Antifibrillarin Autoantibodies Present in Systemic Sclerosis and Other Connective Tissue Diseases Interact with Similar Epitopes

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Summary

Autoantibodies specific against fibrillarin, a 34-kD nucleolar protein associated with U3-snRNP, are present in patients with systemic sclerosis (SSc). To understand the mechanisms involved in the induction of these autoantibodies, we prepared a series of human fibrillarin recombinant proteins covering the entire molecule and analyzed their interaction with the autoantibodies present in various connective tissue diseases. Our results showed that antifibrillarin autoantibodies are present not only in SSc, as previously reported, but also in a variety of other connective tissue diseases. Patients with SSc (58%), mixed connective tissue diseases (60%), CREST syndrome (calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, and telangiectasia syndrome) (58%), systemic lupus erythematosus (39%), rheumatoid arthritis (60%), and Sjogern's syndrome (84%) showed presence of antifibrillarin autoantibodies. Results obtained from competitive inhibition radioimmunoassay and Western blot analyses with purified recombinant fusion proteins revealed that these autoantibodies react primarily with epitope(s) present in the NH_2- (AA 1-80) and COOH-terminal (AA 276-321) domains of fibrillarin. Autoantibodies reacting with internal regions of fibrillarin are less frequent. Analysis of the hydrophilicity profiles of reactive peptides showed presence of three potential antigenic sites in the NH_2- and two in the COOH-terminal regions. While a hexapeptide sequence NH_2 terminus of fibrillarin is shared with an Epstein-Barr virus–encoded nuclear antigen, the COOH-terminal region shares sequence homology with P40, the capsid protein encoded by herpes virus type 1. Interestingly, these two regions of fibrillarin also contain the most immunodominant sequences, as predicted by surface probability and the Jameson and Wolf antigenic index. These observations suggest that molecular mimicry might play an important role in the induction of antifibrillarin autoantibodies.

Presence of antinuclear antibodies is a hallmark of rheumatic autoimmune diseases (1). Autoantibodies reacting with nuclear proteins like Smith antigen, (Sm)[1], topoisomerase I, centromere proteins, and amino-acyl tRNA synthetase are characteristic for SLE (2), systemic sclerosis (SSc) (3), CREST syndrome (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia syndrome) (4), and polymyositis (5), respectively. Although pathogenicity of these antibodies has not been established, they are useful as markers in the differential diagnosis. In addition to the antinuclear autoantibodies, antibodies reacting with specific nucleolar proteins have been reported in systemic autoimmune diseases (6). For instance, RNA polymerase I (7), 7-2RNP (8), PM-Scl (9) and U3 snRNP–associated fibrillarin (10, 11) are targets of scleroderma autoantibodies. Antifibrillarin antibodies may characterize a subset of patients with diffuse sclerosis and widespread telangiectasia (12). In contrast to humans, production of autoantibodies specific for nucleolar antigens is seldom associated with the spontaneous development of systemic autoimmune diseases in animals. However, antifibrillarin antibodies can be induced in B10.S and SJL mice after injection with mercuric chloride (13, 14).

Fibrillarin is a highly conserved 34-kD nucleolar protein localized in the fibrillar regions of nucleoli (10, 11). It is associated with U3 snRNP complex, which is thought to play a role in the processing of preribosomal RNA (15). Fibrillarin has three distinct domains: (*a*) NH_2-terminal domain of 80 amino acids rich in glycine and dimethylarginine; (*b*) central domain of 90 amino acids containing the RNA binding consensus octomeric sequence; and (*c*) COOH-terminal α helical domain of 151 amino acids (16). The genes coding for fibrillarin of *Xenopus laevis* (17), *Physarum polycephalum*

[1] *Abbreviations used in this paper:* CREST, calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, and telangiectasia syndrome; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyronoside; MBP, myelin basic protein; MCTD, mixed connective tissue diseases; MS, multiple sclerosis; RA, rheumatoid arthritis; Sm, Smith antigen; SS, Sjogern's syndrome; SSc, systemic sclerosis.

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(18), yeast (19), and mammalian species (16, 20, 21) have been cloned and sequenced. The primary structure of fibrillarin from these sources shows a high degree of homology to human fibrillarin.

Antifibrillarin antibodies are currently demonstrable either by immunofluorescence or immunoprecipitation methods. Use of these techniques has shown that a small fraction of scleroderma sera—3.4% (22), 6% (23), and 3.6% (24)—contain antifibrillarin autoantibodies. The major drawback of these methods is their inability to discern the specificity of the antibodies reacting with large macromolecular complexes that were used in such analysis. The nucleolar extracts enriched for U3 RNP complex that were used in immunoprecipitation analyses also contain five other polypeptides with molecular masses of 74, 59, 30, 13, and 12.5 kD, in addition to fibrillarin (25). Presence of autoantibodies against any one of the polypeptides associated with fibrillarin can also bring down fibrillarin by immunoprecipitation via the sera that may contain these antibodies. Furthermore, fibrillarin is also found associated with other less abundant snRNPs such as U8, U13, U14, X, and Y (26). In a recent report, out of seven mAbs obtained from mercuric chloride treated A.SW mice that precipitated U3 RNP and showed positive nucleolar staining of Hep2 cells by immunofluorescence, only four mAbs immunoprecipitated purified human fibrillarin (27).

