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Immunoscreening of phage-displayed cDNA-encoded polypeptides identifies B cell targets in autoimmune disease☆

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Abstract

Characterisation of self-antigens can contribute to an understanding of the aetiology of autoimmune disorders as well as to the development of new therapies and diagnostic methods. The present study was undertaken to investigate the applicability of complementary DNA (cDNA) phage-display technology to the identification of autoantigens recognised by the humoral response in autoimmune disease. Using systemic lupus erythematosus (SLE) as a model system, a pool of patient immunoglobulin G (IgG) was biopanned on a fibroblast cDNA phage-display library constructed in the vector pJuFo. Following three rounds of biopanning, recovered cDNAs were sequenced and then identified using BLAST comparisons with international databases. Both previously reported SLE autoantigens, for example, α-enolase and U1 small nuclear ribonucleoprotein-C (U1snRNP-C), and novel autoantibody targets, including ribosomal protein S20 (RPS20), ribosomal protein S13 (RPS13), ubiquitin-like protein PIC1 (PIC1), and transcription factor-like protein MRG15 (MRG15), were recovered from the biopanning procedure. Radiobinding assays were used subsequently to confirm the reactivity of some putative autoantigens to panels of sera from SLE patients, control patient groups, and healthy individuals. SLE patient sera were positive for reactivity to: U1snRNP-C, 4/15 (27%); α-enolase, 1/15 (7%); RPS20, 3/15 (20%); RPS13, 1/15 (7%); PIC1, 1/15 (7%); and MRG15, 2/15 (13%). Overall, cDNA phage-display technology appears to be applicable to the identification of autoantigens in autoimmune disease.

Keywords: Autoantigen; Autoantibody; Autoimmunity; Phage-display; Systemic lupus erythematosus

Phage-display, based on the pJuFo cDNA cloning system [1–5], permits both the expression of complementary DNA (cDNA) libraries and the covalent attachment of the expressed products as Fos-fusion proteins on the surface of filamentous phage particles, thus allowing the selective enrichment of phage that display immunoglobulin G (IgG)-binding peptides in rounds of biopanning. The cDNAs encoding immunoreactive proteins can be recovered from phage particles by infection of bacterial cultures and then identified by DNA sequencing and database searches. Phage-display technology provides several advantages over conventional immunoscreening of plasmid or λ-phage cDNA libraries performed after transfer of phage plaques or bacterial colonies to nitrocellulose membrane. First, by screening the cDNA library in a fluid-phase, denaturation of the proteins displayed on the surface of the phage is avoided. This might allow the interaction of antibody with conformational epitopes that are recognised by humoral responses in autoimmune disease [6]. In addition, immobilisation of the IgG instead of the cDNA library allows the specific enrichment of phage particles that interact with the antibody immobilised

☆ *Abbreviations:* cDNA, complementary DNA; cpm, counts per minute; IgG, immunoglobulin G; LB, Luria–Bertani; MRG15, transcription factor-like protein MRG15; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PIC1, ubiquitin-like protein PIC1; PM, polymyositis; RA, rheumatoid arthritis; RPS13, ribosomal protein S13; RPS20, ribosomal protein S20; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus; U1snRNP-C, U1 small nuclear ribonucleoprotein-C; SC, scleroderma.

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onto a solid-phase by repeated rounds of selection. Antigens that are present at low abundance can therefore be enriched increasing the possibility of detection and identification. Moreover, as many as 10^{10}–10^{11} individual clones can be screened in a single experiment thereby increasing the chance of isolating autoantigens.

The aim of the present study was to assess the applicability of cDNA phage-display to the identification of autoantigens in autoimmune disease. Characterisation of self-antigens is essential for understanding the immunopathological mechanisms in autoimmunity, which may in turn lead to the development of effective therapies and novel diagnostic assays for autoimmune disease. In this study, we chose to investigate systemic lupus erythematosus (SLE) as the disorder is associated with the presence of autoantibodies targeted against an array of cellular proteins and nucleic acids. These include DNA [7], ribosomal RNA and proteins [8–10], transcription factors [11], heat shock proteins [12], histones [13], and ribonucleoprotein complexes such as Sm-antigen [14] and SSA/Ro [15].

