

Autoantigenicity of DFS70 is restricted to the conformational epitope of C-terminal alpha-helical domain

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Abstract

Autoantibodies against DFS70 (Dense Fine Speckles 70) are found in 30% of Japanese atopic dermatitis patients, and less frequently in patients with other diseases. We have recently reported that they are also seen in 11% of hospital workers, but in only ~2% of patients with systemic rheumatic disease. In this study, in order to investigate the possible pathological role of anti-DFS70 antibodies, fine epitope mapping was carried out using 93 anti-DFS70 autoantibody-positive sera. Immunoblotting using overlapping peptides failed to reveal major linear epitopes. Western blotting using various truncated proteins showed a strikingly uniform epitope distribution on a suspected tertiary structure expressed by DFS70^{349–435}. Some sera showed reactivity only in an immunoprecipitation assay using an in vitro translated DFS70. Circular dichroism analysis revealed that DFS^{349–435} contains an approximately 40% alpha-helical conformation, while an overlapping, non-antigenic peptide is composed of random coiled structures. The skewed single major epitope enabled us to establish a highly quantitative ELISA for the epitope region. Antibody titers showed no significant differences between the diseased group and healthy individuals. We propose that anti-DFS70 antibody may be a natural autoantibody, which might modify or reflect the inflammatory process of various disorders.
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1. Introduction

Autoantibodies against Dense Fine Speckles 70 (DFS70), also identical to lens epithelium derived growth factor (LEDGF) and transcription coactivator p75, were identified in 30% of Japanese atopic dermatitis (AD) patients, and less frequently in patients

with asthma and interstitial cystitis [1]. We have recently reported that autoantibodies to DFS70 were seen in 11% of 597 Japanese hospital workers, who represented 54% of the antinuclear antibody-positive population, but only 1.5% of patients with systemic rheumatic disease had this autoantibody [2]. On the other hand, LEDGF, which is considered to be a secreted protein translocating into the nucleus, was initially cloned as an autoantigen of cytotoxic antibodies found in sera from patients with age-related cataracts [3] and has been found to link with various cellular processes [4]. It was shown to be a DNA-binding transcription coactivator [5], promote cell survival and enhance stress resistance in

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various cell lines, and induce the disassembly of gap junction of lens epithelial cells via activation of protein kinase C gamma [4,6]. LEDGF interacts with HIV-1 integrase and mediates the chromosomal targeting of integrase [7]. The mRNA of p75 was shown to be expressed ubiquitously in a variety of tissues but was most abundant in thymus [5]. Alternative splicing of LEDGF produces a second protein product, p52, from the same gene; 325 of their N-terminal amino acid residues are identical, whereas C-terminal 205 and eight residues are different in LEDGF and p52 [4]. LEDGF and p52 show distinct nuclear distributions and cellular functions, which is considered to be due to the unique C-terminal 205 residue fragment of LEDGF/p75 [5,7–9].

Since autoimmunity to DFS70 appears in patients with various diseases and even in healthy individuals, our interest is whether these autoantibodies are clinically relevant; whether the autoantibodies mediate any pathological processes in these patients, or alternatively, whether the appearance of anti-DFS70 antibodies is a reflection of any immunological events occurring in these patients. To find a clue to these questions, we conducted fine epitope mapping of 93 anti-DFS70-positive sera from patients with various clinical manifestations as well as those from healthy individuals.

2. Materials and methods

2.1. Serum samples

Fifty-nine sera out of 670 sera in the serum bank of the Department of Dermatology, Nagoya University Hospital, were judged to be positive for anti-DFS70 antibody by indirect immunofluorescence assay (IIF) and western blotting assay using HeLa nuclear extract [2], and were selected for further study. The gender and age for all donors are shown in Table 1, and the clinical diagnosis of the patients are shown in Table 2. Thirty-four healthy human sera containing anti-DFS70 antibody were obtained from workers in Social Insurance Chukyo Hospital [2], and their medical history was confirmed for the absence of allergic diseases including

AD, prolonged fatigue, and cystitis-like symptoms. Thirty-six healthy human sera without anti-DFS70 antibody were randomly selected from workers in the same hospital. Informed consent was obtained from all donors. The approval was also obtained from the Ethics Committee of both the hospitals for this research. Diagnostic criteria for AD were those described by Hanifin and Rajka [10]. Patients with chronic fatigue syndrome (CFS) were diagnosed using the criteria of International Chronic Fatigue Syndrome Study Group [11]. Patients with CFS-like symptoms were considered to be those who suffered from prolonged fatigue with one or more symptoms of CFS, but otherwise did not fully meet the criteria. Diagnoses of systemic lupus erythematosus (SLE) and Sjögren's syndrome (SjS) were made in accordance with the revised classification criteria of American College of Rheumatology [12], and Japanese Criteria for SjS [13], respectively. Patients with connective tissue disease (CTD)-like symptoms were patients with one or more manifestations characteristic of SLE, SjS, or scleroderma, but did not fully meet the criteria. Other dermatological diseases were diagnosed according to the clinical and/or histopathological features of the patients.

2.2. Expression and purification of *Escherichia coli* recombinant proteins

The pET-DFS plasmid, full-length DFS70 cDNA cloned in *EcoRI* and *HindIII* sites of pET28a vector (Novagen, Madison, WI), was a kind gift from Dr. Tan (Scripps Research Institute, La Jolla, CA) [1]. This vector harbors a histidine tag at the N-terminal end of the expressed protein to allow rapid purification using metal affinity columns. To construct DFS70^{375–530}, pET-DFS was double digested with *EcoRI* and *StuI* and then ligated with a short linker DNA, aat-tAAGCTTGG (small letters indicate that the nucleotides are single stranded). DFS70^{1–326} was cloned from HeLa cDNA library by nested polymerase chain reaction (PCR) with the following primers: outer primer set, 5'-GCCCCCGGAAACATGACTC-3' and 5'-TTCCAGGTATGTCAACCTAG-3', inner primer

