

Dermatologic and ocular diseases

Autoantibodies to DFS 70 kd/transcription coactivator p75 in atopic dermatitis and other conditions

Robert L. Ochs, PhD,^a Yoshinao Muro, MD,^a Yuanzheng Si, PhD,^b Hui Ge, PhD,^b
Edward K. L. Chan, PhD,^a and Eng M. Tan, MD^a La Jolla, Calif, and Bethesda, Md

Background: Sera of patients with atopic dermatitis (AD) were found to have autoantibodies that reacted with tissue culture cell substrates in immunohistochemistry to display a characteristic pattern of nuclear distribution of dense fine speckles. The sera also recognized a 70-kd protein on Western immunoblots, and the antigen was termed *dense fine speckles 70 kd* (DFS70).

Objective: Because spontaneously occurring autoantibodies could be immune responses to proteins that might be participating in the disease process, it was of interest to identify the antigens driving the autoimmune antibody response.

Methods: A serum containing high-titer antibodies to DFS70 was used to immunoscreen a complementary (c)DNA expression library to isolate cDNA encoding the antigen. After the cDNA was isolated, this was used to express recombinant protein to determine the prevalence of antibody in AD and other conditions.

Results: Thirty percent of patients with AD were found to have antibody to recombinant DFS70 in Western immunoblots. Sixteen percent of patients with asthma and 9% of patients with interstitial cystitis had antibodies of the same specificities. The cDNA encoding DFS70 was identical to a transcription coactivator called p75, which had been shown to be required for RNA polymerase II-dependent transcription. Another important finding was that IgE antibodies to DFS70 were also present in AD sera.

Conclusion: It is suggested that a common basis for the presence of autoantibodies to DFS70 might be related to AD in asthma, interstitial cystitis, and other conditions. A possible role of this antigen-antibody system in pathogenesis remains to be demonstrated, but it appears to be a marker for a subset of patients with AD. (J Allergy Clin Immunol 2000;105:1211-20.)

Key words: Atopic dermatitis, autoantibodies, antinuclear antibodies, asthma, interstitial cystitis, transcription coactivator

Atopic dermatitis (AD) is a chronic, relapsing, pruritic skin disorder that generally first appears in childhood and frequently progresses to asthma, allergic rhinitis, or both in the adult.¹⁻³ There are no reliable laboratory markers for this condition, but patients with AD often have elevated serum IgE levels and allergic reactivity to foods and other common allergens, such as pollens, molds, and insects. There have also been reports of antinuclear antibodies (ANAs) in this condition.^{4,5} The prevalence of AD appears to be on the rise, with 10% to 15% of the population being affected at some time during childhood.^{6,7} We now report the finding of an autoantibody-autoantigen system in 30% of patients with AD, which is also shared to a lesser extent by patients with asthma and interstitial cystitis (IC). IC is a urinary bladder condition in which the classical pathology is characterized by predominant mononuclear cell infiltration of the lamina propria with lymphocytes, plasma cells, and mast cells.⁸

The identification of this antigen-antibody in AD was initiated by an observation in patients with IC of an autoantibody against a nuclear antigen distributed as dense fine speckles in the nucleoplasm of cells in interphase and increased localization in condensed chromosomes in mitosis. In addition, in immunoblotting with tissue-culture extract as the antigen source, sera of such patients reacted with a 70-kd protein. This antigen was termed *dense fine speckles 70 kd* (DFS70), incorporating the immunolocalization of the antigen (DFS) and the estimated molecular size of the antigen in polyacrylamide-SDS gels.⁹ In continuing characterization of this antigen-antibody system, we observed that the antibody was present in higher frequency in patients with AD than in patients with IC. We report that this antigen is a nuclear protein that is identical to a nuclear transcription coactivator called p75 and that the antibody is also present in other conditions. It is possible that the underlying common feature could be the presence of AD in patients with asthma, IC, and other disease states.

METHODS

Patients and antibody control subjects

Sixty-four patients with AD from the Department of Dermatology, Nagoya University School of Medicine, Nagoya, Japan, were

From ^aW. M. Keck Autoimmune Disease Center, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla; and ^bthe Laboratory of Molecular Embryology, National Institute of Child Health and Development/National Institutes of Health, Bethesda.

Dr Muro is currently affiliated with the Department of Dermatology, Nagoya University School of Medicine, Nagoya 466-8550, Japan.

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Reprint requests: Eng M. Tan, MD, Autoimmune Disease Center, MEM 131, The Scripps Research Institute, 10550 N Torrey Pines Rd, La Jolla, CA 92037.

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Abbreviations used

AD:	Atopic dermatitis
ANA:	Antinuclear antibody
DFS70:	Dense fine speckles 70 kd
IC:	Interstitial cystitis
LEDGF:	Lens epithelium-derived growth factor
TBS:	TRIS-buffered saline
USA:	Upstream stimulatory activity

enrolled in our study. Diagnostic criteria for AD were those described by Hanifin and Rajka.¹ Chart records were reviewed for age, sex, duration of disease, eosinophil numbers, IgE levels, presence of respiratory atopy (bronchial asthma or allergic rhinitis), and degree of facial dermatitis. Patients with IC were given that diagnosis by using established criteria⁸ and were part of a large study on clinical features and seroepidemiology reported previously.^{10,11} Patients with asthma were recruited from the Division of Allergy and Immunology, Scripps Clinic, La Jolla, California, and fulfilled the American Thoracic Society criteria for the diagnosis of asthma. Patients with psoriasis without accompanying AD came from the clinical practice of one of the authors (Y. M.). Patients with chronic fatigue syndrome were reported and characterized in a previous study.¹² Normal human sera and sera from patients with systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, and scleroderma were obtained from the serum bank of the W. M. Keck Autoimmune Disease Center, Scripps Research Institute, La Jolla, California.

