The Fragile-X-related Gene FXR1 Is a Human Autoantigen Processed during Apoptosis*

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We describe a new human autoimmune antigen in a patient suffering from scleroderma with high levels of antibodies to nucleolus and cytoplasmic antigens. Using a Chinese hamster ovary cell expression library, we have shown that this antigen corresponds to the autosomal Fragile-X-related gene FXR1. The deduced amino acid sequence from the hamster cDNA is 97, 98, and 58% homologous to the human, mouse, and Xenopus laevis FXR1 genes, respectively. Expression of the hamster cDNA clone in Escherichia coli and antibody production indicates unequivocally the location of the FXR1 protein in the cytoplasm of hamster cells. Affinity chromatography followed by immunofluorescence microscopy analysis and immunoblots demonstrated the presence of autoimmune IgGs to FXR1 in the scleroderma patient. Immunolabeling studies in Jurkat cells, induced to apoptosis by anti-Fas/APO1 serum, indicated that the FXR1 antigens were clearly displaced from their original cytoplasmic location to several punctuated foci, resembling the bleb-like membranous structures characteristic of cells at certain stages of apoptosis. This phenomenon could be part of a putative mechanism in which the FXR1 protein is presented as a target for the autoimmune response in humans.

Human autoimmune sera have proven to be remarkably powerful investigative tools for cell and molecular biologists. In addition to allowing the identification of many cellular proteins, human autoantibodies have also been used to study the details of several intracellular events. One example has been the elucidation of the mechanisms involved in the processing of heterogeneous nuclear RNA to mRNA (1). Thus, autoantibodies to ribonucleoprotein particles have been observed in many patients suffering rheumatoid diseases (2–4). Among others, these include anti-RNP directed against U1RNP (5), anti-Sm directed against U1, U2, U4, U5, and U6 RNPs (6), anti-Ro/SS-A directed against Y-RNPs (7), and anti/Lo/SS-B directed against several cellular RNPs (8). These autoantigens are particularly prominent in disorders such as systemic lupus erythematosus (SLE), scleroderma or systemic sclerosis (SSc), and mixed connective tissue disease (MCTD) (9).

Expression of a variety of adhesion molecules is increased on fibroblasts and endothelial cells in SSc patients, as are circulating levels of the same molecules. In some cases, genetic factors influence the repertory of autoantigens to which the host loses tolerance (10). When Hep-2 cells are used as substrate, virtually every patient with scleroderma (95–97%) will have a positive test result for antinuclear and/or antinucleolar antibody (ANAs) in indirect immunofluorescence (IF) (11). In particular, nucleolar IF has been suggested as a diagnostic possibility for SSc since antibodies that target this region of the cell are unusual in the other connective tissue diseases (3).

In an attempt to characterize scleroderma autoantigens, we have screened sera that produced nucleolar staining by immunofluorescence from a large number of patients suffering autoimmune diseases. Here we describe the identification and molecular characterization of a hamster cDNA for a novel autoantigen that turned out to be FXR1, an autosomal homolog of FMR1. Mutations and CGG expansions in the FMR1 gene are associated with Fragile-X syndrome. Immunological studies presented reveal that the behavior of the FXR1 protein in cells undergoing apoptosis suggests a putative way for this protein to be implicated in the human autoimmune response (12).

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Promega and Amersham Pharmacia Biotech. All radionucleotides were purchased from Amersham Pharmacia Biotech. All tissue culture reagents were from Life Technologies, Inc. Oligonucleotides were obtained from Eurogentec, Belgium. Antibodies were purchased from Boehringer Mannheim. λ ZAP II hamster expression library was obtained from Stratagene (La Jolla, CA). CNBr-activated Sepharose was obtained from Amersham Pharmacia Biotech. All other chemicals were of the highest grade available and obtained from Sigma.

Immunofluorescence Microscopy—Immunofluorescence was carried out on CHO, HeLa, and Jurkat cells using formaldehyde fixation and a brief Triton X-100 lysis as described (13). Rabbit anti-FXR1 sera were generated as indicated below and used at a dilution of 1:500 in PBS. In some cases, affinity purified human autoimmune IgGs was used as a primary serum. Detection of the primary antibodies was with fluorescent isothiocyanate-conjugated secondary antibodies made in goat and used at 1:100 dilution in PBS. Coverslips were counterstained with Hoechst 33528 for DNA, mounted in PBS/glycerol 1:9 (v/v), and observed in a Zeiss Axioskop fluorescence microscope equipped with a 1:63 immersion oil objective.

Isolation of cDNA and Sequencing—3 × 10⁵ plaques of λ ZAP hamster cDNA library were screened to obtain FXR1 cDNAs using a human autoimmune serum named JC. Three positives clones sequenced were shown to be highly homologous to the human FXR1 gene (14). Since no initial clones contained the entire open reading frame encoding FXR1, a 5′-RACE approach was used as described. All plasmids and cDNA fragments were manipulated using standard techniques (15). The nucleotide sequence has been deposited in the GenBank database (GenBank accession number U84941).