Materials and methods

Patient and control sera. Sera from 15 patients (1 male, 14 female; mean age: 43 years) who fulfilled the revised American Rheumatism Association criteria for SLE [16] were used in this study. Control patient groups were: 20 rheumatoid arthritis (RA) patients (8 male, 12 female; mean age: 58 years), 20 scleroderma (SC) patients (4 male, 16 female; mean age: 60 years), and 20 polymyositis (PM) patients provided by Dr. Paul Plotz (National Institutes of Health, Bethesda, MD). Sera from 20 healthy individuals (9 male, 11 female; mean age: 31 years) with no personal or family history of any other autoimmune disorders were used as controls. All sera were kept frozen at −20 °C. Local Ethics Committee approval was obtained and all subjects gave informed consent.

Library construction. An oligo(dT)-primed uni-directional human fibroblast cDNA library (Stratagene, La Jolla, CA) was excised in vivo according to manufacturer's protocol and plasmid DNA containing cDNA inserts was prepared using a Wizard Midipreps DNA Purification System (Promega, Southampton, UK). Plasmid DNA was restricted with enzymes *Xba*I (Promega) and *Kpn*I (Promega) and the cDNA inserts were resolved by electrophoresis in a 0.8% agarose gel [17]. Following purification using a Wizard PCR Preps DNA Purification System (Promega), the cDNA inserts were ligated into *Xba*I- and *Kpn*I-digested pJuFo phagemid [2]. The ligation reaction was electroporated into *Escherichia coli* XL1-Blue cells (Stratagene) as described by the manufacturer and the ligation efficiency was determined by plating aliquots of the electroporated cells onto selective Luria–Bertani (LB) agar [17] containing 50 μg/ml ampicillin and 10 μg/ml tetracycline. To prepare the phage-display library, the electroporated cells were superinfected with 1×10^{12} plaque-forming units of VCMS13 helper phage (Stratagene) at room temperature for 30 min. The culture was subsequently added to 100 ml LB medium [17] supplemented with 50 μg/ml ampicillin, 10 μg/ml tetracycline, and 10 μg/ml kanamycin. After overnight incubation at 200 rpm and 37 °C, the culture was centrifuged at 10,000*g* for 20 min at 4 °C and phage particles precipitated from the supernatant by the addition of 0.2 volumes of 2.5 M sodium chloride/20% polyethylene glycol 4000 at 4 °C for 1 h. Phage were harvested by centrifugation at 10,000*g* for 20 min at 4 °C and resuspended in 2–3 ml phosphate-buffered saline (PBS) (Sigma, Poole, UK). The phage titre was determined by infecting 2 ml log-phase *E. coli* XL1-Blue with an aliquot of the phage-display library for 30 min at room temperature and then plating out samples of the culture onto selective LB agar.

Isolation and biotinylation of IgG. IgG was isolated from the sera of patients by protein G–Sepharose 4 Fast Flow affinity column chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Eluted IgG fractions were concentrated using an Amicon Concentrator (Amicon, Beverley, MA). The concentrated IgG was filter-sterilised with a Millex Filter Unit (Millipore, Bedford, MA) and the final concentration measured by photometry at 280 nm. Biotinylation of the IgG was performed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL), according to manufacturer's protocol. All IgG samples were stored at 4 °C until required.

Isolation of phage displaying IgG-binding peptides (biopanning). A 15-μl aliquot of biotinylated IgG was incubated with 200 μg Dynabeads M-280 Streptavidin (Dynal Biotech, Oslo, Norway), prepared according to the manufacturer in 235 μl sterile water, and incubated at 4 °C for 30 min on a rotating platform to permit antibody–bead binding. The antibody sample used was a pool of 15 SLE patients with each biotinylated IgG at a concentration of 2 mg/ml. To block any non-specific phage binding to the beads later in the procedure, 300 μl blocking buffer, containing 2% dried milk powder and 10% glycerol in PBS, was added to the bead–IgG suspension and incubation at 4 °C continued for 1 h. The bead–IgG complexes were separated from the blocking buffer using a Dynal Magnetic Particle Concentrator (Dynal Biotech), washed twice, and finally resuspended in 150 μl PBS/0.05% Tween 20 before the addition of a 100-μl sample of phage-display library containing 1×10^{10} CFU. The suspension was then incubated overnight at 4 °C to allow interaction of the antibody–bead complexes with peptides displayed on the surface of the phage particles.

