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ORIGINAL ARTICLE

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Inositol 1,4,5-trisphosphate receptors are autoantibody target antigens in patients with Sjögren's syndrome and other systemic rheumatic diseases

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Abstract IP_3R2 and IP_3R3 double knock-out mice present with exocrine dysfunctions such as secretion deficits of saliva and pancreatic juice. Therefore, we investigated whether the presence of antibodies to IP_3Rs could be found in patients with Sjögren's syndrome, and the location of the epitopes. Subjects included 35 primary Sjögren's syndrome, 39 secondary Sjögren's syndrome, 144 rheumatoid arthritis, and 96 other connective tissue disease patients. As controls, 33 healthy subjects were included. Immunoblot was conducted using recombinant proteins IP_3R1, IP_3R2, and IP_3R3 made from full-length cDNA, and core, T604, and EL for epitope mapping. Antibodies to IP_3R1 in sera from patients with primary Sjögren's syndrome, secondary Sjögren's syndrome, and rheumatoid arthritis were found to be positive in 17 of 35 (48.6%), 13 of 39 (33%), and 34 of 124 (27.4%) cases, respectively. These frequencies were significantly higher than the 1 of 33 (3.0%) found in normal healthy subjects. The frequency of anti-IP_3R2 antibodies in rheumatoid arthritis was found to be higher than those found in Sjögren's syndrome, systemic lupus erythematosus, and systemic sclerosis. Anti-IP_3R2 antibodies found in rheumatoid arthritis primarily recognized residues 578–2171 of the internal coupling and regulatory domain. On the other hand, anti-IP_3R1 found in Sjögren's syndrome recognized residues 224–604 of the core protein IP_3R1. Anti-IP_3R1 antibodies were present in 48.6% of primary Sjögren's syndrome and in 3.0% of normal healthy subjects. Anti-IP_3R2 antibodies were detected most frequently in rheumatoid arthritis. Locations of the antigenic epitopes were found to differ among the disease conditions.

Key words Anti-IP_3Rs antibody · Inositol 1,4,5-trisphoshate (IP_3) · Inositol 1,4,5-trisphosphate receptor (IP_3R) · Rheumatoid arthritis · Sjögren's syndrome

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Introduction

Sjögren's syndrome (SS) occurs predominantly in middle-aged women (male : female ratio = 1 : 9). Etiology is as yet unknown, although hereditary, immunological, environmental, and hormonal factors such as decreasing estrogen levels are thought to be involved.[1] Patients with SS clinically present with dry eye, dry mouth, and other systemic manifestation in various organs where T and B lymphocytes infiltrate. They are classified into two groups: primary (sicca alone) and secondary (associated with other connective tissue disease) SS. Seventy percent of patients with SS serologically have antibodies to SS-A/Ro and 20%–30% with primary (P)-SS have antibodies to SS-B/La.[2,3] Autoantibodies to SS-A/Ro recognize a ribonucleoprotein complex composed of small single stranded Y1–Y5 RNAs, and one or more proteins. The SS-B antigen is thought to be a RNA polymerase β termination factor. Elsewhere, antibodies to Ki, recently identified as proteasome (PA 28γ) are found in less than 10 % of patients with SS.[4] Furthermore, antibodies to α-Fodrin have been reported in some patients with SS.[5] Other cytoplasmic antibodies found in SS include those directed against the Golgi complex,[6] early endosome antigen 1,[7] ribosome P, mitochondria, and p97/VCP.[8]

Recently, IP_3R2 and IP_3R3 knock-out mice presenting with a lack of Ca^{2+} release capability from the endoplasmic reticulum, and thus unable to induce secretion of saliva and pancreatic juice,[9] were shown to be clinically similar to patients with SS. Therefore, we investigated the existence of antibodies to IP_3Rs in such patients and in those with other systemic rheumatic diseases, and determined the location of the antigenic epitopes.

Patients and methods

Patients

Patients consisted of 74 with SS (35 primary and 39 secondary), 144 with rheumatoid arthritis (RA), and 96 with other connective tissue diseases (CTD), and 33 normal healthy subjects (NHS). The diagnosis of SS was made by either European or international consensus criteria for SS.[10,11] Diagnoses of RA, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and other diseases were made by standard criteria. Only those patients and doctors who had given their written consent were included in the study.

