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Original Article

Periostin forms a functional complex with IgA in human serum

Junya Ono ^{a, b}, Masayuki Takai ^{a, b}, Ayami Kamei ^b, Satoshi Nunomura ^a, Yasuhiro Nanri ^a, Tomohito Yoshihara ^a, Shoichiro Ohta ^c, Koubun Yasuda ^d, Simon J. Conway ^e, Yasuyuki Yokosaki ^f, Kenji Izuhara ^{a, *}

^a Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, Saga, Japan^b Shino-Test Corporation, Sagami, Japan^c Department of Laboratory Medicine, Saga Medical School, Saga, Japan^d Department of Immunology, Hyogo College of Medicine, Nishinomiya, Japan^e HB Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA^f Cell-Matrix Frontier Lab, Health Administration Office, Hiroshima University, Hiroshima, Japan

ARTICLE INFO

Article history:

Received 19 March 2019

Received in revised form

12 May 2019

Accepted 28 May 2019

Available online xxx

Keywords:

Complex

IgA

Integrin

Periostin

Serum

Abbreviations:

LIGHT homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for binding to HVEM, a receptor expressed on T lymphocytes

KD dissociation equilibrium constants

ka association constant

kd dissociation rate constant

Cys cysteine

ABSTRACT

Background: Periostin is a matricellular protein belonging to the fasciclin family, playing a role for the pathogenesis of allergic diseases by binding to integrins on cell surfaces. Serum periostin is elevated in various allergic diseases reflecting type 2 inflammation and tissue remodeling so that for allergic diseases, periostin is expected to be a novel biomarker for diagnosis, assessing severity or prognosis, and predicting responsiveness to treatments. We have previously shown that most serum periostin exists in the oligomeric form by intermolecular disulfide bonds.

Methods: In this study, we examined how periostin forms a complex in serum, whether the periostin complex in serum is functional, and whether the complex formation interferes with reactivity to anti-periostin Abs.

Results: We found that periostin formed a complex with IgA1 at a 1:1 ratio. The periostin in the serum complex contained at least five different isoforms. However, IgA was not essential for the oligomeric formation of periostin in mouse serum or in IgA-lacking serum. The periostin-IgA complex in human serum was functional, sustaining the ability to bind to $\alpha_V\beta_3$ integrin on cell surfaces. Moreover, periostin formed the complex with IgA broadly, which interferes the binding of the Abs recognizing all of the domains except the R4 domain to periostin.

Conclusions: Periostin is a novel member of the IgA-associated molecules. These results are of great potential use to understand the pathological roles of periostin in allergic diseases and, from a practical standpoint, to develop diagnostics or therapeutic agents against periostin.

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Introduction

Periostin is a matricellular protein belonging to the fasciclin family, composed of the EMI domain in the N terminus, four fasciclin domains (R1–R4) in the middle, and an alternative splicing domain in the C terminus.^{1,2} Periostin is expressed in the periosteum, periodontal ligament, heart valves, and tendons maintaining a steady

state, playing a role in development of these organs.^{1,3} Various stimuli—IL-4/IL-13, TGF- β , histamine, LIGHT (homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for binding to HVEM, a receptor expressed on T lymphocytes), angiotensin II, connective tissue growth factor 2, bone morphogenetic protein 2, mechanical stretch, and cancer-derived factors—are known to induce periostin expression in various pathological conditions.^{1,4,5} Among these factors, IL-4/IL-13, TGF- β , histamine, and LIGHT are important to induce periostin expression in allergic diseases. Periostin binds to several integrins on cell surfaces, activating cells and playing a role as a matricellular protein for the pathogenesis of various allergic diseases including asthma, atopic dermatitis, chronic rhinosinusitis, and allergic conjunctivitis.^{2,4,6}

* Corresponding author. Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, Saga, 849-8501, Japan.

E-mail address: kizuhara@cc.saga-u.ac.jp (K. Izuhara).

Peer review under responsibility of Japanese Society of Allergology.

<https://doi.org/10.1016/j.alit.2019.05.014>

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Most serum periostin is likely derived from bone tissues; serum periostin levels are high during childhood and adolescence, when bone metabolism is active, and decline as bone growth slows.^{7,8} However, serum periostin is elevated in various allergic diseases including asthma, atopic dermatitis, chronic rhinosinusitis, and allergic conjunctivitis reflecting type 2 inflammation and tissue remodeling.^{2,4} This would be mainly attributable to the easy movement of periostin from the producing sites into blood and to the relatively low basal concentrations in serum compared to other extracellular matrix proteins (serum fibronectin/vitronectin: ~100 µg/mL vs. serum periostin ~ tens ng/mL, Ref. ⁴). Therefore, periostin is expected to be a novel biomarker useful in diagnosing diseases, assessing severities or prognosis, and predicting responsiveness to treatments for allergic diseases as well as fibrotic diseases and cancers.^{2,4}

Periostin is a protein whose molecular weight is around 80 kDa.⁹ We have previously shown that most serum periostin exists in the oligomeric form, which migrates at around 250 kDa on SDS-PAGE.¹⁰ The oligomeric form of periostin is assembled by intermolecular disulfide bonds because the oligomeric form is in the non-reducing condition, whereas it is dissociated into the monomeric form in the reducing condition. This finding brings up several questions: (1) whether the oligomeric form of periostin is the homo-oligomer of periostin or the hetero-oligomer composed of periostin and other proteins, (2) whether the oligomeric form of periostin is functional as a matricellular protein, and (3) whether the oligomer formation interferes with the binding of the anti-periostin Abs for diagnostics.

IgA is the most produced isotype of immunoglobulin in the body, and serum IgA is the second most abundant isotype in the circulatory system.^{11,12} Secretory IgA is synthesized in local plasma cells at the basal side of mucosal epithelial cells, then transported to the apical side, playing a critical role in host defense. In contrast, serum IgA is synthesized in bone marrow plasma cells, with anti-inflammatory actions rather than systemic immune responses. It is known that serum IgA can form complexes with α 1-microglobulin,¹³ α 1-anti-trypsin,^{14,15} and fibronectin.¹⁶

In this study, we examined the purified oligomeric form of periostin in human serum, finding that it was the heterodimer of periostin and IgA. The complex of periostin and IgA was functional, sustaining an ability to bind to $\alpha_v\beta_3$ integrin, one of the main periostin receptors, on cell surfaces. Moreover, periostin formed the complex with IgA broadly, which interferes the binding of the Abs recognizing all of the domains except the R4 domain to periostin. These results demonstrate that periostin as a novel member of the IgA-associated molecules and are of great potential use to understand the pathological roles of periostin in allergic diseases and, from a practical standpoint, to develop diagnostics or therapeutic agents against periostin.

Methods

Purification of the periostin complex in human serum

The periostin complex in human serum was purified from the pooled serum of healthy subjects (300 mL, n = 22). The ammonium sulfate precipitation (the 40–50% precipitate fraction) was pre-cleared by Albumin & IgG Depletion Spin Trap (GE Healthcare UK Ltd, Little Chalfont, UK), followed by incubation with rat IgG-conjugated beads. The supernatants were applied to NHS-activated Sepharose 4 Fast Flow (GE Healthcare UK Ltd) conjugated with F(ab')₂ of monoclonal anti-periostin Ab (SS18A) by 250 µg of Ab for 1 mL of the beads. After incubation at 4 °C overnight, the immunoprecipitates were eluted by buffer containing 50 mM glycine (pH 2.7) and neutralized at pH 7.4 with Tris-buffered saline (pH 10.0) with 0.1% TritonX-100. This study was approved by

the Ethics Committee of Saga University (#25–63). All subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

LC-MS analysis

The extracted peptides from purified samples were analyzed using LC-MS. The LC-ESI-TOF-MS system was composed of a Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) and a Triple TOF 5600 + mass spectrometer (m/z100–2000, AB Sciex, Framingham, MA, USA). Data were processed and searched against the human SwissProt database.