Immunofluorescence

Sera from patients and control subjects were diluted 1:100 in PBS and examined by using indirect immunofluorescence for the presence of autoantibodies to nuclear and cytoplasmic antigens on commercially prepared HEp-2 cell-substrate slides (Bion, Park Ridge, Ill) with anti-human IgG coupled to FITC (Caltag, San Francisco, Calif) as the secondary detecting reagent.

IgG subclass identification of autoantibodies was performed by using indirect immunofluorescence on commercially prepared HEp-2 cell substrate slides as described above with affinity-purified FITC-conjugated sheep anti-human IgG1, IgG2, IgG3, and IgG4 (The Binding Site, Birmingham, England) as secondary antibodies.

Immunoblotting

Human MOLT-4 cells (a T-cell line) were obtained from the American Type Culture Collection (Rockville, Md) and grown in suspension culture in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 10 µg/mL gentamicin sulfate. Whole-cell extracts were prepared from MOLT-4 cells by pelleting suspended cells in media, rinsing in PBS, and resuspending the cell pellet in an equal volume of 2× gel electrophoresis sample buffer containing a cocktail of protease inhibitors (catalog #1697498; Boehringer Mannheim, Indianapolis, Ind). The cell extract was then sonicated on ice for 4 to 5 bursts of 10 seconds each until the cells were dispersed. After sonication, the extract was then passed through progressively finer gauge needles (No. 18 to No. 27) to shear the DNA. The extract was boiled for 5 minutes and centrifuged to remove insoluble material, and aliquots were stored at -70°C. This form of cell extract has been used extensively in many previous studies^{11,12} as a source of autoantigens for detection of serum autoantibodies against ubiquitous cellular antigens.

Western blotting was performed essentially as described by Chan and Pollard.¹³ Serum was diluted 1:100, and detection of immunoreactive bands was performed by using chemiluminescence with peroxidase-conjugated goat anti-human IgG (Caltag, Burlingame, Calif) and the ECL chemiluminescence kit (Amer-

sham, Arlington Heights, Ill) according to the manufacturer's directions, followed by autoradiography. For blotting of recombinant DFS70, the detection procedure was identical.

For detection of specific IgE autoantibodies to DFS70, serum samples were diluted 1:10 according to the method of Valenta et al¹⁴ to detect serum IgE levels that are on average 100,000 times lower in amount compared with levels of IgG. IgE antibody was detected by using peroxidase-conjugated goat IgG antibody to human IgE epsilon heavy chain (Cappel, Durham, NC) as the secondary detecting reagent, followed by chemiluminescence and autoradiography as described above for the detection of IgG. Normal control sera were included in every determination to exclude spurious findings in this procedure.

Complementary DNA cloning

DFS70 was originally detected by using autoantibodies from patients with IC⁹ and was given the designation because of a characteristic immunohistochemical staining pattern on HEp-2 (human epithelial line) cells consisting of DFS distributed in the nucleoplasm in interphase cells and with accentuated generalized staining of condensed chromosomes in mitotic cells. These sera also showed reactivity with a 70-kd protein in MOLT-4 extracts in Western blotting. One of these sera, which was high in antibody titer and available in larger quantity, was used to isolate a partial complementary (c)DNA sequence as indicated below. The method for primary screening of a T24 (bladder carcinoma cell line) cDNA expression library was reported previously.¹⁵ Briefly, serum from patient 90-49 was diluted 1:100 and preabsorbed with wild-type λ Zap Express phage without inserts. The preabsorbed serum was then used to screen approximately 300,000 recombinant plaques from a cDNA expression library constructed from T24, a bladder epithelial cell line (American Type Culture Collection), by using iodine 125-staph protein A according to the method of Young and Davis.¹⁶ All screenings were carried out on duplicate filters, and two double-positive clones were obtained. After multiple rescreening, clone DFS6.1 was amplified, purified, and used for sequence analysis. Clone DFS6.1 was a partial cDNA, and to isolate full-length cDNA, multiple overlapping 5' clones were obtained from human placenta cDNAs by using a modified 5' RACE method, as previously described.¹⁷ Clone DFS6.1 was reported in an abstract,¹⁸ and the nucleotide sequence was submitted to GenBank under accession No. U94319 in March 1997.

Sequence and protein analyses

cDNA inserts were analyzed by means of restriction mapping and sequencing. Nucleotide sequence was determined by using dye primer cycle sequencing by using a Model 373A DNA sequencer from Applied Biosystems (ABI, Foster City, Calif). Oligonucleotide primers were synthesized with a Model 394 DNA synthesizer (ABI). DNA and protein sequences were analyzed by the Genetics Computer Group Sequence Analysis Software Package version 7.2 for UNIX computers.¹⁹ Alignment of protein sequences was achieved with the GAP program that used the algorithm of Needleman and Wunsch.²⁰ Molecular mass was calculated by using the Genetics Computer Group program PEPTIDESORT.

