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Simple Screening Method for Autoantigen Proteins Using the N-Terminal Biotinylated Protein Library Produced by Wheat Cell-Free Synthesis

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Abstract: Autoimmune diseases are a heterogeneous group of diseases characterized by immune reactions against either a major or a limited number of the bodies own autoantigens, causing inflammation and damage to tissues and organs. Thus, identification of autoantigens is an important first step to understanding autoimmune diseases. Here we demonstrate a simple screening method for identification of autoantigens reacting with patient serum antibodies by combination of an N-terminal biotinylated protein library (BPL), produced using a wheat cell-free protein production system, and a commercially available luminescence system. Optimization studies using well-characterized autoantigens showed specific interactions between N-terminal biotinylated proteins and antibody that were sensitively detected under homogeneous reaction conditions. In this optimized assay, 1 μL of the translation mixture expressing the biotinylated proteins produced significant luminescence signal by addition of diluted serum between 1:500 and 1:10 000 in 25 μL of reaction volume. For the BPL construction, 214 mouse genes, consisting of 103 well-known autoantigens and 111 genes in the mouse autoimmune susceptibility loci, and the sera of MRL/lpr mouse were used as an autoimmune model. By this screening method, 25 well-known autoantigens and 71 proteins in the loci were identified as autoantigen proteins specifically reacting with sera antibodies. Cross-referencing with the Gene Ontology Database, 26 and 38 of autoantigen proteins were predicted to have nuclear localization and identified as membrane and/or extracellular proteins. The immune reaction of six randomly selected proteins was confirmed by immunoprecipitation and/or immunoblot analyses. Interestingly, three autoantigen proteins were recognized by immunoprecipitation but not by immunoblot analysis. These results suggest that the BPL-based method could provide a simple system for screening of autoantigen proteins and would help with identification of autoantigen proteins reacting with antibodies that recognize folded proteins, rather than denatured or unfolded forms.

Keywords: autoantigen • autoimmunity • biomarker • cell-free protein production • Gene Ontology • high-throughput screening • MRL/lpr mouse • proteomics

Introduction

Autoimmune diseases are generally characterized by the body's immune responses being directed against its own tissues, causing prolonged inflammation and subsequent tissue destruction.[1] A hallmark of autoimmune diseases is the production of autoantibodies such as antinuclear, anti-Sm and anti-dsDNA in systemic lupus erythematosus (SLE),[2] and the presence of RF, hnRNP A2 and calpastatin in rheumatoid arthritis (RA).[3] However, there are still a lot of autoimmune diseases for which antibodies have not been identified.[2] To understand the molecular mechanisms in autoimmune diseases, it is important to identify the relevant autoantigens, and moreover, they could be pathogenic in these diseases. It is widely hypothesized that proteins are the major antigenic targets associated with autoimmune diseases.[2] Therefore, development of methods that allow large-scale screening of autoantigen proteins is indispensable for elucidation and diagnosis of the autoimmune diseases.

To date, autoantigen proteins have been detected as antigenic molecules that are recognized by humoral antibodies, including those in serum.[2] The large-scale screening of au-

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toantigen proteins reacting with patient serum antibodies has been carried out by mainly three technologies: serological proteome analysis (SERPA), serological expression cloning (SEREX) and protein microarray.[4] The utility of SERPA and SEREX for this screening is limited because particular cells and tissues are generally used as antigen resources in these systems and they are dependent on artificial membranes for immunoblotting which do not maintain native protein structure.[5] Recent advances in protein microarray technology have allowed large-scale screening of autoantigens reacting with the sera of patients suffering from autoimmune disorders and cancer.[5−7] However, protein microarray is not yet a commonly used biochemical tool for screening.[8] One issue with protein microarrays is that purified recombinant proteins are required, which demonstrate batch-to-batch variation and limited stability and shelf life.[5] Additionally, it is difficult to maintain the functional form of a protein after their immobilization on a microplate. Many proteins needed to be appropriately oriented for proper functioning.[9] In fact, a number of spotted autoantigens were not always detectable with planar arrays, presumably due to loss of three-dimensional structures, steric interference or electrostatic repulsion.[6]

In this work, we developed a novel autoantigen protein screening method that overcame the following issues highlighted above: (1) utilization of a high-throughput and genome-wide protein expression system, (2) specific protein labeling for assay using unpurified protein samples and (3) high-throughput detection system of properly folded antigen. Toward the first, we recently developed an automated protein production robot utilizing a high-throughput wheat embryo derived cell-free protein production system.[10,11] The combination of an automatic cell-free protein production system and the full-length cDNA allowed for facile construction of a robust protein library.[12] To enhance the utility of the library, per the second issue above, specific labeling of each protein is required for efficient detection. We selected biotin as the labeling compound because it is readily available and demonstrates high specificity for streptavidin binding. The biotinylated protein library (BPL) was constructed using target proteins fused to a biotin ligation site (bls), and expression was performed in the presence of biotin and biotin ligase (BirA).[13] BirA from *Escherichia coli* specifically conjugates a single biotin on the bls. This method was compatible with our high-throughput automated platform. To address the third issue, we selected the luminescent high-throughput protein−protein interaction detection system AlphaScreen.[14,15] This method can directly recognize biotinylated protein in the translation mixture without purification and the use of any potential protein denaturants allowing for antibody detection of natively folded antigens.[15] In this work, we demonstrate a simple BPL-based method for screening of autoantigen proteins reacting with the sera of an autoimmune disease model mouse, MRL/Mp-*lpr*/*lpr* (MRL/lpr), and the detection of the autoantigen proteins by immunoprecipitation, rather than immunoblotting methods often accompanied by protein denaturation.

Materials and Methods

General. The following procedures have been either described in detail or cited previously:[10,16] generation of DNA template by polymerase chain reaction (PCR) using "split-primer"; synthesis of mRNA and protein in parallel; estimation of the amounts of synthesized proteins by densitometric scanning of the Coomassie brilliant blue (CBB)-stained bands or by autoradiography. The wheat germ extract was purchased from Cell-Free Science Co. (Yokohama, Japan). Anti-p53 monoclonal antibody (D01) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse serum for mouse immunoglobulin in Figure 1 was purchased from Calbiochem (Darmstadt, Germany). Other reagents used in this study were described previously.[10,15]

Serum Samples. MRL/lpr mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). All of the mice used in this paper were maintained in clean rooms at the Animal Research Institute, School of Medicine, Ehime University. Sera of female mice were collected from 15 mice and pooled and stocked in −20 °C until use. All experiments were done according to the Guidelines for the Care and Use of Laboratory Animals at Ehime University.

