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Isolation of novel catalytic antibody clones from combinatorial library displayed on yeast-cell surface

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Abstract

A combinatorial library of the Fab fragment of a catalytic antibody able to hydrolyze a non-bioactive chloramphenicol monoester derivative to produce chloramphenicol was constructed on yeast-cell surface. Interesting clones were selected using fluorescence-activated cell sorting (FACS). When binding affinity to a transition-state analog was detected, evolution of the catalytic antibody was carried out in vitro on yeast-cell surface. A number of variants with enhanced catalytic activity and binding affinity were obtained. The results showed that the improvement of catalytic antibody, which can be performed easily on yeast-cell surface using the cell-surface engineering system, is a good example of the application of protein library construction.

Keywords: Combinatorial library; Catalytic antibody; Yeast-cell surface engineering; Molecular display; Molecular evolution

1. Introduction

The investigation of catalytic antibodies used to catalyze chemical reactions has opened a novel field [1–3] and led to excitement in chemical and biochemical communities. Catalytic antibodies will provide a powerful tool for organic synthesis, and for understanding of the transition-state analogs of chemical reactions and the mechanism of enzyme catalysis. Catalytic antibodies, like enzymes, acquire their specifities and activities in an evolutionary process of immune maturation. Analysis of catalytic antibodies may thus help us better understand the relationship between the evolution of binding and catalytic functions in protein. Results obtained in relevant investigations largely confirm the positive relationship between high affinity for transition state analog and efficient catalysis [4,5]. Studies have also elucidated the evolution and sequence diversity of series of related catalytic antibodies [6,7]. In vitro affinity maturation results in the generation of secondary diversified libraries, from which clones with improved affinity can be selected. These secondary and artificial libraries of variants with increased catalytic activity and new substrate specificity have mostly been accomplished using phage display [8,9].

However, recent advances in yeast display technology have made in vitro affinity maturation of antibodies much more efficiently and allowed repertoires to be selected by flow-cytometric cell sorting [10,11]. This selection process with untethered antigen allows quantitative assessment of antibody-displaying yeast cells [12,13]. With phage display, in contrast, screening for improved affinity is less reliable and can result in selection for other parameters.

In our previous research, a catalytic antibody in a heterodimeric Fab format, which could catalyze the hydrolysis of a chloramphenicol monoester derivative 1 and exhibited high stability in binding with transition-state analog 3 (TSA3), was successfully expressed on yeast-cell surface [14] (Fig. 1). Here, we describe a study in which the catalytic antibody 6D9 was used for the affinity maturation of an Fab catalytic antibody with specificity for transition-state analog 3-conjugated FITC, with fluorescence-activated cell

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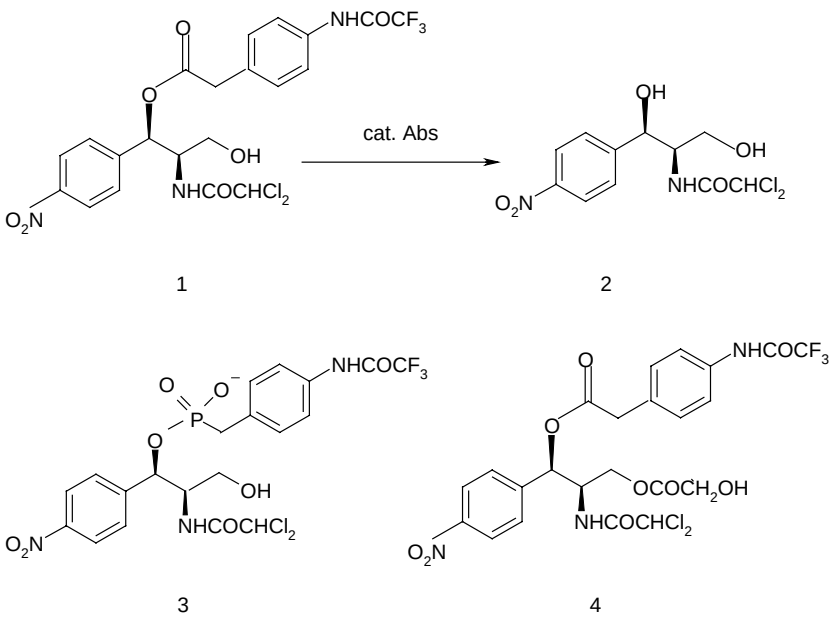

Fig. 1. 6D9 antibody-catalyzed prodrug-activation. The hydrolysis of the chloramphenicol monoester derivative 1 was hydrolyzed by the catalytic antibody 6D9 to produce the bioactive chloramphenicol 2. Hapten 3, a transition-state analog of the hydrolysis, was used for immunization to generate the catalytic antibody. Substrate 4 was used for the assay of catalytic reaction.

sorting (FACS) used for selection of yeast-displayed repertoires, and which produced efficient catalytic antibodies.