Purification of recombinant DFS70

For expression and purification of recombinant protein, the *EcoRI-EcoRI* fragment of clone DFS6.1 and the full-length cDNA were subcloned into pET 28 vector (Novagen, Madison, Wis). The full-length cDNA was obtained by using RT-PCR with T24 cell messenger RNA and primers corresponding to sequences flanking the methionine start codon and 3' stop codon. The sense and antisense primers were 5'-GCAGAATTCGATACATGACTCGC-GATTTC-3' and 5'-AGTGAAGCTTATATTCCAGGTATGTCAA-

CCTACGTT-3', and the sequences incorporated to facilitate subcloning were *EcoRI* and *HindIII* restriction sites (underlined). The pET28 vector has N-terminal fusion tags comprising T7 and 6× histidine. The T7 fusion tag allows for easy detection of recombinant protein with T7 antibodies, and the 6× histidines allow for protein purification because of the high affinity of histidine to nickel. *Escherichia coli* strain BL21-DE3 containing the pET 28a vector with the DFS6.1 insert was grown in LB media containing kanamycin until an optical density at 600 nm of 0.8. Then, 5 mmol/L isopropyl thiogalactose was added to induce recombinant protein expression for 6 hours at 37°C. Cells were harvested by means of centrifugation and broken open by cycles of freezing and thawing in a buffer solution containing 6 mol/L guanidine hydrochloride according to the manufacturer's directions (Qiagen, Chatsworth, Calif). This solution was then passed through a Ni-NTA-Agarose resin (Qiagen, catalog #30210) column where the recombinant protein was bound at elevated pH (pH 8.0) by the affinity of nickel for the 6× His portion, unbound material was washed out, and the recombinant protein was eluted in fractions at a pH of 6.0. Each fraction was checked by using Western blotting for the presence and purity of recombinant protein.

Recombinant proteins and antibodies for transcription coactivators p52 and p75

The upstream stimulatory activity (USA) fraction of HeLa cells containing the transcription coactivator proteins p52 and p75 was described previously.²¹ Subsequent studies resulted in the cDNA cloning and production of recombinant proteins for p52 and p75 and the production of rabbit polyclonal antibodies.²² These reports appeared while these studies were in progress, and it was noted that our plasmid DFS6.1 had complete identity with the corresponding region of p75. Studies were therefore initiated with Ge and colleagues (National Institutes of Health, Bethesda, Md), and reagents were exchanged to determine the reactivity of autoantibodies to p75 and p52 as described in the "Results" section.

Immunoelectron microscopy

Small pieces of mouse intestine were fixed for 1 hour at 4°C with 1% glutaraldehyde buffered in PBS. Pieces were then dehydrated in 70% ethanol and embedded in LR White. After polymerization at 56°C for 2 days, sections were collected on gold grids. For immunolabeling, individual grids (with sections down) were incubated for 30 to 60 minutes with a blocking solution consisting of 2% normal goat serum/0.2% BSA made in TRIS-buffered saline (TBS) containing 1.0% Tween-20 (0.02 mol/L TRIS, 0.15 mol/L NaCl, and 1.0% Tween-20, pH 7.6). Grids were then transferred to drops consisting of a 1:100 dilution of human anti-DFS70 made in TBS/Tween-20 for 1 hour at room temperature and then rinsed with TBS/Tween-20 10 times for 1 minute each. Grids were then blocked for 30 to 60 minutes in TBS/Tween-20 and incubated for 1 hour at room temperature in a 1:50 dilution of goat anti-human IgG linked to 10 nm colloidal gold (Amersham) diluted in TBS/Tween-20. Grids were then rinsed in TBS/Tween-20 10 times for 1 minute each, rinsed in PBS, fixed for 30 minutes at room temperature in 1% glutaraldehyde/PBS, rinsed in PBS 3 times for 5 minutes, rinsed in distilled water 3 times for 5 minutes, counterstained with uranyl acetate and lead citrate, rinsed in distilled water, and blotted dry.

RESULTS

ANAs in patients with AD

Sixty-four patients with AD (30 men and 34 women) were analyzed for IgG ANAs by means of indirect immunofluorescence on commercial human HEP-2 cell

substrates and by means of Western blotting on whole-cell extracts of human MOLT-4 cells (Fig 1). Patients with AD ranged in age from 4 to 43 years (average, 24.4 years), and the duration of disease ranged from 1 to 35 years (average, 13.4 years). Of these 64 patients with AD, 26 (40.6%) had ANAs of one pattern or another, but only 18 (28%) produced a pattern of DSF in interphase nucleoplasm and staining of chromosomes in mitotic cells. The prototypic immunofluorescence staining pattern for one AD serum is illustrated in Fig 1, A, where interphase cell nuclei display fine speckles distributed in the nucleoplasm and aggregation of the speckles in the region of condensed chromosomes in mitotic cells (arrowheads). A prototypic IC serum showed a similar pattern (Fig 1, B). Thirteen of the 18 patients described in Table I were included in the Western blot analysis in Fig 1, C, depicting the initial studies that showed that the majority of patients with DFS-type antibodies appeared to be reacting with an antigen of approximately 70 kd (arrowhead) contained in MOLT-4 cell extracts. Lanes 5 to 22 consisted of 13 sera from patients in Table I with the addition of 5 other patients with AD who did not have the distinctive DFS type of autoantibodies and other control positive and negative sera in lanes 1 to 4. It was noted that some AD sera had antibodies to antigens of other molecular sizes, but the important finding was the occurrence of a common antigen-antibody system in the majority of sera with DFS-type antibodies. The last column of Table I includes data on the 18 DFS sera with respect to antibodies to recombinant antigen, which is described further below. It should be noted from Table I that there was no significant relationship between the presence of anti-DFS70 antibody and parameters of age, duration of disease, eosinophils, and IgE levels or respiratory atopy, but the majority of patients had facial dermatitis.