DNA Template Construction for the BPL. Functional annotation of mouse (FANTOM) as a mouse full-length cDNA resource is purchased from a company (Danaform, Tokyo, Japan). The DNA templates for transcription were constructed by "split-primer" PCR technique described previous reports.[10,17] The first PCR was amplified with 10 nM of each of the following primers: a gene specific primer, 5′- CCACCCACCACCACCAAT-Gnnnnnnnnnnnnnnnnnnn (n denotes the coding region of the target gene), and AODA2303 (5′-GTCAGACCCCGTAGAAAAGA) or AODS (5′-TTTCTACGGGGTCTGACGCT). The second PCR products for protein synthesis were constructed with 100 nM SPu 5′-GCGTAGCATTTAGGTGACACT, 1 nM deSP6E02bls-S1 (5′-GGTGACACTATAGAACTCACCTATCTCTCTACACAAAACA-TTTCCCTACATACAACTTTCAACTTCCTATTATGGGCCTGAAC-GACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAACTCCA-CCCACCACCACCAATG) and 100 nM AODA2303 or AODS. By this "split-primer" PCR, the bls was fused onto the N-terminals of all the genes for protein biotinylation.[13]

Construction of the BPL by the Cell-Free Protein Synthesis System. Cell-free construction of the BPL is based on the previously described bilayer diffusion system in which 1 μL (50 ng) crude cell-free expressed BirA was added to the translation layer and 500 nM D-biotin (Nacalai Tesque, Kyoto, Japan) was added to both the translation and substrate layers.[13,18] *In vitro* transcription and cell-free protein synthesis for the BPL were carried out using the GenDecoder1000 robotic synthesizer (CellFree Sciences Co.) as previously described.[17,19]

Detection of Biotinylated Protein−Antibody Reaction by Luminescence Method. The AlphaScreen assay was performed according to the manufacture's protocol (PerkinElmer Life and Analytical Sciences, Boston, MA). Reactions were carried out in 25 μL of reaction volume in 384-well Optiwell microtiter plates (PerkinElmer Life and Analytical Sciences). For the antigen−autoantibody reaction, the translation mixture expressing the biotinylated protein was mixed with MRL/lpr mouse serum diluted 1:600 in 15 μL of reaction buffer [100 mM Tris-HCl (pH 8.0), 0.01% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin] and incubated at 26 °C for 30 min. Subsequently, 10 μL of streptavidin-coated donor beads and protein A-conjugated acceptor beads (PerkinElmer Life and Analytical Sciences) were added to a final concentration of 20 μg/mL per well and incubated at 26 °C for 1 h in a dark box. Fluorescence emission was measured with the EnVision plate reader (PerkinElmer Life and Analytical Sciences), and the resultant data were analyzed using the AlphaScreen detection program. All repetitive mechanical procedures were performed by a Biomek FX robotic workstation (Beckman Coulter, Fullerton, CA).

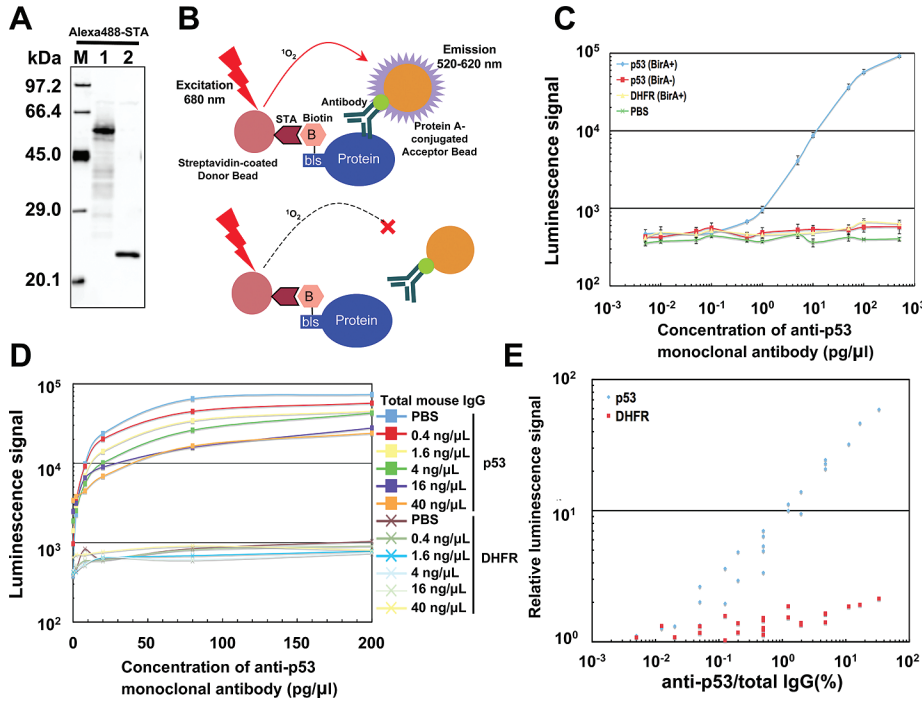

Figure 1. Sensitivity and specificity for detection of biotinylated p53 protein−antibody complex. (A) Biotinylated p53 (lane 1) and dihydrofolate reductase (DHFR) (lane 2) was detected by immunoblotting analysis using Alexa488-STA. M indicates protein molecular weight marker. (B) Schematic diagram of detection of biotinylated protein−antibody interaction by luminescence analysis. When a biotinylated protein and antibody interact (upper panel), Protein A-conjugated acceptor beads bound to antibody and streptavidin (STA)-coated donor beads bound to biotinylated protein are in close proximity. Upon excitation at 680 nm, a singlet oxygen is generated by the donor beads, transferred to the acceptor beads within 200 nm, and the resultant reaction emits light at 520−620 nm. This emission is measured using an EnVision. (C) Detection sensitivity of the antibody concentration measured by luminescence analysis. Translation mixture (1 μL) expressing biotinylated or nonbiotinylated p53 protein and biotinylated DHFR were incubated with various concentration of monoclonal antibody from 5×10^{-3} to 5×10^{2} pg/μL. (D) Biotinylated protein−antibody complex by interaction between biotinylated p53 protein and the monoclonal antibody in the presence of mouse serum was detected by luminescence analysis. (E) Minimum IgG amount in the presence of mouse serum to detect biotinylated p53 proteins. The relative luminescence signals between the specific luminescence and background signals indicated in the *y*-axis.

