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Characterization of the Molecular Interaction between Tropoelastin and DANCE/Fibulin-5

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Fibulin-5 is believed to play an important role in the elastic fiber formation. The present experiments were carried out to characterize the molecular interaction between fibulin-5 and tropoelastin. Our data showed that the divalent cations of Ca^{2+}, Ba^{2+} and Mg^{2+} significantly enhanced the binding of fibulin-5 to tropoelastin. In addition, *N*-linked glycosylation of fibulin-5 does not require for the binding to tropoelastin. To address the fibulin-5 binding site on tropoelastin constructs containing, exons 2–15 and exons 16–36, of tropoelastin were used. Fibulin-5 binding was significantly reduced to either fragment and also to a mixture of the two fragments. These results suggested that the whole molecule of tropoelastin was required for the interaction with fibulin-5. In co-immunoprecipitation experiments, tropoelastin binding to fibulin-5 was enhanced by an increase of temperature and sodium chloride concentration, conditions that enhance the coacervation of tropoelastin. The binding of tropoelastin fragments to fibulin-5 was directly proportional to their propensity to coacervate. Furthermore, the addition of fibulin-5 to tropoelastin facilitated coacervation. Taken together, the present study shows that fibulin-5 enhances elastic fiber formation in part by improving the self-association properties of tropoelastin.

Key words: coacervation, DANCE/fibulin-5, extracellular matrix, molecular interaction, tropoelastin.

Abbreviations: BH, C-terminal half-fragment of bovine tropoelastin encoded by exons 16–36; BTE, bovine tropoelastin; cbEGF, calcium-binding epidermal growth factor; CHO-K1, Chinese hamster Ovary-K1 cells; DANCE, developmental arteries and neural crest epidermal growth factor-like; DTT, dithiothreitol; EMIKIN, elastin microfibril interface located protein; FH, N-terminal half-fragment of bovine tropoelastin encoded by exons 2–15; MAGP, microfibril-associated glycoprotein; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine; T-TBS, Tris buffered saline supplemented with 0.05% Tween 20.

INTRODUCTION

Elastin is a main component of elastic fibers that provides resilience and elasticity to many tissues such as skin, lungs, ligaments and arterial walls. During elastic fiber formation, tropoelastin, the soluble precursor of elastin, is deposited on pre-formed microfibrillar templates. Several studies have indicated that tropoelastin molecules directly interact with the microfibrillar proteins fibrillin-1, -2 (*1*, *2*). Moreover, two structural proteins of elastic fibers; microfibril-associated glycoprotein 1 (MAGP-1) and fibulin-5, bind both tropoelastin and fibrillin-1 (*2–4*). It has been considered that the C-terminus of tropoelastin plays an important role in the interaction with microfibrils because exon 36 of the elastin gene encodes a critical domain for binding microfibrillar proteins (*5–7*). More recently, a peptide mapping study has revealed that the N-terminus of fibrillin-1 binds not only to the C-terminus but also to the N-terminus of tropoelastin encompassing domains 2–18 (*8*). However, the mechanisms of elastic fiber assembly remain unclear.

Fibulin-5, also known as DANCE (developmental arteries and neural crest epidermal growth factor-like), belongs to fibulin family of at least six members. It is localized to the surface of elastic fiber *in vivo* and is characterized by a unique arrangement of structural domains, including N-terminal signal peptide and six calcium-binding epidermal growth factor (cbEGF) like domains (*9*). It also contains two putative Asn glycosylation sites and an RGD sequence in the first cbEGF like domain (*10*, *11*). Studies of fibulin-5 deficient mice have demonstrated that the disorganization of elastic fibers leads to skin laxity, vascular abnormalities and

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emphysematous lung (*12*) and this phenotype resembles the cutis laxa syndrome in humans (*13*). In addition, it is believed that the ability of fibulin-5 to bind integrin receptors on the cell surface, tropoelastin, lysyl oxidase-like-1 (*14*) and fibrillin-1 (*4*) play a critical role in cell-directed elastic fiber formation.

In the present study, we investigated the molecular interaction between tropoelastin and fibulin-5 using recombinant proteins in solid phase binding assays and co-immunoprecipitation experiments. Our data showed that the molecular structure of cbEGF-like domains in the fibulin-5 molecule played an essential role for the interaction with tropoelastin, but post-translational modifications of fibulin-5 did not. Furthermore, the whole molecule of tropoelastin was required for the interaction with fibulin-5 and the molecular interaction was enhanced by an increase of temperature and sodium chloride concentration. These data indicated that the conformational structure of tropoelastin, including coacervation was important for interactions with fibulin-5. Consistent with an allosteric effect of fibulin-5, its addition of fibulin-5 to tropoelastin enhanced coacervation. These data provide new insights into the initial steps of elastic fiber assembly.

