*Original Research*

# The antigen-binding fragment of anti-double-stranded DNA IgG enhances F-actin formation in mesangial cells by binding to alpha-actinin-4

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## Abstract

**Anti-double-stranded DNA (dsDNA) IgG causes renal damage in patients with lupus nephritis by cross-reacting with multiple autoantigens, including alpha-actinin-4, in mesangial cells (MCs). However, how the cross-reactions play a role in mesangial phenotypic abnormalities is not well understood. Here, we investigated the effects of the fragment antigen-binding (Fab) of anti-dsDNA IgG3 on the biochemical properties of alpha-actinin-4. Experiments revealed that anti-dsDNA Fab specifically binds to alpha-actinin-4, but not G-actin. The binding by anti-dsDNA Fab sequentially increases the positive charge of alpha-actinin-4 and inhibits the affinity of alpha-actinin-4 to calcium ions. By the low shear viscosity and a co-sedimentation assay, we found that the alpha-actinin-4-induced F-actin gelation improves when anti-dsDNA Fab is added. However, the Fab control has no such effect on F-actin gelation. Furthermore, the *in vitro* cultured MCs exhibit higher F-actin expression and transforming growth factor-$\beta$1 synthesis after the incubation with anti-dsDNA Fab. Therefore, our results indicated that anti-dsDNA Fab may enhance F-actin formation by the proprietary modification of alpha-actinin-4, which could partially explain the myofibroblast-like phenotype of MCs in anti-dsDNA-positive lupus nephritis.**

**Keywords:** autoantibody, actinin, calcium ion, F-actin, mesangial cell, phenotype

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## Introduction

Anti-double-stranded DNA (dsDNA) antibodies are one of the main pathogenic factors in systemic lupus erythematosus (SLE). It has been demonstrated that the IgG but not the IgM response to dsDNA antigen is responsible for the renal damage in patients with lupus nephritis.[1] Different isotypes of anti-dsDNA antibodies play distinct roles in the pathogenesis of lupus nephritis. In the murine model of SLE, anti-dsDNA IgG3 is more responsible for the development of lupus nephritis.[2] The shift in the subclass profile of anti-dsDNA IgG from IgG3 to IgG1 causes the amelioration of lupus nephritis in MRL-lpr mice.[3] Cross-reaction is an important characteristic of the pathogenic anti-dsDNA antibodies in SLE. Several mesangial compounds have been identified as self-antigens for anti-dsDNA IgG, including laminin, annexin II, ribosomal-P and alpha-actinin.[4–6] Alpha-actinin is attracting more attention because it serves as a self-antigen in multiple autoimmune diseases, including autoimmune hepatitis[7,8] The interaction with alpha-actinin is suggested to be responsible for the nephritogenicity of anti-dsDNA antibodies.[9]

Alpha-actinin is an ubiquitous cytoskeletal protein, and it belongs to the superfamily of filamentous actin (F-actin) cross-linking proteins. At present, four isoforms of alpha-actinin have been identified. Different from the 'muscle' alpha-actinin-2 and -3 which form part of the contractile machinery in smooth muscle cells, the 'non-muscle' alpha-actinin-1 and -4 are more widely expressed[7] and present in mesangial cells (MCs) and podocytes that comprise the glomeruli.[10,11] By interacting with F-actin and certain membrane proteins, the 'non-muscle' alpha-actinins are involved in the organization of cytoskeleton and adherent junctions. The cell membrane-associated alpha-actinin-1 is concentrated in actin stress fiber ends and adherent junctions; the alpha-actinin-4 is co-localized with actin stress fibers and is dispersed in the cytoplasm and nucleus.[7] MCs in lupus nephritis become myofibroblast-like, characterized by the

activation of smooth muscle alpha-actin (alpha-SMA) expression.[12,13] It was indicated previously that the state of polymerization of the actin cytoskeleton regulates the alpha-SMA expression as well as myofibroblast differentiation in MCs.[14] Therefore, the regulation of surface properties of alpha-actinin-4 and its binding to F-actin might affect the phenotypic switch from MCs to myofibroblasts.

The $Ca^{2+}$ sensitivity of the interaction with F-actin is quite elusive on alpha-actinin from non-muscle cells.[15] The dimer structure of alpha-actinin contains four EF-hand motifs that bind $Ca^{2+}$ ions. After calcium binding, the hydrophobic methyl portion of actinin becomes exposed on the protein via conformational changes. The hydrophobic surfaces can in turn bind to basic amphiphilic helices on the target protein. Because alpha-actinin functions as an antiparallel homodimer, it is believed that calcium binding at the carboxy-terminus of one monomer influences the ability of actinin to bind at the neighboring amino-terminus. Consequently, $Ca^{2+}$-sensitive alpha-actinin decreases its activity on F-actin gelation in the presence of micromolar concentrations of calcium ions.[16] Additionally, the affinity of alpha-actinin to calcium ions is largely dependent on the electric charges of actinin.[17] The EF-hand motifs in the alpha-actinin structure supply an electronegative environment for $Ca^{2+}$ ion coordination. Therefore, the alteration of the total charge may affect the $Ca^{2+}$-sensitivity of alpha-actinin.

The fragment antigen-binding (Fab fragment) is a region on an immunoglobulin that binds to antigens. It is composed of one constant and one variable domain from each heavy and light chain of the antibody. This protein retains the specificity of the original antibody, despite removal of the crystallizable fragment (Fc).[18] The Fab fragment reduces non-specific binding that results from Fc interactions in antigen–antibody binding studies. Therefore, the utilization of the Fab fragment of anti-dsDNA IgG is conductive to elucidating the interaction between this antibody and alpha-actinin-4. The purpose of this study was to investigate the effects of anti-dsDNA Fab on the protein properties of alpha-actinin-4, which may further influence the F-actin cross-linking induced by alpha-actinin-4.