Immunoblotting. Biotinylated proteins were partially purified using streptavidin-coated beads (Streptavidin Sepharose High Performance, GE Healthcare, Buckinghamshire, U.K.). Translation mixtures (150 μL) including biotinylated proteins were mixed with 10 μL of streptavidin-coated beads for 30 min. The resin was washed three times with PBS buffer and then boiled in 15 μL of SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.2% bromophenol blue). After separation by 12.5% SDS-PAGE, the proteins were transferred to PVDF membrane (Millipore, Bedford, MA) by semidry blotting. The membrane was soaked in PBS containing 5% (w/v) skim milk for 1 h and then incubated with serum diluted 1:200 in PBS containing 0.1% (v/v) Tween 20 (PBST) for 1 h. After washing three times in PBST, it was incubated in PBS including goat-antimouse IgG-HRP antibody (GE Healthcare) diluted 1:10 000 for 30 min. After washing three times in PBST, the blots were detected by the ECL plus detection system (GE Healthcare) by using Typhoon 9400 imaging system (GE Healthcare) according to the manufacturer's protocol.

Immunoprecipitation. Fifty microliters of translation mixture expressing biotinylated proteins were incubated in 50 μL of IP buffer [PBS containing 0.1% (w/v) BSA, 0.15% (v/v) Tween 20] with 1 μL of undiluted serum overnight at 4 °C. Immobilized Protein A sepharose (20 μL of 50% slurry, Protein A Sepharose 4 Fast Flow, GE Healthcare) in IP buffer was added to each sample and incubated for 60 min at 4 °C. After centrifugation for 1 min at 900× *g*, samples were washed three times with IP buffer and then boiled for 5 min in SDS sample buffer. After separation by 12.5% SDS-PAGE, the samples were transferred to a Hybond-LFP PVDF membrane (GE Healthcare). After blocking with 5% (w/v) skim milk in PBS overnight at 4 °C, the membranes were soaked in PBS buffer containing 10 μg/mL streptavidin Alexa Fluor 488 conjugate (Alexa488-STA) (Invitrogen, Carlsbad, CA) and were washed three times with PBST. The biotinylated proteins on membrane were detected by Typhoon 9400 imaging system (GE Healthcare) according to the manufacturer's protocol.

Results

Sensitivity and Specificity for Detection of Antigen−Antibody Interaction Using Biotinylated p53 Protein. We adapted that AlphaScreen technology toward detecting interactions between antigen protein and antibody. To validate this technique, we used p53 protein, a well-characterized antigen protein.[20] Biotinylated or nonbiotinylated recombinant p53 and biotinylated recombinant dihydrofolate reductase (DHFR), serving as negative control, were synthesized by the wheat cell-free system (Figure 1A). For the analysis of antigen protein−antibody interaction, the translation mixture was used without any purification. In the AlphaScreen system, interaction of the biotinylated protein and antibody in sera results in a biotinylated protein−antibody complex that is captured simultaneously by the streptavidin-coated donor beads and the protein

A-conjugated acceptor bead. The resultant proximity of the acceptor and donor bead generates the luminescent signal upon irradiation at 680 nm. This is illustrated in Figure 1B.

For biotinylation of the target protein, the N-terminus of the target was fused to the bls, and the cell-free system was supplemented with BirA and biotin.[13] This biotin ligation method yields a biotin labeling on the bls, indicating a specific recognition of the target protein by AlphaScreen. To investigate the specificity and sensitivity of the antibody detection, translation mixtures expressing biotinylated or nonbiotinylated p53 protein were incubated with various concentrations of monoclonal antibody, ranging from 5×10^{-3} to 5×10^{2} pg/μL. This luminescence method specifically detected interaction of monoclonal antibody and the biotinylated p53 from the unpurified translation mixture, whereas nonbiotinylated p53 and biotinylated DHFR did not produce a significant luminescent signal (Figure 1C). In this condition, the biotinylated p53 was detected by anti-p53 antibody at concentrations as low as 0.5 pg/μL. Next, we investigated whether this luminescence method could detect the biotinylated protein−antibody complex in the presence of mouse serum. Translation mixture expressing biotinylated p53 protein was incubated with various concentrations of monoclonal antibody from 2 to 200 pg/μL and mouse immunoglobulin from 0.4 to 40 ng/μL. Figure 1D showed that this method could specifically detect the immunocomplex of biotinylated p53 protein and monoclonal antibody in the presence of an excess of mouse immunoglobulin. In addition, comparison between the specific luminescence and background signals indicated that biotinylated p53 could be detected at more than 0.05% of anti-p53 antibody in mouse serum (Figure 1E). These results indicate that this system is a highly specific and sensitive method for detection of interaction between biotinylated recombinant protein and antibody in whole serum.

Detection of Autoantibodies against Hars and LmnB2 Proteins in sera of MRL/lpr Mice. We next tested our protocol for the well-characterized autoantigens histidyl-tRNA synthetase (Hars)[21] and lamin B2 (LmnB2)[22] in the autoimmune disease model mouse MRL/lpr.[23] To determine the assay conditions using serum samples, biotinylated recombinant Hars and LmnB2 proteins were used (Figure 2A) to detect autoantibody in the sera of MRL/lpr mice. Cell-free synthesis of biotinylated Hars and LmnB2 demonstrated yields of 820 and 600 nM, and 43.0 and 56.4% of biotinylation, indicating biotinylated Hars and LmnB2 proteins were 354.4 and 338.5 nM, respectively. Various volumes (0.003 to 4 μL) of translation mixture expressing biotinylated Hars or LmnB2 protein were incubated with the serum of MRL/lpr mouse (final 1:1000 dilution) in 25 μL of reaction volume (Figure 2B). Significant luminescent signals were observed at additions of biotinylated Hars or LmnB2 proteins between 0.01 and 1 μL, which corresponds to biotinylated protein concentrations between 0.14 and 14 nM or 0.13 and 13 nM, respectively. Also serum dilutions between 1:500 and 1:10 000 produced high luminescence signal in 25 μL of reaction volume using 1 μL of the translation mixtures (Figure 2C). These results mean that five micro litter of serum and 200 μL of cell-free translation mixture expressing biotinylated proteins would be sufficient for 200 assays. Taken together, these results suggest that the luminescence method using cell-free expressed biotinylated proteins would be useful for screening the reaction of autoantigen proteins with autoantibodies in serum.