cDNA cloning of DFS70: Sequence identity to the transcriptional coactivator p75

To characterize DFS70 and to determine whether it was a novel protein recognized by the sera of patients with both AD and IC, we used the more abundant serum from patient 90-49 with IC (Fig 1, C, lane 1) to clone the partial cDNA of DFS70. We first isolated poly A(+) RNA from human T24 bladder epithelial cells. From this starting material, a cDNA expression library was constructed and screened with serum, and the positive clones obtained were subcloned and purified to homogeneity. Clone DFS6.1, containing a 2-kb insert, was then cloned and sequenced (Fig 2, A). Analysis of this sequence showed an open reading frame of approximately 1 kb, coding for 351 amino acids with a predicted molecular weight of 40 kd (Fig 2, B). A search of the gene sequence database at that time indicated no identity with previously reported sequences. The sequence of cDNA clone DFS6.1 from protein DFS70 was deposited in GenBank under accession No. U94319¹⁸ in 1997.

An overlapping clone No. 52 was obtained from human placental cDNAs by using 5'RACE (Fig 2, A). Independent confirmation was provided from RT-PCR

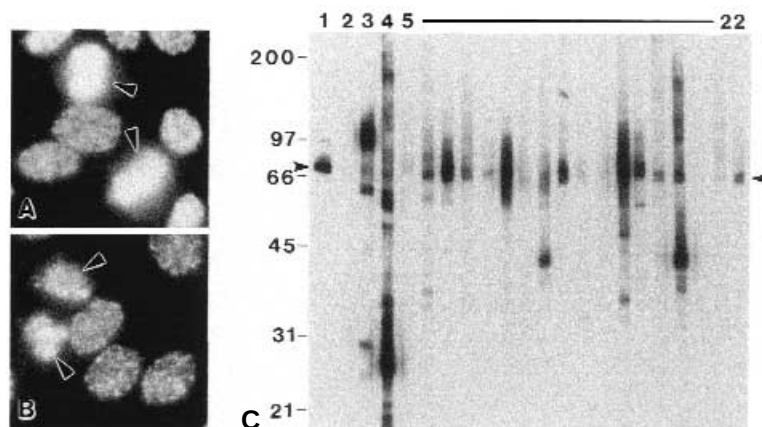


FIG 1. Indirect immunofluorescence (**A** and **B**) and Western blotting against MOLT-4 cell extracts (**C**) of sera from patients with AD and IC. The major ANA immunofluorescence pattern in both groups of patients is characterized by a DFS nucleoplasmic staining pattern in interphase cells and staining of condensed chromosomes in mitotic cells (*arrowheads*). **A** shows staining of an AD serum, and **B** shows staining of a prototype IC serum. As determined by means of Western blotting, a common reactive band was a 70-kd polypeptide (*arrowheads*), which we have termed DFS70. In addition to the reactivity at 70 kd that is shared by many sera, some sera show their own unique extra reactivities. *Lane 1*, Prototype patient with IC (90-49); *lane 2*, normal human serum; *lanes 3 and 4*, sera from patients with scleroderma and lupus, respectively, showing reactivity with other autoantigens present in the cell extract; *lanes 5-22*, sera from 18 patients with AD, all of whom reacted with positive signals at 70 kd, except for *lanes 5 and 20*.

TABLE I. Clinical and laboratory data on 18 patients with AD and DFS70-staining pattern as determined by means of immunohistochemistry

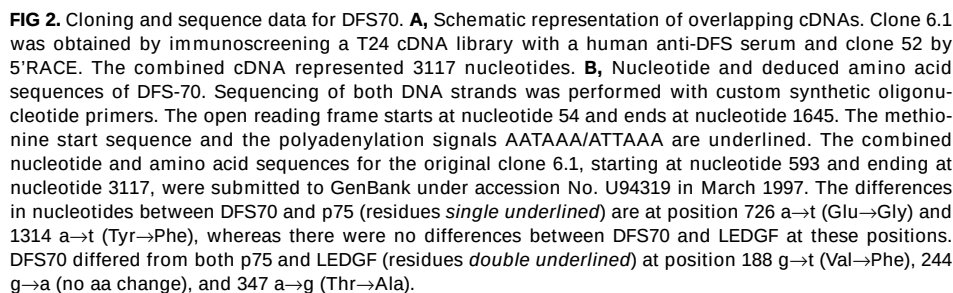
Patient	Sex	Age (y)	Duration of disease (y)	Eosinophils/ mL of blood	IgE (IU/mL of blood)	Respiratory atopy	Facial dermatitis	Antinuclear antibodies Pattern	Titer	Anti-DFS70*
6	F	20	15	ND	ND	BA/AR	Yes	DFS/chr	1280	Positive
8	F	4	2	400	67	No	Yes	DFS/chr	>1280	Positive
10	F	23	22	289	1859	No	Yes	DFS/chr	320	Positive
12	M	19	14	1463	13,226	BA/AR	Yes	DFS/chr/NBs	640	Positive
13	F	20	16	659	4224	BA/AR	Yes	DFS/chr	640	Positive
15	F	29	14	1440	65	No	Yes	DFS/chr/No	>1280	Positive
16	F	22	13	566	6900	No	Yes	DFS/chr	320	Negative
28	M	23	23	629	5024	AR	Yes	DFS/chr	320	Positive
36	F	20	6	213	488	AR	Yes	DFS/chr	1280	Positive
38	F	15	7	743	2189	No	Yes	DFS/chr	320	Positive
39	F	20	20	1296	6270	AR	Yes	DFS/chr	1280	Positive
41	F	19	9	885	196	No	Yes	DFS/chr/NBs	640	Positive
68	F	20	8	568	1579	No	Yes	DFS/chr	640	Positive
77	F	27	26	975	1891	No	No	DFS/chr	ND	Negative
78	M	21	9	87	1714	No	Yes	DFS/chr	ND	Negative
81	M	26	10	205	25	No	No	DFS/chr	320	Positive
88	F	19	3	300	84	AR	Yes	DFS/chr/NBs	640	Positive
99	F	32	27	152	272	AR	Yes	DFS/chr	640	Negative