MATERIALS AND METHODS

Production of Recombinant Human Fibulin-5—Production of recombinant human fibulin-5 was performed according to a previously described procedure (*15*). Briefly, human fibulin-5 cDNA was inserted into the pcDNA 3.1/V5-His-TOPO expression vector (Invitrogen, Carlsbad, CA, USA). CHO-K1 cells (ATCC, CCL-61) were stably transfected with this plasmid (CHO/F5). After 24-h incubation, the culture media were replaced with CHO-SFM-II (Invitrogen, Carlsbad, CA, USA) and the cultures were incubated for 24–72 h. At the end of incubation period, the culture media were collected and concentrated using Amicon Ultra-15 (Millipore, Bedford, MA, USA). The product was used in the experiments as conditioned media or was purified using Ni^{+}-NTA columns with a Duo-Flow system (Bio-Rad Labs, Hercules, CA, USA).

Purification of Recombinant Bovine Tropoelastin—A recombinant full-length bovine tropoelastin (BTE) was prepared as previously described (*16*). To generate the constructs containing the cording region corresponding to the exons 2–15 of BTE and exons 16–36 of tropoelastin, bovine elastin gene was amplified using the following primer sequences: bovine exon 2 forward (5′-GGAGGGGT CCCAGGAGCTGTTCT-3′), bovine exon 15 reverse (5′-TCAAAGCTTCGCTGCTGCTTTAGC-3′), bovine exon 16 forward (5′-GGTGCTGGAGGAGCCGGAGTTC-3′) and bovine exon 36 reverse (5′-TCACTTTCTCTTCCGGCCA CAG-3′). The N-terminal half and the C-terminal half of BTE were abbreviated as FH and BH, respectively. The products were inserted into a bacterial ex pression pTrcHis-TOPO vector (Invitrogen, Carlsbad, CA, USA). These proteins were obtained by overexpression from the plasmid and purified the same method as for BTE. Purified recombinant proteins were resuspended in SDS–PAGE sample buffer including 100 mM dithiothreitol (DTT). Samples were run on 7.5% SDS–PAGE gels and subjected to western blot analysis.

Assessment of N- and O-Linked Glycosylation—To determine whether the recombinant fibulin-5 proteins contained *O*-linked sugars, Jacalin beads bound to agarose (Vector Laboratories, Inc., Burlingame, CA, USA) were used. The Jacalin lectin specifically recognizes *O*-linked sugars, preferring the structure galactosyl-β-1, 3-*N*-acetylgalactosamine (*17*). Conditioned medium were pre-cleared with agarose beads at 4°C for 60 min. The agarose beads were pelleted and Jacalin agarose beads were added to the supernatant and incubated at 4°C for 24 h. The supernatants were collected and all beads were washed five times with cold PBS. Each sample was resuspended in SDS–PAGE sample buffer [62.5 mM, Tris, pH 6.8, 0.4% (w/v) SDS, 10% (v/v) glycerol and 0.003% (w/v) bromphenol blue] including 100 mM DTT. Samples were run on 7.5% SDS–PAGE gels and subjected to western blotting analysis.

Western Blot Assay—Recombinant proteins or aliquots of conditioned media from transfected cells were electrophoresed on a 7.5% SDS–PAGE gels. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH, USA) for immunodetection using a Mini-Protean II transfer apparatus (BioRad Labs, Hercules, CA, USA) according to manufacturer's instructions. Membranes were blocked with Tris buffered saline supplemented with 0.05% Tween 20 (T-TBS) containing 5% (w/v) non-fat milk and then anti-BTE monoclonal antibody (BA4) (Sigma, St. Louis, MO, USA), anti-V5-epitope monoclonal antibody (Invitrogen Japan K. K, Chuo-ku, Tokyo, Japan), or anti-His-G monoclonal antibody (Invitrogen Japan K. K, Chuo-ku, Tokyo, Japan) diluted in T-TBS. After membranes were washed with T-TBS, anti-mouse IgG conjugated horseradish peroxidase (ICN/Cappel, Costa Mesa, CA, USA) diluted in 5% (w/v) non-fat milk/T-TBS. Membranes were thoroughly washed and treated with ECL reagent (Amersham, Buckinghamshire, UK); each membrane was exposed to an X-ray film (Kodak, Rochester, NY, USA) for fluorography.