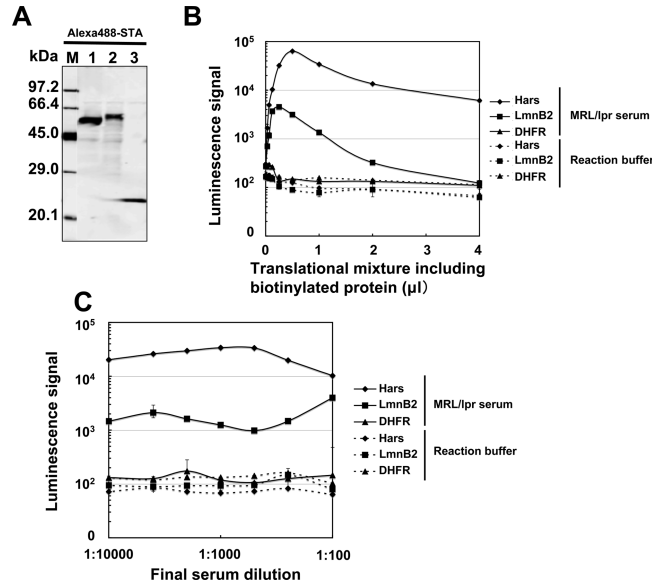

Figure 2. Detection of autoantibodies against Hars and LmnB2 proteins. (A) Biotinylated histidyl-tRNA synthetase (Hars) (lane 1), and lamin B2 (LmnB2) (lane 2) and DHFR (lane 3) proteins were detected by immunoblotting analysis using Alexa488-STA. M indicates protein molecular weight marker. (B) Various volumes (0.003 to 4 μL: representing 2.5−3280 nM Hars, 1.8−2400 nM LmnB2 and 6.4−8520 nM DHFR) of translation mixture expressing biotinylated Hars, LmnB2 or DHFR proteins were incubated with serum of MRL/lpr mouse sera (final 1:1000 dilution) in 25 μL of reaction volume. (C) Serum dilution between 1:100 and 1:10 000 was incubated with 1 μL of the translation mixtures in 25 μL of reaction volume.

Construction of the BPL by the Wheat Cell-Free Protein Production System. It has long been thought that comprehensive screening using a protein library is a strong tool for identification of antigen proteins.[12,24,25] The scheme for the BPL-based screening is shown in Figure 3A. To construct the N-terminal BPL, we selected 226 genes (Supplementary Table 1, Supporting Information) that included well-known autoantigen proteins and proteins coded by genes in the mouse autoimmune susceptibility loci[26] from the mouse full-length cDNA resource (FANTOM).[27,28] For biotinylation, a bls was fused onto 5′ site of a target gene by "split-primer" PCR.[13] Using the PCR, 222 (98.2%) out of 226 genes were successfully amplified and of those, 217 (96%) were transcribed. Synthesis of biotinylated proteins was performed on the GenDecoder1000,[19] and expression confirmed by SDS-PAGE combined with immunoblot analysis using Alexa488-STA (Figure 3B). Finally 214 clones (94.6%) were produced as biotinylated proteins (Supplementary Table 1, Supporting Information) at maximum and minimum concentrations of 500 and 10 nM respectively (data not shown). From our results in Figure 2B, the immunoresponse of biotinylated proteins could be detected below 0.2 nM by the luminescence method, indicating that all 214 proteins are at concentrations viable for screening. Therefore, we used these proteins as the BPL for screening of autoantigen proteins.

BPL-Based Screening of Autoantigen Proteins Using the MRL/lpr Mouse Sera. To identify autoantigen proteins reacting with antibodies in serum of autoimmune disease mice, the BPL and sera from pools of MRL/lpr or normal mouse sera (NMS)