The bead–IgG complexes were washed extensively with PBS/0.05% Tween 20 to remove any unbound phage. Bound phage were eluted from the bead–IgG complexes with 150 μl of 100 mM hydrochloric acid (adjusted to pH 2.2 with solid glycine), and the beads then magnetically separated from the supernatant that contained the phage particles. Neutralisation of the phage suspension was accomplished by the addition of 9 μl of 2 M Tris buffer. The phage were subsequently used to infect 2 ml exponentially growing *E. coli* XL1-Blue (Stratagene) for 30 min at room temperature. Aliquots of the infected cells were plated onto selective LB agar, in order to determine the number of phage eluted and to allow the isolation of pJuFo phagemid containing cDNA inserts.

To generate phage for a further round of selection, the infected *E. coli* XL1-Blue culture was superinfected with VCSM13 as detailed above. Phage were prepared from the infected culture, titred as described above, and stored at −20 °C. This first round library enriched in phage displaying IgG-binding peptides was used in a second round of selective enrichment as detailed above. In all, four rounds of biopanning were undertaken.

Polymerase chain reaction (PCR) amplification and sequencing. Phagemid was prepared from overnight cultures of *E. coli* XL1-Blue using a Wizard Minipreps DNA Purification System (Promega). Each phagemid was subjected to PCR amplification using the oligonucleotide primers 1192 Forward and 1500 Reverse (Life Technologies, Paisley, UK) which flank the *Xba*I and *Kpn*I cloning sites, respectively, in pJuFo and are detailed in Table 1. Briefly, 50 ng samples of phagemid DNA were subjected to 36 cycles of PCR amplification in a DNA Thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT) using previously detailed reaction conditions [18]. The PCR amplification products were analysed by electrophoresis in 0.8% agarose gels to confirm the presence of a cDNA insert and purified according to a Wizard PCR Preps DNA Purification System (Promega) prior to DNA sequencing. Sequencing of purified PCR amplification products was performed according to a Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, OH) with [γ-^{32}P]ATP (ICN Pharmaceuticals, Basingstoke, UK) and primer 1309 5′-CCGAAATCGCGAACCTGCTG-3′

Table 1
Oligonucleotide primers used in PCR amplification

Primer	Primer sequence[a]
1192 Forward	5′-CCGCTGGATTGTTATTACTCGCTG-3′
1500 Reverse	5′-TGCAAGGCGATTAAGTTGGGTAAC-3′
PIC1 Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGTCTGACCAGGAGGCA-3′
MRG15 Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGCCTAAATTCCAGGAG-3′
RPS13 Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGCTTACTCCTTCACAG-3′
RPS20 Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGGCTTTTAAGGATACC-3′
α-Enolase Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGTCTATTCTCAAGATC-3′
U1snRNP-C Forward	5′-GGTAATACGACTCACTATAGGGAGAGCCACC**ATG**ATGATGCCCAAGTTTTATTGT-3′
1474 Reverse	5′-CCTCAT**CTACTTA**TGTAAAACGACGGCCAGTGAATTGT-3′

[a] The ATG translation start codons are shown in bold type and the T7 promoter site is underlined in the specific forward primers. The translation stop codons are shown in bold type in the 1474 Reverse primer.

(Life Technologies) which lies upstream of the *Xba*I cloning site in pJuFo. Sequenced DNA was subjected to electrophoresis in 6% polyacrylamide/7 M urea gels with glycerol tolerant gel buffer (USB). The cDNA sequences were identified using BLAST compari sons with international databases at the network service of the National Centre for Biotechnology Information (National Institutes of Health, Bethesda, MD).

In vitro-coupled transcription and translation. Recombinant pJuFo phagemid were subjected to PCR amplification exactly as described elsewhere [18], but using a specific forward primer and the 1474 Reverse primer (Life Technologies) as detailed in Table 1. This allowed the incorporation of a T7 promoter at the 5′ end of the PCR amplification products, which were then purified using a Wizard PCR Preps DNA Purification System. One microgram samples of the PCR amplification products were transcribed and translated in vitro, with concomitant incorporation of [^{35}S]methionine into the protein products, in a TnT T7 Coupled Reticulocyte Lysate System (Promega) as detailed elsewhere [19]. The reactions were stored at −20 °C until needed. The percentage incorporation of [^{35}S]methionine was determined by trichloroacetic acid-precipitation according to manufacturer's protocol. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of in vitro translated products was performed in 12% polyacrylamide gels as previously detailed [19]. Gels were stained, destained, soaked in Amplify scintillant (Amersham Pharmacia Biotech, Little Chalfont, UK), and dried under vacuum as described elsewhere [19]. Autoradiography was carried out at −70 °C using Fuji RX X-ray film (Genetic Research Instrumentation, Dunmow, UK). Protein molecular weight markers were from Sigma.