Detection of autoantibodies

Antinuclear antibody (ANA) and anticytoplasmic antibody were detected by indirect immunofluorescence, as described in detail elsewhere.[12] Anti-SS-A/Ro, SS-B/La, U1RNP, Sm, Scl 70, Ki, Ku, rRNP, Wa and p95c/p97/VCP were screened by double immunodiffusion.[13] Anti-SS-A/Ro, SS-B/La, U1RNP, Sm, Scl 70 (Topo-isomerase 1), and anti-mitochondrial antibodies were further confirmed by enzyme-liked immunosorbent assay using commercial kits. Immunoprecipitation was used for the confirmation of anti-Ki, Ku,[14] rRNP, Wa,[15] WS,[16] and p97/VCP.[8]

Immunoblotting for anti-IP_3Rs antibodies

Microsome fractions were prepared from Sf9 cells overexpressing mouse IP_3Rs as described previously.[17] Proteins were separated on 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% skim milk in PBST (phosphate-buffered saline plus 0.05% Tween-20) prior to overnight incubation at 4°C with test sera (1:300). Subsequently following three washes with PBST, the membrane was incubated with antihuman IgG (h&l) antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA; 1:2000) for 1h at room temperature. The reaction was visualized using ECL Western blotting detection reagents (GE Healthcare, Slough, UK). The signal intensities were calculated using Scion Image software (Scion, Frederick, MD, USA), and the recorded values were based on the average of at least three measurements. The signal intensities themselves were semiquantitative, so we expediently counted as positive, sera that yielded band intensities exceeding 75.6 for anti-IP_3R1, 48.0 for anti-IP_3R2, and 51.0 for anti-IP_3R3 (twice or more the average intensities obtained for the corresponding bands of NHS).

Preparation of recombinant proteins of the IP_3-binding core domain (core), the IP_3-binding domain (T604), and the N-terminal cytoplasmic region (EL) of mouse IP_3Rs

The IP_3-binding core domain (core) and the IP_3-binding domain (T604) of mouse IP_3Rs were expressed in *Escherichia coli* BL21 codonplus (Stratagene, La Jolla, CA, USA) and purified on a HiTrap heparin HP column (GE Healthcare) as described elsewhere.[17] The cDNA encoding the N-terminal cytoplasmic region of mouse IP_3R1 (1–2217, EL_{m1}) was inserted into pBlueBac4.5 baculovirus transfer vector. The cDNA encoding the N-terminal region of mouse IP_3R2 (1–2171, EL_{m2}) was inserted into pFastBac1 baculovirus transfer vector. Recombinant baculovirus carrying EL_{m1} was generated with the Bac-N-Blue transfection kit (Invitrogen, Carlsbad, CA, USA). Recombinant baculoviruses

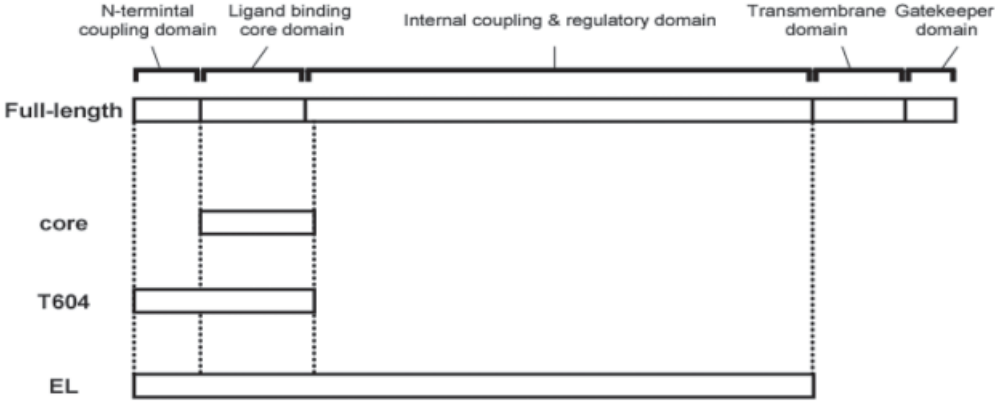

Fig. 1. Schematic domain structure of full-length IP_3R and recombinant protein used for epitope mapping in this study. The five structural domains of IP_3R1[27] are shown. Core, the IP_3-binding core domain, is located within amino acid residues 224–604 of mouse IP_3R1 and IP_3R2. T604, the IP_3-binding domain, is located within amino acid residues 1–604 of mouse IP_3R1 and IP_3R2. EL, the N-terminal cytoplasmic region, is located within amino acid residues 1–2217 of mouse IP_3R1, or within amino acid residues 1–2171 of mouse IP_3R2

carrying EL_{m2} was generated with the Bac-to-Bac Baculovirus Expression system (Invitrogen). Recombinant viruses were amplified and used for expression in Sf9 cells. Sf9 cells were cultured and transfected as described previously.[18] The soluble fraction containing recombinant protein was prepared as described previously[18] (139. 1). Proteins were separated on 7.5% SDS–PAGE and immunoblotted as described above.