ND, Not done; BA, bronchial asthma; AR, allergic rhinitis; chr, mitotic chromosome staining; NBs, staining of unidentified nuclear bodies; No, nuclear staining.

*Western blotting against recombinant DFS70.

by using primers upstream of the methionine start and translation stop codons. The amplified cDNA was subcloned into pET28 for sequencing analysis, as described in the "Methods" section. The complete cDNA and deduced amino acid sequences are shown in Fig 2, B. A GenBank search at this time showed that DFS70 was identical to transcription coactivator p75²² and lens epithelium-derived growth factor (LEDGF).²³ The 2- to

4-amino acid differences among these proteins (Fig 2, B) may reflect errors in reverse transcriptase function during the cDNA synthesis step for establishing the cDNA expression libraries because these are single-nucleotide differences. It should be noted that different cell lines (T24 in this report), HeLa,²² and lens epithelial cell²³ cDNA libraries were used for isolating the different cDNA clones.



cloned into pET vector 28a (pET-DFS) that has a 5' T7 fusion protein tag and a 6× histidine tag. Recombinant DFS70 protein expressed from clone pET-DFS was bound and specifically eluted from a nickel affinity column and was detected with either T7 antibody (Fig 3, A, lane 2) or the serum from patient 90-49 with IC used to originally isolate clone 6.1 of DFS70 (lane 4). Purified

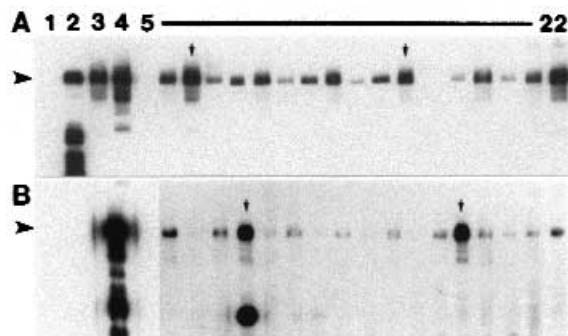


FIG 3. A, Western blot detection of IgG autoantibodies to recombinant DFS70. Arrowhead indicates the position of recombinant DFS70. Lane 1, Normal human serum; lane 2, T7 antibody; lane 3, antibody from patient with IC (UB78); lane 4, antibody from patient with IC (90-49); lanes 5-22, sera from 18 patients with AD showing 16 of 18 lanes with positive signals of varying intensities. **B,** Western blot detection of IgE autoantibodies to recombinant DFS70. Lane 1, Anti-Sm serum from a patient with lupus; lane 2, anti-DNA topoisomerase I serum from a patient with scleroderma; lane 3, normal human serum; lane 5, normal human serum; lane 6, serum from patient with IC (90-49) used as a positive control; lanes 7-22, sera from 16 patients with AD. Lane 4 was developed with IgG antibody to the T7 fusion tag of recombinant protein to show expression of fusion protein. The arrowhead indicates the position of recombinant DFS70. The sequence of sera used in the top and bottom panels do not correspond to the lanes, but the same serum was used in lane 7 of **A** and lane 9 of **B** (arrows), and another serum was used for lanes 16 and 18 (arrows), respectively.

TABLE II. Patient groups reactive with recombinant DFS70 as determined by using Western blotting

Patients	Reactivity
Normal control subjects	0/39 (0%)
AD	19/64 (29.6%)
Asthma	8/50 (16%)
IC	9/103 (8.7%)
Psoriasis	1/22 (4.5%)
Chronic fatigue syndrome	2/60 (3.3%)
Systemic lupus erythematosus	0/36 (0%)
Rheumatoid arthritis	0/30 (0%)
Sjögren's syndrome	2/29 (6.9%)
Scleroderma	1/40 (2.5%)

recombinant DFS70 was then used to confirm the presence of IgG antibodies originally detected by using MOLT-4 cell extracts. Fig 3, *A*, lanes 5 to 22, included 13 patients from Table I who had positive Western blotting results against a 70-kd protein contained in MOLT-4 cells, and these were also positive against recombinant DFS70. In addition, the figure depicts 3 other AD sera not from Table I that were positive and 2 AD sera that were negative (the latter in lanes 5 and 17). All 64 AD sera were subsequently examined in Western blotting against recombinant DFS70, and 19 (29.6%) were positive (Table II). In summary, of 18 sera showing a putative DFS staining pattern by immunohistochemistry, 14 were positive in Western blotting against recombinant DFS70, and of the 46 AD sera without this pattern in immunohistochemistry, 5 were positive in Western blotting.

