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ORIGINAL ARTICLE

Autoantibodies to RuvBL1 and RuvBL2: A Novel Systemic Sclerosis–Related Antibody Associated With Diffuse Cutaneous and Skeletal Muscle Involvement

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Objective. **To identify and characterize a novel systemic sclerosis (SSc)–related autoantibody directed against a complex consisting of RuvBL1 and RuvBL2 (RuvBL1/2) and to assess its clinical correlations.**

Methods. **We first analyzed 316 consecutive patients with SSc who were evaluated at Kanazawa University Hospital. Controls included 290 patients with other connective tissue diseases, interstitial lung disease alone, or autoimmune hepatitis, and 50 healthy subjects. Autoantibody specificities were analyzed using RNA and protein immunoprecipitation assays. Autoimmune targets were affinity purified using patients' sera and subjected to liquid chromatography mass spectrometry. SSc patients in another institution in Japan and the University of Pittsburgh cohort were also included in analysis for evaluating clinical correlations.**

Results. **By protein immunoprecipitation assay, 6 SSc sera (1.9%) reacted with doublets with molecular weights of ~50 kd. Liquid chromatography mass spectrometry of the partially purified autoantigen and additional immunoblot-based analyses revealed that this antibody specificity recognized RuvBL1/2. Anti-RuvBL1/2 antibody was exclusively detected in SSc patients. SSc patients with anti-RuvBL1/2 in both the Japanese and Pittsburgh cohorts consistently had higher frequencies of SSc in overlap with myositis and diffuse skin thickening than those without anti-RuvBL1/2. Compared with other autoantibodies related to SSc/myositis overlap (anti–PM-Scl and anti-Ku), anti-RuvBL1/2 was distinctive in terms of its associations with older age at SSc onset, male sex, and a high frequency of diffuse cutaneous involvement.**

Conclusion. **Anti-RuvBL1/2 antibody is a novel SSc-related autoantibody associated with a unique combination of clinical features, including myositis overlap and diffuse cutaneous involvement.**

INTRODUCTION

Systemic sclerosis (SSc; scleroderma) is a connective tissue disease characterized by excessive fibrosis of skin and internal organs, immune system activation, and microvascular damage. There are variable clinical presentations ranging from limited to diffuse cutaneous involvement.

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Significance & Innovations

- We have identified a novel systemic sclerosis (SSc)–related autoantibody reactive with a complex consisting of RuvBL1 and RuvBL2 (RuvBL1/2) that is involved in many important cellular processes, such as transcription and DNA repair.
- Anti-RuvBL1/2 antibody is highly specific to SSc, but its prevalence is very low, ranging from 1–2%.
- Anti-RuvBL1/2 antibody is associated with a unique combination of clinical features, including older age at SSc onset, a higher proportion of men, and higher frequencies of myositis overlap and diffuse skin thickening.
- Detection of anti-RuvBL1/2 antibody is useful for the diagnosis and clinical subgrouping of SSc patients.

While the etiology of SSc remains unclear, autoimmunity is considered to be involved in the pathophysiology. In particular, serum antinuclear antibodies (ANAs) are detected in more than 95% of patients and are a hallmark of the disease (1,2).

A variety of ANAs have been reported to be specific for SSc, including anti–topoisomerase I (anti–topo I), anticentromere, anti–RNA polymerase III (anti–RNAP III), anti-Th/To, and anti–U3 RNP antibodies. They are present at diagnosis and are almost mutually exclusive to each other. Importantly, ANAs are closely associated with distinct clinical subsets. For example, anti–topo I, anti–RNAP III, and anti–U3 RNP are associated with diffuse cutaneous SSc (dcSSc), while anticentromere and anti-Th/To are detected mainly in patients with limited cutaneous SSc (lcSSc) (1–4). These SSc-related antibodies have close associations with distinctive internal organ involvement, such as interstitial lung disease (ILD), scleroderma renal crisis, and pulmonary arterial hypertension. In addition, anti–U1 RNP, anti-Ku, and anti–PM-Scl are frequently found in SSc patients who have features of another connective tissue disease (overlap syndrome). Therefore, detection of SSc-related antibodies is useful not only in diagnosis, but also in prediction of subsequent organ involvement and prognosis. These SSc-related autoantibodies are identified in ~80% of the entire SSc population, suggesting the possibility that other autoantibodies remain undiscovered. Indeed, a recent study by some of the authors has identified a new SSc-related ANA reactive with U11/U12 RNP that correlates with severe ILD (5).

We routinely examined ANAs using immunoprecipitation (IP) assays in sera with various autoimmune diseases. During this screening process, we found several SSc sera that commonly reacted with doublets with molecular weights of ~50 kd. Using a series of biochemical and molecular analyses, autoantigens targeted by this antibody specificity were identified as a complex consisting of RuvBL1 and RuvBL2 (RuvBL1/2). Further clinical evaluations have revealed that the anti-RuvBL1/2 antibody is a new SSc-related autoantibody associated with diffuse skin thickening and skeletal muscle involvement.

PATIENTS AND METHODS

Patients and controls. We enrolled 316 consecutive Japanese patients with physician-confirmed SSc at Kanazawa University Hospital between 1995 and 2009. Two hundred ninety-one patients (92%) fulfilled the 1980 American College of Rheumatology (ACR) preliminary criteria for SSc (6). Disease controls were randomly selected from among all patients who visited Kanazawa University Hospital during the same period and were evaluated for ANA specificity, including 60 with systemic lupus erythematosus (SLE), 20 with polymyositis (PM), 80 with dermatomyositis (DM), 30 with rheumatoid arthritis (RA), 80 with ILD without features suggestive of connective tissue disease, and 20 with autoimmune hepatitis (AIH). All patients with SLE and RA fulfilled the respective ACR criteria (7,8), while those with PM or DM satisfied definite or probable PM or DM according to the Bohan and Peter criteria (9). The diagnosis of AIH was based on the criteria proposed by the International Autoimmune Hepatitis Group (10). Fifty healthy individuals were used as controls. We used another SSc cohort from Keio University Hospital that included 272 consecutive Japanese patients with SSc first evaluated between 1995 and 2006. Of these, 240 (88%) fulfilled the ACR preliminary criteria for SSc (6).

