted) and the color was developed in TBS, 17% methanol, including 0.02% (v/v) H2O2 and 0.5 mg/ml 4-chloro-1-naphthol (Bio-Rad).

Electron microscopy after rotary shadowing

Based on Brinckmann *et al*. (2005), the purified rF1F and rF3F were adjusted to concentration of 0.25 mg/ml and dialyzed against 100 mM NH_4HCO_3. The samples were diluted with 10 mM NH_4HCO_3 to a final protein concentration of 60 µg/ml and were mixed with glycerol to a final concentration of 50% (v/v) glycerol. Then 80 µl of the samples were sprayed onto freshly cleaved mica and dried under high vacuum (~9 nbar, Edwards Auto 306) for 3 hours. Rotary shadowing was performed by platinum evaporation for 15 s at 50 mA and 2.5 kV at an angle of 5° and a distance of 12 cm. The samples were rotated at 120 rpm, followed by coating with coal for stabilization for 2 s at 100 mA and 2.5 kV at an angle of 90°. The replicas were floated onto a very clean surface of distilled water and then supported with 400-mesh copper grids. Replicas were examined at 100 kV in a transmission electron microscope (Zeiss TEM 109).

Results and Discussion

Microfibrils are heterogeneous and macroaggregate proteins. The microfibrils components include the superfamily of fibrillins and latent TGFβ-binding proteins (LTBPs), as well as the structurally unique families of fibulins, emilins, microfibrillar-associated glycoproteins, and microfibril-associated proteins (Ramirez *et al*., 2004). Microfibrils arsenal has wide range of "interaction" repertoire. Elucidation of these interactions has pivotal impact on understanding their roles in both health and diseases. Here we comparatively study the interactions of multifunctional domains of both fibrillin-1 and fibrillin-3 to fibulin-2 and heparin and their molecular shapes using EM.

In the present investigation, the N-terminal wild-type recombinant polypeptides rF3F was designed and constructed to harbor the multifunctional domain with 3 EGF-like domains, hybrid domain, cbEGF-like domain and TB module in addition to Pro/Gly-rich domain and six His residues (Fig 1). The rF1F and rF3F have been stably transfected into HEK 293 cells and produced proteins were purified using their His-tag affinity to HiTrap Chelating Ni-column (Fig 2). The purification procedure was monitored by SDS-PAGE. Proteins were transferred to PVDF membrane and the recombinant proteins were detected using mAb-His6 antibodies (Fig 2, Inserts). The efficiency of rF1F expression was better than that of rF3F. The fractions with pure recombinant proteins were pooled and used in the subsequent experiments.

The purified rF1F and rF3F proteins were used to study the binding of fibulin-2 and heparin. Two methods were used to study these bindings. First, Solid-phase binding experiment was used. Figure 3 shows the binding of Fibulin-2 to both rF1F and rF3F. Similarly, heparin binding to rF1F as well as rF3F showed in figure 4. This result was further confirmed with the second method, blot overlay assay (Fig 5).

In previous study the fibulin-2 binding to fibrillin-1 was bound to the first hybrid domain in the N-terminus of fibrillin-1 (El-Hallous *et al*., 2007). Due to the high similarity between both fibrillin-1 and fibrillin-3, both have a highly conserved first hybrid domain, consequently and according to presented results, we suggest that fibulin-2 interacts with 1st hybrid domain of fibrillin-3. Many reports shed the light on the important role of fibulin-2 in matrix remodeling during development (Olijnyk *et al*., 2014) and cancer diseases (Baird *et al*., 2013; Fontanil *et al*., 2014 and Missan *et al*., 2014). Extensive study for heparin binding with fibrillin-1 has been previously indicated "active association" between heparin and the N-terminus of fibrillin-1 (Cain *et al*., 2005). Here we showed similar behavior with fibrilin-3 N-terminus using solid-phase binding experiment and blot overlay assay. The similarity in the behavior of both fibrillin-1 and -3 in fibulin-2 and heparin binding demonstrates that the second cbEGF domain, which is absent in fibrillin-3, is not involved in these binding. These results were expected because it was previously demonstrated that, the cbEGF in fibrillin-1 has no effect on the binding of both fibulin-2 and heparin (Reinhardt *et al*., 1996a).

