Isolation of Novel Autoantigens by Lambda Phage Surface Display

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Mikio NIWA

Isolation of Novel Autoantigens by Lambda Phage Surface Display

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Mikio NIWA

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Abstract

The appearance of antibodies against components of the individual's own body, called autoantibodies, is the most important feature of autoimmune diseases. However, autoantibodies are raised against various molecules, and it is revealed that in systemic autoimmune diseases many autoantibodies recognize intracellular molecules, such as cytoplasmic proteins and nuclear proteins, and these antigens are called intracellular autoantigens.

To date, intracellular autoantigens have been discovered by conventional methods such as immunoprecipitation and expression screening of cDNA libraries. However, both methods require tedious processes, making it difficult to carry out a number of independent screenings, and the probability of success is low. Phage display using filamentous M13 phage was proposed by G. P. Smith in 1985. In this new method, a foreign DNA product is displayed on the surface of the phage particle. The desired phage-expressed autoantigens can be enriched specifically from large cDNA libraries by repeat binding to target autoantibodies and phage amplification. However, although this technique is expected to work well in the search for autoantigens, the discovery of intracellular autoantigens using this method has not been reported. One of the reasons for this unexpected result is that proteins with low membrane permeability tend not to be displayed on the surface of the phage.

To overcome this problem, Maruyama developed a novel bacteriophage lambda surface display vector, λfoo (fusion on outside). It is expected that both secreting and intracellular proteins can be expressed while maintaining their native conformation and function, because foreign proteins need not pass through a membrane when λfoo phage assembly occurs in cytoplasm.

In this study, I would like to demonstrate that intracellular autoantigens can be

selected from cDNA library made by λfoo vector using sera of autoimmune diseases. Moreover, the first challenge so far will be to discover novel autoantigens.

The strategy for searching for autoantigens is as follows: 1) mRNA is purified from a cultured human cell line or lesional tissue from disease patients, then cDNA is synthesized and incorporated into λfoo vector and a cDNA library is constructed by the *in vitro* packaging method for forming phage particles. 2) a phage expressed autoantigen is enriched from the cDNA library by autoantibodies in the patient's serum. 3) cDNA inserts in selected phages are determined and searched within the DNA data-base. 4) The reactivity of the selected phages to serum from patients of target disease is compared to that of other relative diseases and of healthy donors. Finally, I evaluate whether the protein expressed by the selected phage is disease-specific or not.

In the first chapter, as a model experiment, I carried out a search for phages encoding intracellular autoantigens recognized in sera from patients with Sjögren's syndrome (SjS) from cultured cell cDNA libraries made with λfoo. As a result, I successfully isolated cDNA clones that encode some intracellular autoantigens recognized by SjS sera. Among these, four clones expressed autoantigens previously identified in SjS, namely SS-B/La, hRPA-70, NOR-90, and Ki-67 antigen, and three clones, SMN, pM5, and TFG, encoded proteins previously not known as autoantigens in any autoimmune disease. In particular, I speculate that the anti-SMN antibody may be a novel anti-nuclear antibody. These results were the first demonstration that novel autoantigens can be found in a cDNA library using the phage display technique.

Moreover, in the second chapter, I isolated novel autoantigens by using synovial fluids and sera from patients with rheumatoid arthritis (RA). As a result, three proteins, sorcin, IGFBP-4, and SHLP-1, were found as novel autoantigens that are specific for RA. Interestingly, it should be noted that autoantibody against IGFBP-4 may participate in the

progression of RA.

In this study, I prove that cytoplasmic proteins can be efficiently selected as autoantigens of autoimmune diseases from the cDNA phage display library using the λfoo vector. Besides, I could determine some novel autoantigen/autoantibody sets. Thus, this technology is expected to elucidate the etiology and mechanisms producing autoantibodies in autoimmune diseases.

Abbreviations

ALS	acid-labile subunit
ANAs	anti-nuclear autoantibody
βGal	β-galactosidase
BCOADC-E2	branched chain 2-oxo-acid dehydrogenase complex-E2
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
IGF	Insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin-G
MCTD	mixed connective tissue disease
NAA	natural autoantibody
NOR-90	nucleolus organizer region autoantigen
NuMA1	type-1 nuclear mitotic apparatus protein
OA	osteoarthritis
PBC	primary biliary cirrhosis
pfu	bacteriophage plaque-forming unit
RA	rheumatoid arthritis
RF	rheumatoid factor
RPA	human replication protein A
SF	synovial fluid
SHLP	small humanin like peptide
SjS	Sjögren's syndrome
SLE	systemic lupus erythematosus

SMN	survival motor neuron gene product
snRNP	small nuclear ribonucleoprotein complex
SSc	systemic sclerosis
TFG	TRK-fused gene product
UPR	unfolded protein response
XGal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

General Introduction

What is autoimmune disease?

By default, the immune system remains inactive based on the principle of "immune tolerance," meaning it does not respond to a self-antigen. An autoimmune disease causes aberrant behavior and dysfunction in various tissues in one's own body by a breakdown in tolerance. There are still many questions regarding the mechanism behind immunological tolerance failure, but several hypotheses have been proposed, including apoptotic abnormality, abnormality of regulatory T-cells, and the molecular mimicry mechanism (Lleo et al., 2010).

An antibody turning on one's own body tissues is called an autoantibody. The existence of autoantibodies has also been reported in the serum of healthy persons, and this is called natural autoantibody (NAA). The functions of NAA are not clear, but NAA may participate in various activities, such as regulation of immune response, and removing aged or damaged tissue in healthy persons (Silosi et al., 2016). However, natural autoantibodies are particularly of the IgM isotype, have low affinity, and are poly-specific while autoantibodies in autoimmune disease tend to be isotype IgG, show high affinity and specificity, and have a characteristic function, such as removing alien substances, viruses and bacteria. However, even though autoantibodies are an important phenomenon in all autoimmune diseases, the mechanism by which autoantibodies affect the clinical condition is still unclear, as is the reason why a particular autoantibody is produced in a specific disease.

Autoantibodies to intracellular antigens

Autoimmune disease have long been classified as organ-specific or systemic, depending on whether or not the autoimmune response is directed against one organ or apparatus, as in autoimmune Hashimoto's thyroiditis (Burek, 2010).

Autoantibodies are targeted against various molecules, such as nucleic acids, polysaccharides and proteins, and these antigens are ubiquitously located, in nucleus or cytoplasm, on membranes or in the extracellular space. However, it is conspicuous that many autoantibodies in systemic autoimmune diseases recognize intracellular molecules, in particular nuclear molecules. The latter are called anti-nuclear antibodies (ANAs).

These intracellular antigens raise the question how the immune response is mounted, why they are specifically targeted in certain disease subsets and inherently, how they are exposed to B- or T-cells so these can mature and produce autoantibodies. To understand these questions, several hypotheses have been explored. Among these hypotheses, three representative models are displayed in Fig. 1: (A) exposure by abnormal clearance of apoptosis/necrosis; (B) epitope modification; (C) molecular mimicry (Suurmond et al., 2015).

However, intracellular autoantigens are expressed ubiquitously in various cell types, and specific subsets are preferentially targeted in different systemic autoimmune diseases. For example, SS-B/La in Sjögren's syndrome (SjS), double-stranded DNA in systemic lupus erythematosus (SLE) and centromere-associated proteins in systemic sclerosis (SSc) are autoantigens recognized by disease-specific autoantibodies (Tan, 1989; Rahman et al., 2008; Nihtyanova et al., 2010). Thus, it is useful to find intracellular antigens and their autoantibodies for diagnosis, classification and to establish for criteria disease activity.

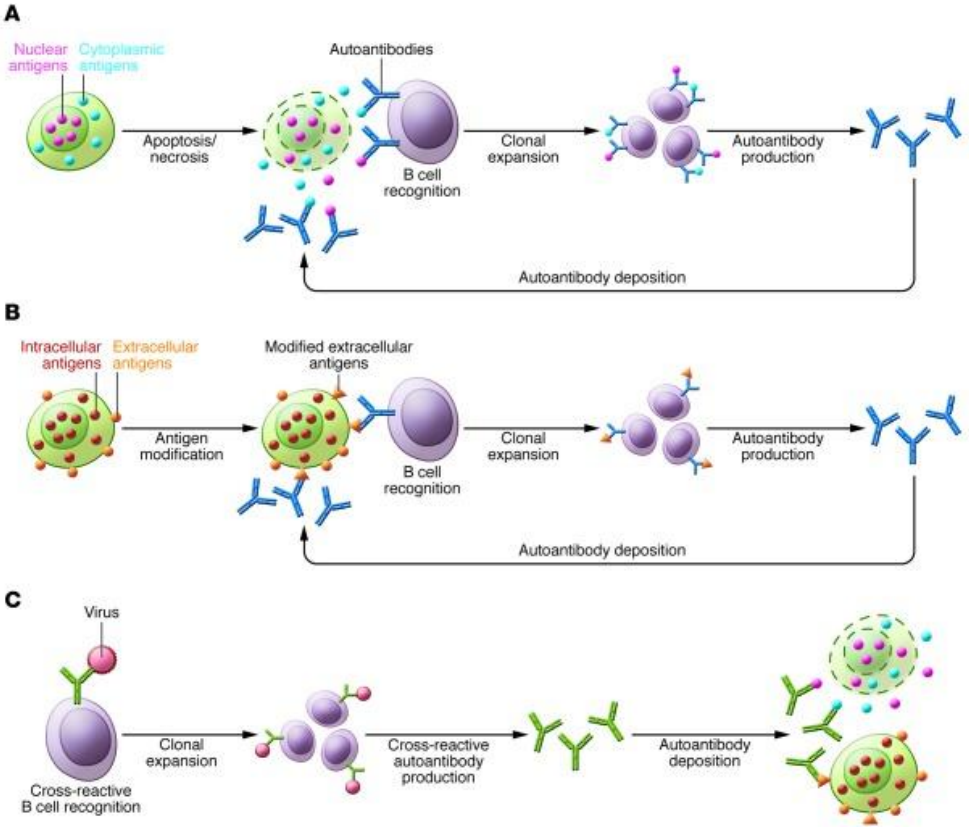

Fig. 1 Mechanisms for autoantibody production: apoptosis, antigen modification, and cross-reactivity.