Solid Phase Binding Assay—Micro-titer plates (FALCON, Becton Dickinson Co., Franklin Lakes, NJ, USA) were coated at 4°C overnight with 1.6×10^{-7} M BTE, FH, BH or 10 μg/ml bovine serum albumin in bicarbonate buffer (14 mM sodium bicarbonate, 6 mM sodium carbonate), respectively. Plates were washed several times with PBS containing 5% Tween 20 and then were incubated with 5% (w/v) non-fat milk/PBS at room temperature for 60 min to block non-specific binding. After blocking, fibulin-5 conditioned media diluted in 0.5% BSA/PBS (dilution buffer) were added and incubated at 37°C for 60 min. After washing extensively, plates were incubated at 37°C for 60 min in anti-V5-epitope monoclonal antibody diluted 1 : 5,000 in dilution buffer and then plates were washed again. Secondary anti-mouse IgG horseradish peroxidase conjugate diluted 1 : 5,000 in dilution buffer was incubated with the blots at 37°C for 60 min. Bound protein was quantified with a colorimetric assay using TMB-substrate reagent for 30 min at room temperature. Plates were read at a wavelength of 450 nm.

Immunoprecipitation—BTE, cloned into the MluI-XbaI site of pCI-neo expression vector (Promega, Madison,

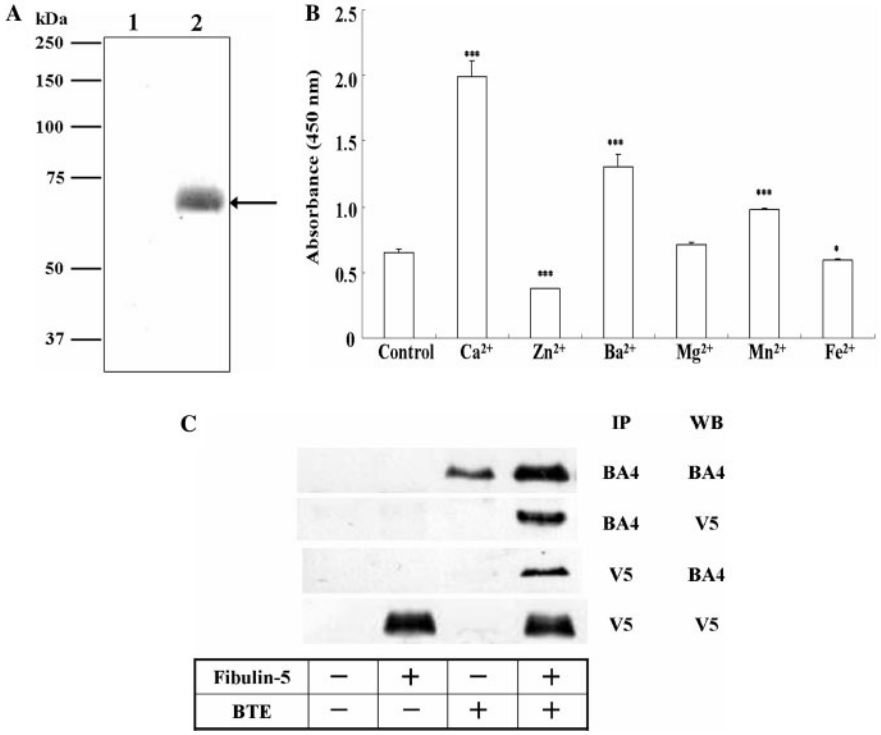

Fig. 1. **Expression of fibulin-5 and binding to tropoelastin in solid phase and solution phase.** (A) Western blot analysis of serum-free conditioned medium using anti-V5 monoclonal antibody, fibulin-5-transfected CHO-K1 cells (CHO/F5) stably expressed fibulin-5 (lane 2). There was no cross-reactivity of the antibody with proteins from untransfected CHO-K1 cells (lane 1). (B) Solid-phase binding assay showed the molecular interaction between fibulin-5 and tropoelastin in the presence of 10 mM various divalent cations. The bar indicates means ± SEM., $n = 3$ $^{***}P < 0.001$ versus control. (C) Full-length bovine elastin gene was transiently transfected into CHO-K1 or CHO/F5 cells. Aliquots of conditioned medium were subjected to immunoprecipitation for tropoelastin or fibulin-5 using BA4 or anti-V5-epitope monoclonal antibody. After immunoprecipitation, the samples were detected by western blot analysis using BA4 or anti-V5-epitope monoclonal antibody. Lane 1, CHO-K1 cells; Lane 2, CHO/F5 cells; Lane 3, ELN-transfected CHO-K1 cells; Lane 4, ELN-transfected CHO/F5 cells.