Table 1
Patient and control profiles

	Sera from Department of Dermatology, Nagoya University Hospital	Sera from hospital workers of Chukyo Hospital	Anti-DFS70 antibody-negative sera from hospital workers
Number	59	34	36
Male/female	1/58	6/28	13/23
Age (year)	7–66	21–45	30–59
Mean age $\pm$ standard deviation (year)	31.8 $\pm$ 14.2	29.3 $\pm$ 7.7	44.1 $\pm$ 9.2
Median age (year)	27.5	27	43.5
With/without clinical manifestation	55/4	0/34	0/36

Table 2
Clinical diagnoses of the patients

Diagnosis	Number of patients (n = 55) ^a
Atopic dermatitis	6
Chronic fatigue syndrome	8
CFS-like symptoms ^b	10
FMS-like symptoms ^c	1
Alopecia areata	4
Systemic lupus erythematosus	2
Sjögren's syndrome	4
Dermatomyositis	1
Discoid lupus erythematosus	1
Raynaud's disease	1
CTD-like symptoms ^d	11
Urticaria/angioedema	4
Pernio	2
Photosensitive dermatitis	2
Erythema nodosum	2
Pemphigus	1
Erythema multiforme	1
Siccant dermatitis	1
Others	1

^a Several patients had multiple diagnoses, thus the total number of diagnoses in this table exceeds 55.

^b Patients who suffer from prolonged fatigue with one or more symptoms of CFS, but do not fully meet the criteria.

^c Patients with one or more manifestations characteristic of fibromyalgia syndrome (FMS), but do not fully meet the criteria.

^d Patients with one or more manifestations characteristic of SLE, SjS, and scleroderma, but not fully meet the criteria.

sets, 5'-CGAATTCATGACTCGCGATTTCAAACC-3' and 5'-AAGCTTAGGCCTGCTCAGTTTCCATT-TGTTCC-3'. The resulting PCR fragment was ligated to a TA-cloning vector, pT7blue-2 T-vector (Novagen), and then subcloned between the *EcoRI* and *HindIII* sites of pET28a. To generate DFS70^{323–530}, a PCR fragment was amplified from pET-DFS using primer sets, 5'-AAGAATTCCAAATGGAACTGAGCAG-3', and 5'-CAGTCGACTGGCTAGTTATCTAGTG-3', and TA cloned to pT-Adv using an AdvanTage PCR cloning kit (Clontech, Palo Alto, CA). The insert was subsequently double digested with *EcoRI* and *Sall*, and then subcloned between the *EcoRI* and *XhoI* sites of pET28a.

Five DFS70-deletion mutants were fused with glutathione *S*-transferase (GST) at their N-terminus. As for DFS70^{323–375}, DFS70^{323–530} was double digested by the *BamHI*/*StuI* and the fragment was subcloned between the *BamHI* and *SmaI* sites of pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ). Insert fragments of DFS70^{323–407}, DFS70^{349–435}, DFS70^{349–450}, and DFS70^{349–464} were generated by PCR from pET-DFS as a template, using following primer sets (see below). PCR products were cloned to pT-Adv, double digested with *EcoRI* and *NotI* and subcloned between the corresponding sites of pGEX-4T-1. The 3' primers were: 5'-TGCGGCCGCAACTTTGAATCGCCGTAT-3'

(DFS70^{323–407}), 5'-TGCGGCCGCCCACGGAATCTC-CTTACC-3' (DFS70^{349–435}), 5'-TGCGGCCGCG-GTTTTATTCGCTTCCTCATGC-3' (DFS70^{349–450}), and 5'-AGCGGCCGCCCCCTGTTTGTTCTTCTC-3' (DFS70^{349–464}). The sequence of the 5' primer for DFS70^{323–407} was 5'-AAGAATTCCAAATGGAAAC-TGAGCAG-3', and for the other constructs, a common 5' primer, 5'-AGAATTCGATTCTCGACTTCAAA-GG-3', was used. All insert fragments of deletion mutants were sequenced to rule out PCR errors and ligation errors.

The histidine-tagged proteins were expressed in *E. coli* cells, BL21[DE3] (Stratagene, La Jolla, CA), and purified using TALON metal affinity resins (Clontech, Palo Alto, CA) under a non-denaturing condition according to the manufacturer's protocol. GST-fusion proteins were expressed in *E. coli* cells (JM109, Takara, Tokyo, Japan) and purified using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) under a non-denaturing condition according to the manufacturer's protocol. For use in the circular dichroism assay, DFS70^{323–407} and DFS70^{349–435} were expressed in JM109 and purified under a non-denaturing condition with or without mild detergents. GST was removed from the fusion proteins by cleavage of the fusion proteins by human thrombin (Sigma-Aldrich). Thrombin was subsequently removed using Benzamidine Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) and the recombinant proteins were buffer-exchanged in phosphate-buffered saline (PBS) with Econo-pac 10DG columns (Bio-Rad, Hercules, CA) and concentrated with a Centricon YM-10 (Millipore, Danvers, MA). The approximately >90% purity of the final samples was confirmed by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining as described elsewhere [14].

2.3. Overlap peptide array

Overlap peptide synthesis and immunoblotting were performed as described with slight modification [15]. In short, 194 peptides spanning DFS70 residues 326–530 were synthesized as a series of peptides of 12 amino acids with the overlapping by 11 amino acids, and blotted on cellulose membranes. Four decapeptides of unrelated sequence were also blotted as controls. Membranes incubated with the sample human sera or control rabbit sera at 1:100 dilution after blocking with TPBS/SM (5% skim milk and 0.05% Tween-20 in PBS). The antigen-antibody complexes were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG, or HRP-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark) at 1/1000 dilution. Color development was carried out with Konica Immunostain (Konica Co. Ltd., Tokyo, Japan).

2.4. Western blotting

Approximately 5 µg of the recombinant proteins were applied on each mini gel for western blotting. The quantities of each recombinant proteins were estimated by comparison to serially diluted series of standard bovine serum albumin in Coomassie-stained SDS-PAGE gels, and the relative quantities of each construct were further adjusted to be equal by their reactivities to anti-histidine-tag antibody or anti-GST antibody. SDS-PAGE and the transfer of proteins from 12.5% or 17.5% polyacrylamide gel onto polyvinylidene difluoride (PVDF) membrane were performed as described previously [2].

For the epitope mapping studies, strips of membrane were incubated with the sample sera at 1/100 dilution. HRP-conjugated rabbit anti-human IgG (Dako) was used as the second antibody at 1/1000 dilution. Color development was carried out with Konica Immunostain (Konica Co. Ltd.).