Western blotting

Western blotting was performed as previously described.¹⁷ Periostin was detected using 5 µg/mL of biotin-conjugated anti-periostin Ab (SS19C, SS17D or SS24D) for primary Ab and poly HRP-conjugated streptavidine (Stereospecific Detection Technologies, Baesweiler, Germany) for secondary Ab. IgA was detected using HRP-conjugated anti human IgA (ThermoFisher Scientific, Waltham, MA, USA) or mouse IgA (Abcam, Cambridge, UK). The chemiluminescence was enhanced by ECL solution (GE Healthcare UK Ltd), and colorimetric DAB signals were detected by a FluorChem FC2 imager (Alpha Innotech, Kasendorf, Germany). We confirmed that Abs using in this study does not recognize F(ab')₂ of SS18A, the Ab used for immunoprecipitation, IgG and IgM.

Immunoprecipitation

NHS-activated Sepharose beads were conjugated with either anti-periostin Abs (SS18A, SS19A, and SS19D), anti-human IgG (Abcam), anti-human IgM Ab (Abcam), or anti-human IgA Ab (Abcam). One hundred µL of either recombinant periostin (lacking exons 17, 18, and 21) or the periostin complex in human serum were incubated with 100 µL of the Ab-conjugated beads or Pierce Jacalin Agarose (ThermoFisher Scientific) in 1 mL Tris-buffered saline containing 0.5% casein at 4 °C overnight. After centrifugation, the supernatants were applied to ELISA. We confirmed that the supernatant after immunoprecipitation by SS18A does not contain the residual SS18A.

ELISA

Human and mouse periostin was measured using ELISA kits (SS18A × SS17B for human, SS19A × SS19C for mouse) as previously described.¹⁸ Human and mouse IgA was measured using a Human IgA ELISA Kit (Abcam) and a Mouse IgA ELISA Kit (Abcam), respectively.

Generation of monoclonal anti-periostin Abs

Several types of recombinant periostin proteins, shown in [Supplementary Figure 1 and 2](#), were prepared using *Drosophila* S2 cells, as previously described.¹⁹ Monoclonal anti-periostin Abs were generated using recombinant proteins as an antigen, as previously described.¹⁰ We determined the recognition sites of the generated monoclonal anti-periostin Abs by immobilized ELISA. Among the monoclonal anti-periostin Abs using the isoform lacking exons 17, 18 and 21 as an antigen, we denoted the Abs specifically recognizing the junctional regions of exons 16 and 19 and exons 20 and 22 as SS17D and SS24D, respectively. Animal studies were undertaken following the guidelines for care and use of experimental animals of the Japanese Association for Laboratory Animals Science

(1987) and were approved by the Saga University Animal Care and Use Committee of the University of Saga (#29-06-2).

Mice

C57BL6 background *Rag2*^{-/-} mice were purchased from Taconic Biosciences (Rensselaer, NY, USA) and maintained. To prepare *Rag2*^{-/-}, *Rag2*^{+/-}, and *Rag2*^{+/+} mice, we crossed male *Rag2*^{+/-} mice with female *Rag2*^{+/-} mice. BALB/c background *Postn*^{-/-} mice and their wild type littermates were prepared as previously described.²⁰ All experiments were performed at Saga Medical School following the guidelines for care and use of experimental animals required by the Japanese Association for Laboratory Animals Science (1987) and approved by the Saga University Animal Care and Use Committee (Saga, Japan).

Serum derived from IgA-deficient patients

Serum of IgA-deficient patients was purchased from TRINA BioReactives AG (Naenikon, Switzerland) and ProMedDx (Norton, MA, USA). Further information and the IgA concentrations of these patients are depicted in [Supplementary Table 1](#).

Cell adhesion assay

Cell adhesion assay was performed as previously described with slight modifications.²¹ Briefly, 96-well plates (Thermo Fisher Scientific) were coated with either 1% BSA, 10 µg/mL recombinant human periostin (R&D Systems, Minneapolis, MN, USA), or 10 µg/mL (as the concentration of periostin) periostin complex purified from human serum at 4 °C for 16 h, and then wells were blocked with 1% BSA in serum-free DMEM. For the inhibition assay, each 50 µg/mL anti-periostin Ab were added to BSA- or serum periostin-coated wells. Mock- or β₃-transfected SW480 cells on which α_v and β₅ were endogenously expressed were prepared as previously described.²¹ Five × 10⁴ cells in 100 µL of serum-free DMEM containing 0.5% BSA and 0.25 mM MnCl₂ were seeded onto each well. Plates were centrifuged at 10×g for 5 min followed by incubation for 1 h at 37 °C. Unbound cells were washed away, and the adherent cells were fixed with 1% formaldehyde and were stained with 0.5% crystal violet. The stain was extracted with 2% Triton X-100 and was quantified by measuring the absorbance at 620 nm with a microplate reader (Tecan, Männedorf, Switzerland).

Surface plasmon resonance sensor analysis

The interaction between anti-periostin Abs and recombinant monomeric periostin protein or serum periostin complex purified from human serum was analyzed by Biacore X100 (GE Healthcare UK Ltd). All binding studies were performed in a running buffer, phosphate buffered saline containing 0.05% tween-20 (pH7.4) at 25 °C. Antigens were diluted with the running buffer, and a serial dilution series was created with a two-fold dilution factor. A mouse antibody capturing kit (GE Healthcare UK Ltd) was used to prepare the Biacore CM5 chip surface. Briefly, rabbit anti-mouse polyclonal Ab was first immobilized onto a CM5 sensor chip using the amine coupling method according to the manufacturer's instructions, followed by capturing mouse or rat anti-periostin monoclonal Abs for 1 min at a speed of 5 µL/min. Different concentrations of antigens were then injected for 2 min at a speed of 30 µL/min. The dissociation of bound antigens from the Abs was evaluated by generating kinetics sensorgrams for 3 min with single-cycle kinetics. The reference surface was generated by the flow cells by treatment without ligands. All biosensor data processing and the analyses were performed using Biacore X100 Evaluation Software

version 2.0.2. Kinetic parameters were evaluated using a 1:1 binding model with mass transfer using global R_{max} parameters, and the association and dissociation rate constants (k_a and k_d) as well as dissociation equilibrium constants (K_D) were calculated.

Statistical analysis

Results are presented as means ± SD. Statistical analyses were performed using a paired Student t test for the coupled comparisons and Dunnett test for the multi-coupled comparisons. *P* < 0.05 was considered statistically significant.

Results

Identifying the composition of the periostin complex in human serum

To identify the composition of the periostin complex in human serum, we adopted the procedure shown in [Figure 1A](#). After we removed excess albumin and immunoglobulins using Albumin & IgG Depletion Spin Trap and then nonspecifically bound proteins using control rat IgG-conjugated beads, we immunoprecipitated the periostin complex by SS18A. The purity of the periostin complex (240 kDa) was estimated to be more than 90% by protein staining ([Fig. 1B](#), left panel). We recovered more than 80% of periostin from the initial materials. When this complex was reduced, four major bands appeared: 58 kDa, 64 kDa, 74 kDa, and 82 kDa ([Fig. 1B](#), right panel). Western blotting for periostin showed that the periostin complex (240 kDa) in non-reducing condition contained five sizes of periostin—74 kDa, 79 kDa, 82 kDa, 86 kDa, and 91 kDa—in reducing condition ([Fig. 1C](#)). It is of note that some bands overlapped, but some did not, between the protein staining and Western blotting in reducing condition, suggesting that some bands detected in the protein staining are different from periostin. We then cut the 240 kDa bands in non-reducing condition and eluted the protein followed by applying it to LC/MS analysis, which showed that the 240 kDa band contained several peptides corresponding to only periostin and IgA (data not shown). Moreover, Western blotting for IgA showed that the periostin complex (240 kDa) in non-reducing condition and the 58 kDa protein in reducing condition contained heavy chains of IgA ([Fig. 1D](#)). These results demonstrate that IgA is a component of the periostin complex in human serum.