WI, USA) behind an engineered Kozak consensus sequence, was transfected into CHO-K1 or CHO/F5 cells using the Fugene 6 transfection reagent according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were cultured for 24 h in serum-free CHO-SFM-II. The medium was collected and centrifuged to remove any detached cells. In some experiments, the amount of BTE, FH or BH was adjusted to 1.6×10^{-7} M in PBS, respectively and fibulin-5-conditioned medium was added to each of the solutions. After the incubation at 37°C for 1 h, the mixtures were cooled by placing at 4°C for 1 h. The medium as described earlier or the mixtures were incubated for 1 h at 4°C with 2 μg of BA4 or anti-V5-epitope monoclonal antibody, followed by incubation with immobilized Protein A-Sepharose (PIERCE Rockford, IL, USA) overnight at 4°C. The immunoprecipitates were collected by centrifugation, were then washed with PBS containing 0.5% Tween 20. The immunoprecipitates were eluted by boiling the samples in 1 × SDS–PAGE buffer containing 100 mM DTT. The samples were electrophoresed on 10% SDS–PAGE gels and subjected to western blot assay using anti-His-G or anti-V5 monoclonal antibodies.

Coacervation—Coacervation of BTE, FH and BH dissolved in PBS at the concentration of 1 mg/ml was assayed in various concentrations of NaCl or in the presence of recombinant fibulin-5, sample was prepared as following; 2 or 4 μg/ml of recombinant fibulin-5 solution was added to equal volume of 2 mg/ml of BTE solution at 4°C, by monitoring turbidity through light scattering at 400 nm using a UV spectrometer and JASCO V-550 spectrophotometer (Nihonbunkou, Tokyo, Japan) with an attached re-circulating water bath and software package V-500 for Windows (JASCO, Tokyo, Japan). Light scattering was monitored continuously while the solution was warmed from 15°C to 60°C at a rate of 1°C/min.

Statistical Analysis—All assays were performed in triplicate and repeated at least twice to confirm observed results. Data were statistically analysed using ANOVA. The results were considered statistically significant when the *P*-value was <0.05. All data are shown as the mean value ± SEM (standard error of the mean).

RESULTS

Expression of Fibulin-5 and Solid Phase Binding to Tropoelastin—In order to produce recombinant proteins, human fibulin-5 tagged with a V5 epitope at the C-terminus was expressed in CHO-K1 cells and recombinant BTE was expressed in bacteria. Western blot analysis demonstrated that the fibulin-5 protein was stably expressed in CHO-K1 cells (Fig. 1A). It is well

known that fibulin-5 strongly binds to tropoelastin in the presence of Ca^{2+} (*12*), however, no other comparable study has examined the ability of other divalent cations to facilitate molecular interactions between fibulin-5 and tropoelastin. The effect of divalent cations on the binding of fibulin-5 to tropoelastin was determined by solid phase binding assay. Our data showed that the presence of 10 mM Ca^{2+}, as expected, significantly enhanced the binding. In addition, the presence of 10 mM Ba^{2+} and 10 mM Mg^{2+} also significantly increased the molecular interaction with tropoelastin, albeit to a lower level than Ca^{2+} (Fig. 1B). The presence of EDTA significantly decreased the binding in a concentration-dependent manner as previously reported (*12*) (Data not shown).

In order to confirm the molecular interaction between fibulin-5 and tropoelastin in a solution, immunoprecipitation assays were performed using culture media containing both tropoelastin and fibulin-5. BTE cDNA in pCIneo mammalian expression vector was transiently transfected into CHO-K1 cells stably expressing fibulin-5 to produce media for these experiments. Fibulin-5 or tropoelastin was recovered by co-immunoprecipitation using the indicated antibodies and then was identified by western blot analysis (Fig. 1C). Tropoelastin-fibulin-5 complexes could be immunoprecipitated using antibodies to either of the two proteins.

Effect Of Post-translational Modification in Fibulin-5 on the Molecular Interaction to Tropoelastin—*O*-linked glycosylation of fibulin-5 was assessed by its ability to be recognized by Jacalin, which is a lectin that specifically recognizes *O*-linked sugars according to previously reported (*1*). Fibulin-5 did not bind to Jacalin (Fig. 2A). We confirmed that fibulin-5 was *N*- glycosylated by demonstrating reduced electrophoretic mobility of fibulin-5 following treatment of cells with tunicamycin, a known inhibitor of *N*-linked glycosylation (Fig. 2B). We also found that *N*-glycosylation of fibulin-5 did not affect its binding to tropoelastin (Fig. 2C).