The IgG subclass was determined for 16 AD sera that were reactive for DFS70. All antibodies were of the IgG1 subclass, and none were IgG4 (data not shown). Two sera also contained antibodies of the IgG2 subclass, and 3 others contained antibodies of the IgG3 subclass.

Detection of IgE autoantibodies to DFS70

We were interested in whether we could detect specific IgE autoantibodies to recombinant DFS70 by means of Western blotting. Because blood levels of IgE are approximately 100,000 times less than those for IgG, we performed IgE Western blotting at serum dilutions of 1:10 compared with normal dilutions of 1:100 for IgG, and the signals obtained also necessitated much longer autoradiographic exposure times (20 minutes for IgE antibody versus 5 seconds for IgG antibody). Fig 3, *B*, illustrates our results. In both patients with IC and patients with AD, we could detect specific IgE autoantibodies to DFS70 by means of Western blotting. It was noted that in general there was a positive relationship between signals for IgG and for IgE antibodies in that a strong signal for IgG antibody correlated with a strong signal for IgE antibody. (Fig 3, *A*, lane 7, and Fig 3, *B*, lane 9, are from one serum, and Fig 3, *A*, lane 16, and Fig 3, *B*, lane 18, were from another serum.) Because of the limitation in sera available, not all AD sera could be analyzed in this pairwise fashion, but the 16 AD sera examined in Fig 3, *B*, came from the larger group of 18 sera studied in Figs 1, *C*, and 3, *A*. In addition, normal human serum and sera from patients with lupus and scleroderma were included in this study.

Other patient groups reactive with recombinant DFS70

Table II summarizes the results of Western blotting against recombinant DFS70 protein in various groups of patients and normal control subjects. None of the normal healthy control subjects had positive results for IgG autoantibodies reactive with recombinant DFS70 compared with 30% of patients with AD. This result compares with a positivity of 16% for patients with asthma, 8.7% for patients with IC, 4.5% for patients with psoriasis, 3.3% for patients with chronic fatigue syndrome, 0% for patients with lupus, 0% for patients with rheumatoid arthritis, 6.9% for patients with Sjögren's syndrome, and 2.5% for patients with scleroderma.

Human autoantibodies to DFS70 bind transcription coactivator p75 but not p52

During the course of the studies described above, a report by Ge et al²² described the isolation of cDNAs encoding transcription coactivators p52 and p75, which these authors showed were required for transcriptional activation in human cell-free systems containing RNA polymerase II and general initiation factors. p52 is a protein of 333 aa and is a splice variant of a gene that also encodes protein p75 of 530 aa with the additional amino acids of p75 located in the C-terminal region. Our initial DFS6.1 partial cDNA clone encoded for the C-terminal

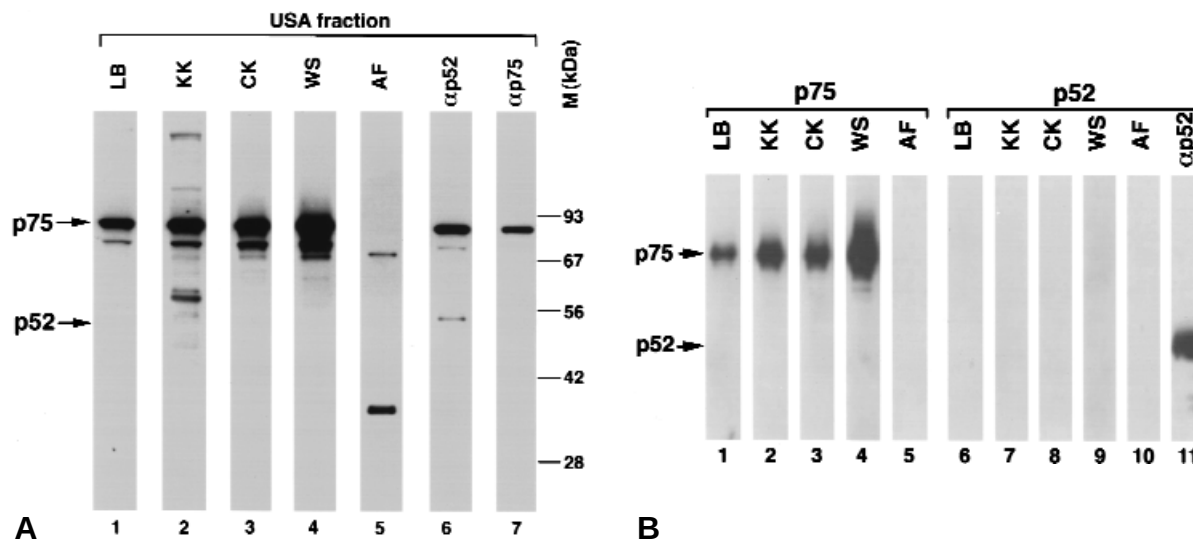


FIG 4. Autoantibodies from patients with AD specifically recognized p75 transcription coactivator but not its isoform p52. **A**, Western blot analysis of a partially purified p75- and p52-containing fraction (USA) from a HeLa cell nuclear extract (Ge and Roeder, 1994) with sera from patients with AD (lanes 1-4), sera from an anti-DFS70 negative control patient (lane 5), and polyclonal antibodies generated by immunization with recombinant p52 (lane 6) and the C-terminal region of p75 protein (lane 7). Signals corresponding to the human p75 and p52 are indicated by arrows. **B**, Western blot analysis of recombinant p75 (lanes 1-5) and p52 (lanes 6-11) with antiserum from the identical patients described in **A**. Lane 11 is a positive control representing the reactivity of polyclonal anti-p52 to recombinant p52. Signals corresponding to the positions of p75 and p52 are indicated by arrows.