(A) Intracellular autoantigens are exposed into the extracellular environment, if clearance mechanisms are insufficient after apoptosis or necrosis. And then, these antigens may be recognized by B cells and autoantibody production. (B) Modification of intracellular proteins generates novel epitope, to which B cells have not been tolerized. (C) Autoantibody production arises from responses to foreign antigens, which cross-react with intracellular proteins. This figure was cited from the reference (Suurmond et al., 2015)

Screening method for discovering autoantigens

Immunoprecipitation and expression screening from a cDNA library have been utilized as conventional method to discover autoantigens. However, in spite of both methods requiring tedious processes, non-specific results tend to be obtained, it is difficult to carry out a number of independent screenings, and the probability of success is low.

As an alternative method, phage display using filamentous M13 phage was proposed in 1985 (Smith, 1985). This approach is based on two pivotal concepts. The first is that a protein or peptide is displayed on the surface of a phage particle and the genes encoding it are contained within the same particle. The second concept is that individual phages can be selected from large libraries by interaction of the displayed protein with a bindable probe, such as proteins, polysaccharides, lipids, nucleic acids and chemicals. Then, selected phages can be amplified by infecting bacteria. A phage display library is easier to make than a chemical library, may contain enormous diversity (more than 10^{11}), and amino acids sequences can be determined with ease and precision by reading DNA sequences. Therefore, this technique was expected to work well for searching for autoantigens, but no cytoplasmic autoantigen has been reported using a phage display made with the M13 filamentous phage. This unexpected result is probably caused by the life cycle of M13 filamentous phage. In M13, foreign DNA product is fused to the N-terminal of the phage's coat protein pIII. However, this fusion protein maintains its infectivity, and has to transfer into the periplasmic space across the inner membrane of the host *E.coli* cell, when phage assembly occurs (Fig. 2). Thus, proteins with low membrane permeability tend not to be displayed on the surface of phage. Besides, the environment in the periplasmic compartment is oxidizing, hence imprecise disulfide bonds possibly occur, resulting in a non-native conformation and function of the intracellular protein (Barbas et al., 2001).

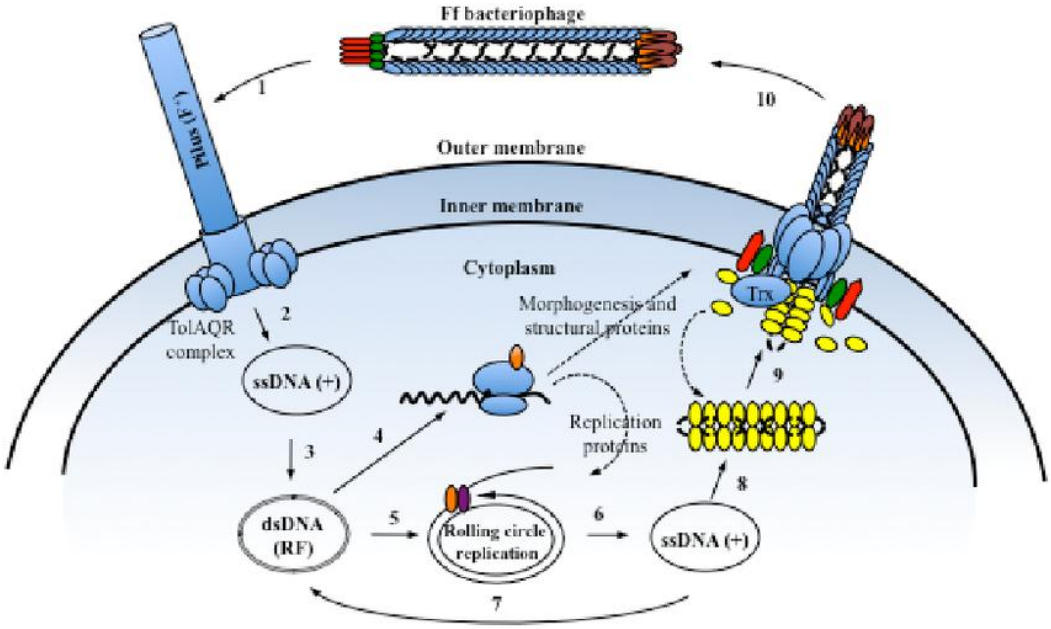

Fig. 2 Life cycle of M13 filamentous phage. This figure was cited from the reference (Pacheco et al., 2012)

To overcome this problem, Maruyama developed a novel bacteriophage lambda surface display vector, λfoo, in which a foreign protein is produced as a chimeric protein fused to the C-terminal phage coat protein D (Maruyama et al., 1994; Mikawa et al., 1996). It is expected that both secreting and intracellular proteins can be expressed while maintaining their native conformation and function, because a foreign protein does not need to pass through membrane when λfoo phage assembly occurs in the cytoplasm (Fig. 3).

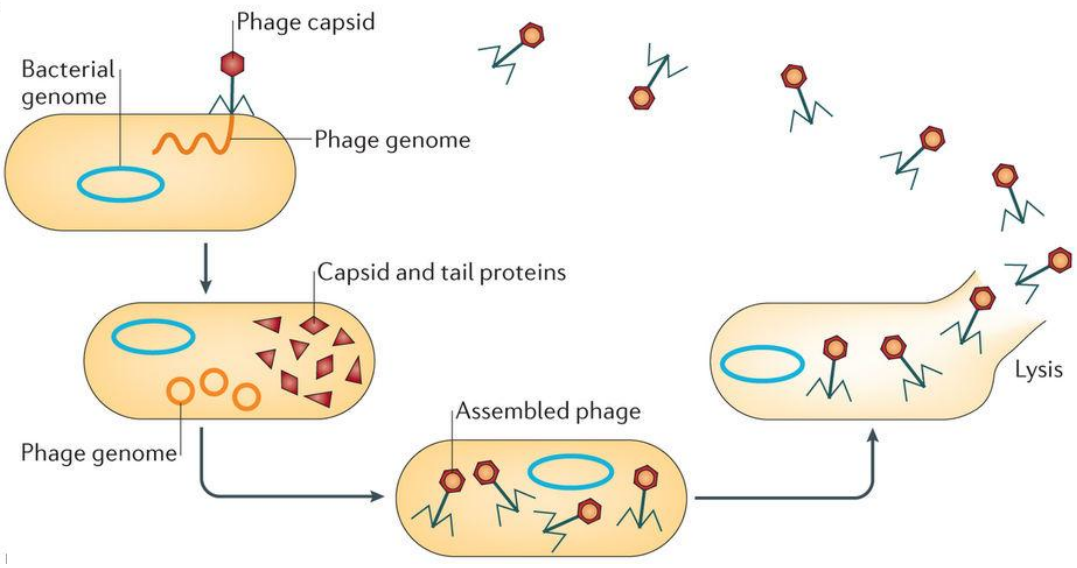

Fig. 3 Life cycle of lambda phage. This figure was cited from the reference (Feiner et al., 2015)

Objective of this research

With this new λ phage display system, I would like to demonstrate whether intracellular autoantigens can be selected from a cDNA library using the serum of autoimmune disease patients, and moreover to discover novel autoantigens, which is the first challenge.

In chapter 1, as a model experiment, I explored the approach of affinity isolation of cDNA clones encoding intracellular autoantigens from HeLa and HepG2 cDNA libraries made with λfoo, using sera from patients with SjS. In chapter 2, I isolated novel autoantigens by using specimens from a patient with RA.

Chapter 1

Affinity selection of cDNA libraries by λ phage surface display

Summary

Bacteriophage λ surface display was used to isolate cDNA clones encoding autoantigens recognized by sera from patients with Sjögren's syndrome. I made cDNA libraries from human HeLa and HepG2 cells, using an expression vector, λfoo. By repeating affinity selection of the libraries with the sera immobilized in microtiter wells, I isolated three clones that encode novel antigens as well as four clones previously known as SjS autoantigens. The newly identified autoantigens include TRK-fused gene product (TFG), survival motor neuron gene product (SMN) and pM5, which has a similarity to the metal-binding domain of human fibroblast collagenase. Thus, the bacteriophage λ surface display is powerful to isolate cDNA clones by affinity screening.

Introduction

cDNA library screening requires tedious processes including preparation of a number of membrane filters blotted with approximately a million of bacterial colonies or bacteriophage plaques, which are subsequently searched for by using probes such as labeled proteins or DNA. In order to streamline the screening of cDNA libraries, Maruyama et al. have devised a bacteriophage lambda surface display vector, λfoo, in which a foreign protein is produced as a chimeric fusion protein to the phage coat proteins (Maruyama et al., 1994; Mikawa et al., 1996). Therefore, λfoo cDNA libraries can be screened by affinity chromatography with molecules immobilized on the surface of solid matrices including microtiter wells and agarose beads. Similar vectors based on λ phage have also been developed (Sternberg and Hoess, 1995; Dunn, 1995; Santini et al., 1998), and many functional prokaryotic and eukaryotic proteins have been produced on the surface of the vector phage. The vectors have also been applied to mapping linear and conformational epitopes by affinity screening of random fragments of antigens expressed on the phage surface (Kuwabara et al., 1997; 1999; Moriki et al., 1999). In this chapter, I have further explored an approach to the affinity isolation of cDNA clones encoding autoantigens from HeLa and HepG2 cDNA libraries made with λfoo, using sera from patients with SjS as probes. Since many autoimmune sera recognize conformational epitopes of autoantigens, I may be able to test λfoo for its ability to express conformational epitopes on its surface and for affinity selection of such clones from complex libraries.