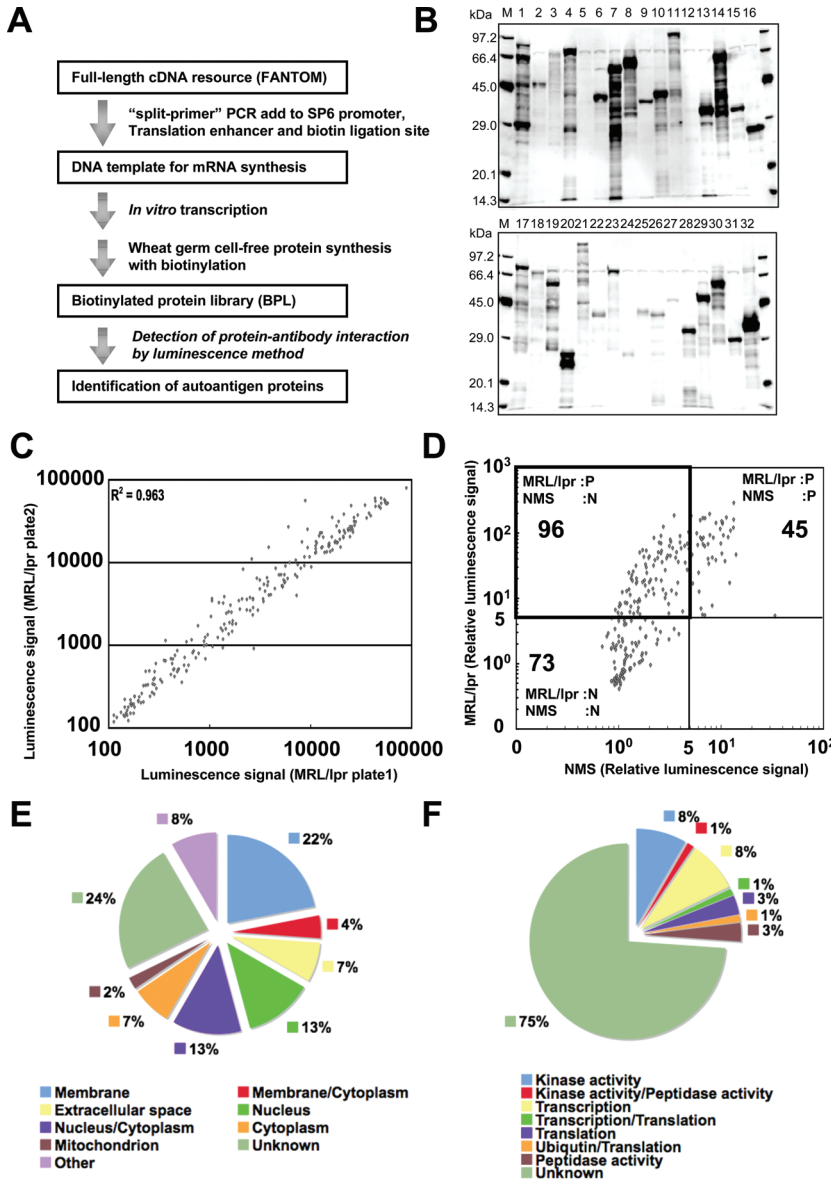

Figure 3. BPL-based screening of autoantigen proteins using the MRL/lpr mouse sera. (A) Schematic of the BPL-based screening method. (B) Thirty-two randomly selected biotinylated proteins of the BPL were detected by immunoblotting analysis using Alexa488-STA. (C) Scatter plot showing the luminescent signals in each well of two independent screening data sets using MRL/lpr mouse sera. The *x*-axis indicates luminescence signals in MRL/lpr plate 1 whereas the *y*-axis represents those in MRL/lpr plate 2. (D) Each data point represents luminescence signals using MRL/lpr mouse sera or normal mouse sera (NMS). The *x*-axis indicates luminescence signals in NMS whereas the *y*-axis represents those in MRL/lpr mice. (E, F) Ninety-six proteins identified as autoantigen proteins were grouped by protein localization in cells (E) Membrane (GO:0016020), Nucleus (GO:0005634), Cytoplasm (GO:0005737), Extracellular region (GO:0005576) and Mitochondrion (GO:0005739) and biological function/process (F) Kinase activity (GO:0016301), Peptidase activity (GO:0008233), Ubiquitin (GO:0005551), Translation (GO:0006412) and Transcription (GO:0006350) according to Gene Ontology Database. Minor groups less than 3 proteins were belonged to "Other" group. More detailed information on individual proteins was indicated in the Table 1.

were used. In each well of a 384-well plate, a translation mixture expressing biotinylated protein was incubated with either serum for 30 min, and subsequently a mixture of donor and acceptor beads was added to each well (see Figure 1B). After incubation, antigen−antibody reaction of the BPL was detected by the luminescence assay as described above. As shown in the scatter plot (Figure 3C), the intensity of paired luminescent signals in each well of two independent screening data sets (plate 1 and 2) using MRL/lpr mouse sera showed a linear distribution with a R^2 of 0.963, indicating reproducibly sufficient score for screening. We then compared the luminescent signals of the BPL reacted with MRL/lpr sera and NMS (Figure 3D). In this assay, only 141 of the 214 proteins in the BPL were identified as positive clones, which was indicated by a luminescence signal 5-fold higher than the average background signal. Only 96 proteins in the 141 positive clones reacted with MRL/lpr sera, whereas the remaining 45 proteins interacted with both sera. From these results, 96 proteins were identified as autoantigen proteins in MRL/lpr mice (Table 1, upper left panel in Figure 3D). In these MRL/lpr autoantigen proteins, 25 well-known autoantigens were included, and 71 out of 96 clones were coded in the genetic loci on chromosome 10; 40 cM, chromosome 15; 18 cM and chromosome 19; 49 cM,[26] indicating that this screening identified new MRL/lpr sera

Table 1. List of 96 Identified Proteins As Autoantigen Proteins

gene symbol	source[a]	M_w (kDa)	MRL/lpr[b]	NMS[b]	Gene Ontology[c]	
					cellular location	biological function/process
Agpat3	Chr 10_40	43.3	49.7	2.6	Me	
Bcr	Chr 10_40	35.3	6.4	1.5		K
Unc5b	Chr 10_40	103.7	42.3	3.6	Me	
Pyp	Chr 10_40	33.0	6.3	1.3		
Cdc2a	Chr 10_40	34.1	11.9	3.6	N	K
Cnnm2	Chr 19_49	96.6	64.4	4.4	Me	
Thop1	Chr 10_40	78.0	38.9	2.3	C	K, P
Hhex[d]	Chr 19_49	30.0	54.7	1.9	N	Tc
Palm	Chr 10_40	41.6	59.4	4.1	C, Me	
Gnaz	Chr 10_40	40.8	72.2	5.0	Me	
2610028F08Rik	Chr 15_18	28.3	17.9	1.7		
Ank	Chr 15_18	54.3	55.7	1.7	Me	
Nov	Chr 15_18	38.8	20.4	1.5	E	
Neurl	Chr 19_49	36.0	58.0	4.8		
Gpam	Chr 19_49	93.4	54.2	2.6	Mi, Me	
Ncald	Chr 15_18	22.2	5.9	1.1		
Wnt8b	Chr 19_49	40.5	13.8	2.7	E	
Timp3	Chr 10_40	24.2	19.8	2.3	Me, E	K
Osr2	Chr 15_18	30.6	17.2	1.5	N	
Nnp1	Chr 10_40	54.6	51.3	3.7		
Nfic	Chr 10_40	48.8	80.4	3.2	N	Tc
Pfkl	Chr 10_40	85.4	8.3	3.1	C	K
Slc18a2	Chr 19_49	55.8	72.5	2.5	C, Me	
Sgta[d]	Chr 10_40	34.2	23.0	1.3		
Hps6	Chr 19_49	87.3	59.5	3.4		
Sgpl1	Chr 10_40	63.7	90.9	4.0	Me	
Pdxk	Chr 10_40	35.1	8.1	1.0	C	K
Pwp2h	Chr 10_40	102.9	36.3	2.7		
Psap	Chr 10_40	61.1	42.2	2.4	Mi, E	
Fzd6	Chr 15_18	79.1	92.0	2.6	Me	
Ilvbl	Chr 10_40	68.2	45.8	3.0	Me	
Itgb2	Chr 10_40	84.9	41.7	2.9	Me	
Cstb	Chr 10_40	11.0	18.9	1.4	N, C	
Gstt2	Chr 10_40	27.6	25.1	1.3	N, C	
Bsg	Chr 10_40	29.7	20.4	1.3	Me	
Cpn1	Chr 19_49	52.1	6.1	1.0	E	P
Eif3s6	Chr 15_18	52.0	8.7	1.2		
Timm9	Chr 10_40	10.4	14.2	2.2	Mi, Me	
Ndufs7	Chr 10_40	24.7	11.8	2.9	Mi, Me	
Psd	Chr 19_49	12.4	51.6	2.4	Me	
Tfam	Chr 10_40	28.0	17.8	2.0	N, Mi	Tc
Ppap2c	Chr 10_40	31.2	51.2	1.6	Me	
Gpx4	Chr 10_40	22.1	18.1	1.5	N, C, Mi, Me	
Pcbd1	Chr 10_40	12.0	44.4	2.0	N, C	Tc
Ins1	Chr 19_49	12.2	30.1	1.9	E	
Mrpl54	Chr 10_40	15.4	8.5	1.0	Mi	
Oaz1	Chr 10_40	25.1	11.4	1.3		
Cxxc6	Chr 10_40	25.6	32.1	1.5		
Sdc2	Chr 15_18	22.1	42.5	2.0	Me	
Npm3	Chr 19_49	19.0	13.1	1.2	N	Tc
Eif4ebp2	Chr 10_40	12.9	7.6	1.0	Tl	
Ddt	Chr 10_40	13.1	5.1	0.9	C	
Pah	Chr 10_40	51.8	32.1	2.2		
Peo1	Chr 19_49	77.0	6.8	0.9	Mi	
Cabin1	Chr 10_40	65.2	13.8	1.3	N	
Lilrb4	Chr 10_40	37.5	9.0	1.4	Me	
Casp7	Chr 19_49	34.1	7.6	1.3	C	P
Matk	Chr 10_40	53.6	72.9	4.0	C, Me	K
Egr2	Chr 10_40	49.8	74.7	3.2	N	Tc
Slc1a6[d]	Chr 10_40	60.8	126.0	2.7	Me	
Adn	Chr 10_40	28.1	63.8	3.1		
Gna11	Chr 10_40	42.0	69.7	4.7		
Tbxa2r	Chr 10_40	37.1	43.9	1.8	Me	
Trhr	Chr 15_18	44.6	188.7	3.5	Me	
Ube2g2	Chr 10_40	33.0	16.0	1.8		U, Tl
Madcam1	Chr 10_40	43.6	33.5	1.8	Me	
Pcdh15	Chr 10_40	129.9	70.6	2.6	C, Me	
Efna2	Chr 10_40	23.6	5.3	1.0	Me	
Sema5a	Chr 15_18	120.3	19.1	1.8	Me	
Aire	Chr 10_40	18.0	8.6	1.2	N, C	Tc, Tl
Fgf8	Chr 19_49	24.7	11.1	1.8	E	
Snrpd2	AA*	13.6	13.4	3.3	N	
Hmgn2	AA	9.4	85.7	2.2	N, C	
Mcrs1	AA	51.7	45.5	3.2	N	
Hnrpa2b1	AA	32.5	19.5	2.1		
Hars	AA	57.4	104.9	4.8	C	Tl
Rpo1−3	AA*	15.1	6.4	4.2		