351 aa of p75, and it was of interest to determine how AD sera reacted with the splice variants p75 and p52. Five human sera, 4 from patients with AD, and an autoimmune serum containing autoantibodies to other cellular antigens were coded and analyzed for reactivity in the Ge laboratory with the USA fraction of HeLa cells that contained both p52 and p75. The Western blotting data are shown in Fig 4, A. All the AD sera reacted with p75 but not with p52, whereas the irrelevant autoimmune serum AF reacted with the USA fraction to give two unrelated bands of unknown identity. Rabbit antibodies raised against p52 and p75 fractions were used as positive controls, and it can be seen that anti-p52 reacted with both p52 and p75, whereas anti-p75 reacted with p75 alone. This could be expected because anti-p52 was raised with full-length p52 as immunogen, whereas anti-p75 was raised with the unique C-terminal region of p75. The human sera were further analyzed in Western blotting with recombinant p75 and p52 (Fig 4, B), with the results confirming the observations made with the USA fraction.

Rabbit antibodies to recombinant p75 and p52 bind to recombinant DFS70 and display DFS localization

It was demonstrated that rabbit antibodies raised against recombinant transcription factors p75 and p52 were both able to bind to recombinant full-length DFS70, as shown in Fig 5, A. When the rabbit antibodies were used in immunohistochemistry, they displayed identical patterns of immunolocalization compared with human

anti-DFS70. A pattern of DFS in interphase nuclei and generalized staining of condensed chromosomes in mitotic nuclei was observed. (Compare Fig 5, B, for human autoantibodies versus Fig 5, C, and 5, D, for rabbit antibodies to p75 and p52, respectively.)

Localization of DFS70 at the ultrastructural level

A high titered human anti-DFS70 was selected for immunoelectron microscopy, and the targets were small pieces of mouse intestine fixed at 4°C with 1% glutaraldehyde, as described in the "Methods" section. Mouse intestine was used as the substrate because p75 has been shown to be ubiquitously expressed at the mRNA level in many different tissues, including small intestine,²² and numerous studies have shown that human autoantibodies react with cellular antigens, which are highly conserved between mouse and man.²⁴ Immunoelectron microscopy demonstrated that DFS70/p75 was localized in interphase chromatin and appeared to be concentrated over areas of condensed chromatin in the periphery of the nucleus (Fig 6) and also in perinucleolar chromatin. The nucleolus and the interchromatin regions contained relatively few colloidal gold particles.

DISCUSSION

As shown in Table I, 18 patients with AD had what appeared to be a characteristic DFS staining pattern, as determined by means of immunohistochemistry, but 4 of these sera were negative in Western blotting against

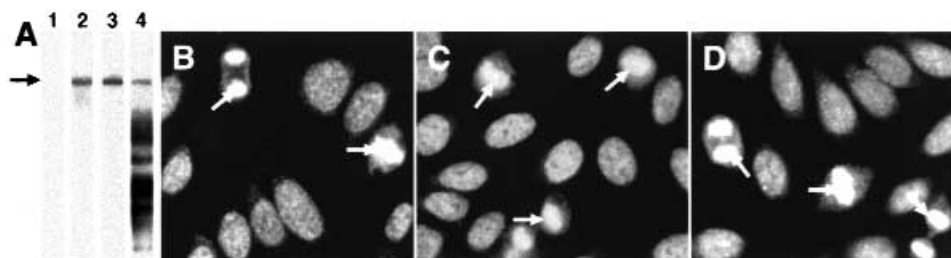


FIG 5. Rabbit antibodies to recombinant p75 and p52 bind to recombinant DFS70 and display a DFS localization pattern. **A**, Western blotting against recombinant DFS70. *Lane 1*, Normal human serum; *lane 2*, human autoimmune serum against DFS70; *lane 3*, rabbit antibody made against recombinant p75; *lane 4*, rabbit antibody made against recombinant p52. The arrow indicates the position of recombinant DFS70 reactivity. **B**, Immunohistochemical staining on human HEp-2 cells with the human autoimmune serum against DFS70 shown in *lane 2* of **A** compared with rabbit anti-p75 (**C**) and anti-p52 (**D**). All 3 antibodies gave rise to DFS nuclear staining and generalized staining of chromosomes in mitotic cells (arrows).