Statistical analysis

Data among the over three disease conditions were compared using the Mann–Whitney *U*-test with Bonferroni correction.

Results

Intensities of anti-IP_3R1, IP_3R2, IP_3R3 antibodies found in Sjögren's syndrome and other disease conditions

Mean intensities of anti-IP_3R1 antibodies found in P-SS, S-SS, and NHS were 80.9, 81.8, and 37.8, respectively. Those found in RA, SLE, and PBC were 61.0. 54.3, and 91.2, respectively. With the exception of PBC, anti-IP_3R1 reactivity was found to be strongest in SS. Both the mean titers of P-SS and S-SS were significantly higher ($P < 0.05$) when compared to the mean titers of NHS (Fig. 2a).

Elsewhere, mean intensities of anti-IP_3R2 antibodies found in P-SS, S-SS, and NHS were 55.1, 41.4, and 24.0, respectively. Those found in RA, SLE, and PBC were 57.4, 55.9, and 53.7, respectively. The reactivity observed in RA was higher than those in P-SS and S-SS. Here, only the mean titter of P-SS was significantly higher ($P < 0.01$) when compared to the mean titer of NHS (Fig. 2b).

Finally, mean intensities of anti-IP_3R3 antibodies found in P-SS, S-SS, and NHS were 86.7, 65.0, and 25.5, respectively. Those found in RA, SLE, and PBC were 64.2, 63.5, and 78.5, respectively. Generally, reactivities found in P-SS and S-SS were higher than those in RA and SLE. Both the mean titers of P-SS and S-SS were significantly higher ($P < 0.01$) when compared to the mean titer of NHS (Fig. 2c).

Incidences of anti-IP_3R1, anti-IP_3R2, and anti-IP_3R3 found in SS and in other disease conditions

Reactivities twice the mean intensity obtained for NHS were judged positive. Using such a criterion, the prevalence of anti-IP_3R1 antibodies among P-SS, S-SS, and NHS was 17/35 (48.6%), 13/33 (33.3%), and 1/33 (3.0), respectively. Prevalences found in RA, SLE, SSc, and PBC were 34/124 (27.4%), 6/26 (23.1%), 6/17 (35.2%), and 4/6 (66.7%), respectively. Prevalence of anti-IP_3R2 antibodies among P-SS, S-SS, and NHS was 13/35 (37.1%), 14/39 (35.9%), and 2/33 (6.1%), respectively. Prevalences found in RA, SLE, SSc, and PBC were 56/124 (45.2%), 10/26 (38.5%), 5/17 (29.4%), and 4/6 (66.7%), respectively. Prevalence of