2.5. Immunoprecipitation

Biotinylated DFS70 was produced from pET-DFS plasmid using TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) and Transcend Colorimetric Translation Detection System (Promega) according to the manufacturer's manual.

Immunoprecipitation was performed as described elsewhere [16] with minor modifications. In short, 10 µl of patients' sera were mixed and incubated with the 10 µl in vitro translation products, 40 µl 50% slurry of Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), and 240 µl IP buffer (PBS containing 0.5% IGEPAL CA630). The IgG-bound proteins retained by the sepharose were run on 12.5% SDS-polyacrylamide gel and transferred onto PVDF membrane as described above, and the biotinylated protein was subsequently developed with Western Blue Substrate (Promega).

2.6. Circular dichroism

DFS70^{323–375} and DFS70^{349–435} were prepared as described above. To investigate the secondary structural differences of these peptides, their far-UV circular dichroism spectra were recorded on a J-720W (JASCO, Tokyo, Japan). Cuvettes with a path length of 0.1 cm were used for the measurements. The first measurements were carried out using the peptides, DFS70^{323–375} and DFS70^{349–435}, at the concentration of 0.09 mg/ml in PBS, that were purified under the presence of IGEPAL CA630. Eight scans in the range between 200 and 250 nm were obtained at 4 °C by taking points every 0.1 nm with 1 nm slit width. To obtain more precise spectra, the second measurement was done with

DFS70^{349–435} at the concentration of 0.15 mg/ml dialyzed to ultra pure water, containing 0.2 M NaCl adjusted to pH 7.2, that was purified without IGEPAL CA630. In the second measurement, eight scans in the range between 185 and 250 nm were obtained at 4 °C by taking points every 0.1 nm with 1 nm slit width. Based on the obtained molar ellipticity, the secondary structure content was estimated using the computer program k2D, which is available at <http://www.embl-heidelberg.de/~andrade/k2d.html>[17].

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described with some modifications [2,15,18]. Wells of microtiter plates (Nunc Immunoplate Maxisorp; Nunc, Roskilde, Denmark) were coated with purified DFS70^{323–530} diluted in PBS (0.1 µg/100 µl/well), overnight at 4 °C. The wells were washed three times with PBS and blocked with 200 µl of PBS containing 5% skim milk for 2 h at room temperature, then washed three times with TPBS (0.05% Tween-20 in PBS). Sample sera diluted at 1/100 in TPBS/SM were added to each well (100 µl/well) and incubated for 1 h at room temperature. The wells were washed three times with TPBS, then 100 µl of HRP-conjugated rabbit anti-human IgG, diluted at 1/1000 in TPBS/SM, was added and incubated for 1.5 h. The wells were washed three times with TPBS and twice with PBS, and then developed for 30–60 min using 2,2'-azino-dia(3'-ethylbenzthiazoline-6-sulfonic acid). The substrate solution was described in the previous report in detail [18]. The absorbance was determined at 410 nm.

Each serum was applied to the protein-coated wells in duplicate, and also to two additional wells that were coated only with PBS. O.D. readings from these background wells were subtracted from those of corresponding sample wells, and the average subtracted value of the duplicates was used for the following calculations. Any minus value was treated as a zero value. Linear standard curves were generated for each plate using serial dilutions (1/100, 1/400, 1/1600 and 1/6400) of a representative serum (#345) as a reference. We defined the #345 serum as 64 ELISA units and calculated the unit values of each sample. The cut-off value was determined as mean + 3SD of 36 examples arbitrarily chosen among the anti-DFS70 antibody-negative sera by the IIF and western blotting.

2.8. Statistical analysis

Statistical analyses were carried out by StatView 5.0 J (2002 SAS Institute Inc., Cary, NC). Spearman's rank test, impaired *t* test, or Fisher's exact test was applied for their applicable data analysis (see Section 3). *p* values less than 0.05 were considered significant.

3. Results

3.1. The vast majority of anti-DFS70 positive sera did not recognize the linear continuous epitopes of DFS70

The profiles of the sera used in this study are described in Tables 1 and 2. In order to determine the fine epitope specificity of these autoantibodies, they were screened by a synthetic peptide array (Fig. 1). Series of peptides of 12 amino acid residues were synthesized with overlapping by 11 amino acids, and blotted in nitrocellulose membranes. Because a previous report [1] had shown that the autoepitope of DFS70 lies in the C-terminal 205 residues that are not shared with its splicing isoform, p52, we generated 194 peptides from 326–337aa (QQNKDEGKKPEV) to 519–530aa (TEISLKDSTLDN) (Fig. 1A). A pilot experiment using rabbit serum immunized with a DFS70 C-terminal peptide, SSEERETEISLKDST, which corresponds to 513–527aa, showed a fine epitope specificity of the rabbit anti-DFS70 serum to the original antigen peptides (Fig. 1B, C). However, among the 59 human sera from our department's serum bank (Table 1, the left column) we examined, only one serum, #657, which showed faint reactivities against some C-terminal

peptides of DFS70 (Fig. 1E). The peptides with which #657 serum reacted with were 515–526aa (EERETEISLKDS), 516–527aa (ERETEISLKDST), 517–528aa (RETEISLKDSTL), 518–529aa (ETEISLKDSTLD), and 519–530aa (TEISLKDSTLDN). The other 57 sera failed to react to any peptides compared to the background staining (Fig. 1D, F). One serum, #600, had high background signals and was omitted from the study.