SjS is a chronic autoimmune disease characterized by lymphotic infiltrates of salivary and lachrymal glands with progressive destruction of the parenchymal tissue leading to a reduction or complete loss of secretory function. Sera from patients with SjS often contain autoantibodies reacting with nuclear and cytoplasmic components (Fayyaz et al., 2016).

Among them, anti-SS-B/La autoantibody is used as a diagnostic marker for SjS (Tan, 1989). The SS-B/La antigen, a 50 kDa protein, binds RNA polymerase III transcripts as part of their maturation process (Gottlieb and Steitz, 1989). In the present, anti-SS-A/Ro and anti-SS-B/La are used for diagnostic criteria; however, their sensitivity and specificity are not sufficient for the diagnostic of SjS. Although less frequently observed, SjS sera also recognize many other autoantigens including human replication protein A (RPA) (Garcia-Lozano et al., 1995), RNA polymerase I transcription factor hUBF/nucleolus organizer region autoantigen (NOR-90) (Fujii et al., 1996), and the cell proliferation-associated protein Ki-67 antigen (Bloch et al., 1995). Meanwhile, many studies are carried out for the relationship between autoantibodies and clinical manifestation in SjS (Fayyaz et al., 2016). Therefore, novel sets of autoantigens and autoantibodies open a window for not only the diagnostic approach, but also the therapeutic method.

Isolation of autoantigens recognized by sera from patients with an autoimmune disease has been traditionally carried out through time-consuming processes by either immunoprecipitation of autoantigens, or immuno-screening of cDNA expression libraries constructed with plasmid or phage vectors. In this chapter, I describe an innovative approach, affinity screening of λfoo surface display libraries, to the isolation of cDNA clones encoding autoantigens recognized by SjS sera. Using sera from 51 patients with SjS as probes, I have screened HeLa and HepG2 cDNA libraries constructed with λfoo, and have found seven clones encoding three unknown and four known proteins as autoantigens.

Materials and Methods

Bacteria, cell lines, phage vector and sera

The following *Escherichia coli* strains were used; JM105 (Yanisch-Perron et al., 1985), TG1 (Mikawa et al., 1996), and Q447 (Kuwabara et al., 1999). The bacteriophage surface expression vector λfoo (Mikawa et al., 1996) was modified to accommodate two *Sfi* I recognition sites in its multiple cloning site in order to simplify the process of cDNA library construction (Christian et al., 1992). The following double-stranded synthetic oligonucleotides,

5'-GATCCCCGGGTACCGAGGCCGCCTCGGCCGAGCTCGAATTCGGCCGGCCATAGCGGCCGC

3'-GGGCCCATGGCTCCGGCGGAGCCGGCTCGAGCTTAAGCCGGCCGGTATCGCCGGCGTTAA,

in which *Sfi* I recognition sequences are underlined, were inserted into λfoo DNA digested with *Bam* HI and *Eco* RI. The resulting vector was designated as λfoo2SfiI. Sera from 51 patients with SjS were provided by Scripps Reference Laboratory, The Scripps Research Institute, La Jolla, CA, and used as probes for screening of cDNA libraries. The following sera were obtained from the laboratory serum bank of Nara Medical University, Nara, Japan; 21 sera from patients with SjS, 8 sera from patients with systemic lupus erythematosus (SLE), 8 sera from patients with primary biliary cirrhosis (PBC), 6 sera from patients with systemic sclerosis (SSc), and 72 sera from healthy people. Human cultured cell lines, HeLa and HepG2, obtained from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified eagle medium (DMEM; Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) fetal calf serum (Life Technologies), 2 mM L-glutamine, 100 units/ml penicillin-G and 100 μg/ml streptomycin sulfate in a 5% CO_2/95% air incubator.

cDNA library construction

Total RNA was prepared from ~3 x 10^8 HeLa or HepG2 cells through standard procedures using guanidine thiocyanate treatment followed by CsCl centrifugation. From approximately one mg of the total RNA, poly(A)$^+$ RNA, about 25 μg, was enriched by a single cycle of oligo(dT)-cellulose column chromatography as described previously (Sambrook et al., 1989). The first strand cDNA was synthesized from 5 μg of the poly(A)$^+$ RNA using SuperScript II reverse transcriptase (Life Technologies) and 2.5 μg of random hexamers (Sigma, St. Louis, MO). The second strand of the cDNA was synthesized using a kit (Promega, Madison, WI) according to the manufacturer's protocol. Using T_4 DNA ligase (New England Biolabs, Beverly, MA), the resulting double-stranded cDNA was ligated with the mixture of following *Sfi* I adaptors;

adaptor 1, 5'-CGGCCAGGATCTGGTGGT

3'-GGAGCCGGTCCTAGACCACCA

and adaptor 2, 5'-CTGACTGACTGAGGCCATAG

3'-GACTGACTGACTCCGGT.

Unligated adaptor oligonucleotides were removed from cDNA by passing the ligation mixture through a Sephacryl S-400 (Amersham Pharmacia Biotech, Piscataway, NJ) spin column. The resulting cDNA was ligated with one μg of the λfoo2SfiI phage vector DNA digested with *Sfi* I. After incubating overnight at 16ºC, the ligation mixture was packaged using MaxPlax (Epicentre Technologies, Madison, WI), and was amplified by infection of the *E. coli* strain Q447. General procedures for the cDNA synthesis, adaptor ligation and phage DNA packaging were previously described (Ausubel et al., 1987).

Affinity selection of cDNA library

Microtiter wells (Immulon 4; Dynatec Laboratory, Chantilly, VA) were coated overnight at 4°C with 50 μl of 20 μg/ml protein A (Sigma) in phosphate-buffered saline

(PBS; 137 mM NaCl/8.1 mM Na_2HPO_4/2.68 mM KCl/1.47 mM KH_2PO_4) containing 0.05% (wt/vol) sodium azide. After pre-blocking the wells with 50 µl of PBS containing 1.0% (wt/vol) bovine serum albumin (BSA) and 0.05% sodium azide, patient sera diluted 1/100 in PBS were added to the wells and incubated overnight at 4ºC in order to capture IgG to the wells. Unbound serum proteins were removed by washing the wells twice with blocking buffer [PBS/0.1% (vol/vol) Tween-20/0.25% BSA/5% (wt/vol) non-fat dry milk/0.1% sodium azide].

The cDNA libraries were grown with TG1, and after complete lysis the library phage was precipitated by the addition of 7% (wt/vol) polyethylene glycol (PEG; Mw, ~8000; Fisher, Fair Lawn, NJ), 0.6 M NaCl and 2 mM $MgCl_2$ at final concentrations. After re-suspending the phage precipitate in λ-dil (Maruyama et al., 1994), an aliquot, ~2 x 10^{10} pfu, was applied to the microtiter wells coated with patient sera, and incubated overnight at 4°C. Unbound phage was removed by washing the well three times with 200 µl of washing buffer (PBS/5% non-fat dry milk/0.5% Tween-20/0.1% sodium azide) for 5 min at room temperature, and then three times with 200 µl of λ–dil for 5 min at room temperature. Bound phage to the well was eluted with 50 µl of collagenase solution [20 units collagenase (Sigma) in λ-dil supplemented with 10 mM $CaCl_2$] for 2 h at 37°C. Phage titers were assayed as described previously (Sambrook et al., 1989).

Phage culture, plaque staining and DNA sequencing

Phage was cultivated either in CY liquid medium (Maruyama et al., 1994) or on an *E. coli* lawn on an agar plate. General manipulation of phage was described previously (Sambrook et al., 1989). Immediately before the affinity selection, phage was cultured with a suppressor-positive strain, TG1. After affinity selection of cDNA libraries, an aliquot of eluted phages from the wells was infected JM105 and plated on agar containing 20 µg/ml 5-

bromo-4-chloro-3-indolyl ß-D-galactoside (XGal) at a final concentration. The λfoo2SfiI vector phage formed a blue plaque while recombinant fusion phage formed a white plaque on the plate. Plaques formed on the plate were lifted onto a nitrocellulose filter (Shleicher & Schuell, Keene, NH) and stained with patient sera as previously described by Kuwabara et al. (1997). Phage particles picked from the plaque were directly used for DNA sequencing as described previously (Kuwabara et al., 1997).

Phage ELISA

Microtiter wells were coated overnight at 4ºC with rabbit polyclonal anti-λfoo phage antibody (a gift from Taka Yamori, The Cancer Institute, Otsuka, Tokyo, Japan) at 2 μg/ml in PBS containing 0.05% sodium azide. Meanwhile, fusion phages were grown with TG1 until complete lysis in order to express foreign proteins on its particle surface. This phage culture was diluted 1/10 in ELISA buffer [PBS/5% non-fat dry milk/0.1% (vol/vol) Triton X-100/0.1% sodium azide], and incubated overnight at 4ºC in the microtiter wells pre-blocked with PBS containing 1.0% BSA and 0.05% sodium azide. After washing the wells three times with ELISA buffer, patient sera diluted 1/1000 in PBS were bound to the wells for 1 h at room temperature. After washing the wells six times with ELISA buffer, secondary antibody, alkaline phosphatase-conjugated mouse anti-human IgG (Sigma) diluted 1/10,000 in PBS, was added to the wells and incubated for 1 h at room temperature. After the wells were washed four times with ELISA buffer and then twice with AP buffer (100 mM Tris-HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$), a substrate for alkaline phosphatase, 2 mg/ml SIGMA-104 in AP buffer, was added to the wells and incubated for 1 h at room temperature. Alkaline phosphatase activity captured to the wells was estimated by measuring optical densities (OD) at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). This measurement was repeated three times for a patient serum, using the vector phage as a negative control.