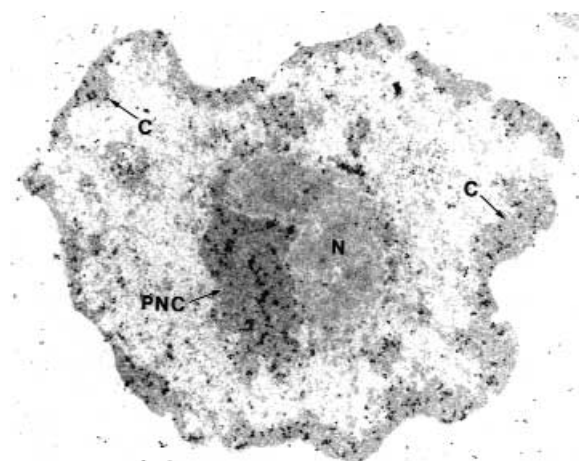


FIG 6. Immunoelectron microscopy revealed that DFS70 was concentrated in condensed chromatin of interphase cells. Colloidal gold particles are concentrated over areas of condensed chromatin (C) and over perinucleolar chromatin (PNC). The nucleolus (N) and interchromatin regions contained fewer colloidal gold particles.

recombinant DFS70. It is possible that if these 4 sera did have antibodies to DFS70/p75 transcription coactivator, the epitope or epitopes recognized by the 4 sera might not be primary sequence regions of the protein but are conformation-dependent epitopes that are lost during denaturation in the polyacrylamide gel electrophoresis-Western blotting procedure. Alternatively, immunohistochemistry might be more sensitive than immunoblotting for this antigen-antibody system. It is also of interest that 5 patients with AD who did not display the prototypic DFS70 staining pattern were found to have antibody to recombinant DFS70 in Western blotting. We reason that this could be related to other autoantibodies in these sera, which might modify the DFS staining pattern. In the future, development of an enzyme-immunoassay method with recombinant protein might be able to address some of these questions.

The DFS70/p75 autoantibody/autoantigen system showed the highest prevalence in 3 disease conditions (ie, AD, 30%; asthma, 16%; and IC, 9%). It has been well documented that AD is frequently associated with bronchial asthma and that children with AD are more likely to have asthma in later life than children without AD.^{3,6} In addition to the fact that ANAs are present in higher than normal frequency in patients with IC, there are several reports that allergic disorders are a major symptom complaint in IC. In a recent study on 374 patients, food allergies were reported in 25.1%, and hay fever was reported in 24.9%.¹⁰ Earlier studies have also pointed out the common occurrence of allergic disorders in IC and the role played by mast cells.²⁵⁻²⁷ None of these studies reported on the frequency of AD, and it might be possible that in IC, some of the patients with allergic diathesis might also have AD, which could be a common thread running through the 3 different disease conditions.

It is of interest that the putative function of the DFS70/p75 antigen has been reported to be a transcription coactivator.²² There are two isoforms that are spliced products of the same gene and encode for p75, as well as a truncated p52 kilodalton protein that lacks the C-terminal region of p75. In studies showing that both these proteins functioned as transcription coactivators in an in vitro transcription system dependent on RNA polymerase II and general transcription factors, the shorter isoform p52 was more active for most activation domains compared with p75. Human autoantibodies recognized exclusively the p75 isoform, indicating that the epitope was located in the C-terminal region of p75, which is absent in p52. We and others^{28,29} have advanced the concept that spontaneously occurring autoantibodies are antigen-driven, and if this is the case for the autoimmune response to DFS70, the immunogen that is driving the immune reaction is the larger isoform p75. At the present time, the significance of this finding is unknown, but although p75 is a less active transcription coactivator, it is possible that its involvement in transcription might be related to certain specific activation domains that were

not used in the reported studies.²² In this respect future studies could address the question of whether p75 might enhance transcription activity for certain genes predisposing to the development of atopy.³⁰

Our studies using autoantibodies from patients with AD and IC and antibodies from rabbits immunized with p75 show that DFS70/p75 is localized in the nucleoplasm by means of immunohistochemistry. This is consistent with the function of p75 as a transcription coactivator associated with RNA pol II. Immunoelectron microscopy studies also show that DFS70 is localized in the nucleus and preferentially in heterochromatin areas. This is in contrast to the reports by Singh et al³¹ on the same protein they call LEDGF, which was initially reported to be a cytoplasmic protein²³ and subsequently reported to be present in the cytoplasm of lens epithelial cells at 4°C, 15°C, and 28°C and in the nucleus and nucleolus at 37°C.³¹ Its localization in the cytoplasm at temperatures lower than 37°C is nonphysiologic, and the significance of this is unknown. These authors identified LEDGF and isolated a cDNA encoding this protein with the serum of a patient with age-related cataracts. Some of the sera from patients with age-related cataracts appeared to contain antibodies to LEDGF. The interesting feature is that cataracts and other ocular complications have been reported in certain patients with AD,^{32,33} and in one report lens opacities were noted in 23.8% of 133 eyes examined.³²

We have proposed the notion that spontaneously occurring autoantibodies might be regarded as messengers from the immune system that report abnormal events involving cellular components participating in disease-related mechanisms and that these cellular components might be the antigens driving the immune response.²⁸ The autoantibodies themselves might not be playing a pathogenic role but can be used to identify the cellular components participating in such disease processes. However, the question that needs to be addressed is whether the autoimmune process in AD involving DFS70/p75 is playing any role in pathogenesis. The current study does not address the question of the cell-mediated immune system because cellular infiltrates consisting of increased numbers of lymphocytes, monocytes, macrophages, and mast cells are present in atopic lesions.^{3,7} An important study would be examination of skin biopsy specimens from AD lesions to determine whether there might be abnormal expression of DFS70/p75 mRNA and protein. Such studies could not be done because the present work was carried out only with serum specimens, and further studies along these lines are needed. We did find IgE autoantibodies to DFS70/p75 in both patients with AD and patients with IC, and this finding lends some support to the possible pathogenic significance of the present findings in view of several studies showing prominence of IgE-positive Langerhans cells in AD lesions.⁷

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