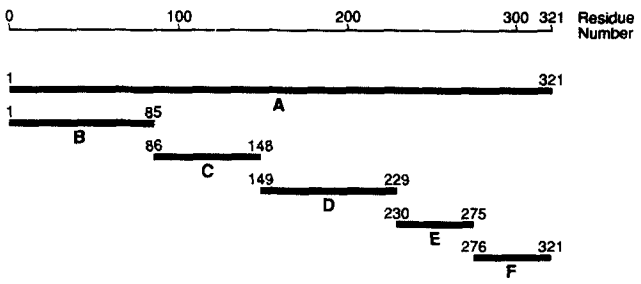
Therefore, we have developed a reliable and sensitive solid-phase RIA, using purified human recombinant fibrillarin proteins, first to study the frequency of antifibrillarin autoantibodies present in scleroderma patients and then to determine whether they are present in other connective tissue diseases. Our results demonstrate that antifibrillarin antibodies are present in high frequency not only in SSc but also in a variety of other connective tissue such as diseases like mixed connective tissue disease (MCTD), SLE, CREST syndrome, rheumatoid arthritis (RA), and Sjogern's syndrome (SS). By preparing a panel of six overlapping fibrillarin fusion proteins, we have also determined whether these autoantibodies are directed against a common epitope(s) or a characteristic set of epitopes in each disease. Our results showed that the autoantibodies from various connective tissue diseases exhibited a similar immunochemical specificity suggesting that the same or similar epitopes are recognized. However, in contrast to autoantibodies present in SSc, MCTD, SLE, and CREST syndrome, which reacted primarily with the epitopes located at the NH_2-terminal region, autoantibodies present in RA and SS reacted equally well with the epitopes present at the NH_2- and COOH-terminal regions.

Materials and Methods.

Sera. Sera were obtained from patients with various connective tissue diseases such as SSc, CREST syndrome, MCTD, RA, SS, and SLE and control healthy subjects were stored at −20°C until use (the patients were classified by the criteria described by the American Association of Rheumatism). A total of 237 serum samples (56 SSc, 81 SLE, 22 MCTD, 11 CREST syndrome, 38 RA, 15 SS, and 14 healthy subjects) were used in this study.

Antigens. Recombinant human fibrillarin fusion proteins, human Sm antigen (kindly provided by Dr. H. Dang, University of Texas, San Antonio, TX), rat collagen I (from Dr. R. Holmdahl, University of Uppsala, Uppsala, Sweden), human myelin basic protein (MBP) (from G. Hashin, Saint Lukes Hospital, New York), and dsDNA were used as antigens in the direct-binding and competitive inhibition RIA. Recombinant fusion proteins containing five contiguous moieties of human fibrillarin were obtained by cloning the corresponding cDNA sequences into pGEX-2T λ expression vector (28).

Expression Cloning of Human Fibrillarin cDNA. The coding region of human fibrillarin cDNA (cDNA clone kindly provided by Dr. J. Aris, University of Florida, Gainseville, FL) and the DNA sequences corresponding to amino acid residues 1-321, 1-85, 86-148, 149-229, 230-275, and 276-321 of fibrillarin were amplified with oligonucleotide primers by PCR (29). The amplified DNA fragments were subcloned into pGEX-2T expression vector, which expresses glutathione-S-transferase (GST). The cloned cDNAs were expressed as GST fusion proteins. Schematic representation of the fibrillarin regions that were cloned is shown in Fig. 1. The following primers were used for PCR amplification: (*a*) primer 01: 5'-atgaagccaggattcagtccc-3'; primer 321: 5'-gttcttcaccttgggggtgg-3'; (*b*) primer 01: 5'-atgaagccaggattcagtccc-3'; primer 85: 5'-aatgtgatggtggagccgcat-3'; (*c*) primer 86: 5'-attcttccccgactggtttcc-3'; primer 148: 5'-gattgctgctgctagcttggag-3'; (*d*) primer 149: 5'-atcctgggtggtgtggacca-3'; primer 229: 5'-cattgcgatgagcatgcggta-3'; (*e*) primer 230: 5'-atgctggatctgatctttgctg-3'; primer 275: 5'-ggctgaggctgtggagtcaa-3'; and (*f*) primer 276: 5'-gaggccgtgtttgcctccga-3'; primer 321: 5'-gttcttcaccttgggggtgg-3'. The 5' and 3' end primers contained BamHI and EcoRI sites, respectively, at the 5' end of the sequence. The amplified DNAs were digested with restriction enzymes, ligated to BamHI–EcoRI–cut pGEX-2T plasmid, and the BL-21 competent cells were transformed. The bacteria expressing the recombinant plasmids were selected on ampicillin plates. The recombinants were grown in 1 ml Lubria broth (LB) medium and induced with 1 mM isopropyl β-D-thiogalactopyronoside (IPTG) for 5 h at 37°C. The bacterial lysates were run on SDS–polyacrylamide gels, and the recombinants expressing appropriate molecular size fusion proteins were selected. Plasmid DNAs were sequenced by the dideoxy chain termination method (30), with an oligonucleotide primer 5'-gcatggcctttgcaggg-3', corresponding to 855-871 of the pGEX sequence, to confirm that the inserts were cloned in frame.



Schematic diagram showing regions of human fibrillarin cloned as fusion proteins

Figure 1. Schematic diagram showing regions of human fibrillarin expressed as fusion proteins. DNA fragments coding for defined regions of fibrillarin A–F generated by PCR were cloned into pGEX-2T expression vector to produce six in-frame fusion proteins as described in Materials and Methods. Fibrillarin proteins containing 13 hydrophilic regions were kept intact to preserve the potential antigenic sites formed by linear amino acid sequences.

Antigen-binding Studies. Direct-binding assay. Microtiter plates were coated with recombinant fusion proteins or GST (as control) at 5 μg/ml in carbonate buffer, pH 9.0, at 4°C, overnight. After washing, the plates were blocked with 3% BSA in PBS for 1 h at room temperature, washed three times, and incubated with 1:400 dilution of sera for 2 h. After thorough washing, 5×10^4 cpm of ^{125}I-labeled rabbit F(ab')$_2$ anti-human γ antibody in 50 μl was added and incubated for a further 2 h. After washing, the bound radioactivity was measured in a gamma counter. Cpm bound to GST coated plates were subtracted from the cpm bound to fibrillarin fusion protein-coated plates and expressed as specific cpm bound.