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† The abbreviations used are: SSc, systemic sclerosis; IF, immunofluorescence; PBS, phosphate-buffered saline; CHO, Chinese hamster ova.
cleotide sequence of overlapping clones was verified by DNA sequencing
(16).

Expression of Hamster FXR1 cDNA and Antisera Production—An
N-terminal region (residues 1–222) of the hamster FXR1 cDNA was
expressed by the pET-3a system in Escherichia coli using an NdeI-site
at the 5' end and a BamHI-site at the 3' end included in the polymerase
chain reaction primers used for cloning. The expressed protein (34 kDa)
was analyzed by SDS gel electrophoresis, isolated from inclusion bodies
after cell sonication, and used as immunogen for polyclonal antibodies
production in rabbits. Western blots were performed with recombinant
truncated FXR1 and CHO whole cell extracts with both human auto-
imune serum JC (diluted 1:200) and rabbit anti-FXR1 sera (diluted
1:500). Detection of the primary antibodies was with peroxidase-conju-
gated secondary antibodies used at 1:2000 dilution in PBS. Develop-
ment of the blot membranes was with 4-chloro-1-naphthol.

Affinity Chromatography of Human Autoimmune Anti-FXR Immu-
noglobulins—The FXR1 expressed polypeptide was bound to CNBr-
activated Sepharose according to the instructions of the manufacturer.
The resin was washed with PBS and equilibrated with PBS containing
0.5 mM NaCl (final concentration). 3 ml of human autoimmune serum
containing anti FXR1 IgGs were loaded into the affinity column
(resin volume was 1.5 ml), washed extensively with PBS-NaCl buffer,
and eluted with 0.2M glycine, pH 2.5. Aliquots of 250 ml were collected
and equilibrated immediately with 1 M Tris, pH 8.0. IgGs were con-
firmed in the eluate by SDS-gel electrophoresis and used for immuno-
fluorescence microscopy and Western blot analysis without further
treatment.

RESULTS

Cloning and Expression of Hamster FXR1—We have used a human autoantibody from a scleroderma patient to screen a
CHO cDNA expression library and have isolated clones corre-
FIG. 1. Sequence of the hamster
cDNA for FXR1. The nucleotide se-
quence of the FXR1 with deduced amino
acid sequence underneath is shown. A pu-
tative stop codon at position 1880–1882 is
indicated by asterisk. DNA and deduced
protein sequence are in the GenBankTM/
EBI Data Bank (access number
Y12387).

Northern Blot Analysis—Total RNA from CHO culture cells was
extracted using the acid guanidinium thiocyanate/phenol-chloroform
extraction method (17). RNA samples were separated by electrophore-
sis in formaldehyde, 1% agarose in MOPS buffer. Northern blotting (15)
was carried out using a polymerase chain reaction fragment obtained
from the largest clone of the hamster FXR1 cDNA, representing nucle-
etides 1–666.

RESULTS

Cloning and Expression of Hamster FXR1—We have used a human autoantibody from a scleroderma patient to screen a
CHO cDNA expression library and have isolated clones corre-
sponding to the hamster FXR1 gene. The largest CHO FXR1 cDNA characterized is 2141 nucleotides long, encoding a 621 amino acid chain (Fig. 1). A 5'-untranslated sequence region CGG repeat was not found in the sequenced clone. This characteristic CGG repeat was described before as part of the human FMR1 cDNA (18), but it is not known if it is present in the human FXR1 cDNA (19). Overall, we found a 97, 98, and 58% homology of amino acid sequence between hamster FXR1 and human, mouse, and frog FXR1 genes, respectively (Fig. 2).

The highly conserved N-terminal region contains two KH domains that represent RNA binding elements (18). The hamster FXR1 gene also contains an arginine-rich sequence similar to arginine-rich motifs found in several proteins including HIV Rev (20) as described previously for other FXR1 genes. Major differences in amino acid sequences between the hamster and FXR1 genes from other species are found at the very end of the C-terminal region (residues 590–618). It has to be mentioned that the sequence of the mouse FXR1 gene available represents a shorter form of this gene (21).

The hamster cDNA was used in a Northern blot analysis with total RNA from CHO cells (Fig. 3). A prominent transcript of about 2.3 kilobases was detected, which correlates well with that described in HeLa cells (14). However, our blot result does not rule out the possibility of additional FXR1 transcripts with close sizes in CHO cells, as was shown previously for human and mouse tissues, where two FXR1 messengers of 2.4 and 2.2 kilobases were identified as a result of an alternative splicing (14, 21).
cDNA was further used to express the N terminus of the molecule in *E. coli*, and the expressed polypeptide was purified to a high concentration from inclusion bodies. Two polyclonal sera were generated against recombinant FXR1 protein and used in apoptosis studies (see below).