Table 1. Continued

gene symbol	source[a]	M_w (kDa)	MRL/lpr[b]	NMS[b]	Gene Ontology[c] cellular location	Gene Ontology[c] biological function/process
Hars2	AA*	23.4	7.3	1.6	C, Mi	Tl
Hspca	AA	84.8	5.9	3.1		
Vtn	AA	54.8	5.8	1.6	E	
Snrpd3	AA*	13.9	10.2	2.8	N, C	
Hmgn1	AA*	10.1	35.6	4.5	N, C	Tc
Rnps1	AA	40.8	26.0	1.4	N, C	
Fbl	AA	34.2	24.9	1.9	N	
Npm1	AA	32.6	12.6	1.1	N, C	K
Top3b	AA*	96.9	18.9	2.6		
Coil	AA	62.2	9.8	1.6	N	
Casp8	AA	55.4	27.5	2.8	N, C	P
Ybx1	AA	35.7	27.8	4.7	N, C	Tc
Srpk1	AA	73.1	17.7	3.8	N, C	K
Rpa1	AA	71.4	36.6	3.8	N	
Car9	AA*	47.3	8.9	1.5	Me	
Sag	AA	44.9	11.9	2.2		
Dnahc8	AA	122.1	9.2	1.6	C	
Top3a	AA*	107.0	13.1	2.0		
Fbn2	AA	56.6	5.9	1.4	E	

[a] The source of selected gene done by symbol, is as follows: Chr 10_40, genetic loci on chromosome 10_40 cM; Chr 15_18, genetic loci on chromosome 15_18 cM; Chr 19_49, genetic loci on chromosome 19_49 cM; AA, well-known autoantigen; AA*, well-known autoantigen homologue. [b] Relative luminescence signals. [c] According to Gene Ontology (GO) Database (http://www.geneontology.org/), the proteins were classified by cellular localization and biological function/process, is as follows: Me, Membrane (GO:0016020); N, Nucleus (GO:0005634); C, Cytoplasm (GO:0005737); E, Extracellular region (GO:0005576); Mi, Mitochondrion (GO:0005739); K, Kinase activity (GO:0016301); P, Peptidase activity (GO:0008233); U, Ubiquitin (GO:0005551); Tl, Translation (GO:0006412); Tc, Transcription (GO:0006350). [d] Hhex, lane1; Sgta, lane3; Slc1a6, lane4 in Figure 4.

reactive autoantigen proteins. Interestingly, these loci were reported as the susceptibility loci of arthritis. Furthermore, according to Gene Ontology (GO) Database (http://www.geneontology.org/), 73 (76%) of the proteins were classified by cellular localization (Figure 3E) and 25 (26%) classified by biological function/process (Figure 3F). The annotated proteins found were classified as localized with Membrane (22%), Membrane/Cytoplasm (4%), Extracellular space (7%), Nucleus (13%), Nucleus/Cytoplasm (13%), Cytoplasm (7%) and Mitochondrion (2%). Also, the annotated proteins were involved in diverse biological functions/processes such as Kinase activity (8%), Kinase activity/peptidase activity (1%), transcription (8%), Transcription/Translation (1%), Translation (3%), Ubiquitin/Translation (1%) and Peptidase activity (3%). Data analysis showed that 26 and 25 proteins were annotated in localization of nucleus and cytoplasm respectively (Figure 3E, Table 1), and that 9 and 9 proteins were related to cellular events of protein phosphorylation and transcription, respectively (Figure 3F, Table 1). Many nuclear proteins were reported as autoantigens.[2] Interestingly, localization of 38 (39.6%) antigen proteins reacting with antibodies in MRL/lpr mouse sera was annotated in membrane and/or extracellular space. These results suggest that the wheat cell-free system is a viable platform to study folded membrane proteins that function as antigens. The data analysis suggests that MRL/lpr autoantigen proteins are represented by a wide variety of biological functions localized in whole cells, rather than just nuclear proteins. Taken together, these results indicate that the BPL-based screening method would be useful for identification of autoantigen proteins.