Competitive Inhibition Assay. 10 μg/ml of serum IgG, purified by use of protein A-Sepharose or 1 μg/ml of affinity-purified antifibrillarin antibodies, was incubated with various amounts of collagen I, Sm, dsDNA, MBP, or fibrillarin in 50 μl for 1 h at room temperature, and then transferred to fibrillarin-coated plates following the procedure described previously (31). The cpm bound to fibrillarin was measured as above. The inhibition was expressed as a percentage.

Western Blotting. Purified fibrillarin fusion proteins were run on 10% polyacrylamide gels in the presence of SDS (32). Separated proteins were transferred to nitrocellulose filters (33) at 100 V for 4 h at 4°C, blocked with 5% BSA in PBS, and then incubated for 1.5 h with a 1:500 dilution of human sera or 0.1 μg/ml of mouse anti-human fibrillarin mAb in PBS containing 5% BSA and 0.05% Tween 20. After washing with PBS containing Tween 20, filters were incubated for 1.5 h at room temperature with ^{125}I-rabbit F(ab')$_2$ anti-human Igγ or ^{125}I-labeled rat anti-mouse &K mAbs at 2×10^5 cpm/ml. Filters were washed, dried, and autoradiographed as previously described (31).

Indirect Immunofluorescence. NIH 3T3 cells were grown on glass coverslips in 35-mm dishes. After 2 d in culture at 60% confluence, the cells were washed with PBS and then fixed with 2% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature. The fixed cells were permeabilized by exposure to 0.2% Triton X-100 in PBS for 5 min on ice. After permeabilization, cells were rinsed three times for 10 min with PBS and incubated with 50 μg/ml of IgG antibodies or 5 μg/ml of affinity-purified antibodies diluted in 1% goat serum for 1 h at room temperature. The unbound antibody was removed by rinsing three times for 10 min with PBS and then incubated with affinity-purified goat F(ab')$_2$ anti-human IgG antibody conjugated with FITC at a dilution of 20 μg/ml for 1 h. The unbound second antibody was removed by washing with PBS, and the cells were mounted in 90% glycerol, 10% PBS (pH 8.0) containing 4% *n*-propyl gallate (34). The cells were examined under fluorescence illumination or laser light in a microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Expression and Purification of Recombinant Fibrillarin Proteins. To identify the immunodominant B cell epitopes of fibrillarin, the PCR-amplified DNA fragments coding for various moieties of fibrillarin (Fig. 1), each containing one to three hydrophilic regions, were cloned into pGEX-2T expression plasmid. Thus, a panel of six in-frame contiguous fibrillarin fusion proteins were generated as described in Materials and Methods. Bacterial cultures expressing recombinant proteins were induced with IPTG, and the fusion proteins were purified by affinity chromatography on a glutathione-S-Sepharose gel column as previously described (28). Total bacterial lysates and purified recombinant proteins run on SDS-PAGE and stained with Coomassie blue R stain are shown in Fig. 2. The level of induction of recombinant fusion proteins varied depending on the insert sequence. The expected molecular mass of fibrillarin fusion proteins FP-A, FP-B, FP-C, FP-D, FP-E, and FP-F were 60.0, 34.5, 32.5, 34.1, 30.6, and 30.6 kD, respectively. The observed sizes of fusion proteins correspond well to this (Fig. 2). All recombinant proteins were full-length products. Only FP-C fusion protein showed lower molecular mass derivatives, indicating proteolytic cleavage in specific regions of fibrillarin. Sequence analysis of recombinant plasmid DNAs confirmed that the insert DNAs were inframe with the gene coding for GST protein.

Frequency of Antifibrillarin Antibodies in Sera of Patients with Connective Tissue Diseases. The presence of antifibrillarin antibodies in the sera of individuals with various connective tissue diseases was studied by solid-phase RIA with purified fibrillarin fusion protein FP-A. Background binding to GST was determined by parallel assays on GST-coated plates. Cpm bound to GST protein was subtracted from total cpm bound to fibrillarin fusion protein. The specific binding activities of sera from patients with various connective tissue diseases are presented as cpm bound in a scatter diagram (Fig. 3). The sera showing three- to fourfold higher binding than that of the control healthy subjects were considered positive. To confirm the results obtained by RIA and exclude any false positives, we tested all available positive sera on Western blots containing purified fibrillarin fusion protein and GST. The sera were preabsorbed with GST-coupled Sepharose 4B to remove the antibodies that might react with GST. We also included a mouse antifibrillarin mAb and four sera from healthy humans as positive and negative controls, respectively. Representative Western blots are shown in Fig. 4. The results showed a strong positive correlation between RIA and Western blot assay methods. The high sensitivity of the immunoblot analysis method is clearly demonstrated by the strong fibrillarin band signal in the blot incubated with mouse antifibrillarin mAb at 0.1 μg/ml.

The data summarized in the histogram (Fig. 5) show that ~58% of SSc patients have significant titers of antibodies that bind to fibrillarin fusion proteins. A similar percentage of patients with CREST syndrome (58%), MCTD (60%), RA (59%), and SLE (39%) also exhibited the presence of these antibodies. To our surprise, >84% of patients with SS showed high titers of antifibrillarin antibodies. Thus, our data clearly show that antifibrillarin autoantibodies are present at high frequency not only in patients with SSc but also a variety of other connective tissue diseases.