Patient sera were judged to have antibody reactive with autoantigens when the mean OD value was greater than 3 x S.D. (standard deviation) above the mean OD values of the negative control.

Results

cDNA library construction

HeLa and HepG2 cDNA libraries constructed with λfoo consisted of 2.7 x 10^7 and 2.5 x 10^7 plaque-forming units (pfu) of phages, of which 15.3% and 3.0% formed white plaques, respectively, on plates containing the color indicator XGal. The λfoo vector is designed to express α–peptide of *E. coli* β-galactosidase (βGal) so that the vector phage forms a blue plaque on a lawn of *E. coli* expressing βGal ω–peptides, such as the strain JM105, in the presence of XGal as a substrate for βGal. Insertion of cDNA into the cloning site of the vector prevent the expression of α-peptide. Therefore, from phage plaque color I could estimate the pfu of recombinants with inserts in the cDNA libraries; 4.1 x 10^6 and 7.5 x 10^5 pfu of recombinants in the HeLa and HepG2 libraries, respectively. However, a half of the recombinants should be in a reverse orientation, and two out of three clones should be out of reading frames. The libraries were, therefore, estimated to comprise of 6.9 x 10^5 and 1.3 x 10^5 pfu selectable recombinants, respectively. The cDNA libraries were amplified by infection of an *E. coli* strain without an amber suppressor mutation to prevent the production of fusion proteins that might result in biased libraries. The vector is designed to accommodate the amber stop codon TAG between the phage coat protein gpD and a foreign protein encoded by inserted cDNA. Therefore, foreign proteins are produced on the surface of the phage particles as fusion proteins to the coat protein only when the phage is grown with *E. coli* hosts having an amber suppressor mutation, such as the strain TG1, immediately before affinity screening of cDNA libraries.

cDNA library screening

I made nine mixtures from 51 sera from patients with SjS, in which five or six

different patient sera were mixed and used to select clones from the HepG2 cDNA library. For affinity selection, approximately 2 x 10^{10} pfu phages from the library grown with TG1 were applied to microtiter wells coated with the serum mixtures. After extensive washing to remove unbound phages from the wells, bound phages were eluted by collagenase digestion. The vector is designed to have a collagenase recognition site between the coat protein gpD and a foreign fusion protein so that infectious phage particles can be released from the wells by the enzyme digestion. An aliquot, ~500 pfu, of the eluated phages was plated on agar, and phage plaques were immunostained with the serum mixtures after blotting onto nitrocellulose filters. The remaining eluates were amplified by growing with TG1 for subsequent affinity selection. After four rounds of affinity selection using the nine serum mixtures, five mixtures were found to enrich for immunoreactive clones. An example of such successful enrichment is shown in Fig. 4. As the selection cycles proceeded, the fraction of phages formed white plaques in total eluate phages was increased and the number of phage clones stained with the serum mixtures was also increased. I isolated ten serum-reactive clones from each of the second eluates of the five successful enrichment, and analyzed by PCR and DNA sequencing using primers that hybridize to the vector DNA. All the ten clones from each of the five successful selections contained inserts with the same size and nucleotide sequence. It was also found that a clone having the same insert was selected by two separate serum mixtures. Therefore, by this affinity selection four different clones were isolated and designated as Sg1p3, Sg3p11, Sg4p1 and Sg9p22 (Table 1). Using these isolated clones, individual serum in the serum mixtures was searched for and serum that specifically reacted with the clone was identified.

I also carried out affinity selection of the HeLa cDNA library, using 17 individual patient sera and eight mixtures consisting of four or five sera (34 sera in total) from different patients from the seventeen. From selection with the individual 17 sera, two positive clones

were isolated and designated as S7-1 and S12-11 after DNA sequencing analysis. By selection with the 8 mixtures, three were able to enrich for immunoreactive clones. Based on its DNA sequence, three different clones were isolated and designated as Sg2-1, Sg4-1 and Sg7-2. These results are summarized in Table 1.

DNA sequence analysis of isolated clones

Nucleotide sequences of the 5' and 3' ends of the cDNA inserts of the isolated phage clones, >400 nucleotide bases long from both of the boundaries, were compared with sequences in databases, GenBank, EMBL, DDBJ and PDB, using the BLAST software (Altschul et al., 1990). All the clones encoded a portion of seven known proteins (Fig. 5). Among the seven, three clones, Sg7-2, Sg3p11 and Sg4-1, have not previously been reported as autoantigens in any autoimmune diseases while the other four, Sg1p3, Sg2-1, Sg9p22 and S7-1, have been characterized as autoantigens recognized by sera from patients with SjS. Sg7-2 coded for a central portion of a survival motor neuron gene product (SMN), which is also known as a product of spinal muscular atrophy (SMA)-determining gene (Lefebvre et al., 1995). Sg3p11 encoded the carboxyl-terminal portion of the pM5 protein and its 3' untranslated region, which was previously isolated from an A2058 melanoma cDNA library using probes that have homology to the metal-binding domain of human fibroblast collagenase (Templeton et al., 1992). The clones Sg4-1 and Sg4p1 encoded the amino-terminal regions of TRK-fused gene (TFG), which is fused to the 3' end of NTRK1 (one of the receptors for nerve growth factor), generating the TRK-T3 fusion transcript found in papillary thyroid carcinoma (Greco et al., 1995).

The clones Sg1p3 and S12-11 encoded the amino-terminal regions of SS-B/La, which is a ribonucleoprotein in association with RNA polymerase III transcripts (Gottlieb and Steitz, 1989). Autoantibodies to SS-B/La are often found in sera of patients with autoimmune

diseases, SjS and SLE. The clone Sg2-1 encoded the amino-terminal 1/3 of the 70-kDa subunit of human replication protein-A (hRPA-70), which is a highly conserved protein complex with single-stranded DNA binding activity (Erdile et al., 1991). Sg9p22 encoded a portion of nucleolus organizer region-90 / human upstream binding factor (NOR-90/hUBF), which activates RNA polymerase I-mediated ribosomal RNA transcription (Chan et al., 1991). S7-1 encoded the carboxyl-terminal region of the cell proliferation-associated protein Ki-67 antigen, one of the ribonucleoprotein involved in cell cycle. Antibody against the Ki-67 antigen is used as a 'proliferation marker' to measure the growth fraction of cells in human tumors (Gerdes et al., 1983).

Frequency of patient sera that recognize isolated autoantigens

To investigate the frequency of the presence of autoantibodies against the isolated antigens in autoimmune sera as well as in normal sera, I used the phage clones to detect autoantibodies in sera from 72 patients with SjS, 22 with other autoimmune diseases (8 SLE, 8 PBC and 6 SSc) and 72 sera from healthy people by enzyme-linked immunosorbent assay (ELISA; Engvall and Perlmann, 1971) using the phage clones as described in Materials and methods. As shown in Table 2, Sg1p3 (SS-B/La) reacted with 21 out of 72 SjS sera (29%) and one PBC serum out of 22 sera from other autoimmune sera (4.5%). The 21 positive sera include all of 20 sera shown to have anti-SS-B/La antibodies based on immunodiffusion precipitin reaction and ELISA. This attests that phage ELISA is as sensitive as the conventional methods. The Sg2-1/hRPA-70 clone reacted with 2 out of 72 SjS sera (2.8%) and 2 out of 8 SLE sera (25%). Four sera out of 72 SjS sera reacted with Sg7-2/SMN clone (5.5%), and one serum out of 22 other autoimmune sera (4.5%) or one out of six SSc (16.7%). Sg3p11 coding for pM5 reacted with 3 sera from patients with SjS (4.2%), but not with other autoimmune sera. Three other clones (Sg9p22/NOR-90, S7-1/Ki-67 antigen and Sg4-1/TFG)

reacted with only one of 72 SjS sera (1.4%) and with none of 22 other autoimmune sera. Among 72 normal sera examined, only one serum reacted with Sg4-1/TFG, and none reacted with the other clones.

Discussion

In this study using the λfoo phage display, I have successfully isolated cDNA clones that encode seven different autoantigens recognized by SjS sera from HeLa and HepG2 libraries; three proteins, SMN, pM5 and TFG, previously not known as autoantigens, and four proteins, SS-B/La, hRPA-70, NOR-90 and Ki-67 antigen, previously identified as SjS autoantigens. However, the TFG clones Sg4-1 and Sg4p1 were also recognized by serum from a healthy person, and therefore the autoantigen appears not to be specific to SjS. SS-B/La is one of the major target antigens recognized by sera from patients with SjS. The clones Sg1p3 and S12-11 encoded the amino-terminal regions of SS-B/La and the shorter clone Sg1p3 reacted with all the sera tested that contain anti-SS-B/La antibody. This result suggests that the major epitope recognized by sera from patients with SjS resides in the amino-terminal region of SS-B/La. The result is consistent with that from epitope mapping by McNeilage et al. (1992), in which a major epitope has been mapped within the first 107 amino-terminal residues as a discontinuous epitope. Nyman et al. (1989) also have mapped an epitope site in the amino-terminal region, while Bini et al. (1990) determined two epitope sites, one each in the amino- and carboxyl-terminal halves of the protein.