Additional SSc patients were obtained from the Scleroderma Databank at the University of Pittsburgh. All patients had physician-confirmed SSc between 1981 and 2011 (5). To determine the prevalence of anti-RuvBL1/2 antibody, 463 consecutive SSc patients first evaluated during 1994–1995 and 2004–2005 (a total of 4 calendar years) with serum samples available were tested for anti-RuvBL1/2 antibody. Ninety-six percent of these patients fulfilled the ACR preliminary criteria for SSc (6). For analysis of clinical correlations, anti-RuvBL1/2–positive patients identified in the 1982–2005 cohorts were used for comparisons to increase the number of patients. We further included SSc patients with anti–PM-Scl or anti-Ku (without concomitant SSc-related antibodies) first evaluated between 1980 and 2004 with a diagnosis of SSc (11). All sera were obtained with written informed consent, as approved by the individual institutional review boards.

Clinical assessments. Clinical and laboratory information obtained on all SSc patients at the first and followup visits was prospectively collected using standardized data collection forms. SSc patients were classified as having dcSSc, lcSSc, or SSc in overlap (12). The maximum modified Rodnan total skin thickness score (MRSS) during the disease course was recorded. The definitions for organ system involvement attributable to SSc (5,11,13) are listed in Supplementary Table 1 (available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22163/abstract).

ANA assay. ANA was detected by indirect immunofluorescence performed on HEp-2 cell slides (MBL) as the substrate, combined with fluorescein isothiocyanate–conjugated anti-human IgG. In some instances, mouse anti-RuvBL1 (clone 3G4-1F8, Abnova) or anti-RuvBL2

monoclonal antibody (mAb; clone 42, BD Biosciences) diluted at 1:100 was used in combination with fluorescein isothiocyanate–conjugated anti-mouse IgG.

IP assays. An IP assay was performed using extracts of the leukemia cell line K562, as previously described (14). Briefly, 10 μl of patients' sera, or 1 μl of anti-RuvBL1 or anti-RuvBL2 mAb, was mixed with protein A–Sepharose CL-4B (Pharmacia Biotech). For protein analysis, antibody-coated Sepharose beads were incubated with ^{35}S-methionine–labeled K562 cellular extracts, and immunoprecipitated materials were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Radiolabeled polypeptide components were analyzed by autoradiography. For RNA analysis, antibody-bound Sepharose beads were incubated with unlabeled K562 cellular extracts. RNA components were extracted from the immunoprecipitated materials and were applied to urea-PAGE, followed by staining with silver.

Identification of autoantibodies. Autoantibodies in all sera from the Japanese and Pittsburgh cohorts were screened using indirect immunofluorescence and RNA and protein IP assays. Antibodies except anticentromere were identified by IP assays based on precipitating patterns of RNAs or proteins, which were identical to those produced by reference sera commonly used in Kanazawa, Keio, and Pittsburgh. Autoantibody specificities recorded included those to centromere, topo I, RNAP III, Th/To, U1 RNP, U2 RNP, U3 RNP, U5 RNP, U4/U6 RNP, U11/U12 RNP, Ku, PM-Scl, aminoacyl–transfer RNA synthetases, SRP, Mi-2, melanoma differentiation–associated gene 5 protein, transcription intermediary factor 1γ, NXP-2, SAE, SSA, SSB, Sm, and ribosomal. Antibodies to topo I, RNAP III, centromere (MBL), and SSA (Ro 60 and Ro 52; ThermoFisher Scientific) were evaluated using commercially available kits.

Purification and identification of autoantigens. Autoantigens recognized by patients' sera were isolated by affinity purification as described previously (15), with some modifications. Briefly, a mixture of sera from 2 SSc patients containing autoantibodies of interest was incubated with protein A–Sepharose CL-4B overnight at 4°C. The antibody–protein A complex was cross-linked by treatment with dimethyl pimelimidate (Pierce) for 1 hour, and subsequently incubated with K562 cellular extracts. Proteins bound were eluted by treatment with a buffer containing 3 *M* $MgCl_2$ and 1 m*M* dithiothreitol, concentrated, and fractionated on SDS–polyacrylamide gel. After both edges of the gel were stained with silver, a portion of the gel corresponding to the molecular weight of the protein of interest was cut out from the gel, and protein components were subjected to amino-terminal amino acid sequencing by nanoscale high-performance liquid chromatography on a C18 column (MAGIC 2002, Michrom BioResources) coupled to a tandem mass spectrometer (Q-Tof2, Waters Micromass) (16). Sequence data were used to search a compiled protein database that was composed of protein database NCBInr, which is publicly available (http://www.ncbi.nlm.nih.gov/) as of November 7, 2007.

Immunoblots. Antibody reactivities to RuvBL1 and RuvBL2 were further evaluated using 2 different immunoblot-based assays (17). First, protein components immunoprecipitated from K562 extracts by patients' sera were subjected to immunoblots. The K562 cellular lysates were used as a positive control. The immunoprecipitates and the K562 lysates were fractionated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with a 1:250 dilution of mouse anti-RuvBL1 or anti-RuvBL2 mAb. After incubation with horseradish peroxidase–conjugated goat anti-mouse IgG antibodies (eBioscience), antibody binding was detected using an enhanced chemiluminescence kit (Thermo Scientific).