Epitope Specificity of Antifibrillarin Antibodies. RIA was performed by use of fibrillarin fusion proteins FP-B, FP-C, FP-D, FP-E, and FP-F to determine the domains of fibrillarin that interacted with the autoantibodies. The sera exhibiting significant titers of antifibrillarin antibodies were further tested by direct binding assay with purified fibrillarin fusion proteins FP-B to FP-F. Out of 56 SSc sera, 31 showed significant binding to fibrillarin fusion protein FP-A, 30 to FP-B, 5 to

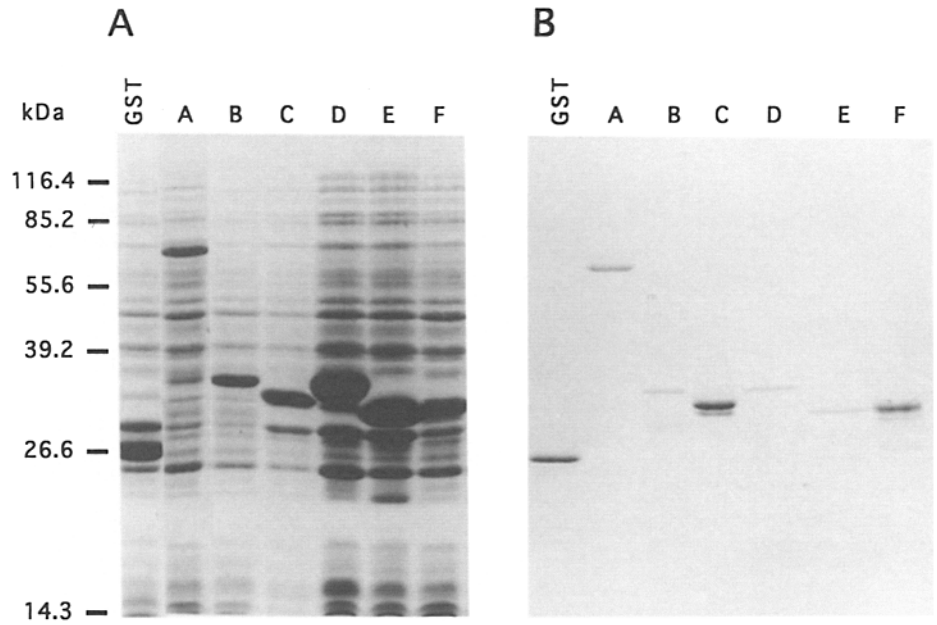


Figure 2. Analysis of recombinant fibrillarin fusion proteins. Coomassie blue–stained polyacrylamide gels show lysates of bacteria expressing fusion proteins A–F (*A*) and the purified fusion proteins (*B*). Fusion proteins were purified by affinity chromatography on glutathione-S-Sepharose gel as described in Materials and Methods. The observed molecular sizes of fusion proteins A–F correspond well to the predicted molecular masses of 60.0, 34.5, 32.5, 34.1, 30.6, and 30.6 kD, respectively.

FP-D, and 8 to FP-F (Table 1). Only 1 sample failed to bind to any fusion protein other than the complete fibrillarin molecule. The titer of antibodies against individual epitopes present in this sera may be low and is not quite sufficient to show above the background when individual fusion proteins were used, or it is a conformational site that was lost as individual fusion proteins were prepared. Sera from MCTD, SLE, CREST, RA, and SS showed high frequency of binding activity to the fusion protein, FP-B, containing the NH_2-terminal region. Comparison of binding profiles of sera from SSc, MCTD, SLE, and CREST disease groups revealed no specific patterns of antibody reactivity. The frequency of antibody reacting with the immunodominant epitope present on FP-B and FP-F did not show significant difference between the above disease groups. However, sera from RA and SS showed a high frequency of binding to the COOH-terminal portion corresponding to amino acid residues 276-321 (FP-F). This is comparable to the binding activity to the immunodominant epitope present on FP-B. This suggests that anti-FP-F antibody may be an additional marker to discriminate RA and SS from other connective tissue disorders. The binding specificity and the pattern of reactivity of autoantibodies were confirmed by Western blot analysis. Samples of positive sera, selected randomly, were tested at 1:500 dilution on Western blots. The binding profiles of several sera were examined in Western blots containing fusion proteins FP-A to FP-F. Representative Western blots are shown in Fig. 6. Distinct patterns of binding by different sera demonstrate that a number of epitopes are recognized by the autoantibodies, confirming the results obtained by direct-binding RIA. This assay also shows that the antibodies demonstrated are specific and do not react with GST. The amount of antibodies bound to the fusion protein FP-B band in Western blots appears to be somewhat lower than that detectable in RIA (data not shown). This may be due to reduced availability of some conformational epitopes present on FP-B after SDS denaturation.

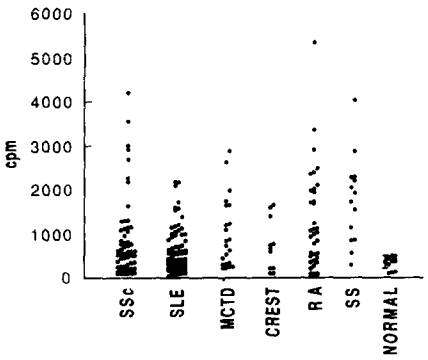


Figure 3. Binding activity of sera from various connective tissue diseases to fibrillarin. The binding activity was measured by RIA with sera at 1:400 dilution as described in Materials and Methods. Optimum dilution of the serum to use in the assays was determined by preliminary assays. Cpm bound to GST was subtracted from the cpm bound to fibrillarin fusion protein. The mean values of triplicate assays are shown in the scatter diagram. Each dot represents an individual serum.