RPA consists of three subunits of 70, 32 and 14 kDa and seems to function in both the initiation and elongation stages of DNA replication (Sibenaller et al., 1998). Recently, using immunoblot analysis, it has been demonstrated that two (70 and 32 kDa) of these subunits react with sera from SjS and SLE patients (Garcia-Lozano et al., 1995, 1996, Yamasaki et al., 2006). Sg2-1 encoding the first 186 amino-terminal residues of hRPA-70 reacted with two sera out of 72 (2.8%) from patients with SjS and two sera out of eight (25%) from patients with SLE. It has also been estimated that the frequency of antibodies against the RPA subunits in sera from patients with SjS is 2-3% (Garcia-Lozano et al., 1995, Yamasaki et al.,

2006), which is consistent with our results. No epitope site in hRPA-70 recognized by SjS sera has been previously analyzed, except for the region, 1-186 amino acid residues, of hRPA-70 encoded by the clone Sg2-1 isolated in this work. NOR-90 has previously been characterized as autoantigens recognized by SjS sera, and autoantibody against NOR-90 has been observed in approximately 7.7% and 2.2% of sera from patients with SjS and SSc, respectively (Fujii et al., 1996). Major epitopes on the NOR-90 molecule have been mapped to two regions encompassing residues 89-310 and 310-633. The clone Sg9p22 encoded a smaller region, amino acid residues 433-510, of NOR-90, which overlapped with one of the epitope regions described above. In the present study, the phage clone Sg9p22 was recognized by 1.4%, one out of 72, of sera tested. This is significantly lower than the frequency observed previously and probably due to the lack of the second epitope site in the clone Sg9p22. The Ki-67 antigen has been found as an autoantigen by screening a λgt11 HepG2 cDNA library with serum from a patient with SjS (Bloch et al., 1995). The isolated cDNA clone encoded amino acid residues 1159-1526 of the Ki-67 antigen, which is far apart from the region, 2823-2916, encoded by the clone S7-1 in this study. These results suggest that at least two epitope sites on the Ki-67 antigen are recognized by SjS patient sera.

Thus, cDNA expression libraries made with the λfoo vector are powerful tools to identify cDNA clones encoding autoantigens using sera from patients with autoimmune diseases as probes. The technique requires only less than five μl of sera from patients, and many independent screenings can be simultaneously carried out using a microtiter plate coated with many sera from different patients. Phage ELISA described in this chapter is as sensitive as conventional methods such as ELISA and immunodiffusion precipitin reaction. It may also be efficient for the detection of autoantibodies against autoantigens in patient sera, since the method does not require purified autoantigens. However, bacteriophage expression vectors including λfoo, λgt 11 and λZAP may not be efficient for epitopes that are post-

translationally modified by such a mechanism as glycosylation or phosphorylation.

In this cDNA library screening, I have successfully isolated at least two clones, Sg1p3 and S12-11 that encode a conformational epitope recognized by SjS patient sera. This particular epitope of SS-B/La has previously been demonstrated to be conformational (McNeilage et al., 1992). Similarly, a large domain encoding a conformational epitope of human blood coagulation factor VIII has also been expressed on the surface of λfoo and recognized by conformation-specific antibodies (Kuwabara et al., 1999). Using lactose as a ligand, a domain of human galectin-3 required for the ligand recognition has been mapped by affinity selection of λfoo libraries (Moriki et al., 1999). Furthermore, many proteins have previously been produced with function on the surface of the λfoo vector phage; *E. coli* β-galactosidase and plant *Bauhinia purpurea* agglutinin (Maruyama et al., 1994). These results indicate that many proteins expressed on the surface of the vector phage retain their native conformation and function. Therefore, cDNA libraries constructed with λfoo may also efficiently be searched for proteins physically interacting with macromolecules including protein, DNA, RNA and polysaccharide immobilized on the surface of solid matrices such as microtiter wells or agarose beads.

Conclusion

In this study using the lambda phage display, I have successfully isolated that encode seven different autoantigens recognized by SjS sera from HeLa and HepG2 libraries; including previously identified as SjS autoantigens, and also novel antigens. The affinity selection using the phage display requires only a small amount, less than five microliter of patient's serum. Furthermore, using a microtiter plate, many sera can simultaneously be used for the screening. Therefore, the approach employed in this work may be an economical and efficient alternative to the conventional approaches to the isolation of cDNA clones encoding autoantigens recognized by sera from patients with not only SjS but also other autoimmune diseases.

Figures and Tables

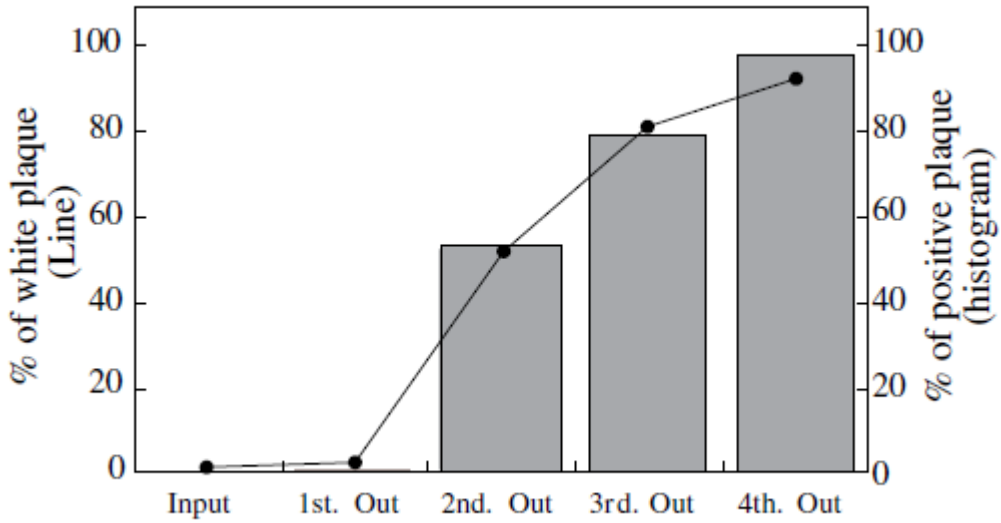

Fig. 4 Affinity selection of cDNA libraries with sera from patients with SjS. Phages from the HepG2 cDNA library (Input; ~2 x 10^{10} pfu, 3% of which formed white plaques) were applied to a microtiter well coated with serum from a patient with SjS, and selected four cycles by affinity selection as described in Materials and methods. After each round of affinity selection, an aliquot of phage eluates was plated with bacteria to assay the fraction of white plaques in total plaques and the number of reactive clones with the patient serum used for the selection. The rest of the eluate was amplified by growing with bacteria for subsequent selection. In this figure, an example of such affinity selection is shown, and the increased fraction of white plaques in total phage plaques is illustrated by a line graph and the increased fraction of reactive clones with a patient serum in the library population by a histogram.

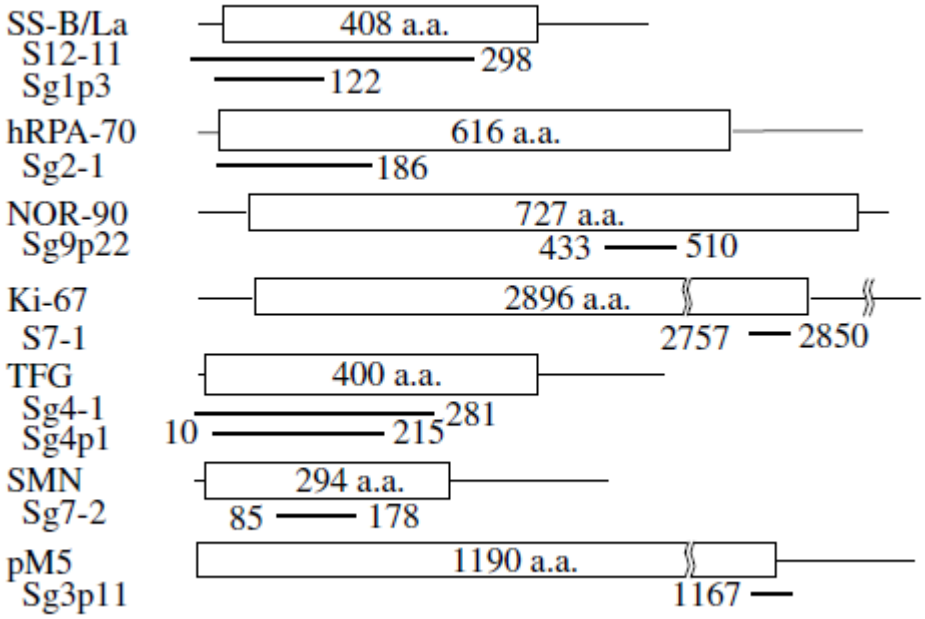

Fig. 5 cDNA sequences encoded by phage clones isolated. The 5'- and 3'-ends of cDNA inserts were determined by DNA sequencing using two oligonucleotide primers that hybridize to the vector DNA. At least 400 nucleotide bases were determined from both of the boundaries. Insert sizes of the clones were also confirmed by PCR. Rectangles indicate coding sequences of autoantigen cDNA based on published sequences. Lines protruded from the rectangles indicate the 5' and 3' untranslated regions on the left and right, respectively. DNA sequences encoded by all the cDNA clones isolated in this study are indicated by lines under the rectangles. The numbers of amino acid residues encoded by the autoantigen cDNA are shown inside the rectangles. The names autoantigens and isolated clones are shown on the left.

Table 1

Summary of cDNA library screening

SjS sera	cDNA library	Clones isolated
Mixed	HepG2	Sg1p3
(9 groups/51 sera)		Sg3p11
		Sg4p1
		Sg9p22
Individual	HeLa	S7-1
(17 sera)		S12-11
Mixed	HeLa	Sg2-1
(8 groups/34 sera)		Sg4-1
		Sg7-2

Table 2

Frequency of antibodies against autoantigens in normal sera and sera from patients with SjS and other autoimmune diseases.