We also used recombinant RuvBL1 and RuvBL2 proteins expressed in *Escherichia coli* (rRuvBL1 and rRuvBL2, respectively; Abnova), which encompassed human full-length amino acid sequences of these proteins fused with glutathione S-transferase (GST), as antigens in immunoblots. These proteins were fractionated and transferred onto nitrocellulose membranes. An edge of the membrane was stained by amide black, and the remaining portion was incubated with a 1:100 dilution of serum samples, or 1:1,000 dilution of mouse anti-RuvBL1 or anti-RuvBL2 mAb. After incubation with alkaline phosphatase–conjugated goat anti-mouse or anti-human IgG antibodies (Cappel), immunoreactive bands were visualized by development with 4-nitro-blue tetrazolium chloride/BCIP (Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA). rRuvBL1 and rRuvBL2 were used as an antigen in ELISA as described previously (18). Briefly, polyvinyl 96-well plates were coated with purified recombinant proteins (0.5 μg/ml) and incubated with a 1:100 dilution of serum samples. Samples were tested in duplicate, and the results were expressed as the optical density at 405 nm.

Depletion of antibodies reactive with rRuvBL1 or rRuvBL2 in serum. Patients' sera were preincubated with an excess amount (1 μg) of rRuvBL1, rRuvBL2, or the mixture of these proteins for 2 hours at 4°C, and were subjected to IP assay or immunoblots.

Statistical analysis. All continuous data are shown as the mean ± SD. The chi-square test or Fisher's exact test was employed for comparison of frequencies, when appropriate. Continuous variables were compared using the Mann-Whitney test.

RESULTS

Detection of a novel SSc-related autoantibody reactive with 50-kd doublets. During our routine assessment of autoantibody profiles using protein IP assay, we noticed

that several SSc sera precipitated strong doublet protein bands at a molecular weight of ~50 kd (Figure 1). Since unlabeled immunoglobulins fractionated on the gels interfered with the electrophoretic motility of these bands, the sizes of the doublet bands varied noticeably and it was difficult to determine the precise molecular weights of these proteins. This pattern of doublet protein IP was not consistent with any reported SSc-related autoantibodies, and was detected in 6 (1.9%) of 316 consecutive SSc patients in the Kanazawa cohort. An identical IP pattern was also found in 4 (1.5%) of 272 consecutive SSc patients in the Keio cohort. In contrast, this antibody specificity was not detected in any sera obtained from a total of 290 patients with SLE, PM, DM, RA, ILD alone, or AIH, or 50 healthy controls, indicating that the anti–50-kd doublet is a novel SSc-specific autoantibody.

Identification of RuvBL1/2 as autoantigens. All 10 sera that immunoprecipitated 50-kd doublets commonly produced a speckled nuclear pattern on indirect immunofluorescence at a titer ranging from 1:160 to 1:1,280 (Figure 2A). This pattern was characterized by condensation of staining during prophase, followed by attenuation of staining in the chromosomal area of the metaphase mitotic cells (Figure 2B). Four (40%) of the positive sera also produced fine granular cytoplasmic staining, but there was no protein commonly immunoprecipitated by those sera. On the other hand, none of the 10 sera with antibodies to 50-kd doublets precipitated small nuclear or cytoplasmic RNA by IP.

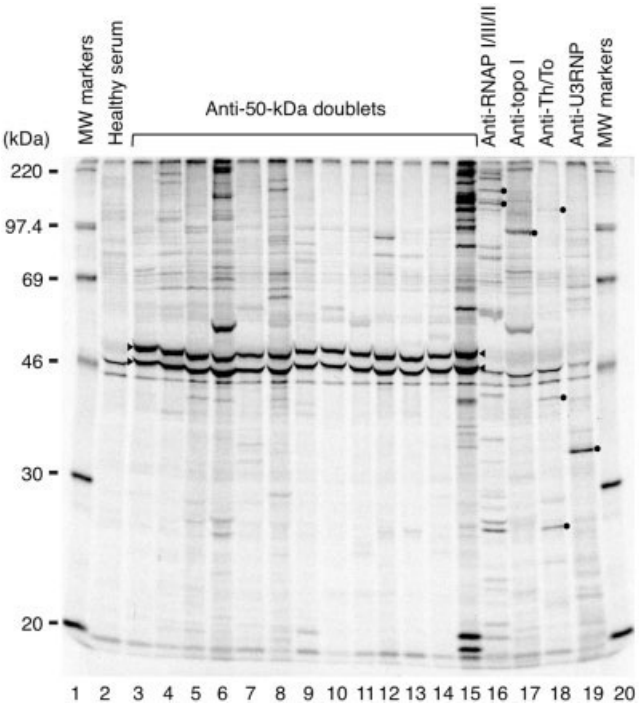

Figure 1. Detection of autoantibodies reactive with the 50-kd doublets by a protein immunoprecipitation assay. Immunoprecipitates from ^{35}S-methionine–labeled K562 cellular extracts were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by autoradiography. Lanes 1 and 20 = molecular weight (MW) markers; lane 2 = healthy serum; lanes 3–15 = sera from systemic sclerosis (SSc) patients positive for the anti–50-kd doublets (RuvBL1/2) from the Kanazawa cohort (lanes 3–8) and Pittsburgh cohort (lanes 9–15); lane 16 = SSc serum with anti–RNA polymerase I/III/II (anti–RNAP I/III/II); lane 17 = SSc serum with anti–topoisomerase I (anti-topo I); lane 18 = SSc serum with anti-Th/To; lane 19 = SSc serum with anti–U3 RNP. **Arrowheads** show the 50-kd doublets. **Dots** in sera positive for anti–topo I, anti–RNAP III, anti-Th/To, and anti–U3 RNP show the main target antigens.