## Materials and methods

### Purification of Fab fragment from anti-dsDNA IgG

The mouse monoclonal anti-dsDNA IgG3 and isotype control were provided by Genbase Biotech Inc. (Guangzhou, China). The antigenic binding to DNA of anti-dsDNA antibody was confirmed by fluorescent microscopy using a blood smear of *Trypanosoma equiperdum*.[19] The isotype control showed no specific fluorescence in this assay. The IgG antibodies were digested using the Fab preparation kit (Fisher Scientific, Pittsburgh, PA, USA) as described previously.[20] In brief, the samples were buffer-exchanged into the kit-supplied digestion buffer containing cysteine and then added to spin columns containing immobilized papain. After a four-hour incubation at 37°C, the digest was collected by centrifugation and applied to the Protein-A columns. After a 10-min incubation, the flow-through fraction was collected. Bound fragments were eluted using the elution buffer, followed by neutralization with 1 mol/L phosphate, pH 9.0. The concentration of Fab purification was measured by absorbance at 280 nm.

### Western blotting

To verify the cross reaction between alpha-actinin and antibodies or Fab fragments, Western blotting was performed as described previously[21] with some modifications. Briefly, alpha-actinin-4 (Novus Biologicals, Littleton, CO, USA) was run on a 4–15% Mini-proTEAN TGX precast gel (Bio-Rad, Shanghai, China). After electrotransfer in Tris/glycine buffer, the polyvinylpyrrolidone membrane was blocked with 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). The anti-dsDNA antibodies/Fab or controls were normalized with kappa chain before dilution in 1% FBS/PBS-Tween. The incubation was carried out for one hour at room temperature (RT), followed by washing three times with TBS-0.05% Tween 20. The biotinylated goat anti-mouse kappa chain (0.25 $\mu$g/mL) was applied to detect the antibodies or Fab binding to alpha-actinin-4. After incubation with horseradish peroxidase-streptavidin solution (1:5000; Thermo Scientific, South Logan, UT, USA) for 30 min, the membrane was developed using an ECL system (Thermo Scientific). The binding to G-actin of antibodies or Fab fragments was detected by the same method when alpha-actinin was replaced by G-actin, which was prepared from pig stomach according to the previous procedure.[22]

### Preparation of immune complex

The conditions of antigen excess for immune complex (IC) formation were determined by immune-precipitation curves, which were prepared by incubating varying amounts of alpha-actinin-4 for one hour at 37°C and for 12 h at 4°C with fixed amounts of anti-dsDNA Fab. All protein determinations were made according to the Hartree method.[23] The presence of formed IC in the reaction solution was determined using a polyethylene glycol (PEG) precipitation assay. In brief, the reaction solution was incubated at 4°C for 12 h with an equal amount of 8% PEG in borate-buffered saline at pH 7.2. The presence of precipitation was analyzed visually. By calculation, the optimal molar ratio of antigen to Fab fragment was found to be 1:1.64, which was utilized for preparing IC with a minimal excess of alpha-actinin-4 or Fab fragment.

### Determination of the isoelectric point

The isoelectric points (pIs) of alpha-actinin-4, anti-dsDNA Fab and alpha-actinin-4/anti-dsDNA Fab complex were determined by the agarose isoelectric focusing (IEF) method.[24] In brief, aliquots of each protein sample (5 $\mu$L), along with standard solution, were applied to the ultrathin layer (0.5 mm) of 0.8% agarose gel (Fisher BioReagent, Pittsburgh, PA, USA) with a pH range of 3.0–10.0. This analysis was performed at 100 V for one hour, 200 V for

one hour and then 500 V for 30 min. Proteins in the gel were detected by Coomassie blue staining.

### Low shear viscometry

The low shear viscosity of F-actin was measured with a falling-ball viscometer as described previously.[25] F-actin (0.15 mg/mL) was polymerized for 30 min at 25°C with alpha-actinin-4 mixed with either anti-dsDNA Fab or control IgG Fab (molar ratio = 1:1.64). The reaction buffer contained 0.1 mol/L KCl, 0.5 mmol/L $MgC1_2$, 0.2 mmol/L ATP, 20 mmol/L Tris-HCl, pH 7.2 and 0.65 mmol/L ethylene glycol tetraacetic acid. The solution was sucked into a 0.1 mL pipette, one end of which was closed. After incubation, a stainless steel ball was dropped into the open end, and the time for the ball to travel a certain distance at a fixed angle of 55° from the horizontal plane was recorded. The apparent viscosity values were obtained by calibrating the viscometer with various concentrations of sucrose solution at 20°C. All values were normalized to control samples with F-actin alone.

### Co-sedimentation assay for binding to F-actin

The interaction of alpha-actinin with F-actin was examined with a co-sedimentation assay.[26] Alpha-actinin-4 was mixed with anti-dsDNA Fab or control IgG Fab (molar ratio = 1:1.64) and then added to 0.1 mg/mL of F-actin and incubated for one hour in 0.2 mL of a reaction solution, as above. The F-actin solutions were centrifuged at 10,000 rpm for two hours at 20°C, and the supernatant was discarded. The tubes were carefully washed with reaction solution to remove the proteins attached to the inner wall. The pellets were dissolved directly in the loading buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subjected to SDS/urea-PAGE. After Coomassie brilliant blue staining, the densitometric ratio of alpha-actinin/actin was determined by a gel scan accessory attached to a DU-68 spectrophotometer (Beckman, Brea, CA, USA).