Stoichiometric analysis of the periostin complex with IgA

Serum IgA is much more abundant than serum periostin (0.8–4.5 mg/mL vs. 10–90 ng/mL, Refs. ^{14,18}). We next examined how the periostin complex is formed with IgA in human serum. When we incubated recombinant periostin or periostin purified from human serum by various Abs, anti-periostin Ab (SS18A) completely immunoprecipitated recombinant periostin or the periostin purified from human serum, but neither anti-IgG nor anti-IgM did so ([Fig. 2A, B, D, E](#)). SS19D, another anti-periostin Ab, recognizing only the monomeric, but not the oligomeric form of periostin,¹⁰ did immunoprecipitate almost 60% of recombinant periostin or 20% of periostin purified from human serum, which is consistent with the ratios of the monomeric form in recombinant periostin and human serum periostin as previously described.¹⁰ Anti-IgA Ab significantly (~80%) cleared periostin purified from human serum, consistent with the oligomeric form of periostin, but not recombinant periostin ([Fig. 2A, B, D, E](#)). SS18A almost completely cleared IgA from periostin purified from human serum, whereas SS19D had only a slight effect on it ([Fig. 2C](#)). Decreased residual periostin ([Fig. 2B](#)) or IgA ([Fig. 2C](#)) by the immunoprecipitation with anti-IgM Ab ([Fig. 2B](#)) or with either anti-

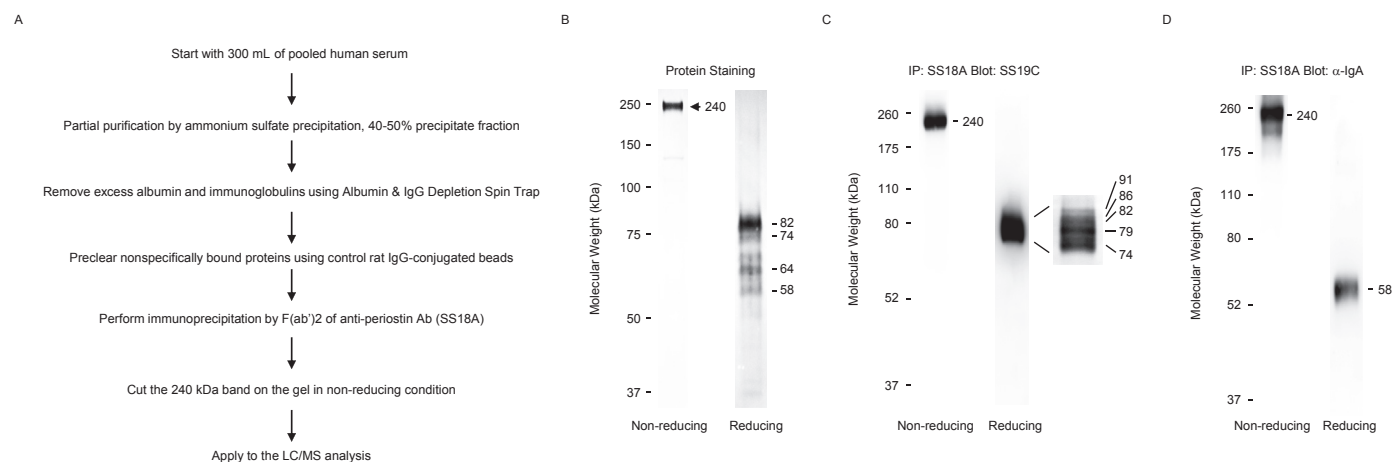


Fig. 1. Identifying the composition of the periostin complex in human serum. **(A)** Procedures to identify the composition of the periostin complex in human serum. **(B–D)** Immunoprecipitated proteins by SS18A developed by protein staining **(B)**, blotted by SS19C **(C)** or by anti-IgA Ab **(D)** in non-reducing (left panel) or reducing (right panel) condition. The arrow in panel B indicates the band cut from the gel and subjected to LC-MS analysis.

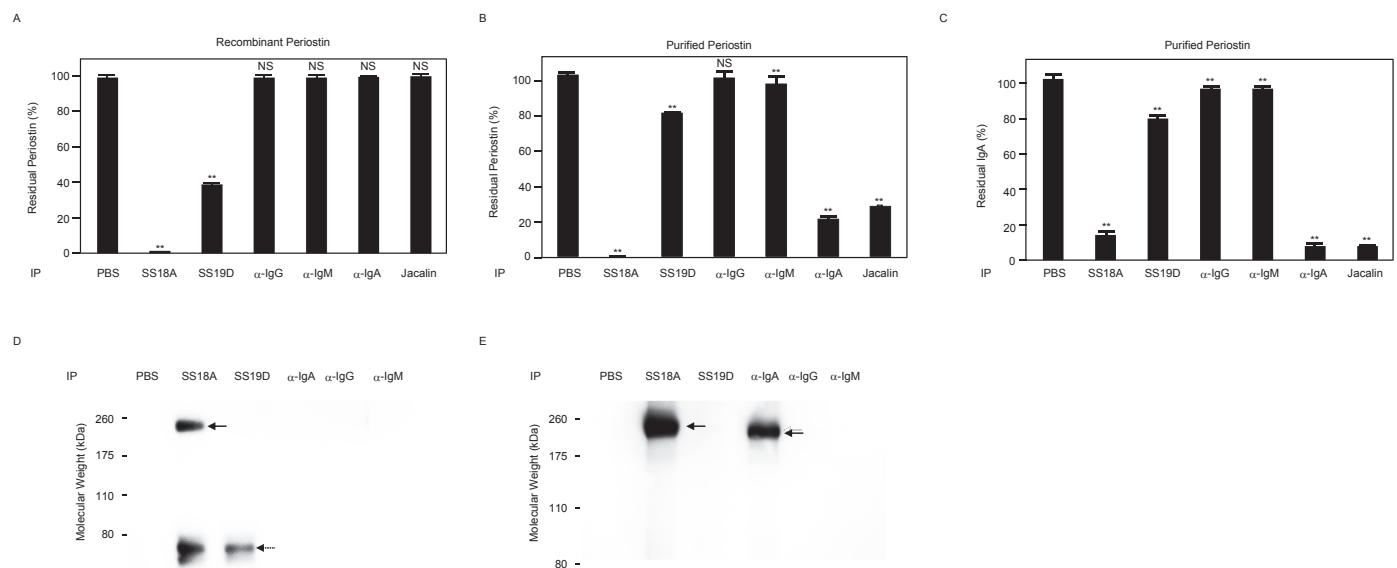


Fig. 2. Stoichiometric analysis of serum periostin-IgA complex. **(A–C)** Recombinant periostin **(A)** or periostin purified from human serum **(B, C)** were immunodepleted by SS18A-, SS19D-, anti-IgG Ab-, anti-IgA Ab-, or Jacalin-conjugated beads. Periostin **(A, B)** or IgA **(C)** concentrations in the supernatants after the immunodepletion are depicted. The statistical significance compared with PBS-treated samples are depicted. ** $P < 0.01$, N.S.: not significant. **(D, E)** Recombinant periostin **(D)** or periostin purified from human serum **(E)** were immunodepleted by SS18A, SS19D, anti-IgA Ab, anti-IgG Ab, or anti-IgM Ab. Western blotting of the immunoprecipitates by the indicated Abs followed by blotting by SS19C in non-reducing condition is depicted.

IgG Ab or anti-IgM (Fig. 2C) with statistical significance would be due to nonspecific effects of Ab-conjugated beads. Moreover, we found that similar concentrations of periostin and IgA were contained in periostin purified from human serum (data not shown). Actually, the sum of the molecular weights of periostin (~80 kDa) and IgA (160 kDa) corresponded closely with the size of the periostin complex in non-reducing condition (240 kDa), suggesting that periostin and IgA would assemble into the complex at a 1:1 ratio. Moreover, serum IgA is composed mainly of IgA1 (85%) and to a lesser extent IgA2 (15%), and Jacalin can bind to IgA1, but not to IgA2. Jacalin-conjugated beads immunoprecipitated periostin or IgA from periostin purified from human serum as well as did treatment with anti-IgA Ab (Fig. 2B, C), suggesting that periostin forms a complex with IgA1, and not with IgA2. These results demonstrate that the periostin complex is composed of periostin and IgA1 at a 1:1 ratio.