The molecular shapes of the purified recombinant rF1F and rF3F used in this study were visualized using rotary shadowing and EM (Fig 6). Recombinant polypeptides of both rF1F and rF3F showed sickle shape folding. Measured lengths were calculated in 3-nm windows, plotted and fitted to Gaussian distribution curve. The mean length for rF1F was 38.3 nm with SE 0.58 nm and $\sigma = 3.6$, while the mean length for rF3F was 39.8 nm with SE 0.89 nm and $\sigma = 47.7$.

Rotary shadowing and negative staining EM are extensively used to examine structure of the recombinant fibrillin proteins. N-terminus of both fibrillin-1 and -2 showed extended thread like shape with occasional kinks and bends (Lin *et al*., 2002).

Our results showed that the N-termini of both fibrillin-1 and fibrillin-3 were folded into similar shapes. These data indicate that fibrillin-1 and -3 are curved at their N-termini and that this shape may be a necessary requirement for direct head-to-tail interactions and may play a role in binding with other proteins in extracellular matrix. The N-terminal polypeptides of Fibrillin-3 can interact with Fibulin-2 and heparin in a similar fashion as compared to fibrillin-1, indicating similar functions for both isoforms. That is why this kind of data is very important to shed more light on the assembly mechanism(s) of fibrillin into the microfibrils.

Abbreviations

cbEGF: calcium-binding EGF; CCA: congenital contractural arachnodactyly; ECM: Extracellular matrix; EGF: epidermal growth factor-like; FBN: fibrillin gene; HRP-Ab: horseradish peroxidase conjugate secondary antibodies; MFS: Marfan syndrome; NEM: N-Ethyl maleimide; LTBPs: Latent transforming growth factor ß-binding proteins; PCOS: polycystic ovary syndrome; PMSF: phenyl methane sulfonyl fluoride; rF1F: polypeptide fragment spanning the region between the N terminus and the second TB of fibrillin-1; rF3F: polypeptide fragment spanning the region between the N-terminus and the second TB of fibrillin-3; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGFß: Transforming growth factor-β.

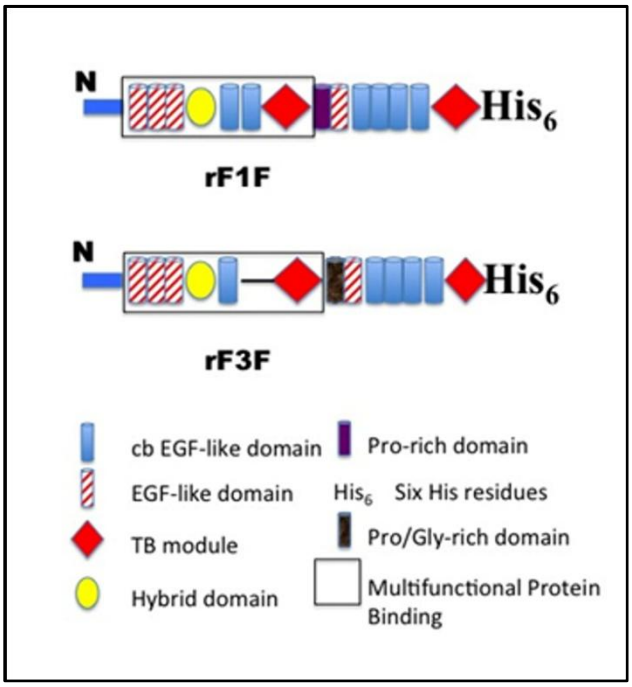

Fig 1. Schematic representation of recombinant humanfibrillin-1 (rF1F) and fibrillin-3N-termini.The modular domains found in the N-termini of both rF1F and rF3F that used in this study are represented. rF1F has been previously designed and generated (Vollbrandt *et al*., 2004).