### Cell culturing and immunocytochemistry staining

Murine MCs were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 5% FBS and maintained at 37°C in 5% $CO_2$ in a humidified incubator. MCs were transferred to six-well ($10^6$ cells/well) or 24-well ($10^5$ cells per well) plates, rested for two days in serum-free DMEM, and then treated with 10 $\mu$g/mL of purified mouse anti-dsDNA Fab. Non-anti-dsDNA Fab-treated or blank MCs were cultured under the same conditions as the controls. After a 48-hour stimulation, the cells and supernatant were harvested for the next experiments. Some cells were plated on gelatin-coated glass cover slips in six-well dishes and treated as above for immunocytochemistry. Coverslips were prepared with cytoskeleton fixing buffer for seven minutes.[27] Goat anti-F-actin IgG (Fisher Scientific) was diluted in PBS containing 2% normal goat serum and incubated for one hour at RT, followed by 30-min incubation with Alexa 488-conjugated secondary antibody (1:500; SouthernBiotech, Birmingham, AL, USA). Images were acquired with an LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

### F-/G-actin ratio assay

To determine the ratio of F-/G-actin, protein extracts from MCs of three groups were subjected to an F-/G-actin assay kit (Cytoskeleton, Denver, CO, USA) based on the manufacturer's protocol.[28] In brief, cells were lysed with lysis buffer and F-actin stabilization buffer and then homogenized using 23 G syringes. The cell lysates were centrifuged at 100,000 *g* for one hour at 37°C. Then the supernatants (G actin) were separated from the pellets (F actin) and immediately placed on ice. The pellets were re-suspended to the same volume as the supernatants using ice-cold $dH_2O$ containing 1% cytochalasin D and incubated for one hour on ice. Equal amounts of the samples (supernatant and pellet) were analyzed by Western blotting with rabbit anti-actin antibody (SouthernBiotech). After three washings, the membranes were incubated with goat anti-rabbit-HRP (SouthernBiotech) for 30 min. Finally, the color was developed by an ECL system (Thermo Scientific) and captured on X-ray film. Bands developed on films were quantitated by ImageJ 1.61u (National Institute of Health, Bethesda, MD, USA), and the fold expression was indicated as the relative protein level. This experiment was repeated three times separately.

### Enzyme-linked immunosorbent assay

The alpha-actinin enzyme-linked immunosorbent assay (ELISA) was performed as previously described.[29] Alpha-actinin (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 20 $\mu$g/mL was coated onto Immulon II 96-well plates (Dynatech Labs, Chantilly, VA, USA) overnight at 4°C. Plates were blocked with 2% FBS for one hour at 37°C and incubated with antibodies or Fab fragments for two hours at RT. After washing with PBS-Tween, plates were incubated with alkaline phosphatase-conjugated goat anti-mouse kappa chain (1:1000, SouthernBiotech) for one hour at 37°C, followed by substrate. The OD value was read at 405 nm. The binding to G-actin was also analyzed by ELISA with G-actin at a concentration of 5 $\mu$g/mL.

The levels of transforming growth factor (TGF)-$\beta$1 were quantitatively assessed using a sandwich ELISA kit (Cell Sciences, Canton, MA, USA), which contained capture antibody, biotin-conjugated detection antibody and standard protein. Cell culture supernatant was collected at the end of Fab fragment stimulation, and centrifuged for 5 min at 3000 rpm. To prevent the binding of TGF-$\beta$1 to plastic material, the supernatants were handled and stored with siliconized pipettes and centrifuge tubes. ELISA was performed according to the manufacturer's instructions. The concentrations of TGF-$\beta$1 were calculated by comparing sample absorbance at 450 nm with data from standard control.

### Realtime polymerase chain reaction

The total RNA was extracted from MCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNA

(cDNA) was synthesized through a reverse transcription reaction. Realtime polymerase chain reaction (PCR) amplification was performed on an ABI 7500 PCR system (Applied Biosystems, Carlsbad, CA, USA) with the cycling conditions as following: 95°C for 30 s and 40 cycles of amplification (95°C for 5 s, 60°C for 30 s). Primers were as follows: for TGF-$\beta$1, 5′-TGCGGCAGCT-GTACATTGACTT-3′ (sense) and 5′-ACTTGCAGGAGCG-CACAATCAT-3′ (antisense), amplifying a 282 bp fragment; for $\beta$-actin, 5′-TCAGCAAGCAGGAGTACGATGA-3′ (sense) and 5′-TGCGCAAGTTAGGTTTTGTCAA-3′ (antisense), amplifying a 117 bp fragment. The TGF-$\beta$1 cDNA templates were quantified by standard curves of diluted standard cDNA. The amount of PCR products was normalized with TGF-$\beta$1 to determine the relative expression ratios for each mRNA. The expression level of the objective gene was calculated according to the formulas:[30] Target gene $= 2^{-\Delta\Delta Ct} \times$ control, where $\Delta\Delta Ct = (Ct^{\text{target gene}} - Ct_{\text{reference gene}})_{\text{treat group}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{control group}}$.

### Statistical analysis

All data are expressed as the means $\pm$ standard error of the mean (SEM). The significance of differences was tested using paired or unpaired Student's *t*-tests. A value of $P < 0.05$ was considered statistically significant.