Specificity of Antifibrillarin Antibodies. To determine whether the autoantibodies present in these disease groups are specific to fibrillarin, we tested the binding activity of autoantibodies by competitive inhibition RIA by use of several autoantigens as competitive inhibitors (collagen I, Sm, dsDNA, and MBP are target autoantigens in SSc, SLE, and multiple sclerosis [MS]). Results of inhibition RIA of four antibodies are shown in Fig. 7. The specificity of affinity-purified antibodies obtained from a SSc serum that was also used in Western blot analysis is shown in Fig. 7 *A*. Preincubation of purified antibodies with chromatographically purified soluble recombinant fibrillarin inhibited the binding of autoantibodies to fibrillarin-coated plates, while other autoantigens used as inhibitors did not show any significant inhibition. Results of inhibition assays

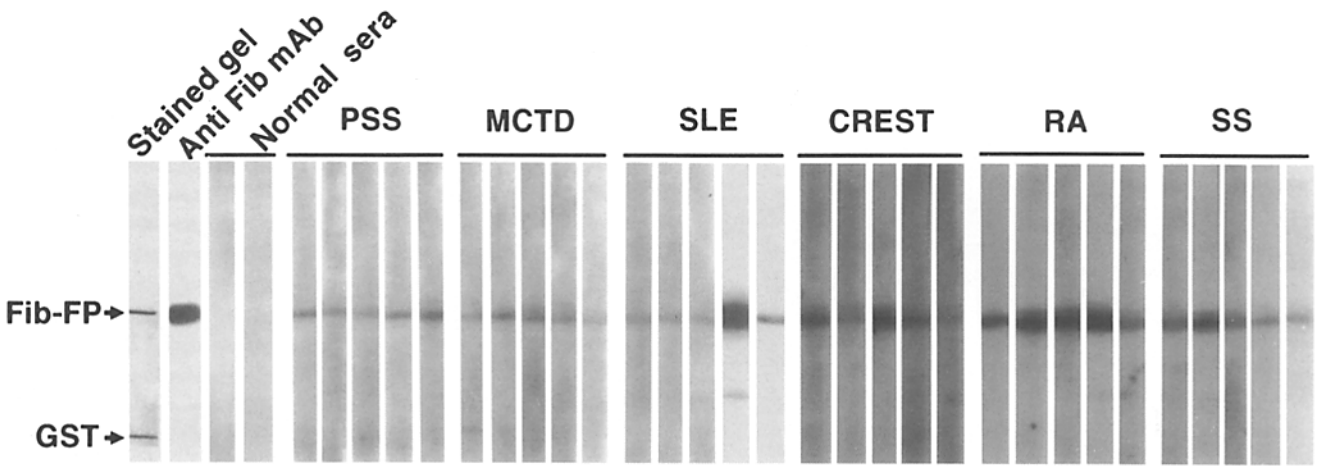


Figure 4. Demonstration of the presence of antifibrillarin autoantibodies in connective tissue diseases. Purified recombinant human fibrillarin and GST were separated on 10% SDS-PAGE, blotted to nitrocellulose, and reacted with sera as described in Materials and Methods. Human sera were used at 1:500 dilution. Mouse anti-human fibrillarin mAb (0.10 μg/ml) and sera from heathy human subjects were used as positive and negative controls. Representative autoradiograms of Western blots reacted with control sera, SSc (*PSS*), MCTD, SLE, CREST, RA, and SS are shown.

of sera from SSc, MCTD, and SLE shown in Fig. 7, *B–D*, further demonstrate that the autoantibodies detected by RIA are specific to fibrillarin and are devoid of polyreactivity. Similar results were obtained with sera from patients with RA and SS (data not shown).

Indirect Immunofluorescence Analysis. Total serum IgG from SSc patients, and affinity-purified antifibrillarin antibodies, from the fusion protein FP-A affinity column were used for determining the pattern of binding to nuclear components on freshly cultured 3T3 cells by immunofluorescence and confocal microscopy. The number of nucleoli per cell vary depending on the cell type used. The 3T3 fibroblasts show two to several nucleoli (10). IgG from SSc patients showed bright nucleolar staining with very weak or no staining of nucleoplasm (Fig. 8, *B*). On the other hand, affinity-purified antifibrillarin antibodies, in addition to brightly staining nucleolar material, also stain the nucleoplasm feebly but consistently. The weaker staining of nucleoplasm by total IgG may be due to the polyclonal nature of the antibodies present. In other words, in these sera, antibodies that are specific for other nucleolar proteins are probably more abundant than antifibrillarin antibodies. Staining of fibrillar regions of the nucleolus was more evident when affinity-purified antibodies were used. Confocal microscopy confirmed that the staining of the fibrillar and granular regions of the nucleolus is characteristic of antifibrillarin antibodies (Fig. 8 *G*).

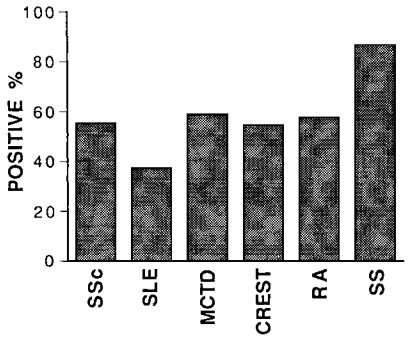


Figure 5. Frequencies of patients with antifibrilllarin antibody activity in connective tissue diseases. The numbers of sera exhibiting two- to four-fold higher binding activity than the mean value of sera from healthy individuals are shown as percentages in the histogram.

Discussion

Autoantibodies reacting with nucleolar proteins are produced in connective tissue diseases such as SSc, SLE, and SS (6). Autoantibodies present in SSc show diverse binding patterns in immunofluorescence staining, suggesting that a variety of autoantigens might be recognized. The molecular nature of some of these autoantigens has been described recently. A distinct nucleolar protein, fibrillarin, associated with U3-snRNP, has been reported as a target autoantigen recognized by autoantibodies present in diffuse scleroderma (10). Immunofluorescence staining with antifibrillarin antibodies shows staining of fibrillar and granular regions of the nucleoli

Table 1. *Epitope Specificity of Anti-Fibrillarin Autoantibodies*

Disease	Fibrillarin	FP-B	FP-C	FP-D	FP-E	FP-F
PSS	31/56	30	0	5	0	8
MCTD	13/22	10	1	6	0	2
RA	22/38	14	0	6	0	17
SS	13/15	8	0	3	0	11
CREST	6/11	4	0	2	0	4
SLE	30/80	22	0	5	0	10