3.2. DFS70 autoepitope is conformation-dependent and highly restricted to the 349–435 amino acid residues, regardless of the clinical settings

Because epitope mapping with the linear peptides had been unsuccessful, we constructed a series of deletion mutants of recombinant DFS70 for further investigation with western blotting (Fig. 2A, B). Most sera reacted to the full-length DFS70 and DFS70^{323–530}. However, DFS70^{1–326}, which contains 1–325aa residues that are identical to the splicing alternative of DFS70, showed positive signals in only 2% (1/59) of the sera (Fig. 2A, C). To further locate the epitope domains, we truncated the N-terminus of DFS70^{323–530}. This protein, DFS70^{375–530}, reacted to only 4% (2/59) of the sera. The GST-fusion protein of the deleted 323–375aa residues (DFS70^{323–375}) showed antigenicity in 5% (3/59) (Fig. 2A, C). In order to define the C-terminal requirement for the autoantigenicity, DFS70^{323–407}, DFS70^{349–435}, DFS70^{349–450}, and DFS70^{349–464} were generated. The former reacted to 15% (9/59) of the anti-DFS70 autoimmune sera, while the rest reacted to 93–95% (55–56/59) of the sera (Fig. 2A, C). Non-fused GST recombinant protein showed no reactivity (data not shown). Therefore we judged that the 375–407aa residues of DFS70 are a prerequisite to the formation of most conformational autoepitopes, and the extension of both amino- and carboxyl-terminals to the 349–435 amino acid residues is generally necessary.

We were then interested in knowing if the distributions of autoepitopes are influenced by the pathological background of the serum donors. Therefore, we categorized the anti-DFS70 antibody-positive sera into two groups: the “diseased group” consisting of 55 sera from our serum bank from patients who had some clinical manifestations at the time of serum collection, and the “healthy group” consisting of four sera from healthy donors in our serum bank plus 34 sera from healthy workers in Chukyo Hospital (Table 1). The clinical status of the Chukyo Hospital workers had been well characterized and their anti-DFS70 antibody properties have been previously reported [2]. We compared the reactivities against the various constructs between the two groups to see whether we could find the epitope progression in accordance with disease expression. Screening of sera from the two groups showed no

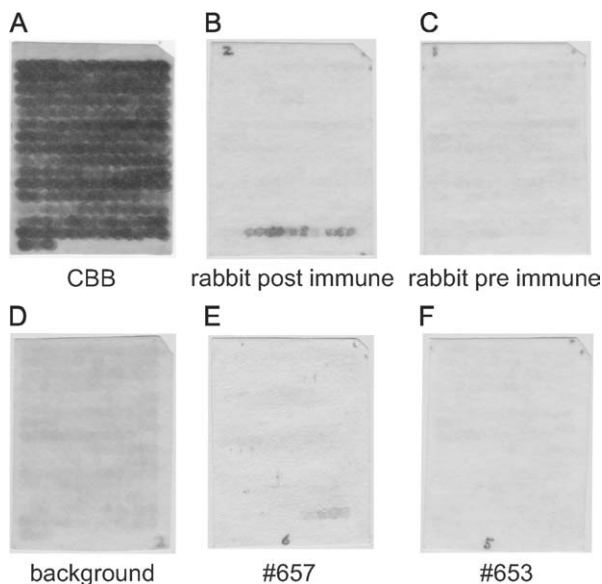


Fig. 1. Epitope mapping of DFS70 using overlapping peptide arrays. Array of peptides spanning DFS70 residues 326–527 were synthesized as a series of peptides of 12 amino acids with the overlapping by 11 single amino acids and blotted on nitrocellulose membranes. Fifty-nine anti-DFS70 antibody-positive sera were diluted 1/100 and used for the assay. A, Coomassie-stained membrane. B, Immunoblotting with a rabbit serum immunized with C-terminal peptide of DFS70. C, Immunoblotting with the pre-immunized serum of the rabbit. D, Background signal levels were determined by incubation without the first antibody. E, Serum #657 showed the presence of linear epitopes. F, Serum #653 is shown as a representative with no signals.