## Results

### Cross-reaction with alpha-actinin/G-actin

In alpha-actinin ELISA, both anti-dsDNA antibody and anti-dsDNA Fab exhibited strong binding affinity to the coated alpha-actinin (Figure 1a). The anti-dsDNA antibody bound to alpha-actinin more tightly than anti-dsDNA Fab when their kappa chain concentrations were normalized to be the same. There was no specific binding between the isotype controls and alpha-actinin. The results of Western blotting also confirmed that anti-dsDNA Fab inherits the antigenic binding to alpha-actinin-4, although the effect is relatively weaker than for the complete anti-dsDNA antibody (Figure 1b). There was no evidence showing that the anti-dsDNA IgG or Fab binds to G-actin in both ELISA and Western blot assay (data not shown).

### Binding to anti-dsDNA Fab modifies the properties of alpha-actinin-4

It has been proven that the conventional IEF method does not dissociate immune complexes.[24] Our results showed that the complex of alpha-actinin/anti-dsDNA Fab appears on the gel as a blurred but affirmative band (Figure 2). These bands have pIs ranging between those of alpha-actinin (5.8) and anti-dsDNA Fab (8.9), with the most visible part at pH 7.5. The distribution of the formed IC might be caused by unstable association between antigen and antibody under the electrophoresis condition. However, the binding to anti-dsDNA Fab increased the pI of alpha-actinin undoubtedly.

As a non-muscle isoform, alpha-actinin-4 is regulated by the binding of calcium ions, in the micromolar range, to the EF-hand domains. The actin binding can be inhibited completely by a high concentration of calcium ions. Figure 3 shows that the gelation of F-actin decreases as the concentration of calcium ions increases gradually. The ratio of bound alpha-actinin to F-actin also exhibited a similar tendency of binding curves. However, the alpha-actinin mixed with anti-dsDNA Fab induced a higher apparent viscosity and ratio of bound alpha-actinin to F-actin than that mixed with IgG Fab control or alpha-actinin alone for the same concentrations of calcium ions ($P < 0.05$). Additionally, to reach the same level of F-actin gelation, the alpha-actinin without addition of anti-dsDNA Fab needed to be incubated at a lower concentration of calcium.

### Anti-dsDNA Fab upregulates the F-actin cross-linking ability of alpha-actinin-4

For a low shear viscosity, the F-actin gelation was elevated as the ratio of alpha-actinin to actin increased (Figure 4a). The addition of anti-dsDNA Fab to the solution induced a higher degree of gelation than IgG control or blank control (alpha-actinin alone) (by paired *t*-test, $P = 0.0217$ and 0.0311, respectively). There was no significant difference between the controls ($P = 0.8251$). This meant that anti-dsDNA Fab enhances the alpha-actinin-4-induced F-actin cross-linking. Additionally, we found that the enhancement of anti-dsDNA Fab is most remarkable at the actinin/actin ratio range of 0.02–0.03. Actually, this range is optimal for the gelation activity of alpha-actinin.[31] The co-sedimentation assay showed that the interaction between alpha-actinin and F-actin is similar to the low shear viscosity (Figure 4b). The ratio of bound alpha-actinin to F-actin was higher in the anti-dsDNA Fab group than in the IgG Fab control ($P = 0.0362$) or blank control ($P = 0.0295$). The reaction curves of all groups remained similar in either the low shear viscosity or co-sedimentation assay.

### Anti-dsDNA Fab enhances F-actin expression in MCs

The confocal laser scanning microscopy revealed the difference of F-actin expression between MCs treated with anti-dsDNA Fab or controls. In Figure 5a, the MCs treated with non-anti-dsDNA Fab (IgG Fab control) or without treatment (blank control) showed normal F-actin expression; however, the anti-dsDNA Fab-treated cells contained abundant actin stress fibers. With a protein assay on the extracts from MCs, we found that MCs, after anti-dsDNA treatment, exhibited a higher F-/G-actin ratio when compared with IgG Fab control-treated or non-treated (blank) cells (Figure 5b). There was no significant difference between the two controls.

### TGF-$\beta$1 expression is promoted in anti-dsDNA Fab-incubated MCs

By realtime PCR, we verified the enhancement efficacy of anti-dsDNA Fab on the gene expression of TGF-$\beta$1 in