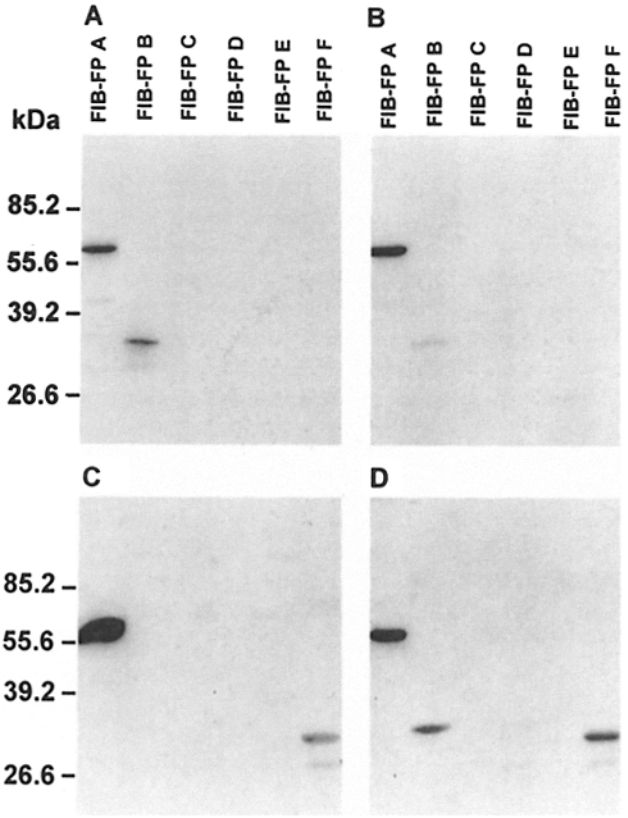


Figure 6. Western blot analysis of autoimmune sera from connective tissue diseases. Selected examples of sera reactive with fibrillarin fusion proteins are shown. Western blots containing recombinant fusion proteins A–F were incubated with a 1:500 dilution of autoimmune sera and developed with ^{125}I-labeled rabbit F(ab')$_2$ antibody specific for human Igγ chain. (*A*) SSc serum. (*B*) MCTD serum. (*C*) SSc serum. (*D*) SLE serum.

(9). The distribution of fibrillarin staining parallels RNA polymerase I staining (7). Our results clearly demonstrate that affinity-purified antifibrillarin antibodies stain fibrillar regions of the nucleolus (Fig. 8). Since the immunofluorescence method does not allow precise identification of the antigens involved, we used a sensitive RIA with recombinant fibrillarin fusion proteins to define B cell epitopes. In this paper, we present data demonstrating that antifibrillarin antibodies are produced not only in SSc, as described previously (12), but also in a variety of other rheumatic diseases. Thus, the frequency of antifibrillarin antibodies occurring in MCTD, CREST syndrome, and RA is comparable to that observed in SSc, while these antibodies are more prevalent in SS (84%) but less common in SLE patients (39%).

There is a striking difference between our results and those previously reported with respect to the frequency of antifibrillarin autoantibodies in scleroderma. While the results obtained by immunofluorescence and immunoprecipitation methods showed an incidence of 3.4–6% (22–24), our results obtained by RIA showed a frequency of 58% among scleroderma patients. Positive sera identified by RIA were also confirmed to be positive in Western blot analysis. High correlation between RIA and Western blot analysis results rules out the possibility of high false positives in RIA. The discrepancy between our results obtained by RIA and those obtained by immunofluorescence and immunoprecipitation methods can be explained by (*a*) higher sensitivity of RIA compared with immunofluorescence and immunoprecipitation methods, and (*b*) use of purified recombinant human fibrillarin in RIA in which all epitopes are available for interaction with antibodies. Thus, use of RIA and Western blot analysis with purified recombinant human fibrillarin allowed the detection

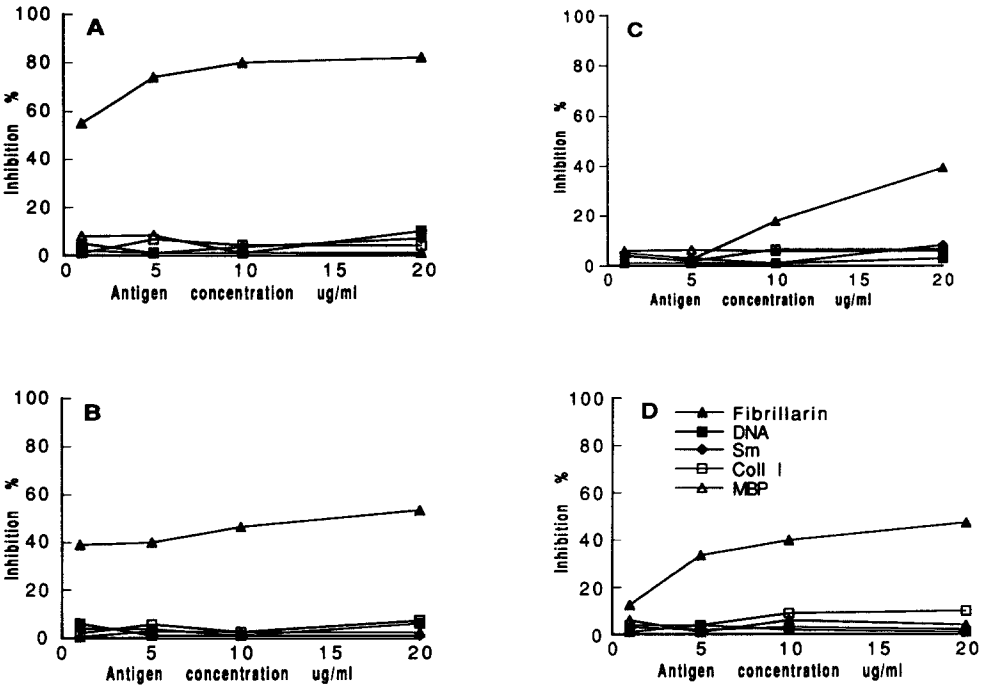


Figure 7. Demonstration of the specificity of antifibrillarin autoantibodies by competitive inhibition RIA. Various concentrations of antigen, 1–20 μg/ml, were mixed with 1 μg/ml of affinity purified antibody or 10 μg/ml of IgG from different sera, preincubated, and then transferred to fibrillarin-coated plates, and the inhibition was calculated as described in Materials and Methods. (*A*) Affinity-purified antibody isolated from an SSc serum. (*B*) SSc serum IgG. (*C*) MCTD serum IgG. (*D*) SLE serum IgG.

