Epitope Screening by Use of a Random Peptide-Displayed Phage Library and Polyclonal Antibody-Coupled Liposomes

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Linear epitopes of sperm whale myoglobin for a rabbit polyclonal anti-myoglobin antibody were determined from a random-peptide displayed phage library by biopanning using antibody-coupled liposomes (AB-MLVs). Two kinds of positive phages displaying peptides (RNFGADAQ and YDLDIGAD) homological to the myoglobin sequence were obtained after 4 rounds of biopanning. Binding affinities of antibodies, which were fractionated from the anti-myoglobin antibody by specificity to both the peptides, were higher than that of an antibody fraction specific to the peptide that was screened by biopanning using an antibody-coated polystyrene tube (AB-Tube). The epitope identified in this study, N(D)FGADAQ has not been reported as an epitope of the rabbit anti-myoglobin antibody. These results suggest that biopanning using AB-MLVs shows much higher selectivity to recover phages displaying peptides with high affinity and specificity than that using the AB-Tube.

Introduction

In recent years, a large number of combinatorial libraries have been produced in genetic engineering fields for several purposes, such as design of new functional proteins, analyses of specific interactions of biomolecules and screening of specific antibodies against target molecules (Stephen and George, 1988; Wilson and Finlay, 1998). Among them, a phage display system utilizing filamentous phages, which can display various peptides or proteins on their particles, is the most popular in such fields due to its usefulness and high versatility. Particularly, screening from random peptide libraries displayed by phages could be a promising way to obtain affinity ligands and antagonists against target proteins and to clarify linear epitopes of antibodies. In this system, however, improvement of biopanning procedures, which consist of adsorption of phages in a library to a target molecule, recovery of the adsorbed phages, infection and amplification of the phages in host cells and phagemid rescue, is highly required to obtain desirable candidates efficiently, because in conventional biopanning procedures using protein-immobilized plastic adsorbents, recovered phages contain many fakes nonspecifically adsorbed.

In a previous work, we developed an efficient biopanning method utilizing protein-coupled liposomes as adsorbents and screened peptides homological to an antigen peptide of an anti-peptide antibody from a random peptide-displayed phage library with high efficiency due to avoiding nonspecific adsorption of phages (Kumada *et al*., 2005).

Epitopes of many antibodies in proteins have been identified to clarify complicated functions of immune systems and to design inhibitory peptides against antibodies to be used as drugs (Atassi and Habeeb, 1977; Frank, 2002; Hujer *et al*., 2004; Kopecky, *et al*., 2005, 2006). Recently, it was reported that phage display systems could be applicable to epitope screening of polyclonal antibodies (Yao *et al*., 1996; Muhle *et al*., 2004). In epitope screening of polyclonal antibodies by this method, however, higher selectivity of the antibodies against specific phages is required because a much smaller number of immobilized antibodies show specificity and affinity for a specific epitope in comparison with that in the epitope screening using monoclonal antibodies.

In this study, epitopes of a polyclonal anti-myoglobin antibody was screened by the use of a random peptide-displayed phage library and antibody-coupled liposomes. The efficiency of screening and binding affinity of selected peptides was compared with those

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in conventional biopanning using an antibody-coated polystyrene tube.

1. Experimental

1.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (Nacalai Tesque Inc.), cholesterol (Chol) (Wako Pure Chemical Industries Ltd.), dicetylphosphate (DCP) (Sigma Aldrich Co.) and 4-(*p*-maleimidophenyl)-butyl-dipalmitoylphosphatidylethanolamine (MPB-DPPE) (Avanti Polar lipids, Inc.) were used for liposome preparation. 2-Iminothiolane·HCl (Pierce) was used for coupling of antibody on liposome membrane. A polystyrene tube (Immunotube, Type 44474) used for the conventional biopanning was purchased from Nalge Nunc International, and Blocking one (Nacalai Tesque Inc.) was used for blocking. Sperm whale myoglobin purchased from Sigma Aldrich Co. was used as a model antigen. It is reported that sperm whale myoglobin is a mixture containing an isotype of myoglobin from skeletal muscle, in which 122nd Asn is substituted with Asp (Edmundson, 1965; Herrera and Lehmann, 1974). Rabbit antiserum was prepared by immunization of myoglobin by the same method as reported previously (Katoh and Terashima, 1994). A polyclonal anti-myoglobin antibody was purified from pooled antisera using an affinity column packed with myoglobin-coupled Sepharose 4B. The concentrations of antibiotics used for supplement of the 2xYT medium were as follows: ampicillin (Amp), 50 mg/L; kanamycin (Kan), 70 mg/L; tetracycline (Tet), 14 mg/L. All other chemicals used were of reagent grade.