**FXR1 Is a Human Autoantigen**—We present immunological evidence that the human autoimmune serum JC used in the screening of the λ ZAP library reacts with the expressed hamster FXR1 antigen (Fig. 4A, center panel). Data included in the same figure also show the blotting with a polyclonal serum generated against the recombinant N-terminal region of expressed hamster FXR1 (Fig. 4B, right panel). Furthermore, this recombinant truncated FXR1 protein was used to make an affinity column to isolate human specific anti-FXR1 immunoglobulins. It is important to state that FXR1 does not appear to be the major autoantigen found in the serum JC. This was demonstrated by immunoblots in CHO whole cell extracts using human serum JC (Fig. 4B, center panel, lane 1), anti-FXR1 affinity purified human immunoglobulins (Fig. 4B, center panel, lane 2), and polyclonal serum to hamster FXR1 (Fig. 4B, right panel, lane 1). These results clearly indicate that: (i) a 70-kDa protein corresponding to the hamster FXR1 antigen is recognized by the human autoantibody, and ii) it is not the major autoantigen of the human serum JC. Another protein of 55 kDa is the autoantigen most probably responsible for the predominant nuclear staining observed by IF with the whole human autoantibody JC (data not shown).

Furthermore, immunofluorescence microscopy of culture cells with affinity-purified human anti-FXR1 IgGs revealed a punctate cytoplasm staining in CHO (Fig. 5b) and HeLa cells (Fig. 5f). This IF staining was identical to that observed using rabbit antibodies to the recombinant FXR1 hamster protein generated in vitro (Fig. 5d and h). Eventually, some weak nuclear staining was observed with the rabbit anti-FXR1 sera. Double IF staining demonstrated unambiguously that the serum from patient JC contains antibodies that co-localized with the serum anti-FXR1 generated in rabbits (data not shown).

**FXR1 Is a Autoantigen Redistributed in Cells Undergoing Apoptosis**—It has been suggested that some mechanistic correlation may exist between the autoimmune phenomenon and apoptosis (12). It was proposed that the selective protein cleavage associated with apoptosis may enhance the immunogenicity of autoantigens by revealing immunocryptic epitopes that are not efficiently generated during antigen processing (22). In order to determine the behavior of the FXR1 protein in cells undergoing apoptosis in vitro, we did immunofluorescence studies of FXR1 localization in Jurkat cells treated with anti-Fas/APO-1 serum (23). The IF results in Fig. 6 with anti-FXR1 serum showed the re-distribution of the FXR1 cytoplasmic antigen to certain specific foci on apoptotic cells. These foci resembled those bleb-like structures observed during the apoptosis phenomenon (12, 22).

**DISCUSSION**

In this report, we have identified and described a new human autoantigen in a patient with scleroderma, by cloning the gene from a CHO cDNA expression library. The cDNA revealed an amino acid sequence homologous to the human autosomal gene FXR1, a gene related to the FMR1 protein responsible for the Fragile-X syndrome in humans (24–26). This syndrome is the most common inherited form of mental retardation. The human, mouse, and frog cDNAs for FXR1 have been previously sequenced (14, 21, 27). The major differences in amino acid sequences we have found between hamster and the other FXR1 man, mouse, and frog cDNAs isolated are in the final 30 amino acid residues of the C-terminal region. This region as described for the FMR and FXR protein family is produced by alternative splicing and corresponds to a nuclear localization signal (28). However, some previous evidence from other groups, and our own IF analysis, do not rule out an additional nuclear binding site for the FXR1 antigen during the cell cycle.

By expressing the hamster FXR1 cDNA and the production
of specific anti-FXR1 antibodies, we demonstrated the autoimmune nature of the FXR1 gene product in a patient suffering from scleroderma. As one of the group of related systemic rheumatic diseases, scleroderma is characterized by a high level of circulating autoantibodies, especially to nuclear antigens (4, 29). The observation that FXR1 protein is a human autoantigen raises questions of possible association between FXR1 (and possibly FMR1) and autoimmune responses in humans. Activation of the immune system is a consistent early event in SSc, and some autoantibodies may be generated in SSc patients through the antigen processing of complexes involved in gene transcription. The generation of autoantibodies and subsequent tissue deposition of immune complexes is thought to trigger the pathogenic consequences of systemic autoimmune disease.

The origin of autoimmune diseases is not yet known but several hypotheses have been put forward and clues have been found (30). Our IF staining results indicated that the FXR1 antigen behaves like other human nuclear autoantigens analyzed (12). It moves from its original cytoplasmic binding site associated with ribosomes to more punctuated foci. These new locations resemble the typical bleb-like membrane organelles found in apoptotic cells. Although the significance of this change of location and the putative cleavage of FXR1 during apoptosis requires more specific studies, the re-distribution of the FXR1 antigen to discrete foci in treated cells (d, f, h), as shown by IF with human autoimmune IgGs to FXR1 (d) and two rabbit polyclonal sera to recombinant FXR1 (f, h), In some apoptotic cells (detail in h), the FXR1 was distributed to the periphery in bleb-like structures, characteristic of membrane changes observed during the process of apoptosis (arrows). Magnification, × 63.

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