The Whole Molecule of Tropoelastin is Required for Interaction with Fibulin-5 in Solid and Solution Phase—To identify the fibulin-5 binding domain on the tropoelastin molecule, we performed solid phase binding and immunoprecipitation assays using FH and BH of recombinant tropoelastin. In those assays, the amount of BTE, FH or BH was adjusted to 1.6×10^{-7} M in PBS, respectively, to compare under the same condition. FH, BH and the combination of FH and BH showed significantly reduced interaction with fibulin-5 in solid phase binding assays (Fig. 3A).

Confirming our solid phase binding studies, both FH and BH tropoelastin half molecules showed severely reduced binding to fibulin-5 in co-immunoprecipitation studies (Figs 3B and C). Coacervations studies, furthermore, demonstrated that the propensity of tropoelastin peptides to coacervate was directly proportional to their binding to fibulin-5 (Fig. 3D). These results suggest that the conformational change of tropoelastin associated with coacervation may also play a critical role in the interaction of tropoelastin with fibulin-5.

Conditions Favouring Coacervation Improve Binding of Tropoelastin to Fibulin-5—Tropoelastin solutions undergo a phase transition called coacervation, which is dependent on temperature, ionic strength, pH and tropoelastin concentration (*18*). These conditions are thought to promote hydrophobic interactions between tropoelastin monomers (*18*). After the incubation of fibulin-5 and tropoelastin at different temperatures or in the presence of 0–0.4 M sodium chloride, we performed co-immunoprecipitation with anti-V5-epitope monoclonal antibody, and products were then identified by western blot analysis. We found that tropoelastin binding to fibulin-5 was temperature dependent (Fig. 4A), and demonstrated that binding (Fig. 4B) and coacervation of BTE (Fig. 4C) are directly proportional to salt concentration.

To confirm the functional role of fibulin-5 on the binding to tropoelastin, coacervation of tropoelastin in the absence or presence of recombinant fibulin-5 was assayed by monitoring turbidity through light scattering using UV spectrometer. Most interestingly, the result showed that coacervation of tropoelastin was started at a lower temperature and was enhanced by the presence of recombinant fibulin-5 (Fig. 5).

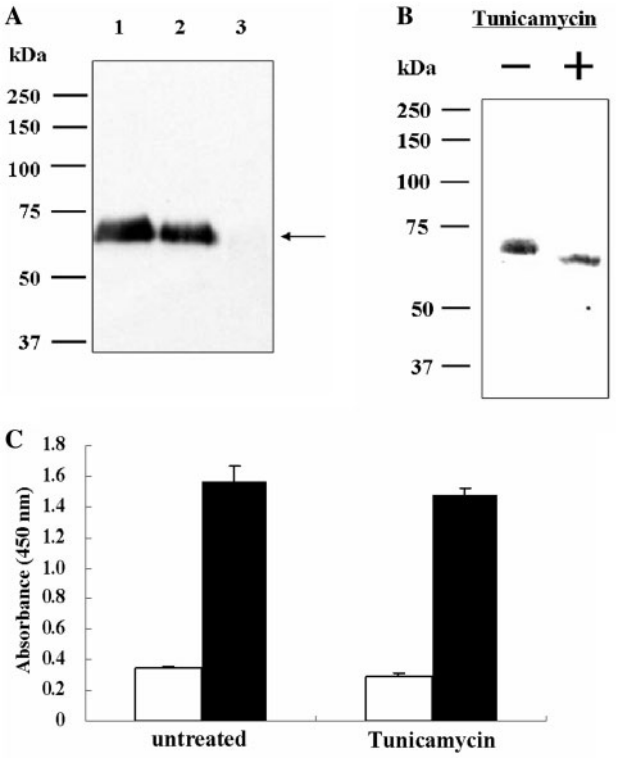

Fig. 2. ***N*-linked glycosylation of fibulin-5.** (A) Fibulin-5 is not *O*-linked glycosylated as demonstrated by its lack of recognition by the lectin Jacalin, which binds *O*-linked sugar moieties. Lane 1, fibulin-5 from conditioned medium used as starting material; Lane 2, unbound fraction to Jacalin beads; Lane 3, bound fraction to Jacalin beads. (B) CHO/F5 cells were incubated for 24 h in the absence (–) or presence (+) of tunicamycin. Western blot analysis using anti-V5-epitope monoclonal antibody for the expressed proteins demonstrates a reduced molecular weight following treatment with tunicamycin indicating that fibulin-5 is *N*-glycosylated. (C) In solid phase binding assays in the absence (open bars) or presence (shaded bars) of 10 mM Ca^{2+}, tunicamycin treatment of cells producing fibulin-5 has no effect on the interaction with tropoelastin. The bar indicates means ± SEM.