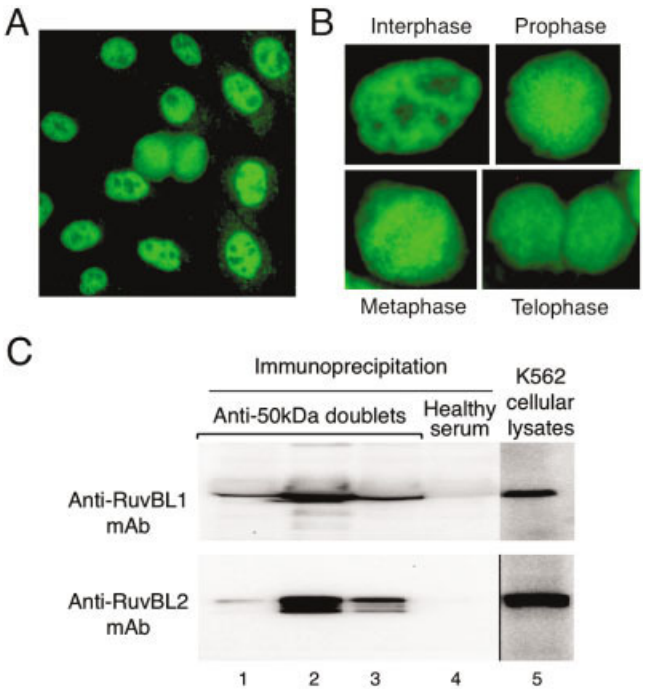

Figure 2. Identification of RuvBL1/2 as the 50-kd doublets. **A,** An indirect immunofluorescent staining pattern on HEp-2 cell slides produced by a systemic sclerosis (SSc) serum positive for anti–50-kd doublets (corresponding to lane 4 in Figure 1). Original magnification × 200. **B,** High-magnification views of HEp-2 cells in various cell cycle phases stained by an SSc serum positive for anti–50-kd doublets. Original magnification × 400. **C,** Detection of RuvBL1 and RuvBL2 in immunoprecipitates using immunoblots. Protein components immunoprecipitated from K562 cellular extracts by 3 representative SSc sera positive for the anti–50-kd doublets (lanes 1–3; corresponding to lanes 3, 4, and 7 in Figure 1) and healthy serum (lane 4) were subjected to immunoblots probed with anti-RuvBL1 (**upper**) or anti-RuvBL2 (**lower**) monoclonal antibodies (mAb). Untreated K562 cellular lysates, instead of immunoprecipitates, were used as controls (lane 5).

To identify autoantigens recognized by antibodies to 50-kd doublets, we partially purified the 50-kd doublets from K562 extracts using the 2 SSc sera samples that showed an ANA titer of ≥1:640 (corresponding to lanes 4 and 7 in Figure 1). When amino acid sequences obtained by liquid chromatography mass spectrometry were subjected to the protein database NCBInr search, the majority of them were derived from human immunoglobulins. However, 8% of 456 sequences completely matched amino acid sequences of the amino-terminal portion of RuvBL1, which is an ATP-binding nuclear protein that belongs to the family of ATPase associated with diverse cellular activities (19). RuvBL1 is present in the nucleus as a complex with RuvBL2, which is a structurally related protein (19). Interestingly, RuvBL1 and RuvBL2 form a double hexamer and interact through their ATPase insert domain (20). The

molecular weights of RuvBL1 and RuvBL2 are reported to be 49 kd and 48 kd, respectively (19), raising the hypothesis that the 50-kd doublets correspond to RuvBL1 and RuvBL2. To test this hypothesis, we examined whether proteins immunoprecipitated by anti–50-kd doublets contained RuvBL1 and RuvBL2 (Figure 2C). The immunoprecipitates of 3 representative SSc sera samples positive for the anti–50-kd doublets contained both RuvBL1 and RuvBL2, but those of healthy serum did not. In total, immunoprecipitates of all 10 SSc sera with anti–50-kd doublets contained both RuvBL1 and RuvBL2. In contrast, all of the 20 control sera from SSc patients negative for the anti–50-kd doublets failed to precipitate any of these proteins. Therefore, the 50-kd doublets targeted by the novel SSc-related autoantibody were confirmed to be RuvBL1 and RuvBL2.

Autoantibodies to the RuvBL1/2 complex are specific to SSc. It has been reported that anti-RuvBL1 antibody is detected in a small proportion of patients with SLE, PM/DM, RA, or AIH by ELISA using a recombinant RuvBL1 fragment expressed in *E coli* (21). This is inconsistent with our findings obtained from IP assay: detection of anti-RuvBL1/2 was exclusive to SSc patients. To examine reasons for this discrepancy, we carried out antibody detection assays using rRuvBL1 and rRuvBL2 individually as antigens. In immunoblots, we occasionally found antibodies reactive with rRuvBL1 or rRuvBL2 in the sera from patients with SSc, PM, DM, SLE, RA, or AIH (Figure 3A). None of those sera reacted with GST alone (data not shown). We used ELISA to further screen a larger number of sera (Figures 3B and C). As a result, antibodies to rRuvBL1 and rRuvBL2 were detected in sera from patients with various diseases, irrespective of the presence or absence of reactivity to RuvBL1/2 by IP. When cutoff values were set at 2 SDs above the mean of healthy controls, anti-rRuvBL1 was detected in 6 (86%) of 7 SSc sera with anti-RuvBL1/2 antibody by IP, but in 8 (5%) of 159 sera without anti-RuvBL1/2 ($P < 0.00001$); anti-rRuvBL2 was detected in 5 with anti-RuvBL1/2 (71%), but in 41 without anti-RuvBL1/2 (26%; $P = 0.03$). These findings suggest that antibodies reactive with RuvBL1/2 detected by IP assay and those reactive with individual rRuvBL1 and rRuvBL2 are distinct repertoires, although anti-RuvBL1/2 antibodies frequently coexisted with antibodies to rRuvBL1 or rRuvBL2.