Clones[a] (antigen)	Sera from patients with		
	SjS (%)	Other autoimmune disease, (%)	Normal (%)
Sg7-2 (SMN)	4/72 (5.5)	1/22 (4.5)	0/72 (0)
Sg3p11 (pM5)	3/72 (4.2)	0/22 (0)	0/72 (0)
Sg4-1 (TFG)[b]	1/72 (1.4)	0/22 (0)	1/72 (1.4)
Sg1p3 (SS-B/La)[b]	21/72 (29)	1/22 (4.5)	0/72 (0)
Sg2-1 (hRPA-70)	2/72 (2.8)	2/22 (9.1)	0/72 (0)
Sg9p22 (NOR-90)	1/72 (1.4)	0/22 (0)	0/72 (0)
S7-1 (Ki-67 antigen)	1/72 (1.4)	0/22 (0)	0/72 (0)

[a]Three clones above the dotted line encode previously unknown proteins as autoantigens and four clones below the line encode SjS autoantigens previously known.

[b]The same results were obtained for the clones Sg4-1 and Sg4p1, and for Sg1p3 and S12-11.

Chapter 2

Novel Autoantigens Recognized by Sera and Synovial Fluids from Patients with Rheumatoid Arthritis: Isolation by Using Bacteriophage λ Surface Display

Summary

To isolate cDNA clones encoding novel autoantigens recognized by sera or synovial fluids (SFs) from patients with RA, I constructed cDNA libraries from synovial sarcoma cells as well as from synovial tissues, using a surface-display vector, λfoo. The libraries were screened by affinity selection using 48 sera and 40 SFs from RA patients as probes immobilized in microtiter wells. As a result, 13 different autoantigens were isolated. These include seven proteins previously known to be recognized by RA sera, two proteins previously unknown to be recognized by RA sera, and four novel autoantigens that are previously unanalyzed in any autoimmune diseases, including an unknown protein. Frequencies of sera that recognize these novel autoantigens were ranged from 12.5 to 24.0% of a panel of RA sera, and 0 to 4.2% of other rheumatic disease sera. The novel autoantigens may serve as diagnostic and prognostic markers for RA as well as for understanding etiology and pathogenesis of RA.

Introduction

RA is characterized by chronic polyarthritis and destruction of multiple joints. Although etiology of RA is not yet known, a number of autoantibodies have been identified in sera from patients with RA, and antigen-driven immune response has been suggested (Smolen et al., 1998; von Landenberg et al., 2000). Despite many serum antibodies identified in RA, rheumatoid factors (RF), which are detected in ~80% of the patients, remain the main serological marker of the disease. Other serum antibodies with high sensitivity and specificity for RA include antibodies against filaggrin (also called antiperinuclear factor, anti-rat esophagus or anti-keratin antibodies), collagen, Sa, A2/RA33, 68-kd protein and gp130/follistatin-related protein (Le Goff et al., 1997; Blass et al., 1995; Tanaka et al., 1998). However, no single marker with sufficient sensitivity and specificity for the diagnosis of RA is currently available. Identification of sensitive and specific markers for RA that are present early in the course of the disease is also receiving considerable attention (Le Goff et al., 1997).

Isolation of autoantigens recognized by sera from patients with autoimmune diseases has been traditionally carried out through time-consuming processes by either immunoprecipitation of autoantigens or by immuno-screening of cDNA expression libraries constructed with plasmid or phage vectors. To identify autoantigens more efficiently, Maruyama et al. have developed a surface display vector based upon bacteriophage lambda, in which an antigen protein encoded by cDNA is produced as a chimeric fusion to the phage coat proteins (Maruyama et al., 1994; Mikawa et al., 1996). Libraries constructed with the vectors can be searched for clones of interest by simultaneously using many different probes immobilized with beads or in microtiter wells (Kuwabara et al., 1997; Moriki et al., 1999).

In this chapter, I describe the application of the lambda phage surface display to the isolation of autoantigens by using RA patient SF and sera as probes. Using 48 sera and 40 SF from RA patients as probes, I have screened synovial tissue and synovial sarcoma cell line, SW982 cDNA libraries constructed with λfoo, and have found four novel autoantigens that are previously unanalyzed in any autoimmune diseases, including an unknown protein.

Materials and Methods

Tissue, cell lines and sera

Synovial tissue from RA patients was provided from Nara Medical University, Nara, Japan. Human synovial sarcoma cell line, SW982, was obtained from American Type Culture Collection (Manassas, VA), and maintained in Leibovits'-15 medium (Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) fetal calf serum (Life Technologies), 2 mM L-glutamine, 100 units/ml penicillin-G and 100 μg/ml streptomycin sulfate in an air incubator in the absence of added CO_2. Sera and SF from patients with RA were provided by Scripps Reference Laboratory, The Scripps Research Institute, La Jolla, California, and by the laboratory serum bank of Nara Medical University. The following sera were also from Nara Medical University; SLE, SjS, SSc, mixed connective tissue disease (MCTD), osteoarthritis (OA) and sera from healthy persons. Diagnoses were made according to a review of clinical charts and information from the attending physicians, and were based on American College of Rheumatology criteria and on SjS classification criteria.

cDNA library construction

Total RNA was prepared from 3 g of synovial tissue from RA patients or ~10^8 SW982 cells by using an RNeasy kit (Qiagen, Valencia, CA), according to the manufacture's protocol. General procedures for poly(A)$^+$ RNA preparation, cDNA library construction were as previously described in chapter 1. Synovial tissue and SW982 cDNA libraries consisted of 3.3 x 10^6 and 1.5 x 10^7 pfu of recombinants with cDNA inserts, respectively, when analyzed by PCR.

Affinity selection of cDNA library

Detailed procedures for affinity selection of the phage libraries were previously described in chapter 1. As probes, patient sera or SF were used after diluted to 1/1000 with PBS and immobilized in microtiter wells. To remove RF from sera or SF prior to immobilization, 20 µl immunoglobulin-G (IgG)-agarose (9.8 mg protein/ml resin; Sigma, St. Louis, MO) was added to the diluted sera or SF and incubated with mild agitation for 16 h in a cold room. Furthermore, to prevent interaction between RF and cDNA clones expressing the Fc domain of IgG, 0.5 µl of recombinant human IgG-Fc protein (12 mg/ml; ICN Pharmaceuticals, Aurora, OH) was added to binding buffer.

Analysis of phage clones

Procedures for phage plaque staining, plaque hybridization, DNA sequencing of cDNA inserts were previously described in chapter 1.

Phage ELISA

ELISA was carried out by using phage particles expressing antigen proteins on the surface. This method called 'phage ELISA' has proved to be as sensitive and specific as ELISA using purified antigen proteins in chapter 1. Detailed procedures for phage ELISA were as previously described in chapter 1. Positive sera were determined when the values were higher than 3 x SD of reference sera.

Results

cDNA library screening

I constructed a cDNA library from synovial tissues of patients with RA, which is a primary target of T- and B-lymphocytes in RA. Using 48 sera and 40 SF from RA patients as probes, the synovial tissue cDNA library was searched for antigens. After two to four selection cycles, most of the sera and SF enriched for cDNA clones encoding the Fc fragment of IgG. This is not surprising because synovial tissues from RA patients become infiltrated by lymphocytes and because most of sera and SF from RA patients contain RF. Therefore, I treated the sera and SF with IgG-agarose beads to remove RF before immobilizing in microtiter wells. During the affinity selection, purified human IgG-Fc proteins were also added to binding buffer in order to inhibit interaction between RF and cDNA clones encoding Fc. By these treatments, the enrichment of the Fc clones was dramatically decreased to less than 1% of that without the treatment. This improved approach allowed us to isolate five different cDNA clones encoding two proteins previously unknown to be recognized by RA sera, type-1 nuclear mitotic apparatus protein (NuMA1) and muscle myosin heavy chain, and three proteins known to be recognized by RA sera, branched chain 2-oxo-acid dehydrogenase complex-E2 (BCOADC-E2), La/SSB and IgG (Table 3). These proteins have previously been analyzed as autoantigens in other autoimmune diseases; NuMA1 in SLE (Andrade et al., 1996), myosin heavy chain in acute rheumatic fever (Horsfall et al., 1992), BCOADC-E2 in PBC (Miyakawa et al., 1999), La/SSB in SjS (Tan, 1989), and IgG in RA and other autoimmune diseases (Smolen et al., 1998).

To eliminate cDNA clones derived from lymphocytes, I also constructed a cDNA library from SW982 cells. Using 44 sera and 22 SF, I performed a single-cycle affinity selection, by which preferential enrichment for abundant cDNA clones with higher affinity

for autoantibodies may be minimized. This selection allowed us to isolate eight different clones; a clone encoding an unknown protein, three clones encoding previously unknown to be recognized by RA sera and four clones known to be recognized by RA sera (Table 3). DNA sequence analysis revealed that the clone Fw29a1 (GenBank accession no. AY557618) encodes an unknown protein matched with a genomic sequence on chromosomes X (Fig. 6). Three proteins, insulin-like growth factor binding protein-4 (IGFBP-4) (Matsumoto et al., 1996) and sorcin (Maki et al., 2002), small humanin like peptide-1 (SHLP-1) (Cobb et al., 2016) encoded by Nw8a1, Sw28b1 and Fw24d2, respectively, have not previously been reported as autoantigens in any autoimmune diseases. The other four clones encoded proteins previously known to be recognized by RA sera and other autoimmune sera with higher frequencies; PDC-E2 in primary biliary cirrhosis (Jones, 2000), CENP-B in SLE (Russo et al., 2000), and 52- and 60-kd Ro/SSA in SjS (Lopez-Longo et al., 1994).