Involvement of periostin isoforms in the periostin complex

It is known that nine isoforms of periostin created by splicing in the C-terminal region are expressed in normal tissues or cancers.²² Some variants are registered at <https://www.ncbi.nlm.nih.gov/gene/10631>. Eight variants in all have been officially reported (Fig. 3A). Given that the periostin complex showed five bands of different sizes—74 kDa, 79 kDa, 82 kDa, 86 kDa, and 91 kDa—on SDS-PAGE (Fig. 1C, 3B), it was reasonable to think that at least five kinds of periostin isoforms are involved in the periostin complex. In order to annotate these bands, we took advantage of two monoclonal anti-periostin Abs, SS17D and SS24D. We confirmed that these two generated by the junction regions between exons 16 and 19, and exons 20 and 22, respectively, as antigens, specifically recognized these regions (Supplementary Fig. 1). SS17D recognized only the 79 kDa band, which was not recognized by

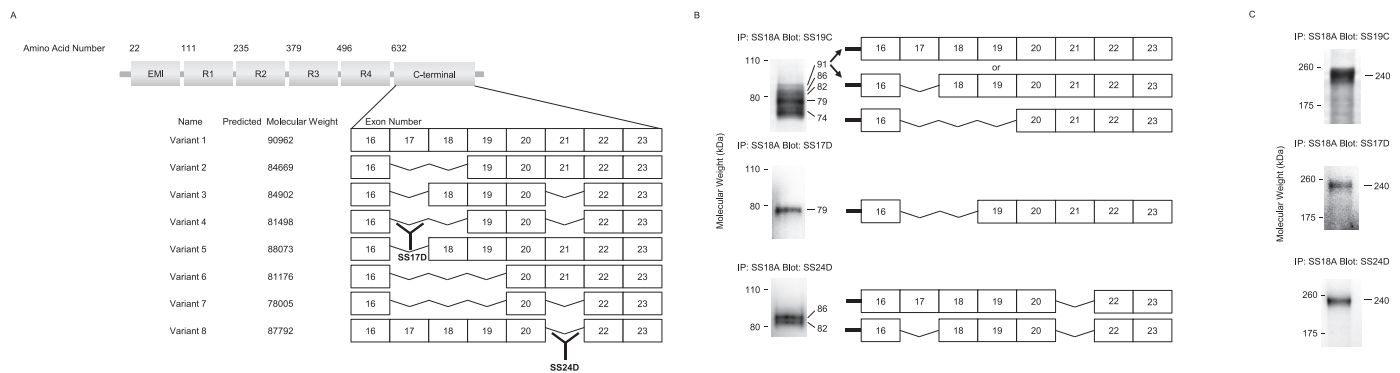


Fig. 3. Involvement of periostin isoforms in the periostin complex. **(A)** The domain structures of each splicing variant of periostin. The predicted molecular weight of each isoform and the recognition sites of SS17D and SS24D are depicted. **(B, C)** Western blotting of periostin purified from human serum by SS19C, SS17D, or SS24D in reducing **(B)** or non-reducing **(C)** condition. The presumed splicing variant of each band is depicted.

SS24D (Fig. 3B), suggesting that this band corresponded to variant 2, having exons 16, 19, 20, 21, 22, and 23. SS24D recognized two bands with 82 kDa and 86 kDa, not recognized by SS17D, suggesting that these bands corresponded to variant 3 having exons 16, 18, 19, 20, 22, and 23 and to variant 8 having exons 16, 17, 18, 19, 20, 22, and 23, respectively. Since the 74 kDa band was not recognized by either SS17D or SS24D and the size was smaller than 79 kDa, it would be variant 6 having exons 16, 20, 21, 22, and 23. The 91 kDa band that was not recognized by either SS17D or SS24D and whose size was greater than 86 kDa would be either variant 1, having all exons, or variant 5, having exons 16, 18, 19, 20, 21, 22, and 23. All five of these bands migrated as the 240 kDa band in non-reducing condition (Fig. 3C). Thus, the periostin complex in human serum contains at least five different isoforms of periostin.

No involvement of IgA in the periostin complex in mouse serum

Serum periostin in mice (0.5–2.0 µg/mL) was significantly higher than in human serum periostin (10–90 ng/mL) as estimated by ELISA (SS19A × SS19C for mouse, SS18A × SS17B for human), probably due to relatively active bone metabolism. In contrast, serum IgA in mice is much less than in humans (~0.27 mg/mL vs. 0.8–4.5 mg/mL Refs. 14,23). It is known that serum IgA from human and from mouse differs in the proportions of monomeric and polymeric forms and in the usage of clearance via the hepatobiliary route.^{11,12} Therefore, we next addressed whether serum periostin in mice forms a complex with IgA as it does in human serum, using wild-type mice and *Rag2*-deficient mice lacking IgA. Serum periostin levels were invariant among wild-type and *Rag2*-deficient mice, but were not detected in *Postn*-deficient mice, whereas serum IgA levels were invariant among wild-type and *Postn*-deficient mice, but were not detected in *Rag2*-deficient mice (Fig. 4A, B). Serum IgA levels were higher in *Postn*^{+/+} mice with the BALB/c background than *Rag2*^{+/+} mice with the C57BL/6 background as previously described.²⁴ The immunoprecipitates by the periostin Ab contained periostin in both wild type and *Rag2*-deficient, but not *Postn*-deficient mice in both non-reducing and reducing conditions, whereas these immunoprecipitates lacked IgA in all three types of mice (Fig. 4C, D). These results suggest that the periostin complex in mice does not contain IgA, in contrast to the human periostin complex, and that periostin would form the oligomer by itself or with some molecule(s) other than IgA.

Oligomer formation of periostin in serum derived from IgA-deficient patients

Given that periostin forms a complex with IgA in human serum, we next sought to learn whether periostin forms oligomers in

serum derived from IgA-deficient patients. The IgA and periostin concentrations of the serum derived from healthy subjects and from IgA-deficient patients used for the analyses are shown in Supplementary Table 1. In IgA-deficient patients, the IgA concentrations were very low, with some samples below the detection level. However, the concentrations of total periostin and monomeric periostin in IgA-deficient patients were almost the same as those of healthy subjects (Supplementary Table 1). The immunoprecipitates of periostin in serum of IgA-deficient patients lacked IgA; however, periostin formed oligomers to the same extent as in healthy subjects (Fig. 5). These results suggest that periostin forms oligomers by itself or with other unknown molecule(s) in IgA-lacking or -decreased serum as well as it does in mouse serum.

Ability of the periostin complex to bind to integrins

We have previously demonstrated that periostin derived from IL-13-stimulated fibroblasts acts as a matricellular protein by binding mainly to $\alpha_v\beta_3$ integrin and complementary to $\alpha_v\beta_5$ integrin on keratinocytes activating NF- κ B.²⁰ This mesenchymal/epithelial interaction is critical for the onset and/or the amplification of allergic skin inflammation. We investigated whether the complex of periostin and IgA is functional, sustaining an ability to bind to $\alpha_v\beta_3$ integrin on the cell surface using mock- and β_3 -transfected SW480 cells on which α_v and β_5 are endogenously expressed.²¹ Integrin α_v and β_3 were expressed on β_3 -transfected SW480 cells (Nanri, unpublished data). β_3 -transfected SW480 cells adhered to both recombinant periostin-coated plates and serum periostin-coated plates, compared to mock-transfected cells, whereas neither mock-transfected nor β_3 -transfected SW480 cells adhered to BSA-coated plates (Fig. 6A). β_3 -transfected SW480 cells adhered to both non-reduced and reduced serum periostin-coated plates, suggesting that the periostin complex binds to $\alpha_v\beta_3$ integrin at the same level as the monomeric periostin.