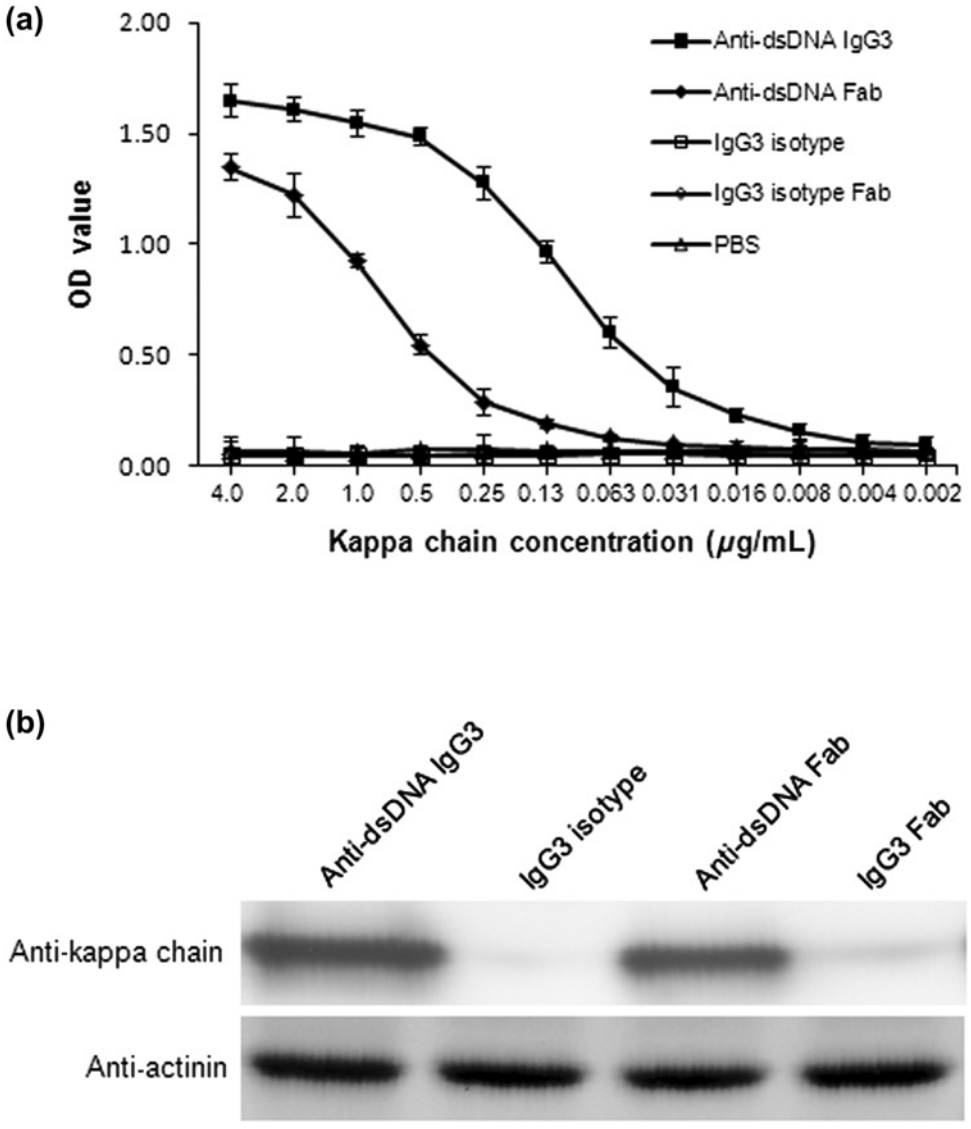


**Figure 1** The anti-dsDNA Fab exhibits the antigenic binding to alpha-actinin. (a) For the alpha-actinin ELISA, anti-dsDNA antibody, anti-dsDNA Fab, and the isotype controls were normalized with kappa chain. The anti-dsDNA antibody showed higher OD values than anti-dsDNA Fab by paired *t*-test ($P < 0.05$). There was no significant difference between the isotype controls and PBS ($P > 0.05$). (b) The Western blot analysis was performed to assess the binding to alpha-actinin-4 of anti-dsDNA antibodies/Fab fragments. The intensity ratio of alpha-actinin-4-bound protein to kappa chain was higher in anti-dsDNA IgG3 than anti-dsDNA Fab. The isotype controls revealed no specific affinity to alpha-actinin-4. Anti-dsDNA, anti-double-stranded DNA, ELISA, enzyme-linked immunosorbent assay, PBS, phosphate-buffered saline; Fab, fragment antigen-binding

MCs (Figure 6a). Compared with the cells with the addition of control Fab fragment or those as blank control, the anti-dsDNA Fab-stimulated MCs exhibited increased fold change of TGF-$\beta$1 mRNA ($P < 0.05$). The control Fab fragment had no such effect on the expression of TGF-$\beta$1 mRNA. The ELISA analysis for MCs' culture supernatants showed that compared with Fab control or blank control, there was significantly more TGF-$\beta$1 production following the anti-dsDNA Fab stimulation ($P < 0.05$) (Figure 6b). No statistical difference was found between the controls ($P > 0.05$).

## Discussion

It was reported that in normal human kidney, alpha-actinin-4 is detected in significant levels compared with alpha-actinin-1.[7] However, to exclude the cross-reaction between anti-dsDNA IgG3 and alpha-actinin-1, we also did both ELISA and Western blotting on them, and found no specific binding of anti-dsDNA IgG to alpha-actinin-1 (data not shown). Our results showed that anti-dsDNA IgG3 binds to alpha-actinin-4 specifically and the Fab fragment digested from anti-dsDNA IgG3 carries on the antigenic affinity of binding to alpha-actinin-4. The slight decrease of anti-dsDNA Fab in binding to alpha-actinin-4 might be related to the contribution of heavy chain constant region to the affinity and specificity of antibodies.[32] Actually, the charge modification of antibodies or antibody-generated fragments is an effective means of altering affinity to antigens.[33,34] Based on such a potential mechanism, the binding of anti-dsDNA Fab may lead to electrical charge alteration of alpha-actinin-4,