Sensitivity and specificity of isolated autoantigens for RA

Among the 13 different antigens isolated, four had not previously been analyzed for their sensitivity and specificity for RA. Using panels of sera from 48 healthy persons and 48 patients with RA or other rheumatic diseases (16 SJS, 16 SLE, 9 SSc, 3 MCTD, and 4 OA), I measured frequencies of the novel antigens recognized by the panels. Frequencies of sera that recognize the novel four autoantigens encoded by Fw24d2, Sw28b1, Nw8a1, and Fw29a1, were ranged from 12.5 to 24.0% of a panel of RA sera, and 0 to 4.2% of other rheumatic disease sera. Interestingly, each of these novel autoantigens has higher frequencies by sera from RA patients than by sera from healthy persons or patients with other rheumatic diseases (Table 4).

Discussion

In this study using the lambda phage display, I have successfully isolated cDNA clones that encode novel autoantigens recognized by sera from patients with RA. Each of these novel autoantigens has higher frequencies by sera from RA patients than by sera from healthy persons or patients with other rheumatic diseases, but the sensitivity of these antigens are not as high as to substitute for RF, which is using as a maker for RA in criteria. However, possibilities remain that these novel antigens may use for activity markers or complementary markers of RF.

One of the cDNA clones that encode novel autoantigens, encodes a 20/24 amino acid polypeptide of SHLP-1, which is a member of the family of small humanin-like peptides (SHLPs) (Cobb et al., 2016). Among the SHLP family, humanin is known as a potent neuroprotective factor (Hashimoto 2001), but the function of SHLP-1 is still unclear. Humanin and SHLP-2 are reported to be decreased in age-related diseases, such as Alzheimer's disease and RA (Cobb et al., 2016). I speculate that the autoantibody against SHLP may reduce SHLP in sera from RA.

Another clone encodes sorcin, which is a penta-EF hand calcium binding protein, which participates in the regulation of calcium homeostasis in cells. Sorcin regulates concentration of calcium ion in the endoplasmic reticulum (ER), allows high levels of calcium in the ER to be maintained, preventing ER stress (Colotti et al., 2014). It is not clear why the autoantibody against sorcin is produced, and the involvement with pathological condition. However, it has been reported that Calnexin, BiP and Grp94 which are calcium binding chaperones in ER, were identified as novel autoantigens in RA and SLE (Weber et al., 2010). Thus, ER-residential calcium binding protein may raise an autoantibody in RA.

Among the novel antigens isolated, IGFBP-4 is of particular interest. Although sensitivity of this antigen is not high, its specificity is very high, and none of sera from other rheumatic diseases and healthy persons recognize the antigen. IGFBP-4 is a potent inhibitor of IGF actions in bone cells (Schiltz et al., 1993, Zhou et al., 2003), which is the most abundant growth factors produced by bone cells and stimulate both bone-cell proliferation and differentiation (Rosen et al., 1994). However, inhibitor action of IGFBP-4 is reassessed because IGFBP-4 knockout mice result in a reduction in body mass (Conover, 2008). The interpretation of these findings is that IGFBP-4 play a role as a reservoir for IGF. The bioactive IGF is released from this reservoir by IGFBP-4 specific protease (Durham et al., 1995). On the other hand, it is reported that a concentration of IGFBP-4 is dramatically increased in RA SF (Matsumoto et al., 1995). The increase of IGFBP-4 in RA SF may be partly due to the antibodies specific to the antigen that may protect a cleavage site by the protease. Thus, specific antibodies to IGFBP-4 detected in sera from RA patients may partly explain RA pathogenesis.

Despite isolation of many cDNA clones encoding antigens recognized by RA sera, I have failed to isolate many other clones encoding proteins previously identified as autoantigens with higher sensitivity and/or specificity for RA, such as filaggrin and collagen. If the specificity of antiperinuclear factor and antikeratin antibodies is directed to citrulline, an amino acid derived by citrullination of arginine and present in large quantities in epidermal filaggrin (Smolen et al., 1998), it may be difficult to isolate cDNA clones encoding filaggrin through the bacteriophage display.

Conclusion

In this study using the lambda phage display, I have successfully discovered four novel autoantigens that have not previously been reported as autoantigens in any autoimmune diseases. Besides, it was revealed that these novel autoantigens are specific for RA. However their specificity for RA is very high, sensitivity of these antigens are not high to substitute for RA in criteria. The approach employed in this work may be an economical and efficient alternative to the conventional approaches to the isolation of cDNA clones encoding autoantigens, more screenings for novel antigens are needed to discover a better marker of RA than RF.

Figure and Tables

Fw29a1

```
TTTAAGACTGACGTTAGTAACCCAGCTCAGAACAGGACCAGAGGGATGGACCGGGATGAC
PheLysThrAspValSerAsnProAlaGlnAsnArgThrArgGlyMetAspArgAspAsp

AGGAAAACTGGAACTTGTGTAACAGACACTAAAAAAACTAGGTGTCAGCGTTTTCTGGAA
ArgLysThrGlyThrCysValThrAspThrLysLysThrArgCysGlnArgPheLeuGlu

GAGGAACTCTGGGTGGAATGATCAGTGTGTAACAGGATATAAAAATGGAAGCTTCTACAG
GluGluLeuTrpValGluSTOP (46 a.a.)

AGACACTGAGCTACAATAAATGTGAAATTTCAGAGTCAGGTGCCAAATCGCCATATATAG
ATTTACAAAAGAAATGGTTCCCGGGCCTGTTCAGCGACTCTGGAGGGTCTCAGCCAGATC
AAAGATAGGAAAGGTCAGAGAGCGAGAAAGGGGAAATAGAGGAAGAGATTTAGGGGGAAG
GGGTAGTAGCTGTGGTGTCTAAGACCCCACCCAAGCCCTTAAAATGAAAGGTTATATGGG
GAAGGATGTATGTGGGAAGGTTTTACCTCTTAACCAAAGTCTAATGAGGGATCCGTCTGA
GCCTGTCTCCTCACCCCCACTCTCCCTACCCTGGCCCTCAAAGGAGGGCTTCTTTACAGT
TAATTGATTTCTTGGAATGGTCCCAAGGGAC (571 bp)
```

Fig. 6 Nucleotide sequence of a cDNA insert of a clone, Fw29a1, which encodes a novel protein. Its amino acid sequence was also deduced and shown underneath the nucleotide sequence.

Table 3

Summary of cDNA clones isolated

cDNA lib.	Clone	Insert size, bp	Antigen	Coding region [a]
Synovial tissue				
Previously unknown to be recognized by RA sera				
	RA123	701	NuMA1	627^{th}-859^{th}
	RA128 [b]	347	Myosin heavy chain	75^{th}-189^{th}
Known to be recognized by RA sera				
	RA114	392	BCOADC-E2	136^{th}-255^{th}
	RA143	968	La/SSB	1^{st}-314^{th}
	RA4	737	IgG	253^{rd}-473^{rd}
SW982 cell				
Unknown protein				
	Fw29a1 [b]	571	on chromosome X	46 a.a.
Previously unknown to be recognized by RA sera				
	Fw24d2 [b]	60	SHLP-1	5^{th}-24^{th}
	Nw8a1	354	IGFBP-4	7^{th}-124^{th}
	Sw28b1	480	Sorcin	7^{th}-167^{th}
Known to be recognized by RA sera				
	Fw19a1 [b]	681	PDC-E2	50^{th}-276^{th}
	Sw8b1	453	CENP-B	464^{th}-599^{th}
	Sw17j1	1735	60-kd Ro/SSA	1^{st}-554^{th}
	Sw17k1	665	52-kd Ro/SSA	1^{st}-215^{th}

[a] Protein fragments encoded by cDNA inserts that are indicated by position of the first and last amino acid (a.a.) residues or length for an unknown protein encoded by Fw29a1.

[b] Isolated by using SF from RA patients.

Table 4

Sensitivity and specificity of autoantigens for RA

Clone	Antigen	Sera [a] from patients with		
		RA (%)	Other RD (%)	Healthy control (%)
Sw28b1	Sorcin	7/48 (14.9)	1/48 (2.1)	0/48 (0)
Nw8a1	IGFBP-4	6/48 (12.5)	0/48 (0)	0/48 (0)
Fw24d2	SHLP-1	6/25 (24.0)	0/48 (0)	not tested
Fw29a1	unknown	9/44 (18.8)	2/48 (4.2)	0/48 (0)

[a] Sera from patients with RA or other rheumatic diseases (RD) were provided by the laboratory serum bank of Nara Medical University, Nara, Japan, and the Reference Laboratory, Scripps Research Institute, La Jolla, CA.

General Discussion

In this study using the λfoo phage display technology, I have successfully isolated cDNA clones encoding many cytoplasmic autoantigens recognized by sera or SF from patients with SjS or RA.

In chapter-1; I isolated seven phages encoding four proteins previously identified as SjS autoantigens, namely SS-B/La, hRPA-70, NOR-90, and Ki-67 antigen, and three proteins, SMN, pM5, and TFG, previously not known as autoantigens in any autoimmune disease.

Regarding the four proteins previously known as SjS autoantigens, two clones encoded epitopes of SS-B/La or NOR-90 previously found by SjS patient sera and the other two clones encoded novel epitopes of RPA-70 or Ki-67. These clones expressed polypeptides of a certain length, not short peptides (less than 20 amino acids length), so that these epitopes may be conformational epitopes. Actually, an epitope of SS-B/La has previously been demonstrated to be conformational (McNeilage et al., 1992) and that epitope is consistent with the region found in this study. Furthermore, many proteins have previously been produced that display their function on the surface of the λfoo phage. Therefore, it is suggested that many proteins expressed on the surface of the λfoo phage retain their native conformation and function.