The binding site on periostin to $\alpha_v\beta_3$ integrin remained obscure because periostin does not have an Arg-Gly-Asp sequence, the typical binding site to integrins.²⁵ We then examined the inhibitory effects on adhesion to serum periostin-coated plates of various monoclonal anti-periostin Abs of β_3 -transfected SW480 cells. We confirmed that the same amount of each Ab bound to periostin (data not shown). The mixture of all Abs completely inhibited the adhesion of the cells to serum periostin-coated plates, whereas the effects of each Ab were variable; SS18A, SS19C, SS16A, and SS17B recognizing R1-R4 domains, respectively, had relatively strong inhibitory effects, whereas SS20A and SS19B recognizing the EMI domain and the C-terminal domain, respectively, had weak inhibitory effects (Fig. 6B). These results demonstrate that the complex of

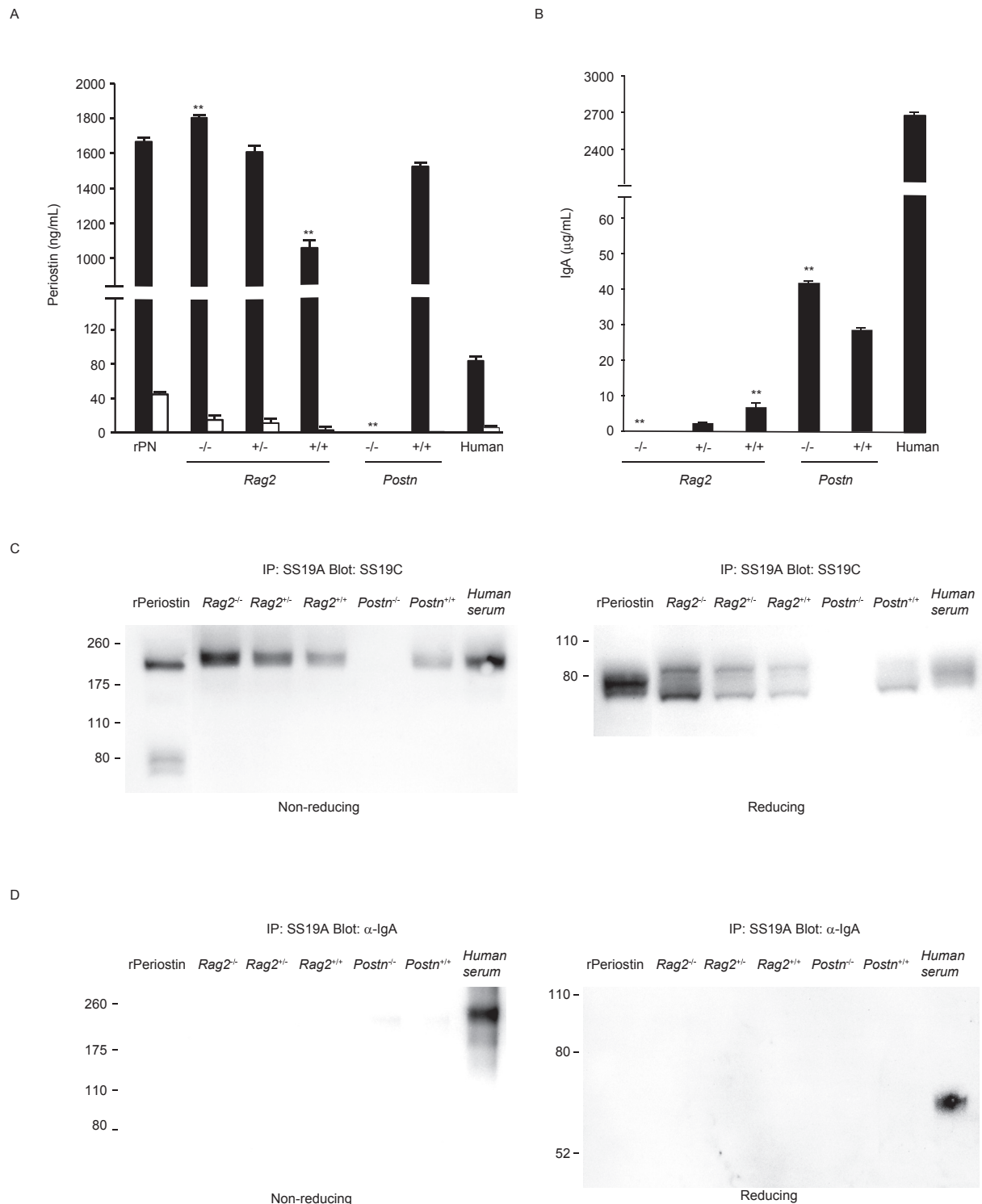


Fig. 4. No involvement of IgA in the periostin complex in mouse serum. **(A, B)** Serum periostin **(A)** and IgA **(B)** levels in the indicated genotypes of mice or human. The statistical significance compared with Rag2^{-/-} mice or Postn^{+/-} mice are depicted. ***P* < 0.01. **(C, D)** Western blotting of recombinant periostin, serum of the indicated mice, or human serum by SS19A followed by blotting by SS19C **(C)** or anti-mouse and human IgA Ab **(D)** in non-reducing or reducing condition.

periostin and IgA sustains an ability to bind to $\alpha_V\beta_3$ integrin on cell surfaces and that periostin would broadly bind to $\alpha_V\beta_3$ integrin, mainly via the fasciclin domains (R1-R4 domains), although we cannot exclude the possibility that large structures of Abs interfered the binding of periostin to $\alpha_V\beta_3$ integrin.

Interference of the periostin-IgA complex formation with the reactivity of anti-periostin Abs

Currently, serum periostin has been applied as a biomarker to diagnose diseases, assess severity or establish prognosis, and