Radiobinding assays. For each assay, an aliquot of the in vitro translation reaction mixture, equivalent to 100,000–200,000 counts per minute (cpm) of trichloroacetic acid-precipitable material, was suspended in 50 μl immunoprecipitation buffer containing 20 mM Tris buffer (pH 8.0), 150 mM sodium chloride, 1% Triton X-100, and 10 μg/ml aprotinin (Bayer, Newbury, UK). Serum was then added to a final dilution of 1:20. After incubation overnight with shaking at 4 °C, 50 μl protein G–Sepharose 4 Fast Flow slurry (Amersham Pharmacia Biotech), prepared according to the manufacturer, was added and incubated for 1 h at 4 °C. The protein G Sepharose–antibody complexes were then collected by centrifugation and washed six times for 15 min in immunoprecipitation buffer at 4 °C. Immunoprecipitated radioactivity was evaluated in an LKB 1217 Rackbeta liquid scintillation analyser (Wallac UK, Milton Keynes, UK). For analysis of immunoprecipitated proteins by SDS–PAGE and autoradiography, the protein G–Sepharose–antibody complexes were resuspended in 50 μl SDS-sample buffer [17] and boiled, and the supernatant was recovered for electrophoresis in a 12% polyacrylamide gel which was processed as detailed above.

Antibody levels were expressed as a relative index. An antibody index for each serum tested in the immunoprecipitation assay was calculated as: cpm immunoprecipitated by tested serum divided by the mean cpm immunoprecipitated by 20 healthy control sera. Each serum was tested in at least two experiments and the mean antibody index was calculated from these. The upper level of normal for the assay was calculated using the mean antibody index + 3 SD of the population of 20 healthy individuals. Any serum with an antibody index above the upper level of normal was designated as positive for antibody reactivity.

Statistical analyses. The frequency of antigen-specific antibodies was compared between patient groups and controls using Fisher's exact test for 2×2 contingency tables. P values < 0.05 (two-tailed) were regarded as significant. Differences between the mean antibody index of patient and control groups were assessed using Student's t tests. P values < 0.05 (two-tailed) were considered significant.

Results

Construction of a fibroblast cDNA phage-display library

The primary size of the pJuFo fibroblast cDNA library was 1.5×10^6 independent clones. Following amplification with helper phage, the titre of the stock phage-display library was assessed by infecting *E. coli* XL1-Blue and plating aliquots of the culture onto LB agar containing ampicillin and tetracycline. A titre of 1×10^{11} CFU/ml was estimated. Twenty of the bacterial colonies, recovered after plating out the infected culture, were grown overnight and the pJuFo phagemid were isolated. Analysis of the phagemid DNA by PCR amplification, using primers 1192 Forward and 1500 Reverse (Table 1), revealed that 18 carried a cDNA insert, indicating that the library contained approximately 90% recombinant pJuFo phagemid. The inserts ranged in size from 0.5 to 1.8 kbp. This library was used for the selective enrichment of IgG-binding proteins from patients with SLE.

Enrichment of phage displaying IgG-binding peptides (biopanning)

The fibroblast cDNA phage-display library was subjected to four rounds of biopanning against a pool of biotinylated IgG from 15 SLE patients. After each

Table 2
Biopanning of the cDNA phage-display library with a pool of SLE patient IgG

Starting library	Phage applied[a]	Phage eluted[b]	Fold increase in % of phage eluted[c]
Unselected library	1.2×10^{10}	1800 (1.5×10^{-5})	—
Enriched library 1	1.5×10^{10}	3200 (2.1×10^{-5})	1.4
Enriched library 2	1.0×10^{10}	80,000 (8.0×10^{-4})	53
Enriched library 3	1.0×10^{10}	82,000 (8.2×10^{-4})	55

[a] Number of phage applied in the biopanning.
[b] Number of phage eluted in the biopanning with percentage in parentheses.
[c] Compared with the percentage of phage eluted in the first round of biopanning.