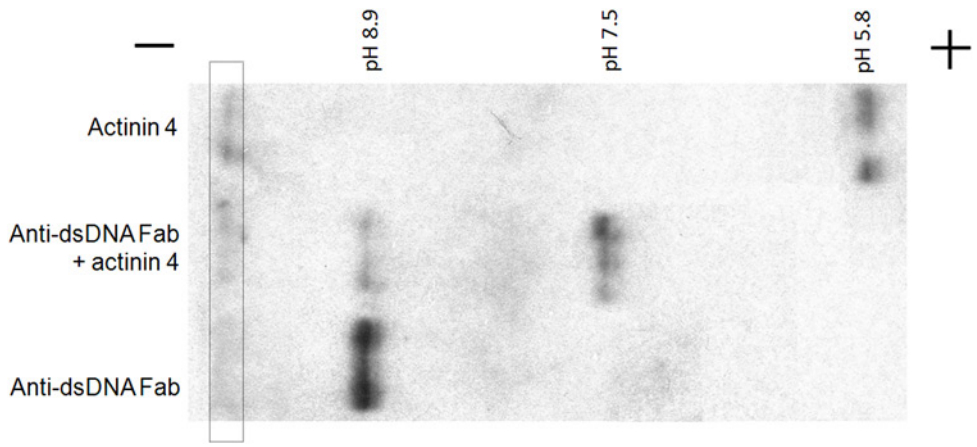


**Figure 2** Anti-double-stranded DNA (anti-dsDNA) Fab increases the isoelectric point (pI) of alpha-actinin-4. The isoelectric focusing patterns of proteins appeared on the gel with distinct pIs of 8.9 (anti-dsDNA Fab alone), 7.5 (complex of anti-dsDNA Fab/alpha-actinin-4) and 5.8 (alpha-actinin-4 alone). The frame indicated the application sites of protein samples

which can further affect the EF-hand-mediated interaction with F-actin.

The previous studies demonstrated that the anti-dsDNA IgG has a positively-charged amino acid chain orientation.

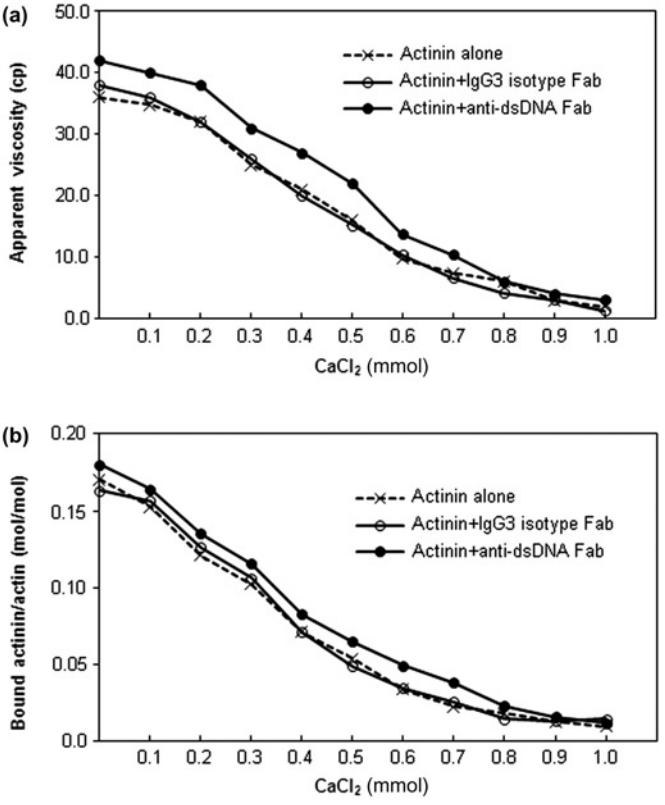


**Figure 3** The $Ca^{2+}$ sensitivity of alpha-actinin-4 after the addition of anti-dsDNA Fab or controls. (a) The low shear viscosity for alpha-actinin binding when anti-dsDNA Fab, IgG control Fab or blank control was added. At each point of $CaCl_2$ concentration, the anti-dsDNA Fab-treated actinin primarily showed higher values of F-actin gelation than the controls. (b) The co-sedimentation assay for the binding to F-actin exhibited results similar to those for a low shear viscosity. The anti-dsDNA Fab treatment increased the F-actin gelation induced by alpha-actinin-4. Anti-dsDNA, anti-double-stranded DNA; Fab, fragment antigen-binding

Liang *et al.*[35] reported that anti-dsDNA antibodies exhibited increased utilization of VH5/7183 genes and highly cationic heavy chain complementarity-determining region (CDR) 3 regions. By replacing arginine/lysine peaks at H96, H98 and H100 with neutral troughs at H95, H97 and H99, the CDR3 regions present an alternating charge. Also, GPIIIa49–66 is a platelet integrin with a strong negative charge, and can be an epitope mimicking dsDNA. The DNA-binding activities of anti-GPIIIa49–66 antibodies are found to be mainly dependent on the positively-charged amino acid in the heavy-chain CDR3.[36] Our results are consistent with these previous findings and showed that the Fab fragment of anti-dsDNA IgG has a high pI of 8.9 because of possessing positive charges. This surface property of anti-dsDNA Fab causes the increase of pI of alpha-actinin-4 when it is bound by anti-dsDNA Fab. Thereafter, the cationic charge of the formed anti-dsDNA Fab-alpha-actinin-4 complex is supposed to affect the interaction between alpha-actinin-4 and F-actin, which takes place in a $Ca^{2+}$-sensitive manner.