1.2 *Escherichia coli* strain and random octapeptide-displayed phage library

Escherichia coli XL1-Blue, which has the Tet^R F plasmid and the chromosomal genotype containing *endA1*, *hsdR17*, *supE44*, *thi-1*, *recA1*, *gyrA96* and *relA1*, was purchased from Stratagene and used as a host for generation of random octapeptide-displayed M13 phage particles. Random octapeptide-deisplayed M13 phages were prepared as reported previously (Kumada *et al*., 2005) and the repertory of peptide-displayed phages in this library was 1.2×10^5.

1.3 Preparation of polyclonal anti-myoglobin antibody-coupled multilamellar vesicles (AB-MLVs)

Polyclonal anti-myoglobin antibody-coupled multilamellar vesicles (AB-MLVs) were prepared by the same method as reported previously (Kumada *et al*., 2005). Briefly, a lipid mixture of 10 μmol DPPC, 1 μmol DCP and 0.5 μmol MPB-DPPE in 5 cm^3 chloroform was dried at 50°C under reduced pressure to make a thin lipid film on the inside wall of a pear-shaped flask. Then, 3 cm^3 of phosphate buffered saline (PBS), pH 7.2, was added, and the flask was shaken at around 50°C to peel off the lipid film to form multilamellar vesicles (MLVs). The MLVs were resuspended in 2 cm^3 of PBS containing 0.25 mg/cm^3 polyclonal anti-myoglobin antibody, which was coupled on liposomes by the use of 2-iminothiolane hydrochloride. The antibody-coupled MLVs (AB-MLVs) were stored at 4°C until use. Antibody concentration in the liposome suspension was measured by the use of DC-protein assay kit using bovine serum albumin as a standard protein. The DPPC amount of AB-MLVs was measured by a kit, phospholipid Test C WAKO (Wako Pure Chemical Industries Ltd.).

1.4 Immobilization of polyclonal anti-myoglobin antibody on the wall of a polystyrene tube (AB-Tube)

One cubic centimeter of 55 μg/cm^3 anti-myoglobin antibody dissolved in PBS was incubated in an Immunotube for 2 h at 37°C. The tube was washed 10 times with PBS and then was blocked with 3 cm^3 of Blocking One for 2 h at 37°C. After washing 10 times with PBS containing 0.05% Tween 20 (PBST), the tube was used for biopanning selection.

1.5 Biopanning selection of peptides specific to polyclonal anti-myoglobin antibody

Biopanning selection of peptides specific to the polyclonal anti-myoglobin antibody was performed by the same method as reported previously (Kumada *et al*., 2005). One cubic centimeter of PBS containing 10% Blocking One and AB-MLVs at the antibody concentration of 10 μg/cm^3 was prepared in a sterile plastic tube (1.5 cm^3) and then centrifuged at 15,000 × *g*. After the supernatant was discarded, AB-MLVs were washed twice with PBS containing 10% Blocking One. AB-MLVs sedimented were then resuspended in 1 cm^3 of 10% Blocking One containing 5×10^8 cfu/cm^3 of the octapeptide-displayed M13 phage library and transferred to a new sterile plastic tube. After incubation at 25°C for 30 min, the suspension was transferred into a new plastic tube. AB-MLVs were washed 9 times by repeated centrifugation using PBS as a washing buffer. AB-MLVs washed were transferred into a new plastic tube and then centrifuged. Supernatant was removed and then AB-MLVs were resuspended in 0.2 cm^3 of 0.1 N HCl to elute peptide-displayed phages attached on the liposome surface. After the suspension was centrifuged, the supernatant containing eluted phages was recovered and immediately mixed with 0.1 cm^3 of 2 M Tris-HCl, pH 8.0. The phages were infected to the precultured *XL1-Blue*, and the infected cells were grown in 50 cm^3 of 2xYT medium (Amp, Tet) until OD at 600 nm reached 0.5–1.0. After superinfection of helper phage VCSM13 (MOI = 20), the recovered phages were amplified in a fresh 2xYT medium (Amp, Tet, Kan) for 16 h at 37°C with shaking at 200 rpm. Supernatant containing amplified phages were separated by centrifugation and concentrated by PEG precipitation. Phage particles were then resuspended to 10^9–10^{10} cfu/cm^3 with PBS containing 10% Blocking