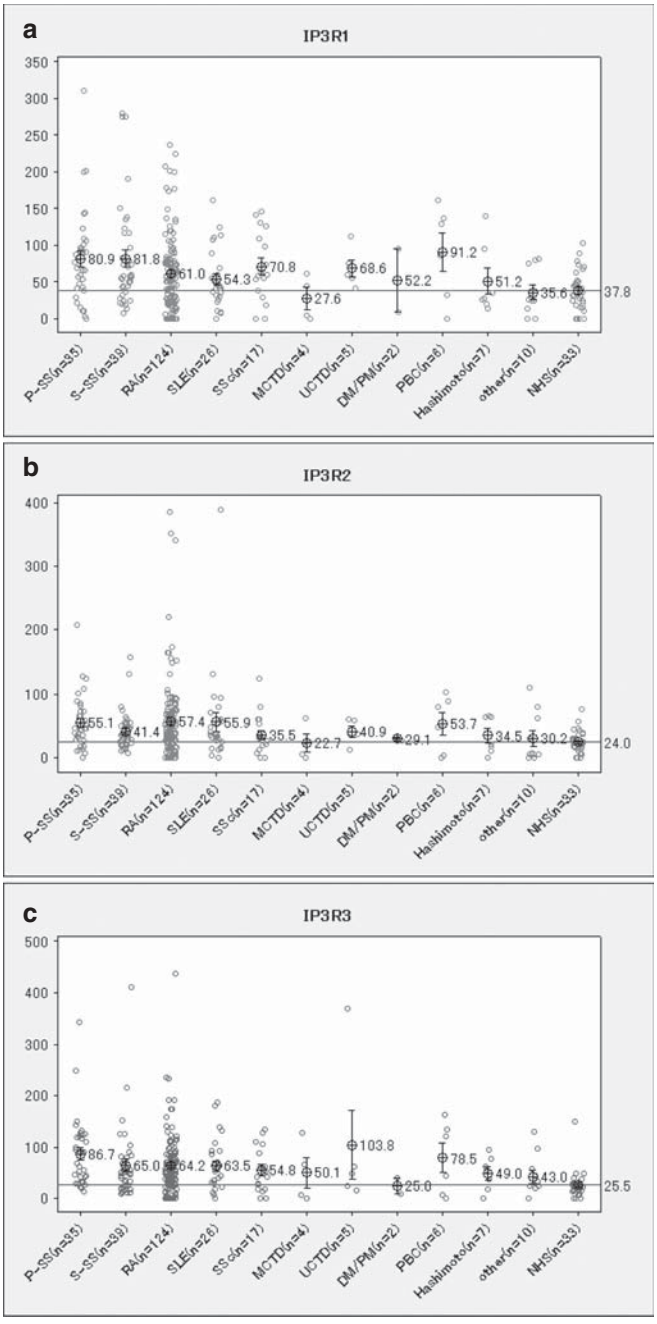

Fig. 2. Titers of anti-IP_3R1, anti-IP_3R2, and IP_3R3 antibodies in the different systemic rheumatic diseases and normal healthy subjects (*NHS*) are shown. The mean titer of anti-IP_3R1 in NHS sera was found to be 37.8. The cutoff value for positivity was arbitrarily set at twice this value (75.6) (**a**). The mean titer of anti-IP_3R2 in NHS sera was 24.0. Likewise, the cutoff for positivity was set at twice this value (48.0) (**b**). Finally, the mean titer of anti-IP_3R3 in NHS sera was 25.5. Twice this value (51.0) was set as the cutoff for positivity (**c**). *P-SS*, primary Sjögren's syndrome; *S-SS*, secondary Sjögren's syndrome; *RA*, rheumatoid arthritis; *SLE*, systemic lupus erythematosus; *SSc*, systemic sclerosis; *MCTD*, mixed connective tissue disease; *UCTD*, unclassified connective tissue disease; *DM/PM*, dermatomyositis/polymyositis; *PBC*, primary biliary cirrhosis

Table 1. Incidence of anti-IP_3R1, anti-IP_3R2 and anti-IP_3R3 antibodies found in patients with SS and other disease conditions

	P-SS 35	S-SS 39	RA 124	SLE 26	SSc 17	MCTD 4	UCTD 5	DM/PM 2	PBC 6	Hashimoto 7	Other 10	NHS 33
γ-IP3R1	17 (48.6)	13 (33.3)	34 (27.4)	6 (23.1)	6 (35.2)	0	2 (40)	1 (50)	4 (66.7)	2 (28.6)	3 (30)	1 (3.0)
γ-IP3R2	13 (37.1)	14 (35.9)	56 (45.2)	10 (38.5)	5 (29.4)	1 (25)	2 (40)	0	4 (66.7)	3 (42.9)	3 (30)	2 (6.1)
γ-IP3R3	17 (48.6)	15 (38.5)	63 (50.8)	14 (53.8)	8 (47.1)	2 (50)	2 (40)	0	3 (50)	4 (57.1)	3 (30)	1 (3.0)

P-SS, primary Sjögren's syndrome; S-SS, secondary Sjögren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; MCTD, mixed connective tissue disease; UCTD, unclassified connective tissue disease; DM/PM, dermatomyositis/polymyositis; PBC, primary biliary cirrhosis; NHS, normal healthy subjects

Table 2. Frequencies of various autoantibodies found in SS and other systemic rheumatic disease