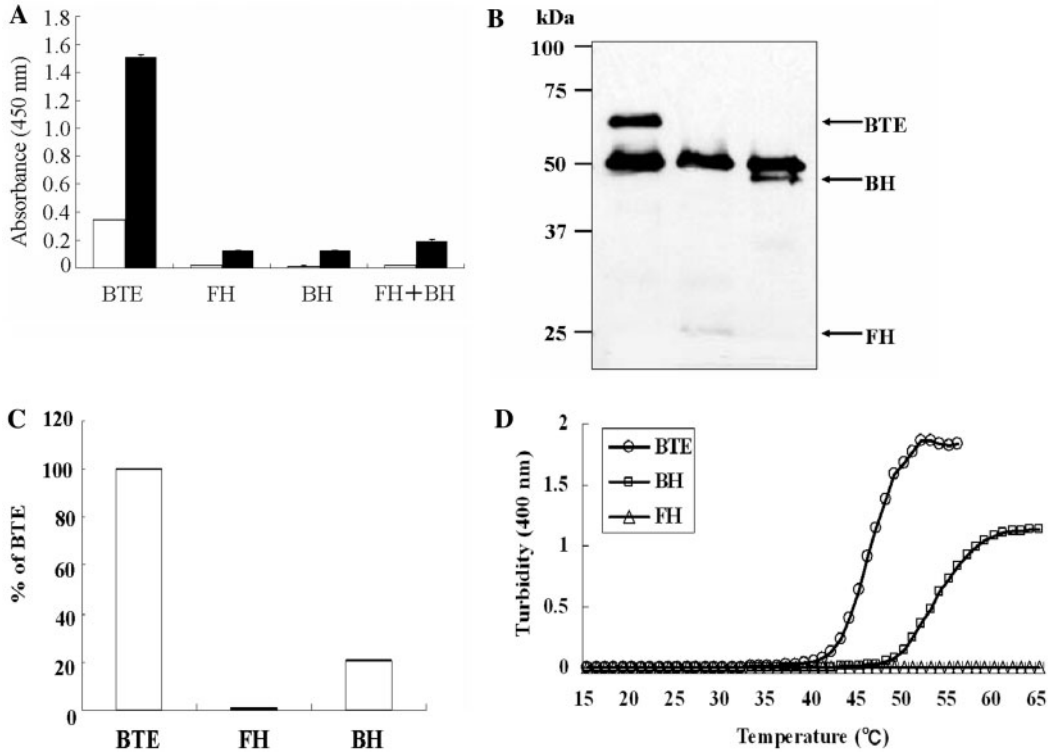

Fig. 3. **Fibulin-5 does not bind to fragments of tropoelastin.** (A) The molecular interaction between fibulin-5 and BTE, FH, BH or combination of FH/BH in the absence (open bars) or presence (shaded bars) of 10 mM Ca^{2+} was performed using solid-phase binding assays. The bar indicates means ± SEM., $n = 3$ $^{***}P < 0.001$ versus control without Ca^{2+} or $^{\#\#\#}P < 0.001$ versus control with Ca^{2+}. (B) Fibulin-5 from conditioned medium was incubated with BTE, FH or BH at 37°C. As in Fig. 4C, western blot analysis for co-immunoprecipitated proteins with anti-His-G monoclonal antibody showed that BTE (left) strongly bound to fibulin-5 in solution phase compared with FH (middle) and BH (right). (C) The area of each of the bands was read using Kodak-1D Image Analysis Software, and the data expressed as a percentage of bound density of BTE. (D) Coacervation of BTE, FH and BH was assayed by monitoring turbidity using light scattering. Each tropoelastin was diluted to a concentration of 1.0 mg/ml in PBS. Light scattering was monitored continuously as the temperature was raised at a rate of 1°C/min from 15°C to 60°C.

DISCUSSION

In the present study, we analysed the molecular interaction between fibulin-5 and tropoelastin, which is a critical step in the process of elastic fiber formation. Our data revealed that neither the N-terminal nor the C-terminal half of tropoelastin is sufficient for interaction with fibulin-5. Furthermore, conditions that facilitate coacervation of tropoelastin also increased binding to fibulin-5. Conversely, addition of fibulin-5 to tropoelastin enhanced coacervation, suggesting that fibulin-5-tropoelastin binding and coacervation are coupled processes.