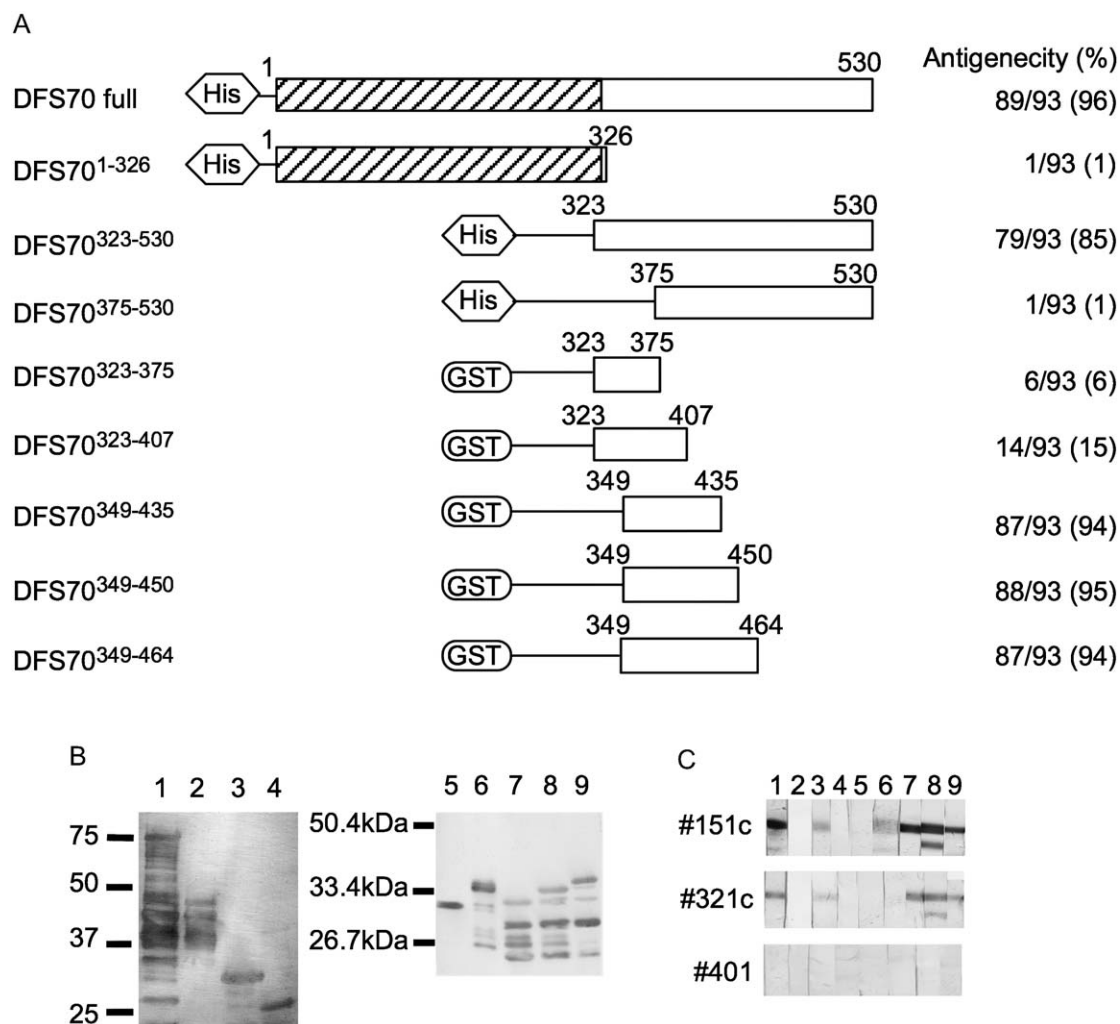


Fig. 2. Epitope mapping of DFS70 using deletion mutants in immunoblotting analysis. A, Diagrams of nine recombinant proteins used in this epitope mapping study. Eight deletion mutants and one full-length DFS70 protein were constructed as described in Section 2. DFS70 full, DFS70^{1–326}, DFS70^{323–530}, and DFS70^{375–530} were expressed in *E. coli* as histidine-tagged proteins. DFS70^{323–375}, DFS70^{323–407}, DFS70^{349–435}, DFS70^{349–450}, and DFS70^{349–464} were expressed in *E. coli* as GST-fusion. Shadows in diagrams of DFS70 full and DFS70^{1–326} indicate 325 amino acid residues identical to p52, a splicing isoform of DFS70. The figures on the right of each diagram show the frequencies of the total 93 examined sera that reacted in the western blotting of each protein. B, Purified proteins were assessed by western blotting using anti-histidine tag antibody or anti-GST antibody. Signals at the position of the highest band of each lane were examined in the following study. Lane 1, DFS full; lane 2, DFS70^{1–326}; lane 3, DFS70^{323–530}; lane 4, DFS70^{375–530}; lane 5, DFS70^{323–375}; lane 6, DFS70^{323–407}; lane 7, DFS70^{349–435}; lane 8, DFS70^{349–450}; lane 9, DFS70^{349–464}. C, Representative results of western blotting using patients' sera. In this figure, #151c and #321c show the common results of anti-DFS70 antibody-positive sera. #401 is an anti-DFS70 antibody-negative serum. The results of the western blotting of 93 sera are summarized in Table 3. The proteins in each lane correspond to those depicted in panel B.

significant intra-group differences (Table 3). In both groups, only a minor population of sera reacted to DFS70^{323–407}, DFS70^{323–375}, and DFS70^{375–530}, however, the clinical settings of the donors of these sera had no common features.

Three sera in the diseased group did not react to the full-length DFS70 in western blotting. Immunoprecipitation assays of these sera were performed using an in vitro translated full-length DFS70, and all of them were positive for this assay (Fig. 3). The addition of 0.05% SDS in IP buffer did not alter the reactivity substantially, however, no sera retained the affinity to

DFS70 when the concentration of SDS was increased to 0.1% (data not shown).

3.3. Antigenic DFS70^{349–435} domain contains alpha-helical and beta-sheet structure

For better understanding of the structural properties of the autoepitope domain of DFS70, circular dichroism analyses were performed. The fused GSTs were cleaved from DFS70^{349–435} and DFS70^{323–407}, and the resulting peptides were monitored at a range of 185–250 nm UV. The observed molar ellipticities were plotted (Fig. 4A).

Table 3
Results of epitope mapping using recombinant mutant DFS70 proteins

Construct	Diseased group (<i>n</i> = 55)					Healthy group (<i>n</i> = 38)					Total (<i>n</i> = 93)				
	–	+/–	++	+++	++++ ^a	–	+/–	++	+++	++++ ^a	+/–	~	++	+++	++++ ^a
DFS70 full	3	2	18	3	52(95)	1	1	11	3	37(97)	89(96)				
DFS70 ^{1–326}	54	0	0	0	1(2)	38	0	0	0	0(0)	1(1)				
DFS70 ^{323–530}	5	1	29	15	50(91) ^b	9	0	19	7	29(76) ^b	79(85)				
DFS70 ^{375–530}	54	0	0	0	1(2)	38	0	0	0	0(0)	1(1)				
DFS70 ^{323–375}	53	0	2	0	2(4)	34	4	0	0	4(11)	6(6)				
DFS70 ^{323–407}	47	1	5	1	8(15)	28	0	3	1	6(16)	14(15)				
DFS70 ^{349–435}	4	2	25	4	51(93)	2	0	22	10	36(94)	87(94)				
DFS70 ^{349–450}	3	2	28	15	52(95)	2	0	26	6	36(94)	88(95)				
DFS70 ^{349–464}	4	0	29	16	51(93)	2	0	24	8	36(94)	87(94)				

^a Numbers in parentheses show proportion of the positive sera in percentile.

^b *p* = 0.0763 in Fisher's test.

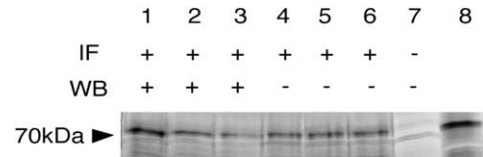


Fig. 3. Immunoprecipitation assays with in-vitro transcription/translation system. Sera were incubated with in vitro translated and biotinylated DFS70, Protein G sepharose, and IP buffer. Proteins retained by the sepharose were separated by SDS-PAGE and detected with streptavidin-alkaline phosphatase conjugate and Western Blue substrate. Lanes 1–3, anti-DFS70 antibody-positive sera with positive result in western blotting of recombinant proteins (lane 1, YC sera; lane 2, #269; lane 3, #271). Lanes 4–6, anti-DFS70 antibody-positive sera with negative result in western blotting of recombinant proteins (lane 4, #329; lane 5, #582; lane 6, #598). Lane 7, control normal serum. Lane 8, loaded in vitro transcribed products. IF, immunofluorescence study; WB, western blotting.

