Epitope Mapping of Anti-α-Fodrin Autoantibody in Juvenile Sjögren's Syndrome: Difference in Major Epitopes Between Primary and Secondary Cases

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ABSTRACT. ***Objective.*** Juvenile Sjögren's syndrome (SS) is an early-onset type of SS. Autoantibody against the N-terminal 120 kDa form of α-fodrin is a specific and sensitive disease marker for both juvenile and adult SS. We investigated the initial and major determinants of α-fodrin in SS.

Methods. Sera were obtained from patients with juvenile SS, 10 with primary SS and 10 with secondary SS. Epitope specificities of IgG antibodies were examined by dot-blot analyses using overlapping fusion proteins of the N-terminal part (561 amino acid residues) of α-fodrin as antigens.

Results. All sera from patients with primary SS reacted with amino acid residues 1 to 98 and 36 to 150, but not with 91 to 199. Epitope mapping using fusion proteins with subfragments, each consisting of about 50 amino acid residues, showed reactivity with amino acid residues 27–80 and 79–132, suggesting that at least 2 epitopes are contained in the first 150 amino acid residues. All 3 cases with neurological complications had additional epitope specificities. Sera from patients with secondary SS showed more diversified specificities and strongly reacted with amino acid residues 1–98 and 334–432, whereas the reactivities to 36–150, a major epitope in primary SS, were minimal.

Conclusion. Major and initial B cell epitopes specifically reside in N-terminal amino acids 36–132 and could be used as a diagnostic tool for primary SS. The epitope subsequently expands to other regions of α-fodrin in association with the development of neurological complications or disease progression. Secondary SS has distinct epitope specificities. (J Rheumatol 2006;33:1395–400)

Key Indexing Terms:
SJÖGREN'S SYNDROME α-FODRIN AUTOANTIBODY
EPITOPE MAPPING EPITOPE SPREADING

Sjögren's syndrome (SS) is an autoimmune disease characterized by a progressive infiltration of lymphocytes and plasma cells to the exocrine glands, predominantly salivary and lachrymal glands, with varying degrees of systemic involvement[1]. SS is classified into 2 groups: primary SS, which occurs alone, and secondary SS, which occurs in association with other collagen vascular diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or scleroderma. Whereas SS commonly affects middle-aged women, juvenile onset SS is not so rare as has been estimated before[2–8]. Most cases of juvenile SS lack sicca symptoms, but share similarity with adult cases in both pathological and laboratory features, suggesting that juvenile SS is an early-onset type of the disease[2-8]. Recent studies using immunoblot analyses have shown that IgG-class autoantibody against the N-terminal portion of the 120 kDa form of α-fodrin, an actin-binding protein, is highly sensitive and specific to both adult and juvenile SS[9-12]. Anti-α-fodrin IgG antibodies appear before either anti-SSA/Ro or SSB/La antibodies become positive, and could be an early diagnostic marker of the disease[10]. In addition, critical roles of α-fodrin-specific T cells in the initiation or progression of the disease have been suggested in both murine models and human primary SS, particularly with short duration from onset of the disease[9,13,14]. Thus, autoimmunity to α-fodrin could be involved in the development of primary SS, although the pathological role of the antibodies remains unknown. On the other hand, anti-α-fodrin antibodies are detected by ELISA in less than 10% to 64% of patients with primary SS, whereas 26% of patients with SLE are positive for the antibodies[15-20]. As well, the antibodies are detect-

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ed in 29% of primary SS and 47% of SLE by immunoprecipitation assay[21]. The discrepancy in the sensitivity and specificity could be attributed to the differences in assay systems[9–12,15–21], classification criteria[12], treatment[19], and possibly age groups[10,11]. Antigens are usually denatured in the Western blot system and differ in structure from those bound to ELISA plates[17]. These facts raise the possibility that the autoantibodies preferentially recognize linear and hidden epitopes of α-fodrin in primary SS. Thus, the identification of initial and major epitopes of the protein could provide insight into both the mechanisms of the disease and the development of more sensitive and specific diagnostic systems. Because anti-α-fodrin antibodies normalize within 3 months of treatment, the antibodies should be assessed in an untreated group[20]. In this study, to identify the initial and major epitopes, we constructed overlapping fusion proteins of α-fodrin and used them for epitope analyses of IgG autoantibodies in untreated juvenile SS.