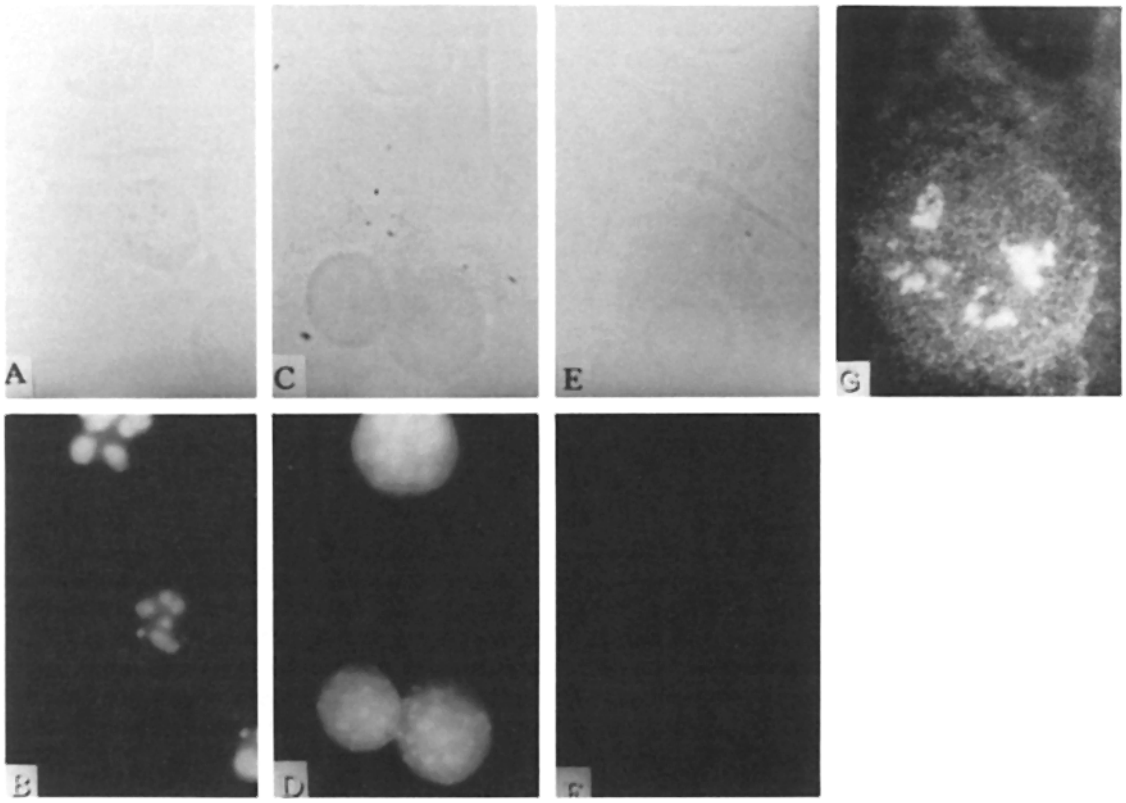


Figure 8. Indirect immunofluorescence staining of 3T3 cells with antifibrillarin antibodies. Freshly grown 3T3 cells were fixed with paraformaldehyde, incubated with SSc serum Ig or affinity-purified antifibrillarin antibodies, and stained with FITC-labeled $F(ab')_2$ goat anti-human IgG antibodies. Phase (*A*, *C*, and *E*) and immunofluorescence patterns (*B*, *D*, and *F*) are shown. (*A* and *B*) Cells incubated with SSc IgG. (*C* and *D*) Cells incubated with affinity-purified antifibrillarin antibodies isolated from a SSc patient. (*E* and *F*) Cells incubated with FITC-labeled second antibody alone. (*G*) Confocal microscope image of the fibrillar regions of nucleoli in cells stained with affinity-purified antifibrillarin antibodies. ×100 (*A–E* and *F*); ×250 (*G*).

of antifibrillarin autoantibodies in the sera of patients with other connective tissue diseases. It is of interest that the presence of antifibrillarin antibodies has been reported in certain malignant diseases such as hepatocellular carcinoma (35).

Antifibrillarin antibodies present in connective tissue diseases are highly specific to fibrillarin antigen. Results from competitive inhibition RIA demonstrate that binding of affinity-purified antifibrillarin antibodies and autoantibodies present in the sera to fibrillarin antigen–coated plates is inhibited only by soluble fibrillarin and not by other autoantigens such as DNA, Sm, collagen type III, and MBP, which are target antigens in SLE, SSc, and MS. These data, as well as low binding to BSA or GST, suggest that antifibrillarin autoantibodies are not endowed with polyspecific properties. The variation in the degree of inhibition between different sera might be due to differences in the concentration, affinity, and epitope specificity of antibodies present in different sera. The data obtained from RIA and immunoblotting of fibrillarin fusion proteins, encompassing the entire fibrillarin polypeptide sequence, show that the immunodominant B cell epitopes are located in the NH_2-terminal glycine–arginine–rich region, since most of the SSc sera, as well as sera from MCTD, SLE, and CREST syndrome, react with FP-A, the complete fibrillarin, and the fusion protein FP-B, which contains the NH_2-terminal moiety. About 15–30% of SSc patients also had antibodies that bound to the COOH-terminal region of fibrillarin, FP-F, while a small percentage of sera also bound to FP-D, the internal helical region. The autoantibodies that bound to the NH_2-terminal portion are distinct from those that bind to the epitopes present in the COOH-terminal region because all the sera that showed binding to FP-B did not bind to FP-F. Western blot analysis confirmed RIA results. In Western blots, the antibodies present in some sera recognized only NH_2-terminal fusion protein, while others recognized only COOH-terminal peptide (Fig. 6). Antibody profiles obtained for RA and SS, by use of recombinant fusion proteins, showed higher correlation of the presence of anti-FP-F antibody with the disease. The variation in the epitope reactivity and specificity of autoantibodies may be influenced by factors such as the duration or severity of the disease, HLA haplotype of the patient, and nature of the therapy.