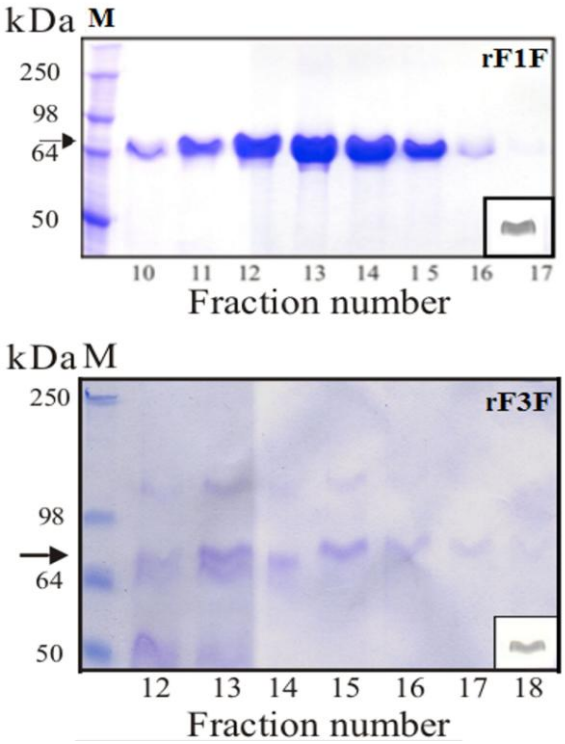

Fig 2. SDS-PAGE for rF1F and rF3F recombinant proteins purification steps. Different fractions of eluted proteins from Hi-Trap Ni-chelating columns were loaded on SDS-PAGE and stained with Coomassie brilliant blue. Followed by protein blotting to PVDF membranes and the recombinant proteins were detected using mAb-His_6 antibodies (Inserts). M: protein marker and the numbers indicated the fractions number.

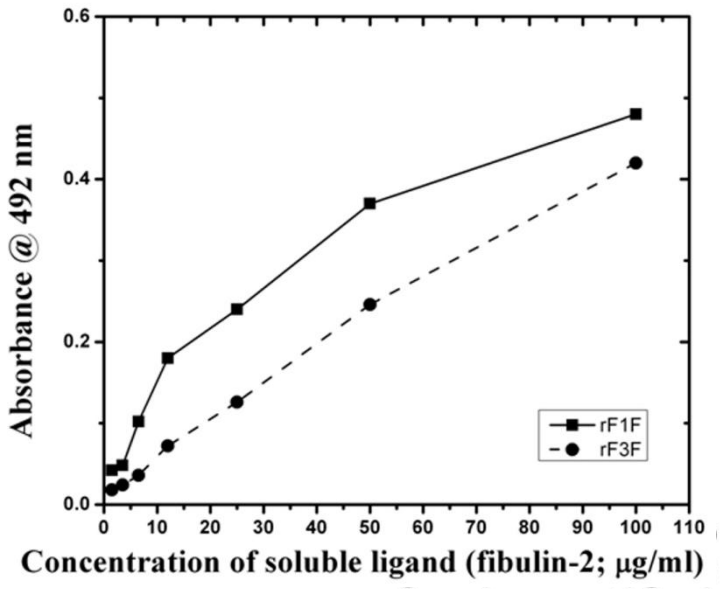

Fig 3. Binding of recombinant rF1F and rF3F to fibulin-2 in a solid phase assay.5 µg/ml of fibulin-2 was immobilized on a multi-well plate, incubated with the recombinant rF1F or rF3F as soluble ligands. Bound ligands were detected using mAb-His_6 antibody. HRP-Ab was used as secondary antibody with 5-aminosalicylic acid for color development which measured at 492 nm using microplate reader.

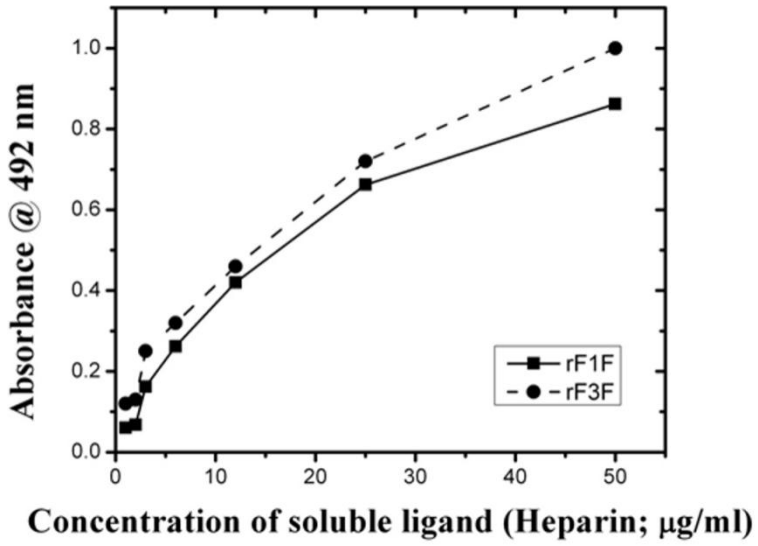

Fig 4. Binding of recombinant rF1F and rF3F to heparin in a solid phase assay.1 µg/ml of heparin was immobilized on a multi-well plate, incubated with the recombinant rF1F or rF3F as soluble ligands. Bound ligands were detected using mAb-His_6 antibody. HRP-Ab was used as secondary antibody with 5-aminosalicylic acid for color development which measured at 492 nm using microplate reader.