MATERIALS AND METHODS

Patients. Ten patients with primary SS, aged 5 to 15 years, (Group I) and 10 patients with secondary SS, aged 7 to 15 years, (Group II) classified according to the Japanese criteria[22] were included in the study (Table 1). All the primary cases had positive findings in both sialography and lip biopsy and were finally classified as having SS according to the American-European criteria for SS[23]. Sera were obtained before treatment with corticosteroids, antimalarials, or immunosuppressive agents, and were stored at –20°C until use. Sera were additionally obtained from a primary SS case (Patient I-5) upon diagnosis of aseptic meningitis and 2 years after remission. Some cases have been described previously[5,10,24]. Control sera were obtained from 6 patients with SLE alone, 7 with juvenile idiopathic arthritis (JIA), 7 with juvenile dermatomyositis, and 30 with no connective tissue diseases.

Production of fusion proteins. Ten nanograms of plasmid DNA (pGEX-JS-1), which encodes the N-terminal 561 amino acids of α-fodrin[25], were used as template for 50 μl of polymerase chain reaction (PCR). Complementary DNA coding 11 overlapping fragments and 5 subfragments of α-fodrin were amplified using the GenAmp 2400 PCR system (Applied Biosystems, Foster City, CA, USA) using primer pairs shown in Table 2. After an initial denaturation at 94°C for 5 min, each fragment was amplified by 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 7 min. PCR products were subcloned into TA cloning vector pCR2.1 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Each clone was examined for nucleotide sequence by ABI Prism GenAnalyzer 310 (Applied Biosystems). DNA fragments with correct sequence were digested with EcoRI, purified by gel extraction, and then subcloned into EcoRI site of glutathione-S-transferase (GST)-fusion protein expression vector, pGEX4T-2 or 4T-3, in-frame. Fusion proteins were expressed in *E. coli*, TB1, in the presence of 1 mM isopropylthiogalactoside (Amersham Pharmacia, Buckinghamshire, UK), and purified with glutathione-Sepharose beads (Amersham Pharmacia) according to the manufacturer's protocol.

Western blotting and dot blotting. One hundred nanograms of recombinant GST-JS-1 or fusion proteins with 11 overlapping fragments or 5 subfragments were electrophoresed on 7.5% or 12.5% sodium dodecyl sulfate-polyacrylamide gels and electrically transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia). For dot-blot analyses, 100 ng of each fusion protein were put onto the nitrocellulose membrane. After blocking with 5% nonfat milk at 4°C for 16 h, membranes were incubated with 1000-fold diluted goat anti-GST antibody or 400-fold diluted human sera at 4°C for 16 h as primary antibody. To prevent cross-reactivity with *E. coli* components or GST, human sera were diluted with the extract of *E. coli* expressing GST

Table 1. Clinical and serological findings of primary and secondary SS cases.