round of enrichment, the number of phage eluted was estimated by infecting *E. coli* XL1-Blue and plating the cells onto selective medium (Table 2). A 53-fold increase in the percentage of phage eluted from the biotinylated IgG was observed after the third round of biopanning, indicating enrichment of the phage-display library. A fourth round of biopanning did not appear to enrich the library further with respect to the percentage of phage eluted. In contrast, increases of only 2-fold were observed after four rounds of enrichment with a pool of IgG samples obtained from healthy controls (data not shown). Following three rounds of biopanning with SLE patient IgG, 40 individual bacterial colonies that contained pJuFo phagemid were grown overnight. Phagemid DNA was recovered from each culture, subjected to PCR amplification with 1192 Forward and 1500 Reverse primers (Table 1) and the products were analysed by agarose gel electrophoresis. Of the 40 phagemids, 36 had a cDNA insert with sizes ranging from 0.5 to 1.8 kbp. The PCR amplification products were purified and sequenced to determine the identity of the cDNA. Analysis of the cDNA inserts using BLAST comparisons of international databases revealed that cDNAs encoding several different polypeptides had been isolated from the third round of biopanning. Eighteen of the cDNAs encoded identifiable proteins and these are listed in Table 3. These included proteins already described as autoantigens in SLE, e.g., α-enolase, as well as previously unreported putative autoantigens, e.g., ribosomal protein S13. Eighteen of the DNA inserts recovered remain unidentified (data not shown) and these might represent as yet unknown antigenic targets in SLE. The potential autoantigens ubiquitin-like protein PIC1 (PIC1), transcription factor-like protein MRG15 (MRG15), ribosomal protein S20 (RPS20), and ribosomal protein S13 (RPS13) were chosen for further analysis in radiobinding assays along with two previously identified SLE autoantigens α-enolase and U1 small nuclear ribonucleoprotein-C (U1snRNP-C).

Radiobinding assays with SLE patient and control sera

Phagemid DNA carrying the cDNA encoding either PIC1, MRG15, RPS20, ribosomal RPS13, α-enolase,

Table 3
Identity of cDNAs isolated after biopanning of a fibroblast cDNA phage-display library with SLE patient IgG

Identity of cDNA by BLAST (database Accession No.)	First amino acid residue of the identified cDNA which is encoded by the cDNA insert[a]	Previously reported as an antigen in SLE or other autoimmune disorders
Ribosomal protein S13 (BC000475)	45	No
Ribosomal protein S20 (BC007507)[b]	1	No
Heat shock protein 90 (D87666)[b]	1	Yes (12,25)
Transcription factor-like protein MRG15 (BC002936)	9	No
Translation elongation factor 1α (BC006102)	119	Yes (26)
α-Enolase (BC004458)	1	Yes (24)
Ubiquitin-like protein PIC1 (U61397)	1	No
Ribonuclease P/Rpp30 (NM006413)	1	Yes (27)
U1 small nuclear ribonucleoprotein-C (NM003093)[b]	1	Yes (30)
Ribosomal protein L24 (XM046136)	1	No
Ribosomal protein L9 (XM049354)	1	No
Zinc finger protein 106 (NM022473)	1	No
Sm-D antigen (J03798)	1	Yes (31)
Sm-B antigen (J04564)	1	Yes (31)
52-kDa SSA/Ro (XM052870)	1	Yes (15)

[a] The ATG start codon of the identified cDNA is designated as encoding amino acid residue number 1.
[b] Sequences represented twice in the cohort of 36 cDNA inserts sequenced.