One and used for the next panning selection. The panning selection described above was repeated 4 times. Sequences of the peptides selected after the second to fourth panning rounds were determined by DNA sequencing of peptide genes by a genetic analyzer (Applied Biosystems).

In the conventional biopanning procedure, 1 cm^3 of PBS containing 10% Blocking One and the random peptide-displayed phages stated above was incubated in an AB-Tube at 25°C for 1 h. The AB-Tube was washed 10 times with PBST and then 200 μ*l* of 0.1 N HCl was added in order to elute phages attached on the surface of the AB-Tube. The recovered phages were immediately neutralized with 100 μ*l* of 2 M Tris-HCl and amplified by the same method as stated above.

1.6 Affinity fractionation of polyclonal anti-myoglobin antibody by the use of peptide-coupled columns

Three peptides among selected peptides by biopanning were synthesized by Pioneer Peptide Synthesizer (Applied Biosystems). They had two additional Lys residues at their C-termini for coupling to CNBr-activated sepharose 4B. One milligram of each peptide was coupled with 3.5 cm^3 of gel according to the method provided by a supplier. After equilibration with PBS, 1.2 cm^3 of 820 μg/cm^3 polyclonal anti-myoglobin antibody was applied to the peptide-coupled column. The column was washed with PBS and then anti-peptide antibodies bound to the peptide-coupled column were eluted with 0.1 N HCl. The fractionated antibody solution eluted was immediately neutralized with 10 vol% of 2 M Tris-HCl, pH 8.0. The antibody concentration was determined by DC protein assay using BSA as a standard protein.

1.7 Enzyme-linked immunosorbent assay (ELISA) using antibody fractionated

PBS solutions containing 40% and 10% Blocking One were used for blocking and dilution of antibodies, respectively. Hundred microlitters of PBS containing 3–200,000 ng/cm^3 sperm whale myoglobin were incubated in each well of a microtiter plate at 37°C for 2 h. The plate was washed, and then blocked for 2 h at 37°C with 300 μ*l* of the blocking solution. The plate was washed and incubated with 100 μ*l* of 5 μg/cm^3 fractionated antibody for 1 h at room temperature. The plate was washed, and incubated with 100 μ*l* of 1000 times diluted HRP-labeled anti-rabbit IgG antibody for 1 h at room temperature. The plate was washed and then color was developed by addition of 100 μ*l* substrate solution containing 0.3 mg/cm^3 ABTS and 0.03% H_2O_2 dissolved in 0.1 M citrate buffer, pH 4.0. After incubation for 15 min at room temperature, absorbance at 405 nm was measured by a micro-plate reader (Bio-Rad Laboratories Inc.).