Patient	Sex	Age at Disease Onset, yrs	Dry Mouth	Dry Eye	Associated CVD	Complications	ANA	RF	SSA	SSB
Primary SS										
I-1	F	5	No	No	Primary	RTA	1:1280	+	+	+
I-2	F	9	No	No*	Primary		1:320	+	+	+
I-3	F	9	No	No*	Primary		1:2560	+	+	+
I-4	F	10	No	No	Primary	RTA	1:40	+	–**	–
I-5	F	8	No	No	Primary	Meningitis	1:1280	+	–**	–**
I-6	F	14	No	No	Primary		1:320	–	+	+
I-7	F	14	No*	No*	Primary	Neuropathy	1:160	+	+	+
I-8	F	7	No	No	Primary		1:2560	+	+	–
I-9	F	14	No	No	Primary	Meningitis	1:320	+	–	–
I-10	F	10	Yes	No	Primary		1:320	+	–	–
Secondary SS										
II-1	F	11	No	No	SLE	Pericarditis	1:2560	–	+	+
II-2	F	10	No	No	MCTD	Hashimoto	1:1280	+	+	–
II-3	F	7	Yes	Yes	SLE	GN	1:640	+	+	–
II-4	F	14	Yes	Yes	SLE	GN	1:2560	+	+	+
II-5	M	11	No	No	JDM	Meningitis	1:1280	+	+	+
II-6	F	11	No	No	SLE		1:1280	–	+	–
II-7	F	14	No	No	SLE		1:1280	–	+	–
II-8	F	14	No	No	SLE		1:1280	+	+	–
II-9	F	12	No	No	JIA		—	+	+	–
II-10	F	15	No	No	JIA		1:320	+	+	+

* Sicca symptoms and ** autoantibodies appeared later. CVD: collagen vascular disease; SLE: systemic lupus erythematosus; MCTD: mixed connective tissue disease; JDM: juvenile dermatomyositis; JIA: juvenile idiopathic arthritis; RTA: renal tubular acidosis; GN: glomerulonephritis; ANA: antinuclear antibodies; RF: rheumatoid factor.

Table 2. Forward and reverse primer pairs for PCR amplification.

Primers	Nucleotide Sequences
Fp1-98F	ATG GAC CCA AGT GGG GTC AAA GT
Fp1-98R	GGC TCC TGA GTT GGC CTG CAC
Fp36-150F	GGC GTC AGA AGC TGG AAG ATT CCT
Fp36-150R	GTC CAT CAC GTC CTC ACA TTC TCG
Fp91-199F	GTG CAG GCC AAC TCA GGA GCC
Fp91-199R	AGC CAT ATC TGT TTG AAA CTC TTC
Fp148-236F	CAG AAC TTG GTG CAG TAC TTA CGA
Fp148-236R	GGC TGC ATT GAC TTC ATC CTG
Fp191-298F	GAA GAG TTT CAA ACA GAT ATT GGC T
Fp191-298R	GTG CTT CCG AAG CAG AGC CTG
Fp236–334F	TGG CAG CGG CTG AAG GCC TGG CT
Fp236-334R	CAC TTG AAT CTG TGT TGC ACT CAG
Fp290-399F	CAG GCT CTG CTT CGG AAG CA
Fp290-399R	CAG GGC TTC AGC CCC AGC CAC ACT
Fp334-432F	AAG CGA GAG GAA CTG ATT ACA AAC TGG
Fp334-432R	GTG ACC AGC AGC AAG CAG TGC CTG
Fp372-480F	AGT GAT GTG GCT GGG GCT GAA GCC CTG
Fp372-480R	GTC CAC CTG CTC AGT GTC CGG TAG AAG AG
Fp432-545F	CAC TAT GCC TCA GAT GAA GTG AGG
Fp432-545R	GGC CAC ATC TTC CAT TGC ATA GTG
Fp470-561F	CTC TTC TAC CGG GAC ACT GAG CAG GT
Fp470-561R	CCG TCA TCA CCG AAA CGC
Fp1-52F	ATG GAC CCA AGT GGG GTC AAA GTG
Fp1-52R	GCA TCT CTT TGA AAG AAC TGG
Fp27-80F	CCG CTT CAA GGA ACT CTC AAC CCT
Fp27-80R	TCC CTG CAA GTT GGT TGG GTC
Fp52-107F	GAT GCT GAA GAG CTG GAG AAA TGG
Fp52-107R	GGT TTC CAG TTT CAT CCA GCT
Fp79-132F	GGG AAA GCT TCA GAA GCA TCA
Fp79-132R	ATT CCC ACT GGC GGT GCA GCT CCA
Fp106-150F	GGA AAC CTG ATG ATC TCA GAA GGA
Fp106-150R	GTT CTG GGC CTG CAG CAA TTT GA