	P-SS	S-SS	RA	SLE	SSc	MCTD
No. (M:F)	35 (2:33)	39 (1:38)	144 (26:118)	34 (1:33)	26 (1:25)	6 (0:6)
Mean age (years)	62 (22–92)	59 (22–84)	62 (21–87)	51 (23–81)	61 (52–85)	49 (37–58)
Anti-						
SS-A/Ro	22 (62.9%)	23 (59.0%)	12 (8.4%)	16 (47.1%)	4 (15.4%)	3 (50%)
SS-B/La	4 (11.5%)	0	0	0	0	0
Centromere	3 (8.6%)	4 (10.3%)	1 (0.7%)	2 (5.9%)	10 (38.5%)	0
U1RNP	0	5 (12.9%)	0	6 (17.7%)	1 (3.9%)	6 (100%)
Ki	1 (2.9%)	4 (10.3%)	0	8 (23.6%)	0	0
Topo1	0	0	0	0	4 (15.4%)	0

P-SS, primary Sjögren's syndrome; S-SS, secondary Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; MCTD, mixed connective tissue disease

anti-IP_3R3 antibodies among P-SS, S-SS, and HHS was 17/35 (48.6%), 15/39 (38.5%), and 1/33 (3.0%). Prevalences found in RA, SLE, SSc, and PBC were 63/124 (50.8%), 14/26 (53.8), 8/17 (47.1%), and 3/6 (50%), respectively (Table 1).

Frequencies of various autoantibodies found in SS and other systemic rheumatic diseases

Frequencies of autoantibodies found in SS and other systemic rheumatic diseases were examined. For statistical purposes, 39 patients with S-SS were reclassified to CTD. Thus, the number of RA, SLE, SSc, and MCTD patients in this study became 144, 34, 26, and 6, respectively. Frequencies of anti-SS-A/Ro antibodies found in P-SS and S-SS were 22 of 35 (62.9%) and 23 of 39 (59%), respectively. Anticentromere antibodies were found in 3 of 35 (8.6%) patients with P-SS, 4 of 39 (10.3%) with S-SS, and 10 of 26 (38.5%) with SSc. Anti-Ki antibodies were found in 1 of 35 (2.9%) with P-SS, in 4 of 39 (10.3%) with S-SS, and in 8 of 34 (23.6%) with SLE (Table 2).

Immunoblot using recombinant IP_3Rs proteins show the different reactivities with sera from patients with SS and RA

Figure 3 represents the different patterns obtained by immunoblot using the recombinant proteins IP_3R1, IP_3R2, and IP_3R3 with representative P-SS, S-SS, and RA patient sera, NHS serum, and rabbit anti-IP_3Rs antibody.[19] Three sera (Nos. 173, 218, 228) from patients with P-SS produced a stronger band against IP_3R1 than against IP_3R2 and IP_3R3. Three sera (Nos. 50, 223, 227) from patients with S-SS yielded a stronger band for IP_3R3 than for IP_3R1 and IP_3R2.

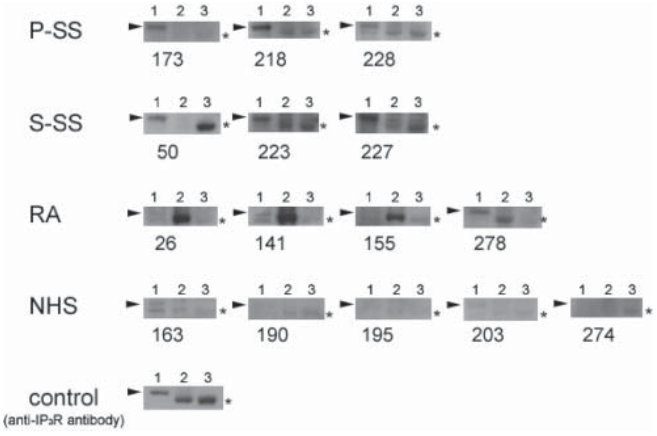

Fig. 3. Immunoblot analysis of the three types of mouse IP_3Rs with sera from patients of various autoimmune diseases, and normal healthy subjects, and with rabbit anti-IP_3R sera[19] as controls. Representative immunoblotting profiles are shown. *Lane 1*, IP_3R1; *lane 2*, IP_3R2; *lane 3*, IP_3R3. Positions of IP_3R1 are indicated by *arrowheads*, and those of IP_3R2/IP_3R3 are indicated by *asterisks*. The number of sera is shown under the immunoblot image. *P-SS*, primary Sjögren's syndrome; *S-SS*, secondary Sjögren's syndrome; *RA*, rheumatoid arthritis; *NHS*, normal healthy subjects

Four sera (Nos. 26, 141, 155, 278) from patients with RA produced a stronger band for IP_3R2 than for IP_3R1 and IP_3R3. Four sera from NHS yielded no significant bands, and a positive control rabbit serum presented with bands specific for IP_3R1, IP_3R2, and IP_3R3.