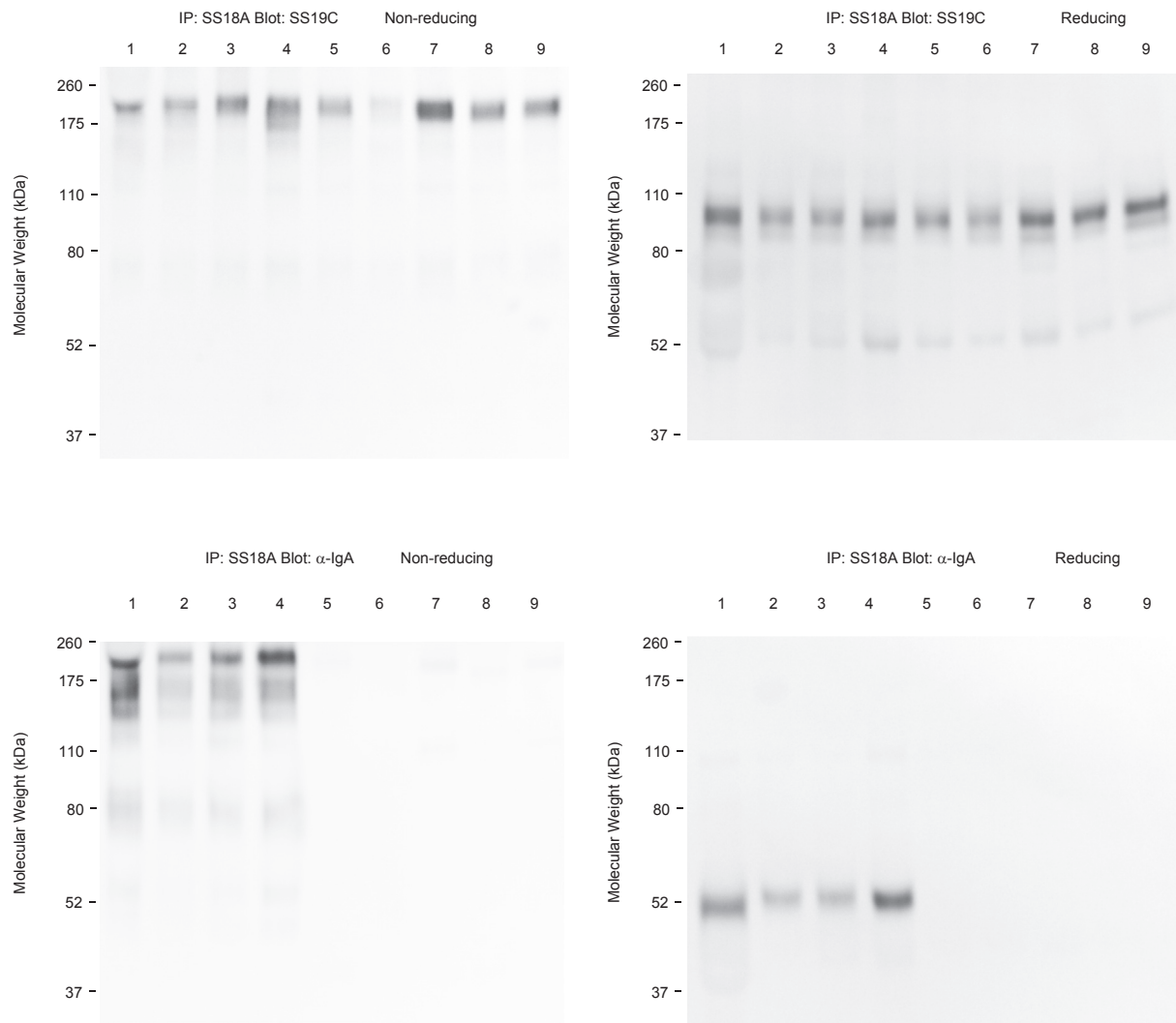


Fig. 5. Oligomer formation of periostin in serum derived from IgA-deficient patients. Western blotting of human serum derived from healthy subjects (lanes 1–4) or IgA-deficient patients (lanes 5–9) by SS18A followed by blotting by SS19C (upper panels) or anti-IgA Ab (lower panels) in non-reducing (left panels) or reducing (right panels) condition.

predict responsiveness to treatments for allergic diseases as well as fibrotic diseases and cancers.^{2,4} Since recombinant periostin is usually used as a standard protein, it is of practical importance to know whether the formation of the periostin-IgA complex interferes with the reactivity of anti-periostin Abs used for diagnostics. Using Biacore analysis, we therefore compared the affinities of various anti-periostin Abs, recognizing each domain of periostin with recombinant protein or with periostin protein purified from human serum. The affinities of Abs recognizing the EMI, R1, R2, R3, and the C-terminal domains to the periostin purified from human serum decreased compared with those to recombinant protein (the EMI domain, 15-fold; the R1 domain, 11-fold; the R2 domain, 2.5–9.1-fold; the R3 domain, 3.5–5.7-fold; and the C-terminal domain, 4.2–7.7-fold). Notably, those of the R4 domain did not change (1.3–1.6-fold, Table 1). The decreased affinities of these Abs, estimated as increase of KDs, were ascribed to both the decreased association constant (k_a) and the increased dissociation rate constant (k_d) values. These results indicated that periostin forms a complex with IgA broadly, which interferes the binding of the Abs recognizing all of the domains except the R4 domain to periostin.

Discussion

In this study, we present evidence that periostin forms a complex with IgA in human serum. The pathophysiological role of formation of the periostin and IgA complex remains obscure. However, our present finding suggests that periostin may play some immunological role associated with IgA. It is known that serum IgA binds to Fc α RI/CD89 or Fc α / μ R on cell surfaces.²⁵ These receptors are expressed on many immune cells such as neutrophils, monocytes, eosinophils, plasma cells, and dendritic cells. On the other hand, it is known that periostin acts as a matricellular protein on immune cells such as eosinophils^{26–28} and mast cells²⁹ activating these cells. Serum IgA may play a role in recruiting periostin to these target immune cells. In this study, we demonstrate that the periostin-IgA complex is functional sustaining an ability to bind to integrin $\alpha_v\beta_3$ (Fig. 6). This may suggest that periostin produced in lesions of allergic diseases circulates in the body and then is involved in the onset of inflammation in lesions other than periostin-producing sites. Moreover, it is known that periostin is secreted in several body fluids such as sputum and tears in asthma and allergic conjunctivitis, respectively.^{30,31} It is of great interest to

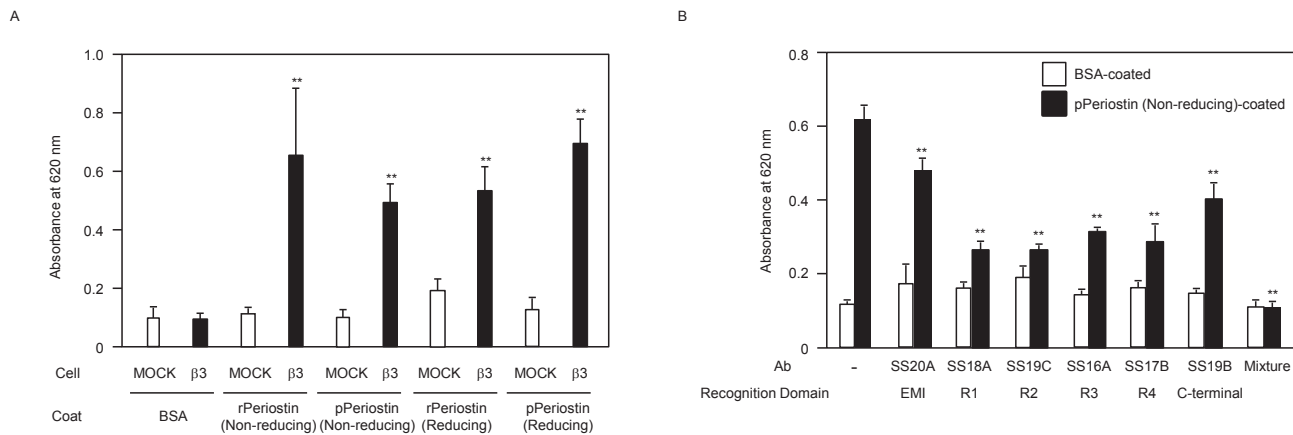


Fig. 6. Ability of the periostin complex to bind to integrins. **(A)** Adhesion of mock- (blank) or $\beta 3$ -transfected (black) SW480 cells to BSA, non-reduced or reduced recombinant (r) periostin, and non-reduced or reduced purified (p) serum periostin. Each bar represents the mean ($\pm$ S.D.) of eight wells in two different experiments. The statistical significance compared with BSA-treated samples are depicted. **(B)** Adhesion of $\beta 3$ -transfected SW480 cells to BSA (blank) or non-reduced purified serum periostin (black) in the presence of SS20A, SS18A, SS19C, SS16A, SS17B, SS19B, or mixture of all Abs. Recognition domain of each Ab is depicted. Each bar represents the mean ($\pm$ S.D.) of six wells in two different experiments. The statistical significance compared with the samples without Ab treatment are depicted. $^{**}P < 0.01$.

Table 1

Comparison of the affinities of anti-periostin Abs recognizing each domain of periostin for recombinant periostin and periostin purified from human serum.

Antibody	Epitope	Recombinant periostin			Periostin purified from human serum		
		$k_a (\times 10^4 \text{ 1/Ms})$	$k_d (\times 10^{-4} \text{ 1/s})$	$KD (\times 10^{-9} \text{ M})$	$k_a (\times 10^4 \text{ 1/Ms})$	$k_d (\times 10^{-4} \text{ 1/s})$	$KD (\times 10^{-9} \text{ M})$
SS20A	EMI	5.67 ± 0.23	12.3 ± 0.14	21.7 ± 0.64	1.03 ± 0.11	32.4 ± 0.53	315.0 ± 28.3
SS18A	R1	4.61 ± 0.01	3.30 ± 0.06	7.17 ± 0.15	2.09 ± 0.08	17.0 ± 1.06	81.4 ± 8.18
SS19C	R2	11.1 ± 1.34	6.97 ± 0.68	6.31 ± 0.13	5.86 ± 0.42	24.6 ± 0.27	42.0 ± 2.56
SS23A	R2	10.0 ± 1.41	7.70 ± 0.99	7.03 ± 0.01	4.99 ± 0.01	8.88 ± 0.15	17.8 ± 0.32
SS28E	R2	7.64 ± 0.46	3.40 ± 0.02	4.46 ± 0.24	5.78 ± 0.10	8.83 ± 0.24	15.3 ± 0.14
SS29A	R2	6.49 ± 0.04	29.9 ± 2.90	46.1 ± 4.74	3.49 ± 0.04	165.1 ± 36.9	417.2 ± 20.9
SS16A	R3	19.1 ± 4.38	6.43 ± 1.25	3.39 ± 0.12	7.94 ± 0.36	15.4 ± 1.04	19.4 ± 2.18
SS27D	R3	13.2 ± 0.35	2.82 ± 0.00	2.15 ± 0.05	9.49 ± 0.27	7.10 ± 0.55	7.48 ± 0.37
SS17B	R4	123.5 ± 7.14	2.57 ± 0.04	0.21 ± 0.04	92.1 ± 7.69	2.97 ± 0.10	0.32 ± 0.04
SS19A	R4	28.0 ± 0.99	1.35 ± 0.39	0.48 ± 0.16	29.6 ± 1.13	1.80 ± 0.24	0.61 ± 0.06
SS26C	R4	25.8 ± 1.41	16.7 ± 0.35	6.47 ± 0.24	23.9 ± 0.10	25.0 ± 1.08	10.5 ± 0.41
SS19B	C-ter	49.1 ± 1.98	6.15 ± 0.47	1.25 ± 0.04	18.2 ± 0.52	9.58 ± 0.31	5.28 ± 0.02
SS21A	C-ter	15.5 ± 2.90	1.09 ± 0.27	0.70 ± 0.04	9.36 ± 0.37	5.07 ± 0.22	5.42 ± 0.02