I also isolated phages expressing three novel autoantigens, SMN, pM5, and TFG, previously not known as autoantigens in any autoimmune disease. The clone encoding the amino-terminal region of TFG only reacted with one serum from a healthy donor and appears not to be a disease autoantigen. The other two clones, encoding SMN and pM5, reacted with more than one SjS serum, however, but not with any serum from healthy donor, so these two proteins may be novel specific autoantigens in SjS.

pM5, also known as nodal modulator 1, was previously isolated from an A2058 melanoma, but the function of pM5 is still not understood well. The survival motor neuron gene product (SMN), which is known as the product of a spinal muscular atrophy (SMA)-determining gene (Lefebvre et al., 1995), is expressed ubiquitously in all cell types, and is found in both cytoplasm and the nucleus. It has been reported that SMN forms macromolecular complexes, termed SMN complex, that are involved in the assembly of small nuclear ribonucleoprotein complexes (snRNPs). This snRNP is known as an autoantigen against anti-Sm autoantibody, one of the represented anti-nuclear autoantibodies detected in SLE. It has often been reported that antibodies are induced against each component of complex proteins, thus the anti-SMN antibody may be a novel anti-nuclear antibody, produced together with the production of anti-Sm autoantibody.

In chapter-2; I have successfully isolated cDNA clones that encode novel autoantigens recognized by SF or sera from patients with RA.

L. J. Cobb et al. revealed new small mRNAs transcribed from mitochondrial DNA and named small humanin-like peptides (SHLPs) (Cobb et al., 2016). Humanin is composed of 24 amino acids, and is the best-characterized potent neuroprotective factor known to be a therapeutic agent for Alzheimer's disease (Hashimoto 2001). SHLP-1 is a member of the family of these small mitochondria-derived peptides, a 24 amino acid polypeptide. SHLP-1 is expressed at a relatively high level in the heart, kidney, and spleen. However, although SHLP-2 and -3 are known to share similar protective effects with humanin, the role of SHLP-1 is still unknown. Humanin and SHLP-2 are reported to be involved in age-related diseases, such as Alzheimer's disease. RA is also an age-related disease, and the SHLP family may be involved in the same manner.

Sorcin is expressed in most human tissues at high levels, in bone, heart, B- and T-lymphocytes, monocytes, kidney, breast, and skin. Sorcin is found ubiquitously, in the

nucleus, cytosols, plasma membranes, and the endoplasmic reticulum (ER). Sorcin participates in the regulation of calcium homeostasis. In particular, sorcin increases the accumulation of calcium (Ca^{2+}) in the ER by activating sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) and by inhibiting ryanodine receptor (RyR) (Colotti et al., 2014). Abnormal Ca^{2+} homeostasis in the ER, e.g., Ca^{2+} depletion, causes ER stress and activates a series of adaptive reactions known as the unfolded protein response (UPR). However, although the problem remains whether autoantibody can access intracellular protein through the cytoplasmic membrane, the anti-sorcin autoantibody may induce the UPR and play a role in the progression of RA. Recently, it has been reported that some calcium binding chaperones in ER are identified as novel autoantigens in RA and SLE (Weber et al., 2010). Therefore, autoantibodies against calcium binding ER-resident chaperones may involve with RA.

In this study, I have also isolated a phage, encoding the N-terminal (7^{th}-124^{th}) of IGFBP-4. Insulin-like growth factors (IGFs) are the most abundant growth factors stored in bone. They stimulate both bone cell proliferation and differentiation, and therefore play an important role in the regulation of bone formation (McCarthy et al., 1989; Rosen et al., 1994). In recent years, it has become known that the effects of IGFs in the bone are regulated by IGFBPs, which are six members of a single protein family (IGFBP-1–6) (Rajaram et al., 1997). IGFBPs bind IGFs with an affinity equal to or greater than the binding of IGFs to their receptors, thus IGFBPs have been considered inhibitors of IGFs (Jones et al., 1995). In general, IGFBPs act in the serum to transport and stabilize IGFs, and as a result prolong the half-lives of IGFs (Mohan et al., 2002). About 75% of the IGFs circulate in serum as a 150 kDa complex, which consists of IGF-I/IGF-II, IGFBP-3, and an acid-labile subunit (ALS) (Mohan et al., 1996). A part of IGFBP-5 also makes this ternary complex, while other IGFBPs are found in a smaller 50 kDa binary complex with IGFs. The larger 150 kDa ternary

complex cannot cross the endothelial barrier, but the smaller 50 kDa binary complexes are able to pass through blood vessels (Rajaram et al., 1997). IGFBP-4 has also been shown to inhibit IGF actions *in vitro*, as do other IGFBPs (Jones et al., 1995; Mohan et al., 1995). However, systemic administration of IGFBP-4 at pharmacological doses caused a significant increase in bone formation *in vivo* (Miyakoshi, 2001). They speculated that IGFs may be shifted away from IGFBP-3 toward IGFBP-4 due to the systemic administration of IGFBP-4, followed by an increase of smaller 50 kDa binary complex. The binary complex of IGF-I/IGFBP-4 may exit the blood circulation and deliver IGF-I into the bone. Finally, IGFBP-4 is proteolytically cleaved, releasing free IGFs, which stimulate bone cell proliferation and differentiation (Fig. 7). It is indicated that autoantibody against IGFBP-4 exists in the serum of RA patients. I speculate that anti-IGFBP-4 autoantibody prevents the transfer of IGF-I from IGFBP-3 to IGFBP-4. As a result, IGF-I will not be delivered into the bone, bone formation will be inhibited, and finally articular destruction will occur. In fact, the autoantibody found in this study recognizes N-terminal (7^{th}-124^{th}) of IGFBP-4, which is consistent with the binding region of IGF-I; therefore the antibody competitively prevents IGF-I binding to IGFBP-4. Thus, the autoantibody against IGFBP-4 may participate in the progression of RA.

In this study, to begin I could prove that cytoplasmic proteins can be selected as autoantigens of autoimmune diseases from a cDNA phage display library made using the λfoo phage vector; besides, some novel autoantigen/autoantibody sets could be found. Furthermore, I speculate that some novel autoantigens may involve with clinical manifestation. I expect that like these findings are useful to pursue the cause of production mechanism of autoantibody and relationship with clinical manifestation, lead the clue to the solution of autoimmune diseases.

Besides, cDNA libraries constructed with λfoo may also be searched efficiently for proteins physically interacting with macromolecules including proteins, DNA, RNA, and polysaccharides immobilized on the surface of solid matrices such as microtiter wells or agarose beads. Therefore, they are expected to exert power in searching for not only antigen/antibody sets, but also in elucidating the interaction between biological molecules such as ligands and receptors or a protein complex. Furthermore, I suggest that describing and efficiently discovering new antigens or ligand/receptor couples like in this study, may increase opportunities for elucidating the protein-protein interaction in the cell and its etiology.

However, bacteriophage expression vectors including λfoo may not be efficient for epitopes that are post-translationally modified by such a mechanism as citrullination, glycosylation or phosphorylation. It is my aim to develop a phage display method in eukaryotes in the future.

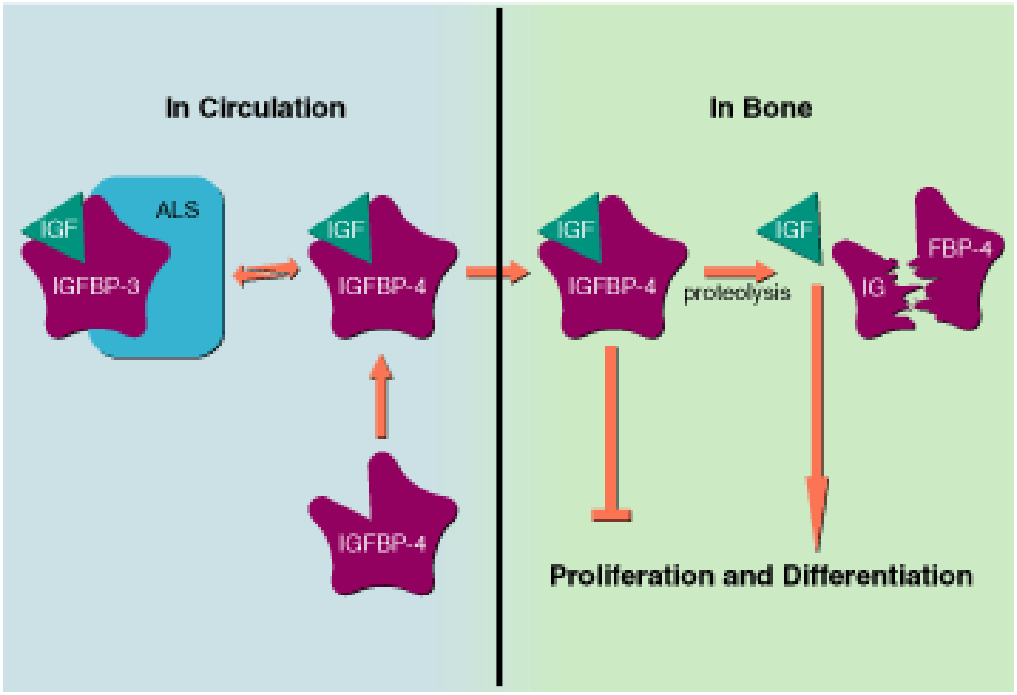

Fig. 7 IGFBP-4 takes up IGF from IGFBP-3 in blood and moves into bone tissue. This figure was cited from R&D Systems website (https://www.rndsystems.com/resources/articles/igfbp-4-and-bone)

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