Rheumatoid arthritis sera containing anti-IP_3R2 antibody recognize the regulatory domain

Ten sera showing the highest intensities to IP_3R2 were selected. Nine of these sera were from patients with RA and

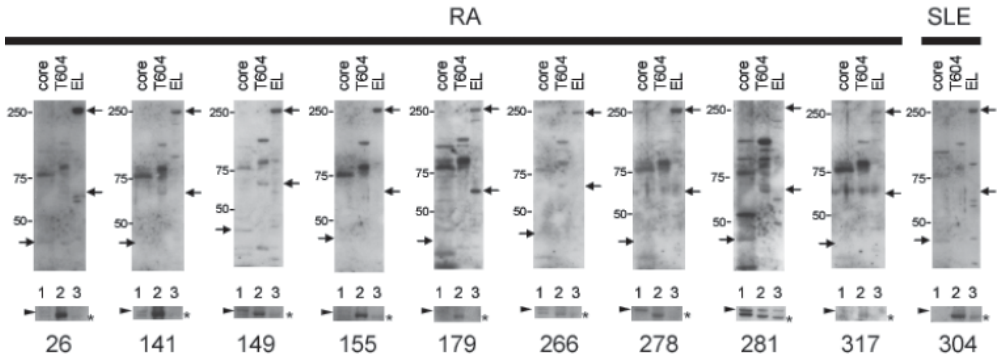

Fig. 4. Immunoblot analysis of portions of mouse IP_3R2 with sera from rheumatoid arthritis (*RA*) and systemic lupus erythematosus (*SLE*) patients. Representative immunoblotting profiles are shown. The band positions of EL, T604, and core are sequentially shown by *arrows*. Bands appearing at the other positions are all nonspecific. Molecular size markers are shown at the *left* (kDa). The lower immunoblot image shows immunoblot analysis with three mouse IP_3R types as antigen. *Lane 1*, IP_3R1; *lane 2*, IP_3R2; *lane 3*, IP_3R3. Positions of IP_3R1 are indicated by *arrowheads*, and those of IP_3R2/IP_3R3 are indicated by *asterisks*. The number of sera is shown under the immunoblot image

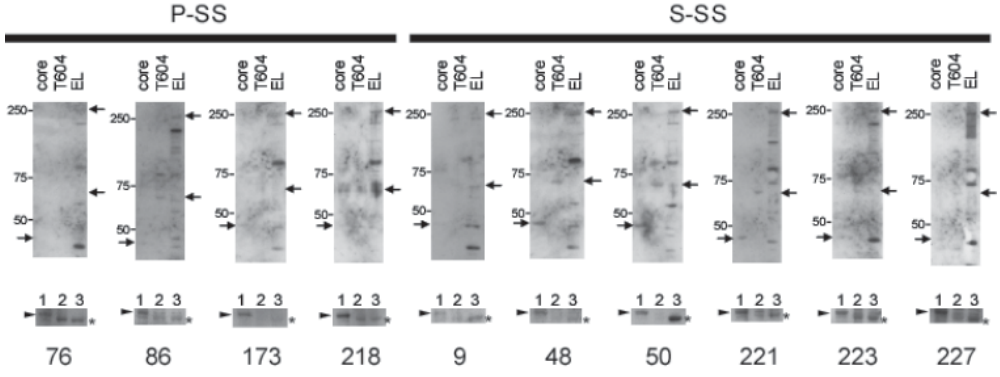

Fig. 5. Immunoblot analysis of portions of mouse IP_3R1 with sera from Sjögren's syndrome patients. Representative immunoblotting profiles are shown. The positions of EL, T604, and core are sequentially shown by *arrows*. Bands appearing at the other positions are all nonspecific. Molecular size markers are shown at the *left* (kDa). The lower immunoblot image shows immunoblot analysis with three mouse IP_3R types as antigen. *Lane 1*, IP_3R1; *lane 2*, IP_3R2; *lane 3*, IP_3R3. Positions of IP_3R1 are indicated by *arrowheads*, and those of IP_3R2/IP_3R3 are indicated by *asterisks*. The number of sera is shown under the immunoblot image. *P-SS*, primary Sjögren's syndrome; *S-SS*, secondary Sjögren's syndrome

1 was from a patient with SLE. All with the exception of serum No. 281 yielded strong bands in the lane containing EL. In contrast, significant bands were not detected in lanes containing the core and T604 (Fig. 4).