Several studies have shown that fibrillin-1, fibrillin-2 (*1*) and MAGP-1 (*19*, *20*) are post-translationally modified including glycation, sulphation, transglutamination. These modifications are thought to facilitate molecular interactions with tropoelastin (*18*). Our data revealed that fibulin-5 has *N*-linked glycosylation, but not *O*-linked glycosylation. However, *N*-glycosylation was not necessary for the binding to tropoelastin. In addition, our data showed that fibulin-5-tropoelastin interaction was not inhibited by the addition of sodium chloride. This result suggests that electrostatic interactions are not required for the interaction of these molecules. Although we have not directly investigated the possibility of sulphation of fibulin-5, the observation that glycosylation of fibulin-5 is dispensable, indirectly indicates that sulphation of sugar side chains in fibulin-5 is not necessary of tropoelastin binding. This is in contrast to evidence that sulphation of fibrillins and MAGP-1 are necessary for incorporation into the extracellular matrix (*19*).

It is thought that the only two cysteine residues and an Arg-Lys-Arg-Lys sequence in tropoelastin participate in the formation of an anti-parallel beta-structured-loop and form a positively charged pocket near the C-terminus of tropoelastin, and these conformational structures is important for the interaction with acidic elastic fiber proteins including MAGP-1 (*21*). Recently, we reported that a frameshift-mutated tropoelastin at the C-terminus of tropoelastin had decreased the binding to fibulin-5 compared with normal tropoelastin (*22*). It has been also reported that full-length tropoelastin molecule is necessary for the binding to MAGP-1 (*3*). Previously our data have indicated that there is no significant difference between normal tropoelastin and exon 16 and 17 missing tropoelastin in the molecular interaction with fibulin-5 (*15*). This result suggest that the between exon 15 and 16 is not junction region. Taken together, we conclude that the whole molecule of tropoelastin is necessary for the molecular interaction with fibulin-5.

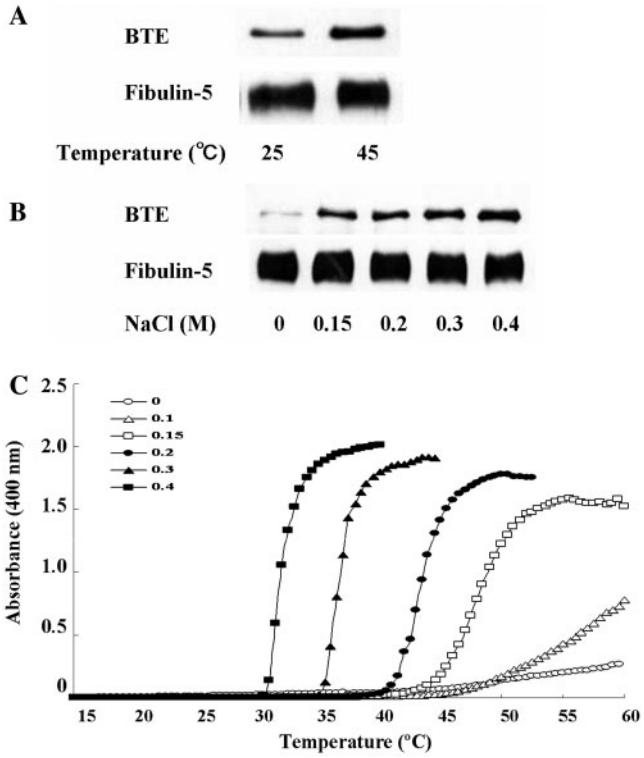

Fig. 4. **The interaction between fibulin-5 and tropoelastin in solution phase.** (A) Immunoprecipitation assay was performed as described MATERIALS AND METHODS section. Co-immunoprecipitated protein was detected by western blot analysis with anti-His-G monoclonal antibody for BTE and anti-V5-epitope monoclonal antibody for fibulin-5. Elevated temperature increased the binding of tropoelastin to fibulin-5. Sodium chloride also enhanced the binding of tropoelastin to fibulin-5 at 37°C (B) and coacervation of BTE in 0–0.4 M NaCl (C). Coacervation of BTE in 0–0.4 M NaCl was assayed by monitoring turbidity using light scattering. BTE was diluted to a concentration of 1.0 mg/ml in PBS. Light scattering was monitored continuously as the temperature was raised at a rate of 1°C/min from 15°C to 60°C.