Swiss-Protein data base search revealed the presence of homology between the NH_2-terminal portion of fibrillarin and RNA binding proteins from various sources and EBV-encoded nuclear protein (Fig. 9). The NH_2-terminal portion of fibril-

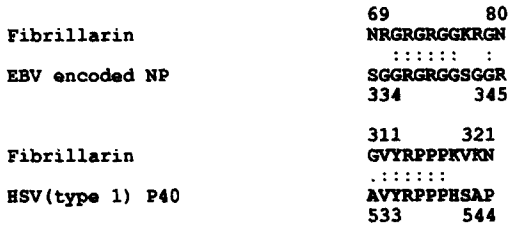


Figure 9. Comparison of amino acid sequences of fibrillarin peptide motifs sharing homology with foreign antigens. Hydrophilic fibrillarin peptide motifs (potential antigenic sites) exhibiting sequence similarity with a nuclear protein encoded by EBV genome and capsid protein P40 encoded by HSV type 1 are shown. :, amino acid identity; ., conserved changes.

larin (fusion protein FP-B) contains three hydrophilic regions corresponding to aa residues 17–28, 33–48, and 66–84. Based on the analysis of surface probability and Jameson and Wolf antigenic index of these regions, as predicted by GCG analysis (Genetic Computer Group, Madison, WI), the aa residue sequence 66–84 appears to be the most immunodominant one in this region. This fusion protein is recognized by a majority of autoimmune sera. Interestingly, this region contains a hexapeptide sequence, GRGRGG, which is shared with EBV-encoded nuclear antigen (36). The next most commonly recognized fusion protein, FP-F, by autoimmune sera, contains the COOH-terminal portion of fibrillarin. This region contains two hydrophilic regions at aa residues 281–306 and 314–321. The first hydrophilic region bears high homology with yeast fibrillarin as well as some self-proteins such as chicken myosin heavy chain and microtubule associated protein 1A (37). The most antigenic peptide in this region is aa sequence 311–319, GVYRPPPK (Fig. 9), bearing sequence homology with capsid protein P40 encoded by HSV type 1 (38).

One may ask how antifibrillarin autoantibodies are induced in patients with various connective tissue diseases. The first possibility is that the peptides that were generated from the nucleolar protein fibrillarin, derived from the endogenous pathway, are transported to the surface, where they are recognized by fibrillarin-specific lymphocytes. In this mechanism, it remains unclear why antifibrillarin antibodies are produced only in patients with connective tissue diseases and not in healthy subjects or in patients afflicted with other diseases. Whether MHC or analogous molecules play a role in presentation of the peptides derived from fibrillarin remains to be determined. The second possibility is that environmental factors, chemicals, or drugs might play a role in presentation of these self-peptides to T lymphocytes, leading to their amplification, and thereby help in the activation of autoreactive B cells. The presence of elevated levels of antifibrillarin antibodies in mercuric chloride–treated mice supports this possibility (13, 14). The third possibility is molecular mimicry, in which the autoantibody response is induced in the host by infectious agents. The classical example is the cross-reactivity of anti-*Streptococcus* A antibodies with cardiac myosin (39). There are increasing examples showing presence of shared antigenic peptide sequences between viral, bacterial, parasitic, and autoantigens (40). Sharing of peptide sequence homology between MBP and viral proteins (41), adenovirus-12 E1B protein and gliadin A (42), gp210 protein reacting with autoantibodies from patients with primary biliary cirrhosis (43), leucine zipper region of C-myc and V-myc with p70 Ku autoantigen (44), RAL-1 antigen of river blindness pathogen *Onchocera volvulus* and 60-kD RO/SS-A autoantigen (45), regions of topoisomerase I with CMV (26) and mammalian type C virus $P30^{gag}$ (46) are well documented. Sharing hexapeptide sequence homologies between fibrillarin and EBV-encoded nucleoprotein and HSV type 1–encoded P40 suggests that molecular mimicry might play a significant role in the induction of autoimmunity and production of autoantibodies reacting with fibrillarin.

The data presented here have twofold importance. First is the demonstration that autoantibodies specific for fibrillarin epitopes are produced in a number of connective tissue diseases in addition to SSc. Second is the demonstration of the presence of autoantibodies against immunodominant epitopes located at the NH_2- and COOH-terminal regions of fibrillarin. In addition, presence of homology between the NH_2-terminal region of fibrillarin and the nuclear protein encoded by EBV on the one hand and capsid protein P40 encoded by HSV and the COOH-terminal region of fibrillarin on the other hand suggests that common latent viruses might play a role in the induction of autoantibodies. Furthermore, correlation between the high incidence of antifibrillarin antibodies and the presence of EBV infection in SS patients supports this. Studies are in progress to isolate mAbs against various fibrillarin epitopes and determine whether the antibodies specific for EBV-encoded nuclear protein or P40 interact with fibrillarin peptides. Such experiments might shed light on the possible role of an antigen mimicry mechanism in the production of autoantibodies against autoantigens.

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