alone. For preabsorption analyses, human sera were preincubated with 10 μg of fusion proteins at 4°C for 1 h and then used as the primary antibody. The membranes were washed 4 times with Tris-buffered saline containing 0.1% Tween-20 (TBST), and then were incubated with 30,000-fold diluted horseradish peroxidase-conjugated (HRP) anti-goat antibody or 25,000-fold diluted HRP-goat anti-human IgG (Biosource, Camarillo, CA). After washing 5 times with TBST, signals were detected by ECL Plus Western blotting detection reagents (Amersham Pharmacia).

RESULTS

Epitope mapping in primary SS. Sera from all 10 primary SS, 9 of the 10 secondary SS, and one of the 6 SLE subjects reacted with GST-JS-1 and were tested for epitope mapping (data not shown). None of the cases of JIA, dermatomyositis, or non-collagen vascular diseases was positive for antibody against GST-JS-1 (data not shown). Each overlapping fusion protein had a molecular weight on SDS-PAGE consistent with the calculated one and was used for the following epitope mapping as an antigen (data not shown). Western blot analyses showed that sera from Patients I-1, I-2, and I-3 reacted with both fusion proteins with amino acid residues 1 to 98 (Fp1–98) and Fp36–150, but not with any other fragments (data not shown). Because only the predicted bands were detected by Western blotting, further analyses were carried out by a dot-blot method. Dot-blot analysis showed that all the sera from 10 patients with primary SS reacted with both Fp1–98 and Fp36–150 (Figure 1). Sera from Patients I-7, I-9, and I-10 showed reactivity to additional fragments but not to Fp91–199. The serum from Patient I-5 showed specificity to Fp1–98 and Fp36–150 at her first visit, and to additional fragments upon the diagnosis of meningitis and later (Figure 2).

To confirm that Fp1–98 and Fp36–150 contain dominant determinants, a preabsorption study was performed. The reactivity of case I-1 serum with GST-JS-1 was partially inhibited by preabsorption of the sera with excess amount of either Fp1–98 or Fp36–150, and was completely diminished by preabsorption with a mixture of both fragments, suggesting that the N-terminal portion of α-fodrin contains at least 2 dominant epitopes (data not shown). To determine the dominant epitopes of α-fodrin more precisely, we constructed 5 subfragments of the N-terminal portion of α-fodrin consisting of about 50 amino acid residues (Table 2). All the sera from primary SS reacted with Fp27–80 and Fp79–132, and weakly with Fp52–107, but not with Fp1–52 (Figure 3). Considering these data together, multiple dominant epitopes are localized to amino acids 36 to 132.

Epitope mapping in secondary SS and SLE. Nine of the 10 secondary SS sera and one of the 6 SLE sera (SLE-3) reacted with recombinant GST-JS-1 (data not shown) and were used for dot-blot analysis. All the sera from secondary SS and SLE-3 showed reactivity with most of the overlapping fragments, including Fp91–199, which was scarcely reactive to sera from