The first measurements showed that the antigenic DFS70^{349–435} peptide had minimum dichroism at 222 nm, which implies a significant alpha-helical content. The less antigenic DFS70^{323–407} peptide showed

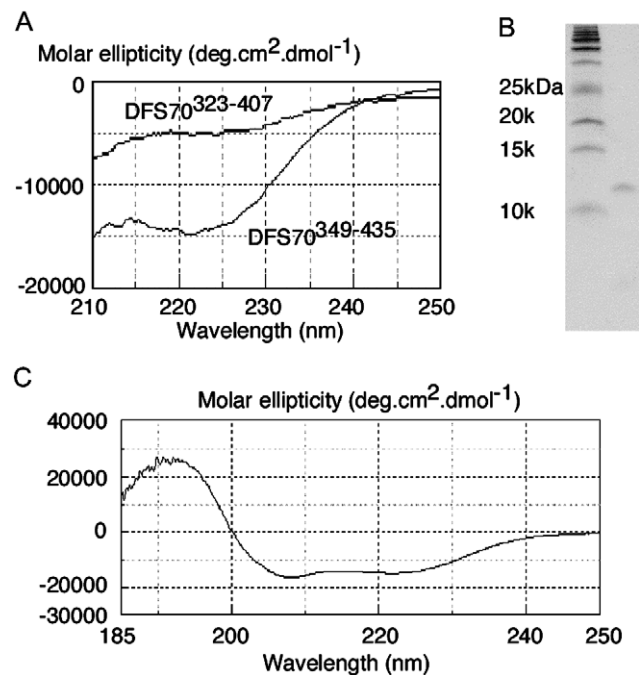


Fig. 4. Estimation of alpha-helical structures in DFS70^{349–435}. A, Comparison of circular dichroism spectrum between antigenic DFS70^{349–435} and less antigenic DFS70^{323–407}. The molar ellipticity of each protein is shown as a function of wavelength (210–250 nm). DFS70^{349–435} is indicative of a high alpha-helical component by the dichroism minimum at 222 nm. B, Purified DFS70^{349–435} used in estimation of secondary structure components. DFS70^{349–435} was expressed and purified without detergents to eliminate the signal noise that appeared in <205 nm in A. The purity of the protein was confirmed in tricine-SDS-PAGE gel stained with Coomassie Brilliant Blue. C, Circular dichroism spectrum of purified DFS70^{349–435}. The spectrum of >210 nm is identical to that of A. The molar ellipticity of each protein is shown as a function of wavelength (185–250 nm).

a significantly different spectrum, that is it had no corresponding peaks at 222 nm nor at any other peak suggestive of secondary structural components. Therefore, even without estimating the secondary structural parameters, it became apparent from the spectrum that the DFS70^{323–407} peptide contains mostly random coiled structures unlike the antigenic DFS70^{349–435} peptide. A reliable estimation of the secondary structure content could not be obtained from the result of the first measurement because of the signal noise at <205 nm, possibly due to the absorption of phosphate moieties in the PBS and trace amounts of detergents that remained in the sample proteins. The second measurement was carried out using DFS70^{349–435} peptide purified without detergents (Fig. 4B, C) and finally dialyzed. Notably, the spectrum at >205 nm was identical to that of the first measurement. The spectrum showed the minimum at 207 and 222 nm and the maximum at 191 nm, as a typical characteristic of the alpha-helix nature. The best estimates of the secondary structure proportions obtained from the data were, 41% in alpha-helices, 17% in beta-sheet, and 42% in the random coil conformation.

3.4. Antibody titers show no significant differences according to the donors' clinical settings

Since the results of the epitope mapping analysis showed a markedly uniform distribution regardless of the donors' clinical settings, we next investigated whether the antibody titers reflected their pathological process. ELISA was performed using DFS70^{323–530} as antigen because *E. coli* expression of recombinant full-length DFS70 typically generates significant populations of short degraded products that lead to low yield and purity [19, our unpublished result]. DFS70^{323–530} was easily expressed and was obtained with high yield in good purity through a single purification procedure with metal affinity resin (data not shown). Anti-DFS70 antibody titers of 45 sera from the diseased group, 37 sera from the healthy group, and 36 anti-DFS70 antibody-negative sera were measured (Fig. 5). The other 11 sera could not be assayed because of the limited sample quantity. The cut-off value of 1.441 ELISA units was determined as mean + 2SD ELISA units of the negative controls; the sensitivity was 90% (74/82) and the specificity was 92% (33/36) in this setting. The measured ELISA units and signal strength of the western blotting using full-length DFS70 showed a significant correlation ($p < 0.0001$, Spearman's rank test), which warranted the use of the deleted form of DFS70 in the ELISA system to evaluate the antibody titers in the sera. The diseased group and healthy group exhibited similar distributions of ELISA titers, and evaluation with impaired *t* test did not show significant differences between the two groups. No significant differences were seen among the groups of any specific

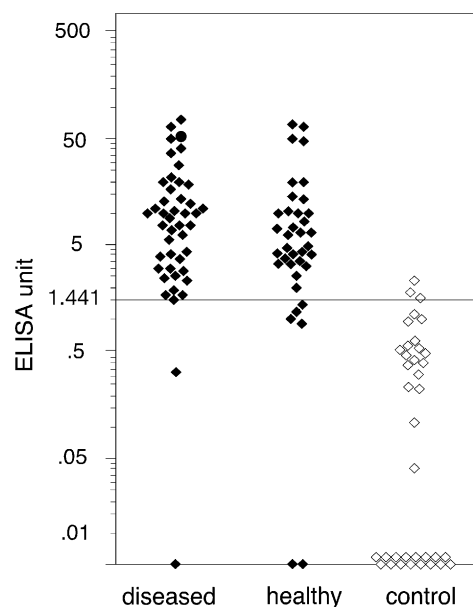


Fig. 5. Anti-DFS70 antibody titers measured by enzyme-linked immunosorbent assay. Sera from 45 diseased and 37 healthy individuals with anti-DFS70 antibody were examined. Sera from 36 healthy, anti-DFS70 antibody-negative individuals were used as negative control. The cut-off level for positivity (average units of negative controls + 2SD) was 1.441 units and is indicated by the broken line. A closed circle depicts #345 as the reference sera. ELISA units as mean value $\pm$ SD were 14.2 ± 17.1 , 11.5 ± 16.8 , and 0.39 ± 0.56 for disease group, healthy group, and negative control, respectively. The reference sera, #345, typically showed OD readings of 1.565–2.817 at 4-fold dilution after 60 min of color development. The mean 60 min-OD values for the negative control group was 0.036 and the standard deviation was 0.051.

diseases (data not shown), and also we could not detect any correlation among the degrees of fatigue or other clinical parameters and the ELISA titers in the patients with CFS or CFS-like manifestations. Although there were more males in the control group and their titers were relatively low, we could find no statistical differences in males and females (data not shown).