One potential explanation for different specificities between antibodies detected by IP assay and recombinant protein-based assays is their recognition of distinct epitopes. The IP detects antibodies recognizing epitopes present on the native RuvBL1/2 complex, whereas assays using rRuvBL1 or rRuvBL2 may detect antibodies reactive with epitopes not present on the native complex. To examine this possibility, we used commercially available anti-RuvBL1 and anti-RuvBL2 mAb, which were generated by immunization of mice with synthetic peptides encoding amino acid sequences unique to each protein. These mAb failed to precipitate RuvBL1/2 by IP assay (see Supplementary Figure 1A, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22163/abstract) and failed to stain the nucleus of HEp-2 cells by indirect immunofluorescence. These characteristics were consistent with patients' sera that reacted with rRuvBL1 and/or rRuvBL2 by immunoblots and ELISA. In addition, SSc sera that immunoprecipitated the RuvBL1/2 complex retained their reactivity even after antibodies reactive with rRuvBL1, rRuvBL2, or both were depleted

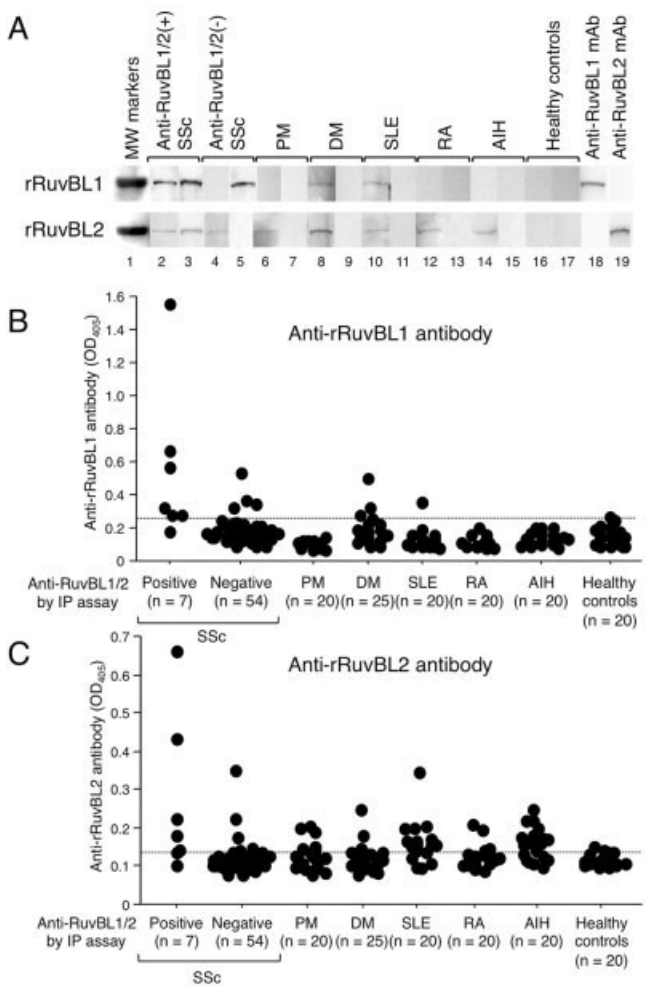

Figure 3. Detection of antibodies to recombinant RuvBL1 and RuvBL2 (rRuvBL2 and rRuvBL2). **A,** Immunoblots using rRuvBL1 (**upper**) and rRuvBL2 (**lower**) as antigens. Systemic sclerosis (SSc) sera positive for anti-RuvBL1/2 antibody by immunoprecipitation (IP) assay (lanes 2 and 3), SSc sera negative for anti-RuvBL1/2 antibody by IP assay (lanes 4 and 5), polymyositis (PM) sera (lanes 6 and 7), dermatomyositis (DM) sera (lanes 8 and 9), systemic lupus erythematosus (SLE) sera (lanes 10 and 11), rheumatoid arthritis (RA) sera (lanes 12 and 13), autoimmune hepatitis (AIH) sera (lanes 14 and 15), healthy control sera (lanes 16 and 17), anti-RuvBL1 monoclonal antibody (mAb; lane 18), and anti-RuvBL2 mAb (lane 19) are shown. Lane 1 indicates molecular weight (MW) markers, and a band corresponds to 75 kd. **B** and **C,** Antibodies to rRuvBL1 and rRuvBL2 measured by enzyme-linked immunosorbent assay in 61 sera from SSc patients, 20 from PM patients, 25 from DM patients, 20 from SLE patients, 20 from RA patients, 20 from AIH patients, and 20 from healthy controls. SSc patients were divided into 2 groups based on the presence or absence of anti-RuvBL1/2 antibodies detected by IP assay. Broken lines show cutoff levels for positivity, which were set at 2 SDs above the mean of healthy controls (0.25 for anti-rRuvBL1 antibody and 0.14 for anti-rRuvBL2 antibody). OD_{405} = optical density at 405 nm.