The existence of calcium ions inhibits the F-actin cross-linking induced by alpha-actinin. The investigation into the effect of anti-dsDNA Fab binding on the $Ca^{2+}$ sensitivity of alpha-actinin-4 could help elucidate the function of anti-dsDNA Fab in the regulation of F-actin cross-linking. The present study showed that alpha-actinin-4 is less $Ca^{2+}$-sensitive when bound by anti-dsDNA Fab. Because of the competitive inhibition of $Ca^{2+}$ ions, the partial loss of $Ca^{2+}$ sensitivity may improve the efficiency of the F-actin cross-linking induced by alpha-actinin. The F-actin cross-linking ability of alpha-actinin-4 is also increased after the addition of anti-dsDNA Fab in the absence of calcium ions. The reaction curves of all groups remained similar in either the low shear viscosity or co-sedimentation assay. It means that anti-dsDNA Fab does not alter the binding behavior of alpha-actinin to F-actin, although the binding ability is improved. Therefore, it suggested that the interaction between anti-dsDNA Fab and alpha-actinin-4 enhances the F-actin cross-linking extracellularly. With regards to the stability of intracellular

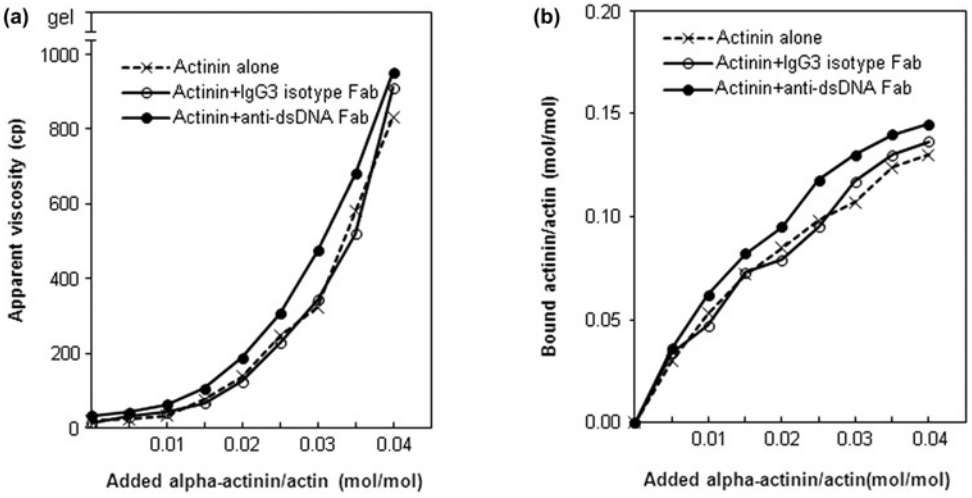


**Figure 4** F-actin binding ability of alpha-actinin in the absence of calcium ions. (a) In low-shear viscometry, the actinin+anti-dsDNA Fab group had elevated gelation value when the ratio of alpha-actinin-4 to G-actin was the same for the control groups (actinin+IgG control Fab or actinin alone). (b) The co-sedimentation assay also indicated a higher ratio of bound actinin to actin on addition of anti-dsDNA Fab when the added actinin-to-actin ratio remained the same. Anti-dsDNA, anti-double-stranded DNA; Fab, fragment antigen-binding

$Ca^{2+}$ concentration under physiological conditions, a similar concept could occur in the formation of cytoplasmic F-actin when anti-dsDNA Fab binds to alpha-actinin-4.

The anti-dsDNA antibodies might possess the potential of penetrating plasma membrane, which is the prerequisite to such presumption. Previously, Jang *et al.*[37] found that four anti-dsDNA mouse monoclonal autoantibodies exhibited the ability to penetrate macrophage cells and subsequently activate proinflammatory reactions. These anti-dsDNA antibodies shared positively-charged amino acids (including arginines) in their CDRs. In fact, Fab fragments induce even more efficient penetration of live cells than complete immunoglobulin.[38] These findings support our suggestion that anti-dsDNA IgG can cross-react with intracellular antigens directly, which also provides the basis for cytoplasmic regulation of anti-dsDNA IgG on alpha-actinin/actin functions. Our results proved that anti-dsDNA Fab incubation enhances the formation of F-actin in MCs. This is not only