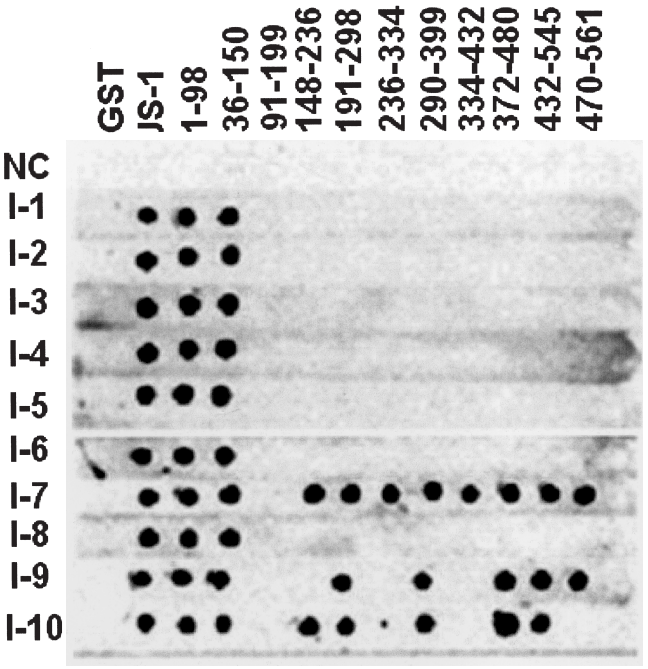

Figure 1. Dot-blot analysis of sera from patients with primary SS using fusion proteins of α-fodrin. Reactivity of each fragment, consisting of about 100 amino acid residues (indicated across the top), was tested with sera from cases of primary SS. GST-JS-1 and GST were used as positive and negative controls, respectively.

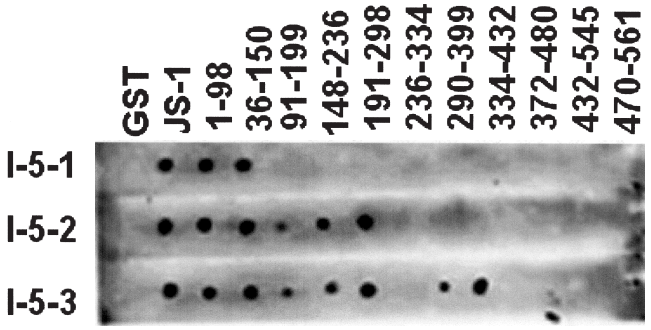

Figure 2. Epitope spreading in a case of primary SS (Patient I-5). Sera were obtained at 3 points: 2 years before the diagnosis of SS (I-5-1), at the time of diagnosis of SS complicated with meningitis (I-5-2), and 2 years after remission of meningitis (I-5-3).

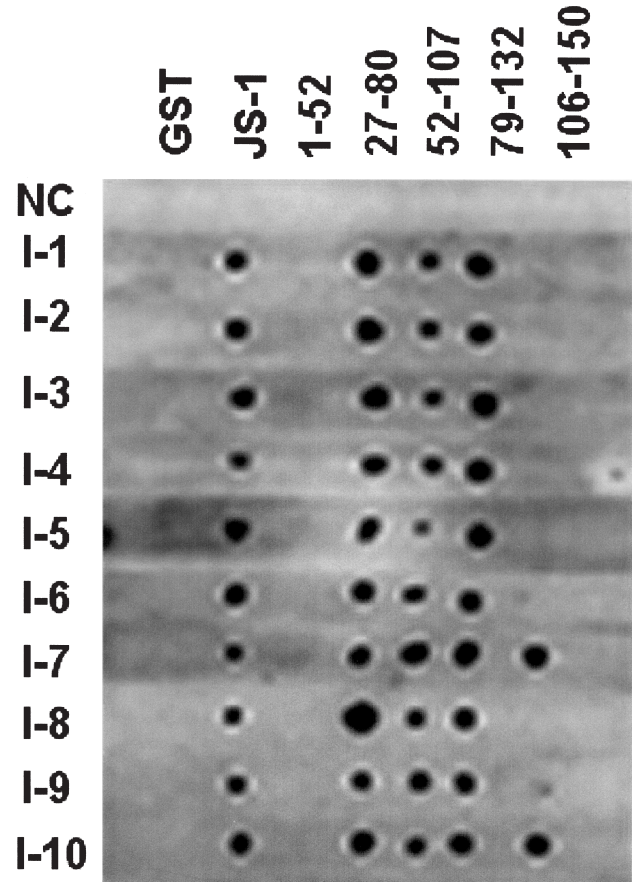

Figure 3. Epitope mapping analyses using subfragments of N-terminal portion of α-fodrin. Reactivity of each subfragment, consisting of about 50 amino acid residues (indicated across the top), was tested by dot-blotting with sera from cases with primary SS. GST-JS-1 and GST were used as positive and negative controls, respectively.

primary SS (Figure 4). In particular, the strongest reactivity was observed with Fp1–98 and Fp334–432.