Table 1. Clinical profiles in SSc patients with and without anti-RuvBL1/2 antibody in 2 independent Japanese cohorts*

Demographic features and organ involvement	Kanazawa cohort		Keio cohort		2 cohorts combined		
	Anti-RuvBL1/2 negative (n = 310)	Anti-RuvBL1/2 positive (n = 6)	Anti-RuvBL1/2 negative (n = 268)	Anti-RuvBL1/2 positive (n = 4)	Anti-RuvBL1/2 negative (n = 578)	Anti-RuvBL1/2 positive (n = 10)	*P*†
Age at SSc onset, mean ± SD years	47.2 ± 14.6	58.0 ± 14.5	42.2 ± 13.6	58.3 ± 7.9	44.9 ± 14.1	58.1 ± 12.1	0.008
Male sex	62 (20)	3 (50)	29 (11)	2 (50)	91 (16)	5 (50)	0.01
Disease classification							< 0.00001‡
dcSSc alone	95 (31)	2 (33)	78 (29)	0	173 (30)	2 (20)	
lcSSc alone	206 (66)	0	142 (53)	2 (50)	348 (60)	2 (20)	
SSc in overlap	9 (3)	4 (67)	48 (18)	2 (50)	57 (10)	6 (60)	
Cutaneous involvement							
Diffuse	102 (33)	5 (83)	93 (35)	2 (50)	195 (34)	7 (70)	0.04
Limited	208 (67)	1 (17)	175 (65)	2 (50)	383 (66)	3 (30)	
Diffuse within overlap, no./total (%)	2/9 (22)	3/4 (75)	15/48 (31)	2/2 (100)	17/57 (30)	5/6 (83)	0.01
Maximum MRSS, mean ± SD							
Diffuse cutaneous involvement	18.4 ± 8.5	20.6 ± 10.3	21.9 ± 6.1	21.0 ± 1.4	20.0 ± 7.4	20.7 ± 7.5	0.8
Limited cutaneous involvement	3.9 ± 3.6	8§	5.0 ± 2.9	5.0 ± 1.4	4.4 ± 3.3	6.0 ± 3.9	0.2
Organ involvement							
Peripheral vasculature	275 (89)	6 (100)	259 (97)	4 (100)	534 (92)	10 (100)	0.8
Skeletal muscle	31 (10)	4 (67)	35 (13)	2 (50)	66 (11)	6 (60)	0.00003
Gastrointestinal tract	143 (46)	3 (50)	172 (64)	3 (75)	315 (54)	6 (60)	0.7
Interstitial lung disease	137 (44)	5 (83)	147 (55)	2 (50)	284 (49)	7 (70)	0.3
PAH	25 (8)	1 (17)	17 (6)	0	42 (7)	1 (10)	0.8
Heart	34 (11)	4 (67)	23 (9)	1 (25)	57 (10)	5 (50)	0.0003
Kidney (renal crisis)	6 (2)	0	11 (4)	0	17 (3)	0	0.7

* Values are the number (percentage) unless indicated otherwise. SSc = systemic sclerosis; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc; MRSS = modified Rodnan total skin thickness score; PAH = pulmonary arterial hypertension.
† *P* values were calculated after the 2 cohorts were combined.
‡ $P = 0.03$ and $P < 0.00001$ in frequencies of lcSSc alone and SSc in overlap between anti-RuvBL1/2–positive and –negative patients, respectively.
§ Only 1 patient had limited cutaneous involvement.

completely (see Supplementary Figure 1B, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22163/abstract). These findings together suggest that anti-RuvBL1/2 detected by IP exclusively in SSc patients recognizes the native complex.

Clinical features associated with anti-RuvBL1/2 antibody in the Japanese cohorts. We first divided SSc patients in the Kanazawa and Keio cohorts into 2 groups according to the presence or absence of anti-RuvBL1/2 and compared clinical characteristics between the groups (Table 1). Since the number of SSc patients with anti-RuvBL1/2 antibody was small in individual cohorts, statistical analysis was conducted by combining the 2 cohorts into one. SSc patients with anti-RuvBL1/2 were older at onset and were more frequently men than those without anti-RuvBL1/2 ($P = 0.008$ and $P = 0.01$, respectively). Of note, 60% of patients with anti-RuvBL1/2 were classified as having SSc in overlap, which was significantly higher than the frequency of SSc in overlap in those without this antibody ($P < 0.00001$). Diffuse cutaneous involvement was more common in patients with anti-RuvBL1/2 than in those without ($P = 0.04$). Interestingly, the majority of patients with SSc in overlap also had diffuse skin thickening. In terms of organ involvement, skeletal muscle disease was significantly more frequent in anti-RuvBL1/2–positive patients than in anti-RuvBL1/2–negative patients ($P = 0.00003$). Heart involvement was also more commonly found in patients with anti-RuvBL1/2 ($P = 0.0003$).

Although some anti-RuvBL1/2–positive sera precipitated additional proteins by IP assay, no known autoantibodies, including SSc- and myositis-related antibodies, were detected, while one was positive for anti-SSA antibody by a commercial kit. No patient received medications that could potentially induce autoantibody production, including statins, antihypertensive drugs, and biologic agents. None of the patients had malignancy diagnosed concurrently with or within 3 years before or after the diagnosis of SSc.

Clinical features associated with anti-RuvBL1/2 antibody in the Pittsburgh cohort. To further examine clinical correlations with anti-RuvBL1/2 antibody, we analyzed SSc patients in the Pittsburgh cohort. By routine screening of SSc patients' sera with IP assay during 1982–2005, 27 SSc sera were found to have anti-RuvBL1/2 antibody. Four positive patients had other SSc-related antibodies (1 with both anti–RNAP III and anti-Ku and 3 with one of each of

Table 2. Clinical profiles in SSc patients with and without anti-RuvBL1/2 antibody in the Pittsburgh cohort*