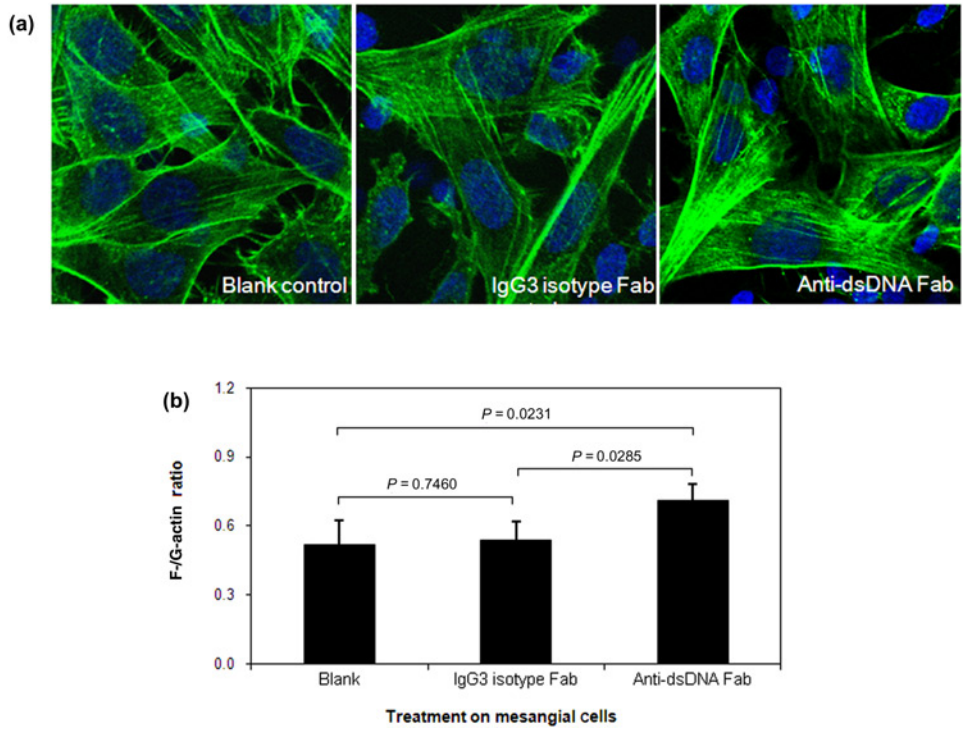


**Figure 5** F-actin expression in mesangial cells. (a) By immunocytochemistry staining, the cells treated with anti-dsDNA Fab showed more and thicker bundles of F-actin compared with those with control Fab treatment or no treatment (blank control). The nuclear counterstaining (blue) was performed with 4′,6-diamidino-2-phenylindole. (b) The results of the F-/G-actin ratio assay affirmed higher F-actin synthesis in mesangial cells after the incubation with anti-dsDNA Fab. Anti-dsDNA, anti-double-stranded DNA; Fab, fragment antigen-binding. (A color version of this figure is available in the online journal)

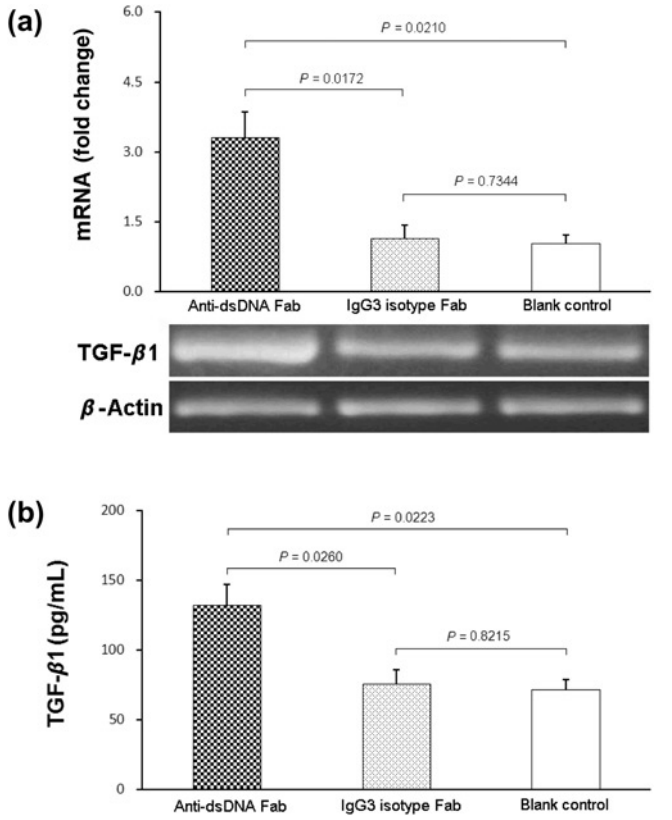


**Figure 6** TGF-$\beta$1 expression in mesangial cells. (a) By realtime PCR, the anti-dsDNA Fab-incubated mesangial cells revealed a higher mRNA level of TGF-$\beta$1 than the controls. (b) The sandwich ELISA was performed to measure the level of TGF-$\beta$1 in culture supernatant. The synthesis of TGF-$\beta$1 by mesangial cells was also enhanced by the anti-dsDNA Fab incubation. TGF-$\beta$1, transforming growth factor-$\beta$1; PCR, polymerase chain reaction; anti-dsDNA, anti-double-stranded DNA; Fab, fragment antigen-binding

strong evidence that anti-dsDNA Fab can penetrate alive cells like other Fab fragments, but also supports the hypothesis that anti-dsDNA Fab regulates F-actin formation intracellularly.

TGF-$\beta$1 is expressed in MCs and has a close relationship with the phenotypic changes of MCs. The increase in TGF-$\beta$1 synthesis leads to a myofibroblast-like phenotype of MCs *in vitro*.[13] Yang *et al.*[39] demonstrated that the assembly of actin cytoskeleton promotes the TGF-$\beta$1/mitogen activated protein kinase-mediated gene expression of plasminogen activator inhibitor-1 in human MCs, which is reported to increase mesangial extracellular matrix (ECM) accumulation.[40] In our study, the TGF-$\beta$1 synthesis in MCs also increases with the stimulation of anti-dsDNA Fab. Here, the reasonable explanation could be that anti-dsDNA Fab might stabilize the actin cytoskeleton by enhancing F-actin formation which subsequently promotes the TGF-$\beta$1 synthesis. Then the up-regulation of TGF-$\beta$1 expression in MCs induces higher secretion of ECM, which is an important event in the phenotypic changes of MCs.[41,42] Therefore, the positive regulation of anti-dsDNA Fab in the cellular F-actin balance is critical to the myofibroblast-like phenotype of MCs induced by anti-dsDNA antibodies.

In conclusion, our results provided evidence that anti-dsDNA Fab enhances the formation of cellular F-actin by the interaction with alpha-actinin-4, which decreases the $Ca^{2+}$ sensitivity of alpha-actinin-4. The anti-dsDNA Fab-enhanced F-actin formation contributes to the increase in TGF-$\beta$1 expression that mediates the phenotypic changes of MCs. This biochemical mechanism may contribute to explaining the myofibroblast-like phenotype of MCs induced by anti-dsDNA antibodies.

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