DISCUSSION

The sensitivities of antibodies to the N-terminal 120 kDa form

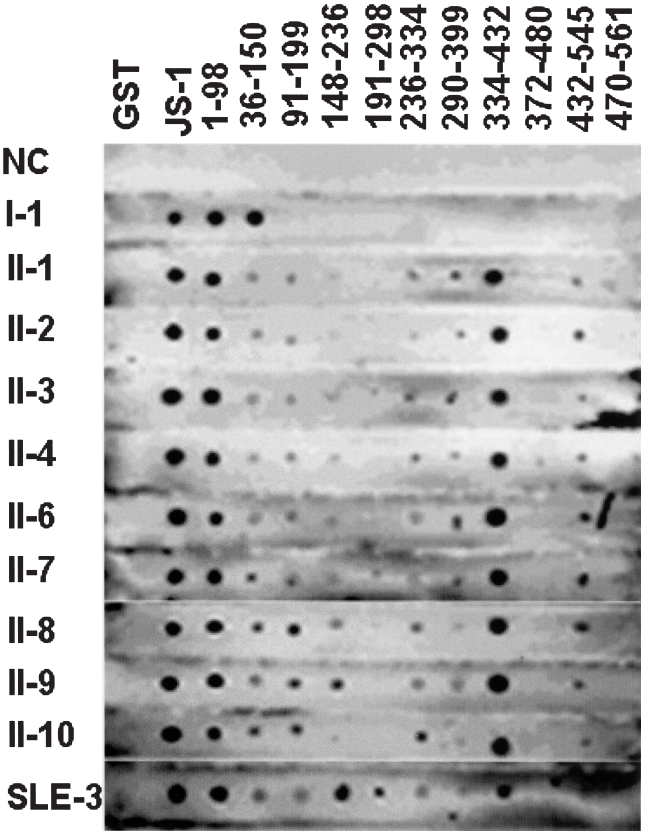

Figure 4. Epitope mapping analysis in cases of secondary SS and SLE. Reactivity of each fragment, consisting of about 100 amino acid residues (indicated across the top), was tested by dot-blotting with sera from secondary SS and a case of SLE (SLE-3). Sera from a primary SS patient (I-1) and a healthy individual were used as positive and negative control (NC) of antibody, respectively. GST-JS-1 and GST were used as positive and negative controls of antigen, respectively.

of α-fodrin in untreated juvenile primary and secondary SS were 100% and 90%, respectively, which are higher than those in adult cases, confirming previous reports in juvenile SS[9-12]. Most cases of juvenile SS lack apparent sicca symptoms and are initially suspected from nonspecific extraglandular symptoms or enlargement of salivary glands associated with elevated erythrocyte sedimentation rate, hypergammaglobulinemia, and autoantibodies, such as antinuclear antibodies, rheumatoid factor, or anti-SSA or SSB antibodies[2-8]. Furthermore, sicca symptoms developed in some of the patients in our study during the followup period (Table 1). Thus, it is possible that juvenile SS is an early stage of the disease, which develops on a strong autoimmune background and accordingly tends to be positive for anti-α-fodrin autoantibodies.

We observed that the initial and major epitopes in primary SS reside in amino acid residues 36–132 of α-fodrin. This portion likely contains at least 2 epitopes, because the reactivity of sera with GST-JS-1 fusion protein was completely inhibited by preabsorption with mixtures of both Fp1–98 and