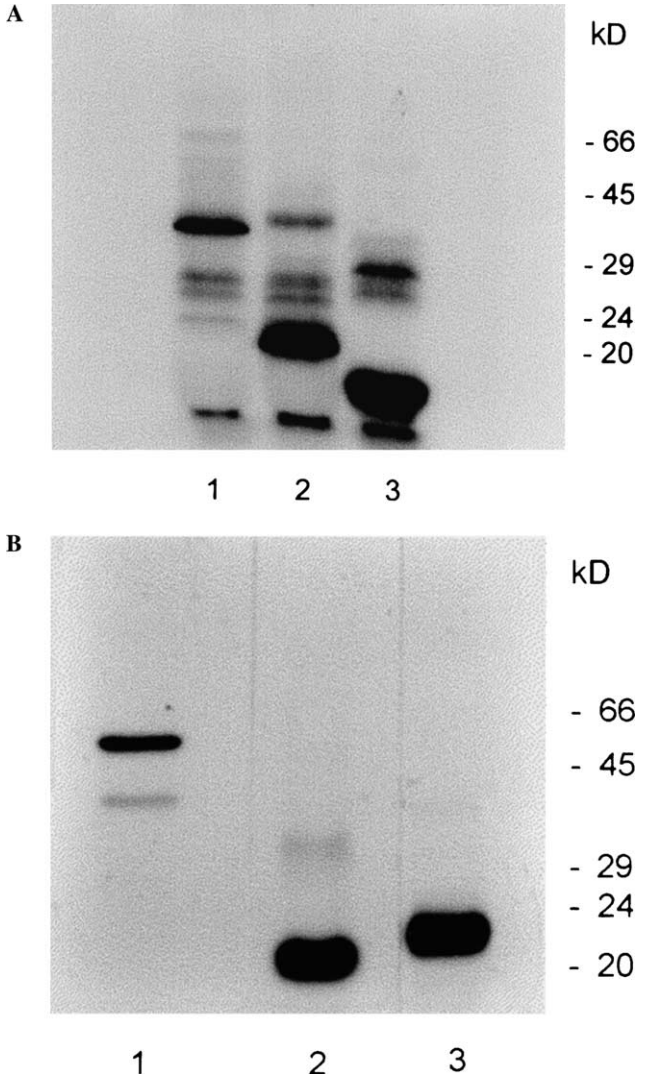

Fig. 1. SDS–PAGE and autoradiography of in vitro translated ^{35}S-labelled polypeptides. Putative autoantigens were produced in vitro in a TnT T7 Coupled Reticulocyte Lysate System and the products were analysed by SDS–PAGE and autoradiography. (A) Lane 1, MRG15; lane 2, ribosomal protein S20; and lane 3, PIC1. (B) Lane 1, α-enolase; lane 2, RPS13; and lane 3, U1snRNP-C. The figures in the right represent molecular weight markers in kDa.

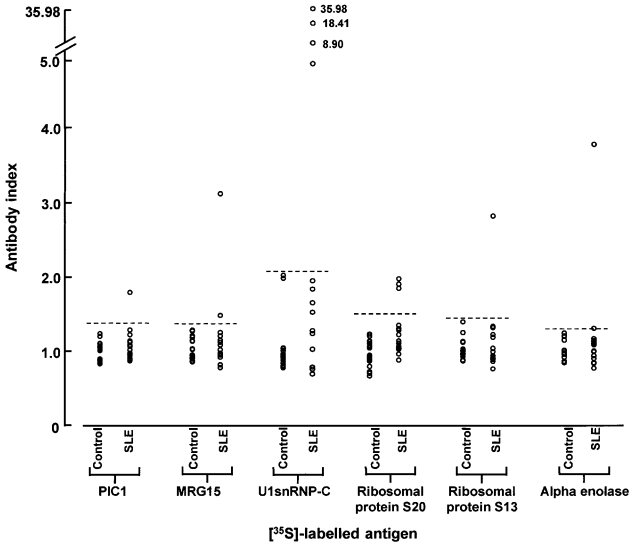

Fig. 2. Antibody indices of the SLE patient ($n = 15$) and control sera ($n = 20$). SLE patient and control sera were analysed in the radiobinding assay as detailed in Materials and methods. The antibody index shown for each serum sample is the mean of at least two experiments. The dotted lines show the upper level of normal for each radiobinding assay: PIC1, 1.39; MRG15, 1.38; U1snRNP-C, 2.11; RPS20, 1.51; RPS13, 1.44; and α-enolase, 1.33.

or U1snRNP-C was subjected to PCR amplification with a specific forward primer and 1474 Reverse primer (Table 1). The purified PCR products were then translated and radiolabelled in vitro, and the protein products were analysed by SDS–PAGE and autoradiography. Figs. 1A and B illustrate the translation products from cDNA encoding RPS13, α-enolase, U1snRNP-C, MRG15, RPS20, and PIC1. Sera from patients with SLE ($n = 15$) and healthy controls ($n = 20$) were tested for their ability to bind to each of the radiolabelled proteins in radiobinding assays, in order to verify immunoreactivity of each individual serum towards each potential autoantigen. An antibody index was assigned for each serum analysed, this being the mean antibody index of at least two experiments (Fig. 2). The upper level of normal for each radiobinding assay was calculated from the mean antibody index + 3 SD of 20 healthy controls. On this basis, none of the 20 control sera was positive for binding to any of the radiolabelled antigens. For the SLE patient sera tested, the frequency of antibody binding to each of the ^{35}S-labelled ligands is summarised in Table 4. Although one or more individual SLE serum samples reacted with each antigen, only in the case of anti-U1snRNP-C antibodies was the antibody frequency in the SLE patient group significantly different from the antibody frequency in the control set ($P = 0.03$). Overall, 8/15 (53%) of the SLE patients tested in the radiobinding assays reacted with at least one antigen ($P = 0.003$), 4/15 (27%) demonstrated binding reactivity to one antigen only ($P = 0.03$), and 4/15 (27%) showed immunoreactivity to two antigens ($P = 0.03$). In two of the patient sera, antibodies were detected to the same two antigens, namely, RPS20 and U1snRNP-C.