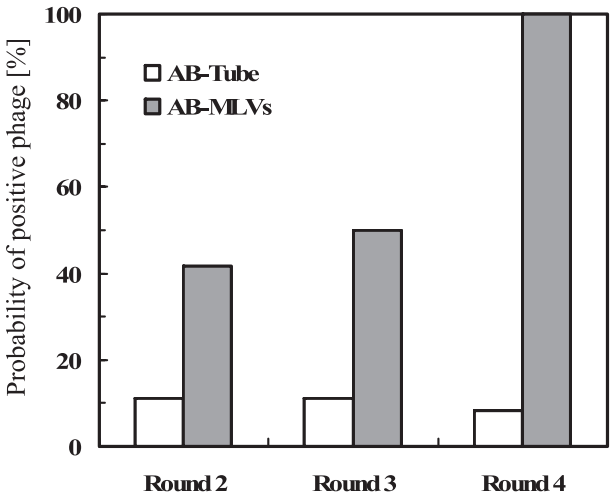

Fig. 1 Percentage of positive phage recovery at each biopanning round

2. Results

2.1 Characteristics of antibody-coupled multilamellar vesicles (AB-MLVs) and antibody-coated polystyrene tube (AB-Tube)

Approximately 6.0×10^7 molecules of the polyclonal anti-myoglobin antibody were covalently coupled on the surface of one liposome particle with an average diameter of 5.0 μm. In each biopanning round, 10 μg of the liposome-coupled antibody was used. Polystyrene tube was coated with approximately 15 μg antibody by use of 55 μg/cm^3 antibody stock solution. The surface area and antibody-immobilized density were similar in AB-MLVs (surface area, 5.1 cm^2; density of immobilized AB, 2.0 μg/cm^2) and AB-Tube (4.8 cm^2; 3.1 μg/cm^2).

2.2 Screening of linear epitopes of polyclonal anti-myoglobin antibody by the use of random octapeptide-displayed phage library

Phages displaying peptides specifically binding to the polyclonal anti-myoglobin antibody were screened from the random octapeptide-displayed phage library by 4 times biopanning selection using either AB-MLVs or AB-Tube. **Figure 1** shows percentages of positive phages recovered after the second to fourth biopanning rounds among the peptides selected and sequenced. A positive phage was defined as a phage displaying a peptide whose amino acid sequence showed higher than 37.5% (3/8) homology with the amino acid sequence of sperm whale myoglobin. Positive phages were efficiently screened with AB-MLVs and its recovery reached 100% after the fourth panning round. **Table 1** shows the amino acid sequences of peptides among 10–12 selected peptides after the fourth panning round. By repeating the biopanning with AB-MLVs, five homological peptides selected after the second round converged to two, RNFGADAQ (peptide A) and

Table 1 Amino acid sequences of peptide selected from the library

(a) AB-MLVs

Amino acid sequence									Homology [%]	Position	Frequency [%]
R	N	F	G	A	D	A	Q	[a]	87.5	122–128	20.0
Y	D	L	D	I	G	A	D	[b]	50.0	122–126	80.0
Y	D	L	D	I	G	A	D	[b]	37.5	141–144	80.0

(b) AB-Tube

Amino acid sequence									Homology [%]	Position	Frequency [%]
L	Y	S	C	H	S	H	V	[c]	37.5	115–119	8.3
I	Y	L	C	H	V	D	P		—	—	16.6
L	Y	V	C	A	S	D	V		—	—	8.3
I	Y	S	C	S	R	D	M		—	—	8.3
I	Y	R	C	V	T	D	V		—	—	8.3
L	Y	V	C	F	S	D	P		—	—	8.3
V	Y	I	C	S	S	D	Y	[d]	—	—	8.3
F	F	V	C	S	P	D	Y		—	—	8.3
N	S	Y	C	S	R	N	S		—	—	8.3
D	C	P	F	M	R	N	S		—	—	8.3
A	V	S	C	L	S	N	S		—	—	8.3

[a]Peptide A
[b]Peptide B (Peptide homological to both 122–126 and 141–144 positions)
[c]Peptide C
[d]Peptide found after 3rd round using AB-MLVs

YDLDIGAD (peptide B), after the fourth round. The peptide A showed 87.5% (7/8) homology to the region N(D)FGADAQ (122–128) of myoglobin. The peptide B was corresponded to both the regions N(D)FGAD (122–126) and DIAA (141–144) with 50% and 37.5% homology, respectively. By using an AB-Tube, on the other hand, a variety of peptides were selected even after the fourth round, although they were composed of considerably similar amino acid sequences (I(L)YXCXXDX) including IYLCHVDP (peptide C) with 16.6% frequency as shown in Table 1(b).