4. Discussion

Anti-DFS70 antibodies have been recently recognized to represent a major ANA subset in the non-rheumatologic population [2,20]. The clinical impact of DFS70 antibodies is marked by the fact that they are one of the most prevalent autoantibodies in AD reported so far [1], and that they are found in more than one half of ANA-positive healthy individuals [2]. However, detailed examinations of these autoantibodies and comparative studies regarding the clinical features of donors have not been done.

Epitope mapping of autoantigens still represents an important topic in the area of autoimmune disease

research, since it may shed light on the basic mechanism leading to immune reactions against self-antigens, and lead to the development of novel diagnostic systems [21,22]. Screening for autoepitopes of DFS70 using short linear peptides with continuous sequences failed to reveal the major antigenic foci of DFS70. Western blot analysis using recombinant truncated proteins revealed that a considerably long peptide within DFS70^{349–435} was necessary for recognition by the majority of anti-DFS70 autoreactive sera. Proteins blotted on PVDF membranes may take denatured linear forms during SDS-PAGE, but they are considered to renature in the following process of immunoblotting. Furthermore, Van Dam et al. reported that the addition of Tween-20 during the incubation period even enhances the renaturing effect of membrane-bound antigens, and results in improved recognition by specific antibodies [23]. Our protocol also included the addition of Tween-20 in the incubation mixture, and we suggest that the results of western blot using recombinant mutant DFS70 proteins reflect the conformational nature of DFS70 autoepitopes. Considering that both DFS70^{323–375} and DFS70^{375–530} showed minimum antigenicity (1% and 6%), and that overlapping DFS70^{323–407} still showed weak reactivity (15%), the autoepitope that extends to 349–435aa residues of DFS70 probably consists of discontinuous epitope components at both sides of this region. The secondary structure prediction by the computer program showed that the alpha-helix contents of DFS70 were concentrated at the 349–435aa residues (data not shown). A circular dichroism analysis proved that this region indeed contained approximately 41% and 17% of alpha-helices and beta-sheets, respectively, while the overlapping peptide, DFS70^{323–407} showed faint signs of structural components. These results would further support the conformational nature of the core autoepitopes of DFS70. A small number of sera reacted only in the immunoprecipitation assay. We suspect that a more strictly native conformation of DFS70 may be required for antigen recognition by these sera, although it is possible that a hitherto unknown post-translational modification may have contributed to the antigenicity.

The conformation-dependency of autoepitopes is a marked feature of many autoantigens, such as proliferation cell nuclear antigen (PCNA), fibrillarin, and Ro-60 [21,22]. The highly conserved nature of the autoepitopes has also been often described in past studies [22], and the antigenic domain of DFS70 is completely conserved in three mammalian species as shown in Fig. 6. These features of autoepitopes, conformation-dependency and a highly conserved nature, might relate to the fact that they are the functional or binding regions of the proteins [22]. Interestingly, Wu et al. reported that this domain contains caspases-3 and -7 recognition motif, DAQD⁴⁸⁶G, near the

autoepitope DFS^{349–435} [24]. Apoptosis may occur as a consequence of stress responses in various cells and is a common phenomenon even in a healthy state as well in pathological conditions. When physiological or pathological stress on keratinocytes or other cells induces apoptosis and the cleavage of DFS70 at DAQD⁴⁸⁶G takes place, it may be possible that putative cryptic epitopes inside DFS^{349–435} are unveiled and drive the production of anti-DFS70 antibodies [20,24]. The C-terminal 205aa residues (326–530aa), which are unique to DFS70, are believed to determine the intranuclear localization of DFS70 [8], to have a DNA-binding domain [4] and to interact with HIV-1 integrase [7].

The unique clinical distribution of the anti-DFS70 antibodies gives rise to the need for reliable and easy-to-access detection systems [2]. Previously, ELISA for the detection of anti-DFS70 antibodies used the full-length form of DFS70/LEDGF or GST-fusion, full-length LEDGF [2,25,26]. However, the expression of full-length DFS70 is accompanied by a considerably large population of degradation products [19] and, in addition, our previous ELISA required a blocking process using *E. coli* extracts to lower the background signals [2]. Past studies have shown that the short epitope peptides of antigen proteins may be equivalent, or even better, alternatives to their wild-type proteins for the detection of autoantibodies [27]. The application of the recombinant protein DFS70^{349–435} in ELISA is indeed a reasonable strategy, for it is easily purified and has significantly improved efficiency compared to its full-length form (data not shown), and it eliminates the need for blocking with bacterial extracts. With the new ELISA, the sensitivity and specificity were 90% and 92%, respectively, while those of our previous ELISA system were 89% and 95%, respectively [2]. Autoantibodies in systemic autoimmune disease often recognize macromolecular complexes and, at the level of the same antigen, target distinct multiple epitopes which are variable among patients [28,29]. However, it is surprising that the autoepitope of DFS70 is very uniform and is restricted to a single region. This result is very advantageous for measuring the antibody by ELISA with the epitope portion.