SDS–PAGE was used to check that the radioactivity immunoprecipitated in the radiobinding assays by each of the antibody-positive SLE sera was due to ^{35}S-labelled antigen. In all cases, antibody-positive sera immunoprecipitated a protein band of the correct size when compared to the in vitro translated radioactive ligand (data not shown).

Table 4
Immunoreactivity of SLE patient sera to ^{35}S-labelled polypeptides

Antigen	SLE sera ($n = 15$)[a]	P value[b]	Antibody indices for SLE patients (mean ± SD)	Antibody indices for controls (mean ± SD)	P value[d]
MRG15	2 (13)[c]	0.18	1.18 ± 0.56	0.99 ± 0.13	0.15
PIC1	1 (7)	0.43	1.07 ± 0.23	1.00 ± 0.13	0.26
RPS13	1 (7)	0.43	1.11 ± 0.50	1.02 ± 0.14	0.45
RPS20	3 (20)	0.07	1.27 ± 0.35	0.97 ± 0.18	0.002
α-Enolase	1 (7)	0.43	1.20 ± 0.73	1.00 ± 0.11	0.23
U1snRNP-C	4 (27)	0.03	5.47 ± 9.66	1.00 ± 0.37	0.046

[a] n = number of serum samples tested.
[b] P value calculated using Fisher's exact test for comparing antibody frequencies in SLE patient and control groups. $P < 0.05$ is considered significant.
[d] P value calculated using Student's t test for comparing mean antibody indices in SLE patient and control groups. $P < 0.05$ is considered significant.
[c] Number of antibody-positive patients with percentage in parentheses.

Table 5
Immunoreactivity of autoimmune disease patient sera to ^{35}S-labelled polypeptides

Antigen	Patient sera					
	SC ($n = 20$)[a]	P value[b]	PM ($n = 20$)	P value	RA ($n = 20$)	P value
U1snRNP-C	4 (20)[c]	0.05	4 (20)	0.11	2 (10)	0.49
MRG15	0 (0)	—	0 (0)	—	0 (0)	—
PIC1	1 (5)	1.00	0 (0)	—	0 (0)	—
RPS13	1 (5)	1.00	0 (0)	—	0 (0)	—
RPS20	0 (0)	—	0 (0)	—	0 (0)	—

[a] n = number of serum samples tested.
[b] P value calculated using Fisher's exact test for comparing antibody frequencies in patient and control groups. $P < 0.05$ is considered significant.
[c] Number of antibody-positive patients with percentage in parentheses.

Radiobinding assays with autoimmune disease patient sera

In order to analyse the disease specificity of the antigens isolated from the biopanning experiment with SLE patient antibody, sera from patients with SC ($n = 20$), PM ($n = 20$), RA ($n = 20$), and healthy controls ($n = 20$) were tested for their ability to bind to radiolabelled antigens PIC1, MRG15, RPS20, ribosomal RPS13, and U1snRNP-C in radiobinding assays. The frequency of antibody binding to each of the ^{35}S-labelled ligands is summarised in Table 5. Antibodies to RPS20 and MRG15 were not detected in SC, PM, or RA patient sera whereas antibody reactivity to both RPS13 and PIC1 was demonstrated in the sera of 1/20 (5%) patients with SC.