Fp36–150, but partially inhibited with each of them. This was confirmed by the finding that primary SS sera strongly reacted with 2 separate subfragments, Fp27–80 and Fp79–132. Two of the 3 cases that showed additional specificities, I-7 and I-9, were complicated by peripheral neuropathy and meningitis, respectively[10,24]. The other case, I-10, had apparent sicca symptoms at presentation. Further, Patient I-5 showed progressively diversified epitope specificities with the development of meningitis. Thus, diversified epitope specificities may be associated with neurological complications or disease progression. Our findings are consistent with the high prevalence of the autoantibody in adult primary SS with neurological complications[26,27]. Up to 20% of cases of primary SS have neurological complications similar to multiple sclerosis (MS)[28]. In addition, anti-α-fodrin antibodies are detected in 13% of patients with MS[26]. Thus, the epitope analyses of α-fodrin in MS may clarify the epitopes related to neurological complications of SS. Greidinger, *et al* have reported dominant epitopes of autoantigens such as SmD1, SSA/Ro, and SmB'/B antigens in human autoimmune diseases, and demonstrated the intermolecular epitope spreading of anti-U1-RNP antibody by a large-scale prospective study[29]. Similar mechanisms may be involved in the production of autoantibody against ß-fodrin, the other component of fodrin in SS[30]. On the other hand, intramolecular epitope spreading has been reported mainly in experimental animals, because in human autoimmune disease the autoantibodies usually appear preceding the onset of the disease[31-35]. Our results suggest the presence of intramolecular epitope spreading associated with neurological complications or progress of the disease in human SS.

In contrast to primary SS, all the sera from secondary SS cases reacted with most of the fragments. Particularly strong reactivity of the sera was observed with Fp1–98 and Fp334–432. It is noteworthy that Fp334–432 was not reactive with the primary SS sera, except for 2 cases with neurological complication (cases I-5-3 and I-7). On the other hand, all sera from secondary SS cases showed only minimal reactivity with Fp36–150, a major epitope in primary SS, suggesting that Fp36–150 is a candidate for a primary SS-specific antigen in the ELISA system. Recent studies have suggested that apoptosis induced by stimulation such as infection triggers an autoimmune reaction against autoantigens[36]. α-fodrin is cleaved to the N-terminal 120 kDa form by apoptosis-activated caspase-3 and calpain, and then acquires antigenicity[13,37-39]. After the cleavage, α-fodrin is translocated to the cell membrane[40,41]. It is possible that the enzymatic cleavage induces conformational change of the antigen and subsequent exposure of hidden epitope(s) at the cell surface of the salivary and possibly the lachrymal gland in primary SS. The different epitope specificity in secondary SS may reflect different degradation pathways from those in primary SS. Cleavage product of α-fodrin is also present in neuronal cells undergoing apoptosis, and is localized to plaques in the central nervous system (CNS) of patients with MS[42]. Additional epitope specificities in primary SS with neurological complications raise the possibility that the degradation pathways are different among the tissues. Indeed, the expression of calpain and its intrinsic inhibitor, calpastatin, is different in a salivary gland cell line, HSY, and a T cell line, Jurkat[13]. Given that α-fodrin is ubiquitously distributed, it is possible that the antibodies detected by ELISA or immunoprecipitation assay in SLE[15-21] are developed against the intact or partly cleaved α-fodrin that has leaked from cells undergoing destruction in other organs. One case of SLE positive for these autoantibodies in our study was complicated by CNS disease[43]. Because this case showed epitope specificities similar to those of secondary SS, secondary SS may develop in the future. Otherwise, anti-α-fodrin antibodies detected in this case could be associated with the neurological complication rather than the underlying SLE.

We found the N-terminal amino acid residue 1–98 reacted with sera from cases of both primary and secondary SS. Major and specific epitopes in primary SS initially reside in amino acid residues 36–132 of α-fodrin and might subsequently expand to other regions of the protein in association with the development of neurological complications or progression of SS disease. The autoantibodies showed distinct epitope specificities in secondary SS, suggesting that secondary SS is different from primary SS in regard to the degradation of the autoantigen. Detection of antibodies against the major determinants, amino acid residues 36–132, rather than intact molecules could be a specific diagnostic test for primary SS, at least in the early phase of the disease.

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