2.3 Characteristics of antibodies fractionated from polyclonal anti-myoglobin antibody with 3 kinds of peptide-coupled columns

Antibodies specific to three peptides (RNFGADAQ (peptide A) and YDLDIGAD (peptide B) selected with AB-MLVs and IYLCHVDP (peptide C) selected with AB-Tube) were fractionated from the polyclonal anti-myoglobin antibody using these affinity columns, on which each of these peptides was immobilized. Percentages of antibodies specific to the peptides A, B, and C in the antibodies were 12.4, 8.5, and 8.3%, respectively. As shown in **Figure 2**, the antigen myoglobin coated on a polystyrene microtiter plate was detected using the fractionated antibodies as primary antibodies in indirect ELISAs. The antibodies specific to the peptides A and B, both of which were selected from the library by the use of AB-MLVs, showed higher affinity against myoglobin than the antibody fraction against the peptide C selected by AB-Tube.

Discussion

By means of AB-MLVs, many positive phages were recovered from the phage library at early panning rounds, and two kinds of homological peptides were finally obtained. One of the possible reasons might be that non-specific adsorption of phages to the liposomes was very little (Kumada *et al*., 2005), because liposome membrane was mainly composed of cell membrane. Even if there would be phages displaying peptides specific to the liposome membrane components, they could be adsorbed specifically on *E. coli* membrane before their liberation from *E. coli*. It should be noted that the region identified by the peptides A and B, N(D)FGADAQ (N(D) (122) to Q (128)) has not been reported as an epitope of rabbit polyclonal anti-myoglobin antibody. Therefore, the biopanning method using AB-MLVs might be a promising method to select novel linear epitopes. On the other hand, AB-Tube finally selected a considerably different type of peptides (I(L)YXCXXDX) from the peptides A and B. Percentages of antibodies specific to the peptides A, B

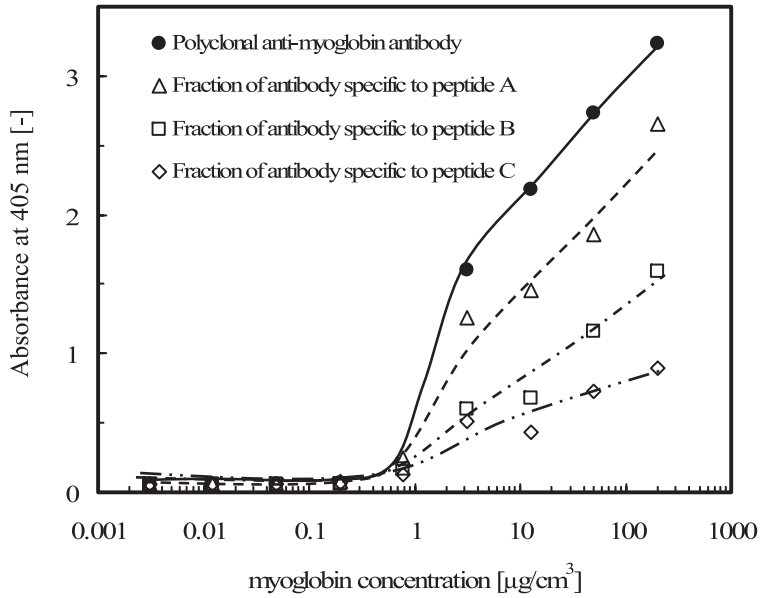

Fig. 2 Binding property of each antibody to sperm whale myoglobin (peptide A:RNFGADAQ; peptide B:YDLDIGAD; peprtide C:IYLCHVDP)

and C were almost the same, while the binding affinity of anti-peptide C antibody was the lowest among them. Interestingly, a peptide VYICSSDY analogous to the peptide C, which was screened after the 4th round by the use of AB-Tube, was also found after the 3rd round by means of AB-MLVs. However, it was eliminated at the next round probably due to lower binding affinity than the peptides A and B. These results suggest that biopanning using AB-MLVs shows much higher selectivity to recover phages displaying peptides with high affinity and specificity against the antibody than that using AB-Tube. Conformational change of antibody physically adsorbed on a hydrophobic polystyrene surface might change characteristics of antigen recognition. Thus, the method using AB-MLVs would be useful to screen antigen peptides for polyclonal antibodies.

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