Validation of Identified Autoantigen Proteins by Immunoblotting and Immunoprecipitation. Recent reports have mentioned the possibility that autoantibodies may react with conformational epitopes.[29−31] These data were obtained by liquid phase immunoprecipitation assays using recombinant proteins.[32,33] Under these situations, autoantigen proteins we found were analyzed by immunoblotting and immunoprecipitation. For this analysis, six proteins were randomly selected (see legend in Figure 4). Immunoblot analysis showed that three proteins reacted with MRL/lpr mouse sera (Lanes 2, 5, and 6 in Figure 4A). Two of these three proteins, LmnB2 (Lane 5) and topoisomerase II alpha (Top2a) (Lane 6), have been well characterized as autoantigen proteins so far.[22,34] Also, six of our identified autoantigen proteins were not detected by immunoblot analysis in the sera of NMS (data not shown). Interestingly, immunoprecipitation analysis revealed antigenicity of all six autoantigen proteins (Figure 4B, C), whereas two proteins randomly selected from nonautoantigen proteins, serving as a negative control, did not show significant reaction to the sera from MRL/lpr mice by both immunoblot analysis and immunoprecipitation (Lanes 7 and 8 in Figure 4A, B). These results suggest that the BPL-based screening method may be useful for identification of autoantigen proteins reacting with autoantibodies recognizing conformational epitopes.

Discussion

To address high-throughput protein production, we have utilized our wheat germ high-throughput protein synthesis system,[10,16] which can produce large numbers of recombinant proteins using a fully automated robot.[19] To create a library of target autoantigen proteins, full-length human and mouse cDNA resources were provided by the Mammalian Gene Collection (MGC) clones (Mammalian Gene Collection Program, http://mgc.nci.nih.gov/) and FANTOM.[27,28] Since the full-length cDNA was provided in plasmids, no additional time-consuming cloning steps were needed for the synthesis of linear DNA templates by PCR for direct entry into the cell-free based protein production system. Additionally, researchers can select and use any appropriate peptide tag for downstream applications, like a bls used in this study, owing to the ease of template construction. In fact, given the advantages of the gateway system and PCR, a recent publication reported successful production of 13 000 His-tagged human proteins by the wheat cell-free system using full-length cDNA resources.[12] Furthermore, because protein purification is a time-consuming-step, an assay system with no purification requirement could dramatically increase the throughput. For that, a specific