Sjögren's syndrome sera containing anti-IP_3R1 antibody recognize core protein

Ten sera producing the highest intensities against IP_3R1 were selected. Four of these were from patients with P-SS and 6 were from patients with S-SS. Most sera yielded only faint to moderate bands, and their cognate epitope was thought to exist in the core protein (Fig. 5).

Discussion

Calcium concentrations are strictly regulated in all biological cells, and one of the key molecules responsible for this regulation is the inositol 1,4,5-trisphosphate receptor (IP_3R), which is known to form a tetrameric Ca^{2+} channel in the endoplasmic reticulum. The receptor is involved in neuronal transmission via Ca^{2+} signaling and for many other functions that relate to morphological and physiological processes in living organisms. Three types of IP_3Rs, derived from three distinct genes, have been identified in mammals. Type1 IP_3R (IP_3R1) is predominantly expressed in brain tissue and plays a critical role in the regulations of motor and learning systems. It is also highly expressed in smooth muscle cells and endothelial cells. The other two types, types 2 and 3 IP_3Rs (IP_3R2 and IP_3R3), are expressed in various tissues and cell lines.[20–22] The three types of human IP_3R have not yet been isolated; for this study full-length recombinant proteins expressed in mice were used as antigens. Amino acid sequences of IP_3Rs are known to be 95% homologous between mouse and human. Among the three types of IP_3Rs, 70% amino acid sequence homology was observed in the IP_3 binding and Ca^{2+} channel sites. However, amino acid sequence of the internal coupling and regulatory domain regions were only 50% homologous.

IP_3Rs are not only located on the endoplasmic reticulum but also are associated with the plasma membrane channels and Na pumps,[23] and are therefore thought to be active target antigens of autoimmune sera from patients with SS and other disease conditions. In general, it is difficult to envision that IP_3R antibody found in sera of patients with SS could have had access to IP_3R on the surface of the endoplasmic reticulum. Initially, we thought this antibody was not related to any specific clinical manifestation in this study. However, if the cell surfaces of endothelial and smooth muscle cells are damaged, circulating anti-IP_3R1 antibody would have access to the cytoplasm, and peripheral neuropathy might be induced due to ischemia. Further, when saliva is secreted from salivary cells, anti-IP_3R2 or anti-IP_3R3 antibodies might enter the cytoplasm and react with IP_3Rs on the endoplasmic reticulum.

Recently, we identified p97/VCP, the most abundant AAA (ATPase) associated with diverse cellular activities,[24] and known to react with autoimmune sera from patients with primary biliary cirrhosis.[8] p97/VCP is formed as a homohexamer and plays important roles in the assembly of the nuclear envelope, endoplasmic reticulum, and Golgi apparatus. This protein in combination with other proteins shows diverse functions.[25] Similarly, IP_3R may combine with adaptor proteins to play important biological roles. For example, anti-IP_3R and anti-SS-A/Ro are frequently found together in SS, in contrast to other autoantibodies directed against U1RNP, centromere antigen, and SS-B/La antigens, which occur individually.

The clinical significance of this IP_3R antibody remains unclear. However, its detection may become useful in establishing the diagnosis of systemic rheumatic diseases, in much the same manner as the detection of anti-SS-A/Ro, since this antibody can be readily identified.[26] Elsewhere, we also detected antibody to p97/VCP in one PBC patient, and antibody to WS in one RA patient associated with SSc, but could not detect any anti-Golgi complex antibodies in any of the SS patients in this study.

Finally, RA sera containing anti-IP_3R2 antibodies were found to react more strongly with IP_3R2 residues 1–2171, as compared to reactivities against residues 1–604 (T604) and 224–604 (core).We have not yet determined the precise location of the antigenic epitope. Regarding anti-IP_3R1 antibodies, some SS sera were able to react with all three core, T604, and EL residues. This leads us to believe that the antigenic epitope might be localized in a common area of the core protein. We plan to conduct further studies to elucidate the structure of each isoform of IP_3R, and to be able to recognize the primary sequence and tertiary structure.

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