investigate whether IgA is associated with periostin also in these body fluids and if so how IgA affects secretion or function of periostin.

We demonstrate that periostin forms a complex with IgA via disulfide bonds (Fig. 1B–D) as well as complexes of IgA with $\alpha 1$ -microglobulin¹³ or $\alpha 1$ -anti-trypsin.^{14,15} The constant region of monomeric IgA has eight cysteine (Cys) residues, six of which form intrachain disulfide bonds.³² Among the six cysteine residues, one Cys471 on the two heavy chains of each IgA monomer forms a disulfide bridge to the J chain. The remaining two cysteine residues, Cys14 and Cys68, form covalent links to the other Cys471 in dimeric IgA. Since serum IgA mainly exists as a monomeric form,^{11,12} Cys14, Cys68, and Cys471 in monomeric IgA are available for forming intermolecular disulfide bonds. In contrast, periostin has eleven cysteine residues—six at the EMI domain (Cys44, Cys60, Cys69, Cys79, Cys80, Cys92), one at the R1 domain (Cys208), two at the R2 domain (Cys311, Cys333), and two at the R3 domain (Cys467, Cys472), but no cysteine residue at the R4 domain. Thus far, it is unclear which cysteine residue in IgA and periostin contributes to forming intermolecular disulfide bonds. It is of particular interest to examine whether free Cys471 on IgA is involved in the formation of the periostin complex because it has been reported that $\alpha 1$ -microglobulin and IgA form a complex via a disulfide bond at Cys34 on $\alpha 1$ -microglobulin and Cys471 on IgA.³³ Further analysis aimed at elucidating this point is needed.

In this study, we demonstrate that periostin can associate with IgA1, but not with IgA2 (Fig. 2A–C). It is known that IgA1 and IgA2 are different in structure and in sugar moiety.³² IgA1 has a hinge region composed of 16 amino acids, whereas it lacks IgA2. The hinge region comprises a repeat of eight amino acids rich in proline, serine, and threonine and is modified by several O-linked oligosaccharides. Moreover, in addition to common N-linked oligosaccharides at Asn263 and Asn459, IgA2 has additional N-linked oligosaccharides attached to Asn166 and Asn337. These differences in the structure and the sugar moiety of IgA1 and IgA2 may affect the association with periostin.

Furthermore, we show that human serum IgA, but not mouse serum IgA, can associate with periostin (Fig. 4). It is known that human and mouse serum IgAs have different characteristics: (1) human serum IgA is monomeric, whereas mouse serum IgA is polymeric; (2) clearance via the hepatobiliary route is important for mouse IgA, but not for human IgA.^{11,12} These differences may be related to a species difference in the affinity between serum IgA and periostin. Particularly, the monomeric or the polymeric status of serum IgA is likely important for the association with periostin, because monomeric IgA can provide more free disulfide bonds, as mentioned above. Our present findings demonstrate that even in a situation in which periostin cannot form a complex with IgA, such as mouse serum or serum derived from IgA-deficient patients, periostin forms the oligomer by itself or with another unknown

molecule(s). This suggests that periostin has the secondary option to form the complex in serum. It is of great interest to see whether periostin forming a complex with IgA or other proteins have different pathophysiological actions *in vivo* due to the different partner proteins.

Thus far, eight variants of periostin have been officially reported, as shown in Figure 3A.²² However, no functional difference of these variants is known, and very little data about the variants in the pathophysiological condition are available, except for a report that the variant splicing out exon 21 is elevated in idiopathic pulmonary fibrosis patients.³⁴ In this study, we show using two monoclonal anti-periostin Abs, SS17D and SS24D, recognizing the junction regions between exons 16 and 19, and exons 20 and 22, respectively, that there exist five dominant variants in serum, among which variants 2, 3, 6, and 8 are included, the remainder being variant 1 or 5 (Fig. 3). This provides us important information about the periostin variant in steady state.

In this study, using several kinds of monoclonal Abs against each domain of periostin, we demonstrate that periostin forms a complex with IgA broadly via all of the domains except the R4 domain (Table 1), although we cannot exclude the possibility that large structures of Abs interfered the binding of periostin to $\alpha_V\beta_3$ integrin. As we have mentioned, the EMI domain and the R1-R3 domains have cysteine residues that can be used for intermolecular disulfide bonds, whereas the R4 domain does not. It is reasonable that the binding sites of periostin with IgA are consistent with the regions in which cysteine residues are available. This manner of the complex formation of periostin with IgA may affect measurement of serum periostin as a biomarker. If the Abs used to measure serum periostin recognize either the EMI domain or the R1-R3 domains, the values may be different between serum samples and recombinant periostin used as a standard. Actually, we used SS18A and SS17B recognizing the R1 and R4 domains, respectively, to measure serum periostin finding that it is upregulated in the patients with asthma, atopic dermatitis, chronic rhinosinusitis, and allergic conjunctivitis.^{2,4} Our present findings suggest that the values of serum periostin estimated by SS18A and SS17B might be different from those of “true” serum periostin. It would be of great clinical importance to compare the values of the IgA-periostin complex and these of periostin alone in serum and to determine how best to apply serum periostin to a biomarker for allergic diseases.

Acknowledgements

We thank Dr. Dovie R. Wylie for critical review of this manuscript. We also thank Ms. Tomoyo Yoshida and Ms. Maki Watanabe for technical assistance and Prof. Atsushi Kawaguchi for statistical analysis. This work was supported in part by JSPS KAKENHI Grant Number #JP16H05343 (to KI) and National Institutes of Health grant HL135657 (to S.J.C).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.05.014>.

Conflict of interest

JO, MT, and AK are employees of Shino-Test Corporation. The rest of the authors have no conflict of interest.

Authors' contributions

JO, MT, AK, SN, YN, TY, and SO performed the experiments and evaluated the data together with KI. KY prepared *Rag2*-deficient mice. YY prepared the transfectants for the SW480 cells. SJC prepared *Postn*-deficient mice. JO and KI wrote the manuscript. All authors commented on the manuscript.