The frequencies of anti-DFS70 antibodies in healthy individuals have differed in past reports from 0% to 21.6% [20]. The discrepancy of the rates may reflect the nature of the population of sera donors included in each study, but also may result from differences in the detection methods employed in each study. For instance, Ochs et al. reported that, among 18 sera which showed the dense fine speckled pattern in immunofluorescence assay, four sera were negative in western blotting using recombinant protein, while five out of 46 sera that were negative in the immunofluorescence assay were positive in the western blotting analysis [1]. From the results of the present study, we recommend that the antibody

		PWWP domain
[Homo sapiens]	MTRDF	KPGDLIFAKMKGYPHWP
[Bos taurus]		-----HETAFGLGPKDIFP
[Mus musculus]	MTRDF	KPGDLIFAKMKGYPHWP
		-----HETAFGLGPKDIFP
[Homo sapiens]		YSENKEKYGKPNKRKGFNEGLWEIDNNPKVKFSSQQAATKQSNASSDVEVEEKETSVSKE
[Bos taurus]		YSENKEKYGKPNKRKGFNEGLWEIDNNPKVKFSSQQAATKQSNASSDVEVEEKETSVSKE
[Mus musculus]		YSENKEKYGKPNKRKGFNEGLWEIDNNPKVKFSSQQAATKQSNASSDVEVEEKETSVSKE
[Homo sapiens]		DTDHEEKASNEVDVTKAVDITTPKAARRGRKRKAQVETEEAGVVTATASVNLKVSPKR
[Bos taurus]		DTDPEEKASNEVDVTKAIDITTPKAARRGRKRKAQVETEEAGVVTATASVNLKVSPKR
[Mus musculus]		DTDQEEKASNEVDVTKAVDITTPKAARRGRKRKAQVETEEAGVVTATASVNLKVSPKR
[Homo sapiens]		GRPAATEVKIPKPRGRPKMVQPCPSESDDIITEEDKSKKKGQEEKQPKKQPKKDEEGQKE
[Bos taurus]		GRPAATEVKIPKPRGRPKMVQPCPSESDDIITEEDKSKKKGQEEKQPKKQPKKDEEGQKE
[Mus musculus]		GRPAATEVKIPKPRGRPKMVQPCPSESDDIITEEDKSKKKGQEEKQPKKQPKKDEEGQKE
[Homo sapiens]		EDKPRKEPDKKEGKKEVESKRKNLAKTGVSTSDSEEEGDDQEGEKKRKGGRNFQTAHRR
[Bos taurus]		EEKPRKEPDKKEGKKEVESKRKNLAKTGVSTSDSEEEGDDQEGEKKRKGGRNFQTAHRR
[Mus musculus]		EEKPRKEPDKKEGKKEVESKRKNLAKTGVSTSDSEEEGDDQEGEKKRKGGRNFQTAHRR
[Homo sapiens]		NMLKGQHEKEAADRRKKQEEQMETEQQNKDEGKKPEVKKVEKKRETSNDSRLQRIHAEIK
[Bos taurus]		NMLKGQHEKEAADRRKKQEEQMETEQQNKDEGKKPEVKKVEKKRETSNDSRLQRIHAEIK
[Mus musculus]		NMLKGQHEKEAADRRKKQEEQMETEQQNKDEGKKPEVKKVEKKRETSNDSRLQRIHAEIK
		DFS70 ³⁴⁹⁻⁴³⁵
[Homo sapiens]		NSLKIDNLDVNRCEALDELASLQVTMQQAQKHEMITLEKKIRRFKVSQVIMEKSTMLY
[Bos taurus]		NSLKIDNLDVNRCEALDELASLQVTMQQAQKHEMITLEKKIRRFKVSQVIMEKSTMLY
[Mus musculus]		NSLKIDNLDVNRCEALDELASLQVTMQQAQKHEMITLEKKIRRFKVSQVIMEKSTMLY
[Homo sapiens]		NKFKNMFLVGEQDSVITQVLNKSIAEQRQHEEANKTKDQGGKGPNNKLEKEQTGSKTLNG
[Bos taurus]		NKFKNMFLVGEQDSVITQVLNKSIAEQRQHEEANKTKDQGGKGPNNKLEKEQTGSKTLNG
[Mus musculus]		NKFKNMFLVGEQDSVITQVLNKSIAEQRQHEEANKTKDQGGKGPNNKLEKEQTGSKTLNG
[Homo sapiens]		GSDAQDGNQPHNGESNEDSKDNHEASTKKKPSSEERETEISLKDSTLDN
[Bos taurus]		GSDAQDSNQPQHNGDSNEESKDNHEASSKKKPSSEERETEISLKDSTLDN
[Mus musculus]		GSDAQESNHPQHNGDSNEDGKDSREASSKTKPGEEREAEISLKESTLDN

Fig. 6. Comparison of amino acid sequence of DFS70 autoepitope domain among three mammalian species. GenBank search showed three mammalian homologues of DFS70: human (Accession Number: NP_150091.2), mouse (Accession Number: CAC34944.1) and calf (Accession Number: AAM90841.1). Multiple alignments showed a complete identity of 48–97 and 253–471 amino acid residues of DFS70 among these species. The former homologous region contains part of the PWWP domain, a domain of protein–protein interactions [4,34]. The core autoepitope domain, DFS70^{349–435}, is located at the center of the latter homologous region. The PWWP domain and antigenic 349–435aa residues are shown in boxed areas.

detection should be sometimes performed by multiple techniques, the combined use of immunofluorescence assay and ELISA and, in cases with inconsistent results between the two methods, additional analyses using immunoprecipitation and western blotting.

While many pathogenic and disease-specific autoantibodies have been discovered, there are also “natural autoantibodies” that are present in the sera of healthy individuals in the absence of deliberate immunization with the target antigen [30,31]. Natural autoantibodies may react with self-antigens that are also targets for autoantibodies in autoimmune disease, such as thyroglobulin, Factor VIII (FVIII), insulin, glutamic acid decarboxylase, single- or double-stranded DNA and histone IIA [30,31]. Anti-FVIII antibodies represent a well-studied model of a natural autoantibody against serum protein. FVIII is a component of the intrinsic pathway of the coagulation cascade, and its deficiency leads to hemophilia. Spontaneous autoantibodies to FVIII may develop in non-hemophilic patients even without signs of associated disease [32]. Natural anti-

bodies to FVIII inhibit FVIII activity in a reference pool of normal plasma used as a source of FVIII, but not the activity of autologous FVIII. This phenomenon has been explained by the existence of ‘protective’ anti-idiotypic antibodies that neutralize the activity of the patients’ pathogenic autoantibodies [32].

We suspect that anti-DFS70 autoantibody may similarly play a pathogenic role in a certain population of patients, although in most carriers such autoantibodies are non-pathogenic. Further studies including the quantification of serum DFS70 levels and investigations on the isotype/subclass [33] the idiotype networks of anti-DFS70 antibodies are needed.

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