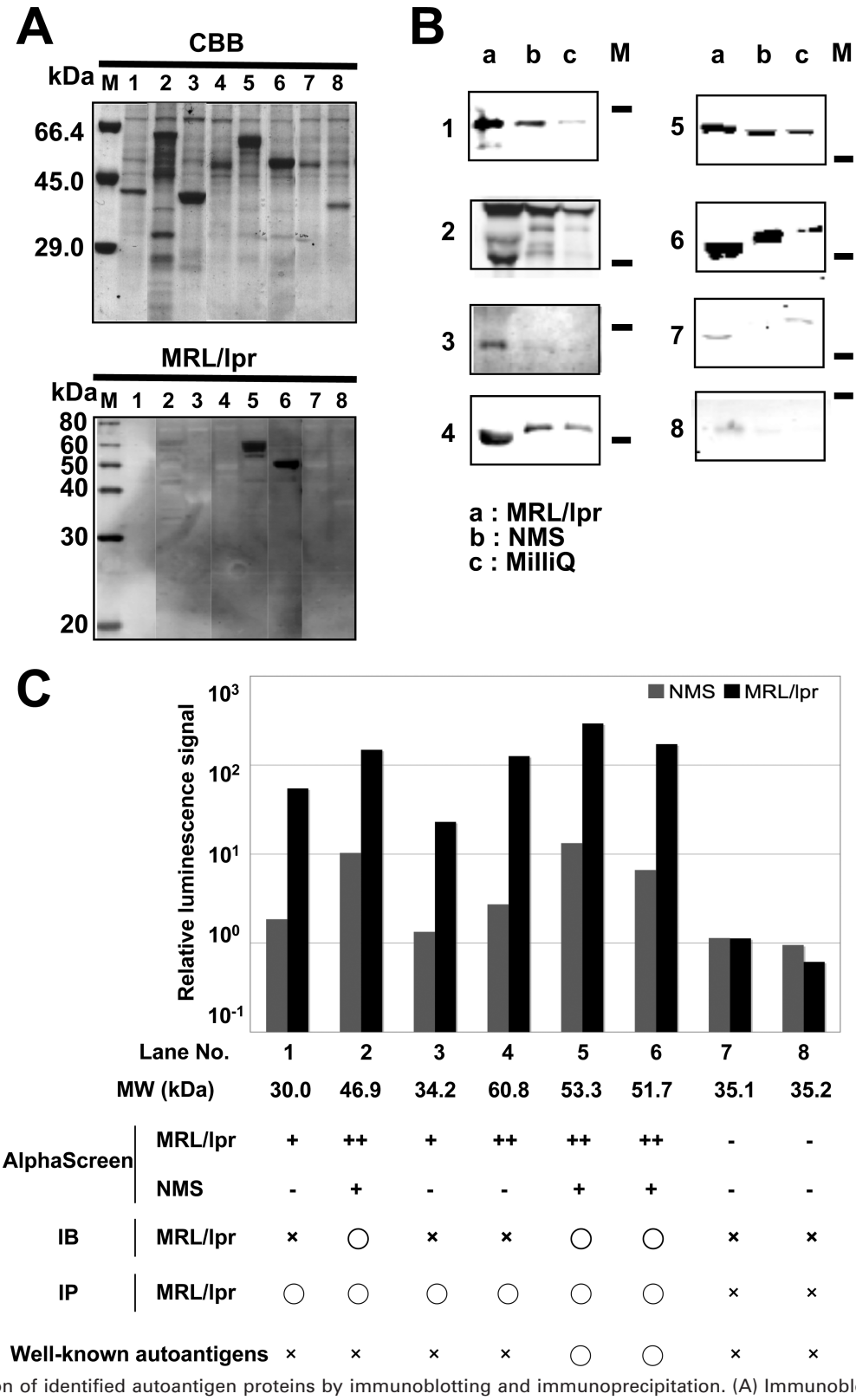

Figure 4. Detection of identified autoantigen proteins by immunoblotting and immunoprecipitation. (A) Immunoblotting analysis by using recombinant proteins. Purified recombinant proteins were separated by SDS-PAGE and stained with CBB (Upper). Purified recombinant proteins were reacted with serum from MRL/lpr mouse (Lower). (B) Immunoprecipitation analysis using recombinant proteins. Translation mixtures expressing biotinylated proteins were incubated with 1 μL of undiluted serum overnight at 4 °C. Immobilized Protein A sepharose was added to each sample, and incubated for 60 min at 4 °C. After washing, proteins were separated by SDS-PAGE, followed by immunoblotting with Alexa488-STA. M indicates a 45 kDa protein molecular weight marker. (C) Whole data of randomly selected proteins. IB, Immunoblotting; IP, Immunoprecipitation. Relative luminescence signal, $10^2 \leq ++$; $5 \leq + < 10^2$; $- < 5$. (A, B, C) Lane 1, Hhex; Lane 2, Tdg; Lane 3, Sgta; Lane 4, Slc1a6; Lane 5, Lmnb2; Lane 6, Top2a; Lane 7, Cs; Lane 8, Car4. Lane 7 and 8 were negative controls. Detailed information on individual proteins was indicated in Supplementary Table 1 (Supporting Information).

protein has to be clearly recognized in a homogeneous condition. In this study, we selected biotin as our target protein label due to the highly specific binding of biotin-streptavidin. Commonly, biotinylated proteins are produced via NHS ester-activated biotins. However, this technique requires laborious purification to remove any nonreacted biotin reagent in the reaction mixture. Therefore, we used a BirA biotin-ligase-based labeling system. By addition of BirA and biotin to the wheat cell-free system, highly specific biotin-labeling is available and the biotinylated proteins can be directly used for assaying[13,15,35] without further purification. Taken together, the biotinylated protein library produced by the wheat cell-free system is suitable for autoantigen screening.

Several autoantigen screening methods such as SERPA, SEREX and protein microarray are currently widely used for identification of autoantigen proteins, each of which has inherent limitations. In this study, we demonstrated improved methodologies that overcome the aforementioned limitations using a wheat cell-free based BPL and luminescence assay that allow detection of autoantigen proteins with autoantibodies in sera. The BPL-based screening revealed that specific antibody interaction were detected at subpicogram scale, with a linear response over a 1000-fold range, and appear to be more sensitive than conventional method, such as ELISA.[6,36] It should be noted that protein microarray could also detect autoantigen at picogram scale, in the linear fashion over a 1000-fold range.[6] While the detection sensitivity of the BPL-based autoantigen protein screening method might be equivalent to microarray based autoantigen protein screening, the folded state of the autoantigen proteins differs. The reports which autoantibodies would recognize conformational epitopes[29,30] might contain an important implication for screening of autoantigen proteins. Although conventional methods use denatured or unfolded proteins, such as dehydrated or detergent-treated proteins, the BPL-based method tested in this study better represents the folded, native form as all procedures are carried out in the solution phase without dehydration or detergent treatment. In addition, the BPL-based screening method using serum dilutions of 1:10 000 could produce high luminescent signal in 25 μL of reaction volume (Figure 2C). Thus, 50 μL of serum would be sufficient to screen 20 000 kinds of human proteins.

Autoimmune diseases were thought to be a complex of both genetic and nongenetic factors influencing susceptibility, severity and response to therapies.[37] Twin and family studies suggest that approximately 60% of susceptibility is due to genetic factors and genes within the HLA locus, particularly HLA-DRB1, which accounts for almost half of the genetic component of susceptibility.[37] Also, genetic analyses identified that other susceptibility locus of RA, SLE, and so on.[38] In this study, we screened proteins encoded by genes on an autoimmune susceptibility loci,[26] and 71 out of 111 clones (Table 1 and Supplementary Table 1, Supporting Information) localized on the chromosomes 10, 15 and 19[26] were found as new autoantigen proteins reacting with the sera of MRL/lpr mice. Additionally, the Gene Ontology (GO) Database may be extremely useful for the screening of autoantigen protein. For example, based on data from the GO Database, localization of 38 (39.6%) autoantigen proteins out of 96 MRL/lpr autoantigen proteins were annotated in membrane and/or extracellular space (Figure 3E). These results suggest that a specific protein library focused on the human autoimmune susceptibility loci and membrane proteins or extracellular spaces classified according to GO Database may be a good target for screening of autoantigen proteins.

A key obstacle for robust genome-wide screening has been experimentally simple techniques and automated technology. The BPL-based screening method is one of the simplest approaches for identification of autoantigen proteins, because all experimental processes, including construction of DNA templates, and interaction and detection of antigen−autoantibodies reactions, were reduced to mixing steps. Synthesis of the biotinylated protein library was accomplished using a fully automated robot,[19] and the biotinylated proteins can be used in subsequent screening steps without purification. The method described here can be used in developed for use in 96, 384 (Figure 3A) or 1536-well microtiter-plate format through the use of appropriate automated liquid handling robots. Therefore this method is suitable for development of a genome-wide screening platform. In conclusion, the BPL-based screening method has a high potential for identification of autoantigen proteins in human autoimmune diseases.

Abbreviations: Alexa488-STA, streptavidin Alexa Fluor 488 conjugate; NMS, normal mouse sera; SEREX, serological expression cloning; SERPA, serological proteome analysis; bls, biotin ligation site; DHFR, dihydrofolate reductase; Lmnb2, lamin B2; Hars, histidyl-tRNA synthetase; Top2a, topoisomerase II alpha; Hhex, hematopoietically expressed homeobox; Tdg, thymine DNA glycosylase; Sgta, small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha; Slc1a6, solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6; Cs, citrate synthase; Car4, carbonic anhydrase 4; BPL, biotinylated protein library; FANTOM, functional annotation of mouse; CBB, coomassie brilliant blue; RF, rheumatoid factor; hnRNP, heterogeneous nuclear ribonucleoprotein; Sm, Smith; GO, Gene Ontology; NHS, *N*-hydroxysuccinimide; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MRL/lpr, MRL/Mp-*lpr/lpr*.

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Supporting Information Available: Supplementary Table 1: List of selected 226 mouse genes and primer sequences used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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