Demographic features and organ involvement	Anti-RuvBL1/2 negative (n = 458)	Anti-RuvBL1/2 positive (n = 27)	*P*
Age at SSc onset, mean ± SD years	44.0 ± 15.5	46.0 ± 15.1	0.5
Male sex	104 (23)	10 (37)	0.1
White race	414 (90)	25 (93)	1.0
Disease classification			< 0.00001†
dcSSc alone	215 (47)	8 (30)	
lcSSc alone	207 (45)	3 (11)	
SSc in overlap	36 (8)	16 (59)	
Cutaneous involvement			
Diffuse	226 (49)	18 (67)	0.08
Limited	232 (51)	9 (33)	
Diffuse within overlap, no./total (%)	11/36 (31)	10/16 (63)	0.04
Maximum MRSS, mean ± SD			
Diffuse cutaneous involvement	26.5 ± 12.0	20.0 ± 8.4	0.02
Limited cutaneous involvement	4.5 ± 3.8	7.6 ± 8.7	0.03
Organ involvement			
Peripheral vasculature	449 (98)	22 (81)	< 0.00001
Skeletal muscle	64 (14)	16 (59)	< 0.00001
Gastrointestinal tract, no./total (%)	239/310 (77)	17/18 (94)	0.08
Interstitial lung disease, no./total (%)	157/374 (42)	11/22 (50)	0.3
PAH, no./total (%)	56/285 (20)	2/15 (13)	0.8
Heart, no./total (%)	70/353 (20)	5/23 (22)	0.8
Kidney (renal crisis)	50 (11)	1 (4)	0.4

* Values are the number (percentage) unless indicated otherwise. Denominators are the number of patients with objective testing for organ involvement. SSc = systemic sclerosis; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc; MRSS = modified Rodnan total skin thickness score; PAH = pulmonary arterial hypertension.
† $P = 0.001$ and $P < 0.00001$ in frequencies of lcSSc alone and SSc in overlap between anti-RuvBL1/2–positive and –negative patients, respectively.

anti–RNAP III, anti-Ku, and anti-Th/To). Myositis-related antibodies were not detected, but anti-SSA antibody was found in 2 sera. Regarding medications that could conceivably induce autoantibody production, we examined those taken prior to the serum sample that demonstrated anti-RuvBL1/2 antibodies. These included, alone or in combination, D-penicillamine (n = 7), methotrexate (n = 8), and anti–tumor necrosis factor biologic agents (n = 2). Angiotensin-converting enzyme inhibitors (n = 2) and statins (n = 0) were infrequently or not prescribed. Only 1 patient had lung cancer 10 months after anti-RuvBL1/2 was identified.

When the prevalence of anti-RuvBL1/2 antibody was calculated in 2 consecutive 2-year periods, including 1994–1995/2004–2005, 5 (1.1%) of 463 consecutive new SSc patients with serum available for testing had anti-RuvBL1/2 antibody.

For assessment of clinical correlations with anti-RuvBL1/2, demographic and clinical characteristics of the 27 anti-RuvBL1/2–positive SSc patients were compared with the 458 consecutive anti-RuvBL1/2–negative SSc patients first evaluated in 1994–1995/2004–2005 (Table 2). In contrast to the Japanese cohorts, there was no difference in age at SSc onset between the groups, but the proportion of men among the anti-RuvBL1/2–positive patients tended to be higher. The distribution of SSc subsets was different between patients with and without anti-RuvBL1/2 ($P < 0.00001$). More than half of the anti-RuvBL1/2–positive patients were classified as having SSc in overlap. Diffuse skin thickening was more common in patients with anti-RuvBL1/2 in general and in those with SSc in overlap compared with those without. In terms of organ involvement, peripheral vascular involvement was less common and skeletal muscle involvement was more common in patients with anti-RuvBL1/2 than in those without ($P < 0.00001$ for both comparisons). There were no differences in frequencies of other organ involvements, including the heart, between these 2 groups.

Comparison of clinical features in SSc patients with 3 autoantibodies associated with SSc/myositis overlap. Anti–PM-Scl and anti-Ku are representative SSc-related antibodies associated with inflammatory myopathy (1,2). To examine potential differences in clinical correlations between anti-RuvBL1/2 and these 2 other antibodies, clinical features were compared between SSc patients who were identified to have anti-RuvBL1/2, anti–PM-Scl, and anti-Ku (Table 3). In this analysis, SSc patients in the Japanese and Pittsburgh cohorts were combined. Anti–PM-Scl was not found in the Japanese cohort, which is consistent with a previous report (22). Age at SSc onset was older and the proportion of men was higher in patients with anti-RuvBL1/2 ($P = 0.0001$ and $P = 0.002$, respectively). Approximately half of the patients were classified as having SSc in overlap in all 3 groups, but diffuse skin thickening was more prevalent in patients

Table 3. Comparisons of clinical profiles in SSc patients with 3 autoantibodies associated with SSc/myositis overlap*

Demographic features and organ involvement	Anti-RuvBL1/2 positive (n = 37)†	Anti–PM-Scl positive (n = 76)	Anti-Ku positive (n = 44)	Overall *P*
Cohort, no.				
2 Japanese institutions	10	0	13	
University of Pittsburgh	27	76	31	
Age at SSc onset, mean ± SD years	59.3 ± 14.4	37.6 ± 17.7	38.4 ± 15.5	0.0001‡
Male sex	15 (41)	8 (16)	8 (18)	0.002§
White race	25 (68)	74 (97)	26 (59)	< 0.00001¶
Disease classification				0.02#
dcSSc alone	13 (35)	12 (16)	15 (34)	
lcSSc alone	5 (14)	28 (37)	10 (23)	
SSc in overlap	19 (51)	36 (47)	19 (43)	
Cutaneous involvement				
Diffuse	25 (68)	22 (29)	20 (45)	0.0004**
Limited	12 (32)	54 (71)	24 (55)	
Diffuse within overlap, no./total (%)	12/19 (63)	10/36 (28)	5/19 (26)	0.02††
Typical DM rash	4 (11)	24 (32)	6 (14)	0.01‡‡
Maximum MRSS, mean ± SD				
Diffuse cutaneous involvement	20.3 ± 8.4	11.0 ± 8.8	22.7 ± 12.4	0.01§§
Limited cutaneous involvement	7.2 ± 5.6	4.6 ± 3.5	5.6 ± 4.8	0.1
Organ involvement				
Peripheral vasculature	32 (86)	68 (91)	40 (91)	0.8
Skeletal muscle	21 (57)	39 (51)	22 (50)	0.7
Gastrointestinal tract, no./total (%)	23/28 (82)	24/46 (52)	29/40 (73)	0.04¶¶
ILD, no./total (%)	18/32 (56)	31/62 (50)	19/44 (43)	0.5
PAH, no./total (%)	3/25 (12)	3/59 (5)	4/28 (14)	0.4
Heart, no./total (%)	10/33 (30)	6/54 (11)	8/40 (20)	0.08
Kidney (renal crisis)	1 (3)	6 (8)	1 (2)	0.9