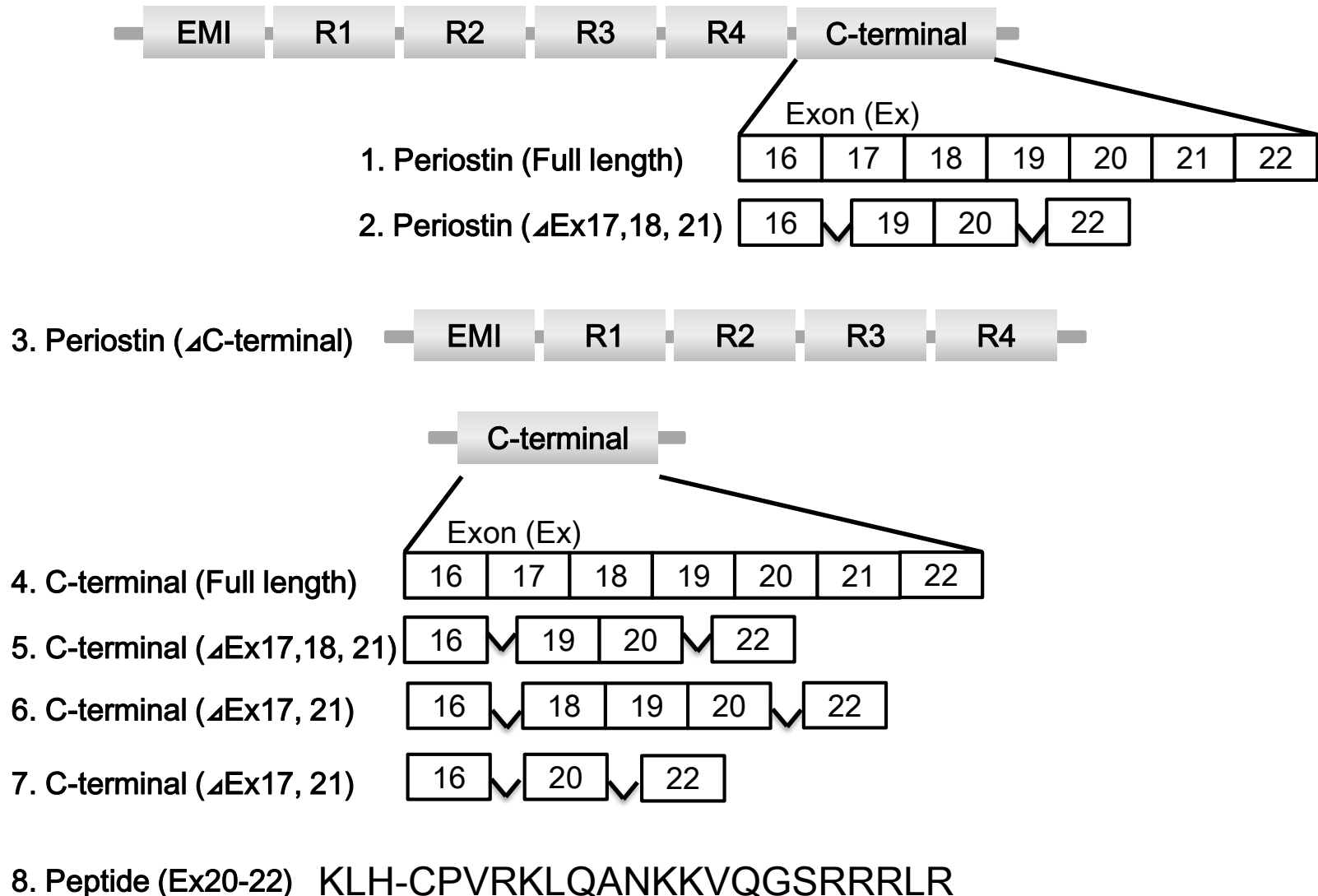
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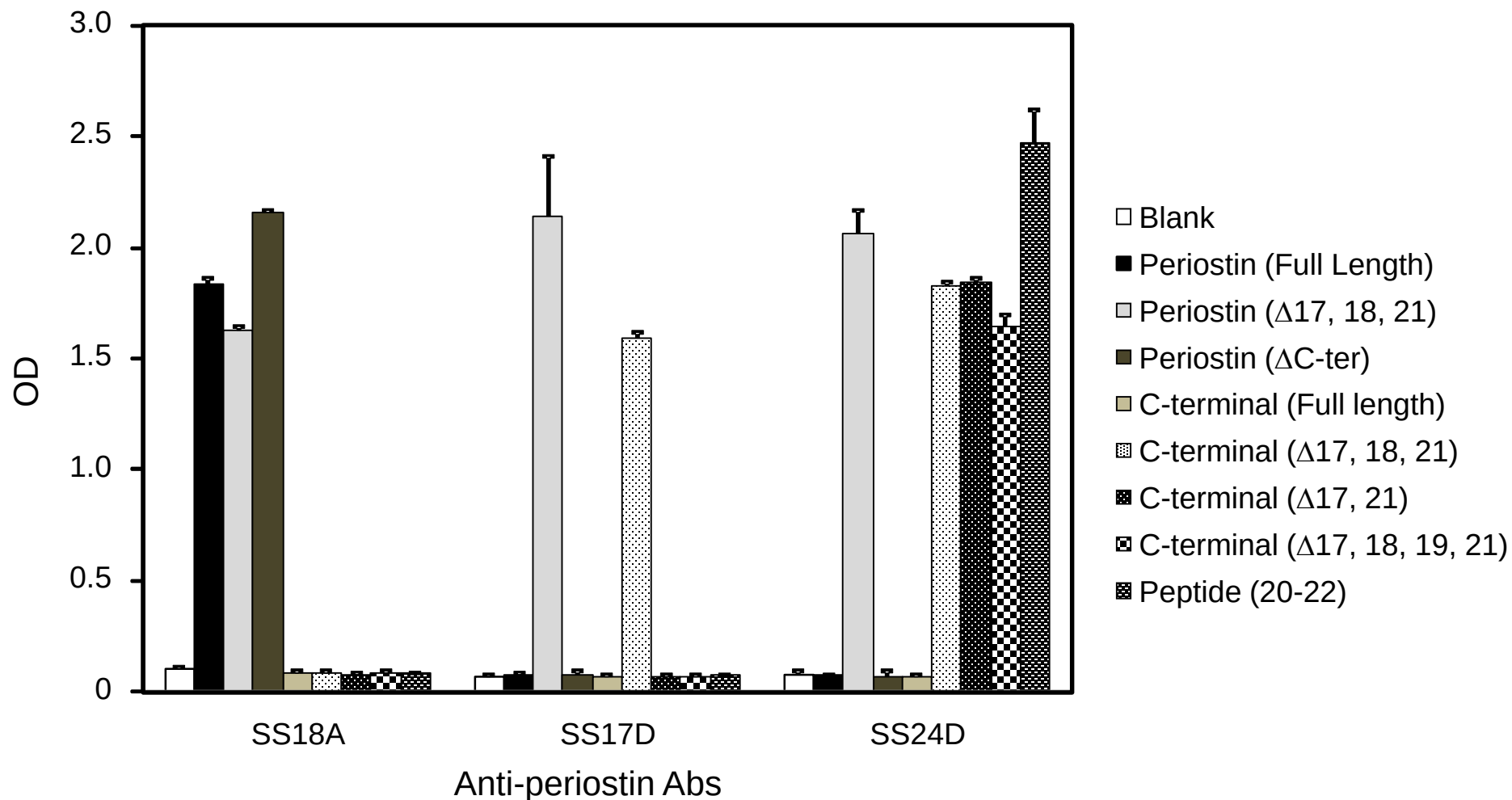
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Supplementary Table 1. The IgA concentrations and periostin of the serum derived from healthy subjects and IgA-deficient patients used for the analyses.

Subject number	Age	Sex	IgA (μg/mL)	Total periostin (ng/mL)	Monomeric periostin (ng/mL)
1	31	F	2456	77	4.2
2	49	M	4907	57	3.6
3	28	M	2848	71	4.4
4	38	F	2705	60	3.8
5	69	F	0.0	48	7.1
6	42	M	7.1	19	4.5
7	34	M	0.3	51	7.0
8	27	M	0.1	52	9.4
9	56	F	0.1	40	5.4



Supplementary Fig. 1. Schematic images of various recombinant periostin proteins used for the experiments.



Supplementary Fig. 2 Reactivities of SS18A, SS17D, and SS24D to various recombinant periostin proteins.

After the indicated various recombinant periostin proteins and the peptide were coated on the plates, 5 μ g of SS18A, SS17D, or SS19D were incubated at room temperature overnight. The reactivities of each Ab to each recombinant periostin protein or peptide are depicted. The antigens recognized by each antibody show a reaction with an absorbance of more than 0.2. Each bar represents the mean ($\pm$ S.D.) of two wells in one experiment.