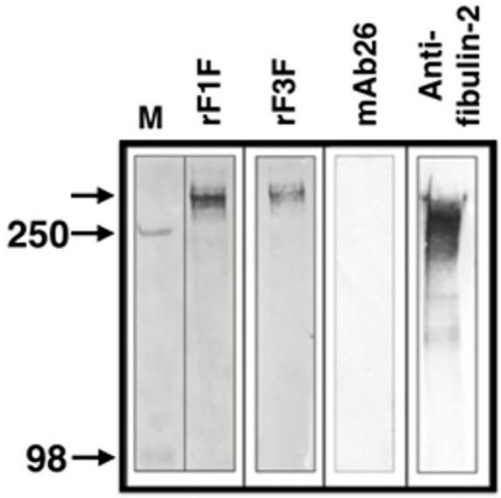

Fig 5. Binding of recombinant rF1F and rF3F polypeptides to human fibulin-2 in a blot overlay assay**.** EDTA extracts of the extracellular layer of human skin fibroblasts were separated under non-reducing conditions by SDS gel electrophoresis then transferred onto nitrocellulose. The soluble binding ligands were used at a constant concentration of 100µg/ml and were detected with specific monoclonal antibodies. Lane M: protein marker; lane rF1F: indicated the soluble used and detected by mAb26. Lane rF3F: indicated the soluble used and detected by mAb26, Lane mAb26: indicated no soluble ligands; Anti-fibulin-2: using specific fibulin-2 antibodies

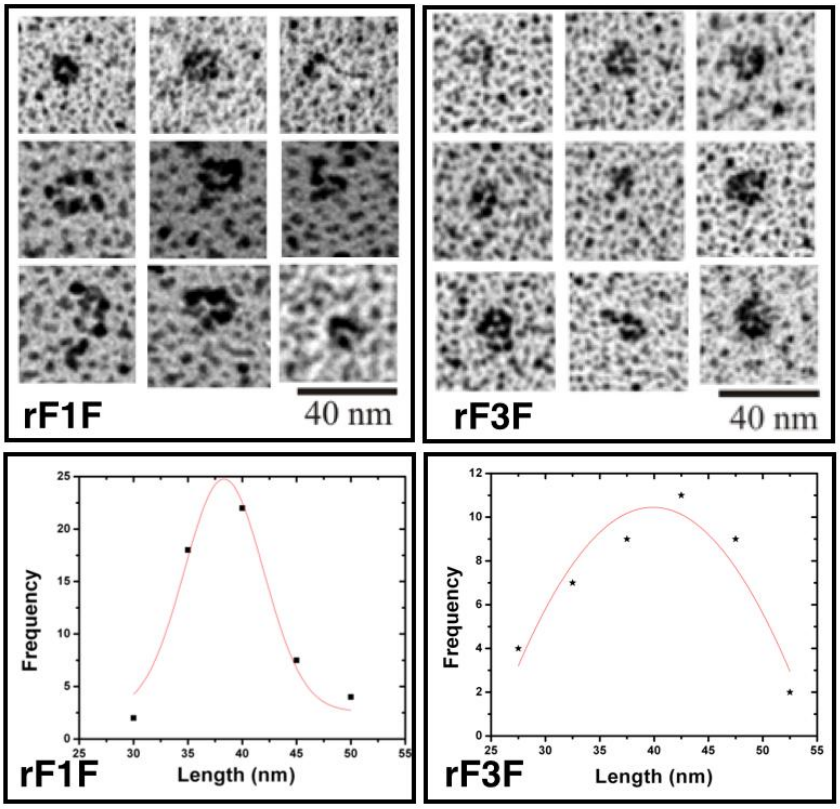

Fig 6. Molecular shapes of recombinant human rF1F and rF3F polypeptides. The purified recombinant rF1F and rF3F are visualized by electron microscopy after rotary shadowing (up). The bar represents 40 nm. Plots represent the measured lengths of rF1F and rF3F (down). Measurements are plotted as numbers of measured molecules with specific length in 3-nm windows (Symbols). Gaussian distributions are shown for each plot (curves).

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