Discussion

Several advantages over the conventional immunoscreening of cDNA libraries attached to nitrocellulose membrane are provided by phage-display technology. For example, screening of the cDNA library in a fluid-phase avoids denaturation of the polypeptides that are displayed on the surface of the phage. This may be important in detecting antibody binding to conformational epitopes [6]. In addition, immobilisation of the IgG instead of the cDNA library allows the specific enrichment of phage particles that interact with the antibody immobilised onto a solid-phase. Antigens that are present at low abundance can therefore be enriched increasing the possibility of detection and identification. Furthermore, as many as 10^{10}–10^{11} individual clones can be screened in a single experiment, thereby enhancing the chances of recovering autoantigens. Biopanning of phage-display cDNA libraries has been employed previously to identify yeast and fungal allergens that bind to human IgE antibodies [1,4,5] and to characterise the ligands for type 1 plasminogen activator inhibitor and transcription factors [20,21]. This strategy has also been used to map the B cell epitopes of several viral antigens [22,23]. In this study, the applicability of cDNA phage-display to the characterisation of self-antigens in autoimmune disease was assessed using antibody from SLE patients as a model system.

Autoantibodies to several of the antigens identified here had been previously reported in SLE, including α-enolase [24], heat shock protein 90 [12,25], translation elongation factor 1α [26], and ribonuclease P/Rpp30 [27]. Anti-U1snRNP antibodies are found in 23% of SLE patients [28], although they are not specific to the

disease [29]. This particular group of antibodies reacts with constituents of the U1snRNP complex designated as polypeptides 70 kDa, A and C [30] and, in this study, a cDNA encoding U1snRNP-C was recovered from the immunoscreening process. We also isolated cDNAs encoding the protein components, namely polypeptides B and D of the U1snRNP. These antigens are recognised by SLE-specific anti-Sm antibodies [31] and are usually present in 4–30% of SLE patients depending on ethnic background [28]. Antibodies to the SSA/Ro antigen are normally found in 24% of SLE patients [28]. In this study, a cDNA encoding the 52-kDa protein of SSA/Ro [15] was identified.

With respect to novel autoantigens, a cDNA encoding a transcription factor-like protein MRG15 [32] was recovered from the rounds of enrichment of the phage-display library with SLE patient IgG. Autoantibodies to other proteins involved in the transcriptional process including RNA polymerase II [33], the transcription factors TFIIB [34] and TFIIF [11], and the transcriptional regulation protein DEK [35], a site-specific 45-kDa DNA binding protein, have been documented to occur in SLE patients. Although their significance to SLE pathogenesis has yet to be reported, autoantibodies that recognise such DNA binding components could adversely affect protein synthesis and/or signal transduction. A number of ribosomal proteins, namely S13 [36] and S20 [37], were also enriched by the biopanning protocol, but these differed from the ribosomal proteins, for example, ribosomal P protein P0 and ribosomal proteins L6 and L7 that have already been described as autoantigens in SLE [8,10,26,38]. A cDNA encoding the ubiquitin-like protein PIC1 [39] was also enriched by the biopanning process. PIC1 has amino acid homology with ubiquitin and autoantibodies to the latter protein have been found in patients with localised scleroderma, systemic sclerosis, and SLE [40,41]. It is possible that the autoantibodies recognise ubiquitin, an autoantigen in various autoimmune disorders [40,41], and might therefore cross-react with PIC1. Several of the DNA inserts recovered in this study remain unidentified and these might represent as yet unknown antigenic targets in SLE.

We did not isolate cDNAs encoding either the protein components, namely polypeptides B′, E, F, and G, of the U1snRNP [31] or the 60-kDa protein of SSA/Ro [15]. More extensive screening of the clones recovered from the biopanning might have revealed cDNAs encoding other known and novel autoantigens, although, to some extent, the small cohort of patients used might have limited the specific cDNAs recovered. It is also possible that a lack of post-translational modification, as is the case with peptides produced in bacterial systems, prevented the recovery of particular antigens during the rounds of enrichment with SLE antibodies. Indeed, methylation and phosphorylation are important in the recognition of some SLE autoantigens [42,43]. Furthermore, other autoantigens might be recovered if a different source of cDNA was used. For example, anti-endothelial cell antibodies are frequently detected in SLE patients [26] and their specific reactivities could be identified using an endothelial cell cDNA phage-display library.

In summary, cDNA phage-display technology has been used successfully to recover both known and novel autoantigens in SLE. The study demonstrates the power of this new method in the isolation of autoantigens and the technique will be useful for identifying autoantigens in other autoimmune disorders, such as vitiligo and thyroid-associated ophthalmopathy, in which the major autoantigens have not yet been characterised.

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