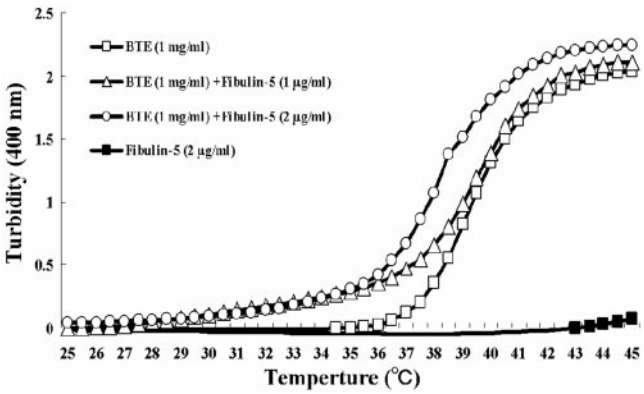

Fig. 5. **Self-aggregation of tropoelastin in the presence of recombinant fibulin-5.** Toropoelastin was diluted to a concentration of 1.0 mg/ml in PBS containing 10 mM Ca^{2+}. Coacervation of tropoelastin was assayed in the presence or absence of recombinant fibulin-5 (1 μg/ml, 2 μg/ml) by monitoring turbidity through light scattering at 400 nm using a UV spectrometer.

It has been reported that EMILIN as an elastin binding protein directly binds to tropoelastin in a solution phase (*23*), but no other elastin binding protein has been shown to bind to tropoelastin in solution. Our data also revealed that tropoelastin strongly binds to fibulin-5 in the solution phase. This result sheds light on the mechanisms of molecular interaction between tropoelastin and fibulin-5 in detail. It is well known that tropoelastin undergoes a phase transition called as coacervation, which is dependent on temperature, ionic strength, pH and tropoelastin concentration (*24*, *25*). It is believed that the process of coacervation is mainly due to interactions between the hydrophobic domains of tropoelastin and promotes the formation of covalent cross-links, such as desmosine or isodesmosine (*26*, *27*). Therefore, coacervation of tropoelastin is thought to play a critical role in elastogenesis (*18*). Recently, it was reported that a fibrillin-1 fragment spanning exons 9–17, which was known to bind to tropoelastin thermodynamically facilitated the coacervation of tropoelastin, resulting in smaller change in entropy and enthalpy values for the coacervation system (*8*). In the present study, we demonstrated that the molecular interaction between fibulin-5 and tropoelastin becomes stronger as reaction temperature or sodium chloride concentration increases. In addition, it is of particular interest that coacervation of tropoelastin correlated with the efficiency of fibulin-5 binding using BTE, FH and BH in a solution phase binding assay. Recently, our data indicated that the binding to fibulin-5 differ among tropoelastin isoforms (*28*). These results suggest that the conformational change of tropoelastin plays a critical role on the interaction with fibulin-5.

Interestingly, conditions favouring tropoelastin coacervation also improved fibulin-5 binding. Fibrillin-1 has recently been reported to promote coacervation (*8*). It is considered that proteins, which are able to bind to tropoelastin, may promote coacervation. Because fibulin-5 interacts with both fibrillin-1 and tropoelastins (*4*, *12*), a fibulin-5–fibrillin-1 complex might cooperatively promote coacervation. Based on these observations, we hypothesize that conformational changes associated with coacervation of tropoelastin are necessary for fibulin-5 binding to tropoelastin. This hypothesis would predict that tropoelastin-fibulin-5 binding and tropoelastin coacervation are coupled molecular processes and that fibulin-5 binding facilitates the coacervation of tropoelastin and thereby enhances elastic fiber formation. This is exactly what our experiments demonstrated. Addition of fibulin-5 to tropoelastin indeed lowered its coacervation temperature consistent with enhanced coacervation of tropoelastin. Our recently data revealed that FH or BH alone, or in combination; do not form cross-linking amino acids such as desmosine (*29*). It has been also reported that fibulin-5 interacts with lysyl oxidase like 1 (*14*). Taken together, molecular interaction between tropoelastin and fibulin-5 may play a more positive role in the alignment and maturation of tropoelastin. Moreover, recent studies on missense variants and mutations in fibulin-5 further support this hypothesis. A G202R variant in fibulin-5 for example enhanced binding to tropoelastin and greatly increased elastin deposition (*30*). Conversely, mutation S227P in fibulin-5 interfered with tropoelastin binding and in the

skin of patients with homozygous S227P mutation elastin globules failed to fuse to form a continuous core of elastic fibers suggesting an elastin polymerization defect (*31*).

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