* Values are the number (percentage) unless indicated otherwise. Denominators are the number of patients with objective testing for organ involvement. SSc = systemic sclerosis; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc; DM = dermatomyositis; MRSS = modified Rodnan total skin thickness score; ILD = interstitial lung disease; PAH = pulmonary arterial hypertension.
† Two anti-RuvBL1/2–positive patients had concomitant anti-Ku antibody.
‡ $P = 0.0001$ and $P = 0.001$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups and between anti-RuvBL1/2–positive and anti-Ku–positive groups, respectively.
§ $P = 0.0002$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups.
¶ $P < 0.00001$ for comparisons both between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups and between anti–PM-Scl–positive and anti-Ku–positive groups.
# $P = 0.01$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups.
** $P = 0.0001$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups.
†† $P = 0.02$ and $P = 0.03$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive or anti-Ku–positive groups, respectively.
‡‡ $P = 0.01$ and $P = 0.03$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups and between anti–PM-Scl–positive and anti-Ku–positive groups, respectively.
§§ $P = 0.001$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups.
¶¶ $P = 0.01$ between anti-RuvBL1/2–positive and anti–PM-Scl–positive groups.

with anti-RuvBL1/2 ($P = 0.0004$). In particular, anti-RuvBL1/2–positive patients with SSc in overlap more frequently had diffuse cutaneous involvement than did patients positive for anti–PM-Scl or anti-Ku ($P = 0.02$). DM rashes were less frequent in patients with anti-RuvBL1/2 and anti-Ku than in those with anti–PM-Scl ($P = 0.01$). The maximum MRSS in patients with diffuse cutaneous involvement was greater in those with anti-RuvBL1/2 than in those with anti–PM-Scl ($P = 0.001$). Gastrointestinal and heart involvement was less common in patients with anti–PM-Scl compared with those patients with the other antibodies ($P = 0.04$ and $P = 0.08$, respectively).

DISCUSSION

We have identified and characterized a novel autoantibody reactive with an RuvBL1/2 complex in a small group of SSc patients. Anti-RuvBL1/2 antibody was detectable by IP assay exclusively in SSc patients. In addition, anti-RuvBL1/2 seldom coexisted with other known autoantibody specificities. Based on these characteristics, anti-RuvBL1/2 should be listed as one of the SSc-related autoantibodies, although its prevalence in SSc patients is low (1–2%). RuvBL1 (also known as RVB1, TIP49, and pontin) and RuvBL2 (also known as RVB2, TIP48, and reptin) are highly conserved eukaryotic proteins and form a double hexamer in the nucleus as a scaffolding molecule. Recent studies have implicated the RuvBL1/2 complex in many cellular processes, such as transcription, DNA repair, chromatin remodeling, and small nucleolar RNP assembly (19).

We also confirmed and extended the finding by Makino et al (21). Specifically, antibodies to rRuvBL1 or rRuvBL2 were detected in a small proportion of patients with vari-

ous connective tissue diseases or AIH. However, these antibodies were distinct from anti-RuvBL1/2 detected in SSc patients, in terms of recognition of the RuvBL1/2 complex by IP assay. It is highly likely that SSc-specific autoantibodies preferentially recognize epitopes present on the native RuvBL1/2 complex, probably a double hexamer, whereas autoantibodies detected nonspecifically in patients with various connective tissue diseases may react with epitopes present on RuvBL1 and RuvBL2, but not on the native complex. In this regard, many SSc-related antibodies have been shown to preferentially recognize autoantigens with native conformations (23–26).

Clinical features associated with anti-RuvBL1/2 included a high frequency of SSc in overlap with skeletal myopathy and diffuse skin thickening. These correlations were originally detected in the Japanese cohorts and replicated in the North American cohort. Therefore, anti-RuvBL1/2 should be included in a group of autoantibodies associated with SSc/myositis overlap, including anti–PM-Scl and anti-Ku. Interestingly, among these 3 antibodies, anti-RuvBL1/2 is associated with a unique combination of clinical features, including an older age at SSc onset and higher frequencies of men and diffuse skin thickening.

Intriguingly, both RuvBL1/2 and Ku are required for the DNA damage response. Specifically, RuvBL1/2 is involved in sensing DNA damage and recruitment of repair proteins at the site of damage (19), while the Ku heterodimer plays a key role in the repair of DNA double-strand breaks by forming a complex with the DNA-dependent protein kinase catalytic subunit (27). Conversely, PM-Scl, also known as an exosome complex, plays a critical role in processing of various RNAs, including ribosomal and small nucleolar RNAs (19,28). RuvBL1/2 is also involved in assembly and trafficking of small nucleolar RNAs (19). These shared features of RuvBL1/2, Ku, and PM-Scl may provide insights into pathologic consequences in SSc and skeletal myopathy.

In summary, we have identified a novel SSc-related ANA reactive with an RuvBL1/2 complex. Anti-RuvBL1/2 antibody is a serologic marker for SSc/myositis overlap with diffuse cutaneous involvement.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kuwana had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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