2. Experimental

2.1. Construction of 6D9 Fab gene for yeast display

Plasmids pICAS [15] and pIRS, a derivative of pICAS which the *Sac*I-*Kpn*I section of pICAS is inserted into pRS406 [16], were used as vectors. pICAS was digested with *Sac*II and *Kpn*I and the Fd fragment was inserted to construct pICAS-Fd. DNA fragments encoding the Lc and Fd genes were amplified from pComb3-6D9 [8] by PCR. The primers used were Fd-f 5′-CCAGGCCGCGGTGCTTGA-ATCTGGGGGAG-3′ and Fd-r 5′-GAGCCGGTACCACC-TTAAGTACAATCCCTGGGCAC-3′. pIRS was digested with *Sac*II and *Nco*I (partial digestion), subjected to insertion of Lc obtained from PCR (primers: Lc-f and Lc-r 5′-CTCGACCATGGGGCGAGCCACCGCCACCACACT-CATTCCTGTT-GAAGCTCTTGAC-3′), and digested again with *Sac*II and *Nco*I digestion to construct pIRS-Lc.

2.2. Display of 6D9 Fab on yeast-cell surface

The yeast MT8-1 transformed with pICAS-Fd and pIRS-Lc, which were constructed and digested, respectively with *Xba*I and *Apa*I, was grown in SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and appropriate supplements) at 30 °C for 32–36 h to OD_{600} = 1.5. Cultures were harvested, washed with PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) that contained 0.1% BSA, and incubated with TSA3-FITC conjugate (10 mg/ml in 20 mM PBS, pH 7.4) for 1 h at room temperature. Labeled cells were washed with PBS (containing 0.1% BSA) and analyzed on a FACS Calibur (Becton Dickinson, Tokyo, Japan).

2.3. Library construction

A combinatorial library of catalytic antibody 6D9 was generated by the introduction of random mutations in the LCDR1 of the light chain and the HCDR3 of the Fd fragment. Two pairs of primers were synthesized; 5′-ATCTCTT-GCAGATCTAGTCAGN NKNN KNNKCATNNKAATGG-ANNKNNKTATTTAGATTGGTTCCTGCAGAAA-3′ and 5′ - GCTTAACTCTAGAATTAACACTCATTCCTGTTGA-A-3′ for the light chain, and 5′-CCAGGCCGCGGTGCTT-GAATCTGGGGGAG-3′ and 5′-GGCTGAGGAGACGGT-GACCGAGGTCCCTGCGCCCCAMMNMMNGAAMN-NCCAMNNGCGMNNMMMMMNMMNATGGGAGAC-T-3′ for the Fd fragment. PCR was carried out with 32 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 56 °C, and extending at 72 °C for 1 min with the templates pIRS-Lc and pICAS-Fd, respectively. The randomized PCR products were digested with *Bgl*II and *Kpn*I for the Lc and *Sac*II and *Bse*RI for the Fd fragment, then cloned with *Bgl*II and *Kpn*I into pIRS-Lc and with *Sac*II and *Bse*RI into the pICAS-Fd vector. The ligation mixture was transformed into *Escherichia coli* DH5α for plasmid amplification. The amplified plasmids were harvested and co-transformed into *Saccharomyces cerevisiae* MT8-1 [17].

2.4. Flow-cytometric sorting and analysis

The yeast-cell library was incubated in SD medium at 30 °C for 48 h. Cells were then harvested and reacted with FITC-labeled TSA for 1 h at room temperature. After washing with PBS containing 0.1% BSA, samples were sorted in exclusion mode with an event rate of ~300 cells/s (FACS). A total of 1×10^7 cells was examined in the sorting, collecting 0.25% of the population. The sorted cells were plated on selective SD medium to isolate individual clones, which were examined for binding affinity with TSA by flow cytometry and for catalytic activity by HPLC.

2.5. Catalytic activity analysis

Cells were precultivated in 5 ml SD medium at 30 °C for around 24 h to $OD_{600} = 1.0$ and transferred to 100 ml SD medium for large-scale cultivation at 30 °C for around 48 h to check catalytic activity. The catalytic activity of Fab was examined in hydrolysis of the substrate 4 with 170 μl cells ($OD_{600} = 560$) in 50 mM Tris (pH 8.0) at 30 °C for 20 min. The reaction was initiated by adding 20 μl of a stock solution (2 mM) of substrate 4 in dimethyl sulfoxide to 170 μl of Fab-displaying cells ($OD_{600} = 560$) and 10 μl Tris buffer. Hydrolysis rates were measured by HPLC with 10 μl injection of the reaction solution. Analytical HPLC was performed on a Waters 600 unit equipped with a Waters 490 multi-wave length detector using a YMC ODS A303 column (YMC Co. Ltd., Kyoto, Japan) eluted with CH_3CN/0.1% aqueous trifluoroacetic acid at a flow rate of 1.0 ml/min with detection at 278 nm. The observed rate was corrected using the uncatalyzed rate of hydrolysis in MT8-1/pICAS control cells without antibody.

2.6. Colony PCR and sequence analysis

Yeast-cell colonies grown on SD plate were used as the DNA template for colony PCR amplification with the two pairs of primers Fd-f and Fd-r (for *Fd*), and Lc-f and Lc-r (for *Lc*). A DNA fragment from colony PCR was purified with PCR kleen spin columns (Bio-Rad Laboratories, CA, USA) and used as template for sequence PCR, with the primer Fd-f for *Fd* sequence analysis and Lc-f for *Lc*. Sequence analysis was completed in an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, CA, USA).

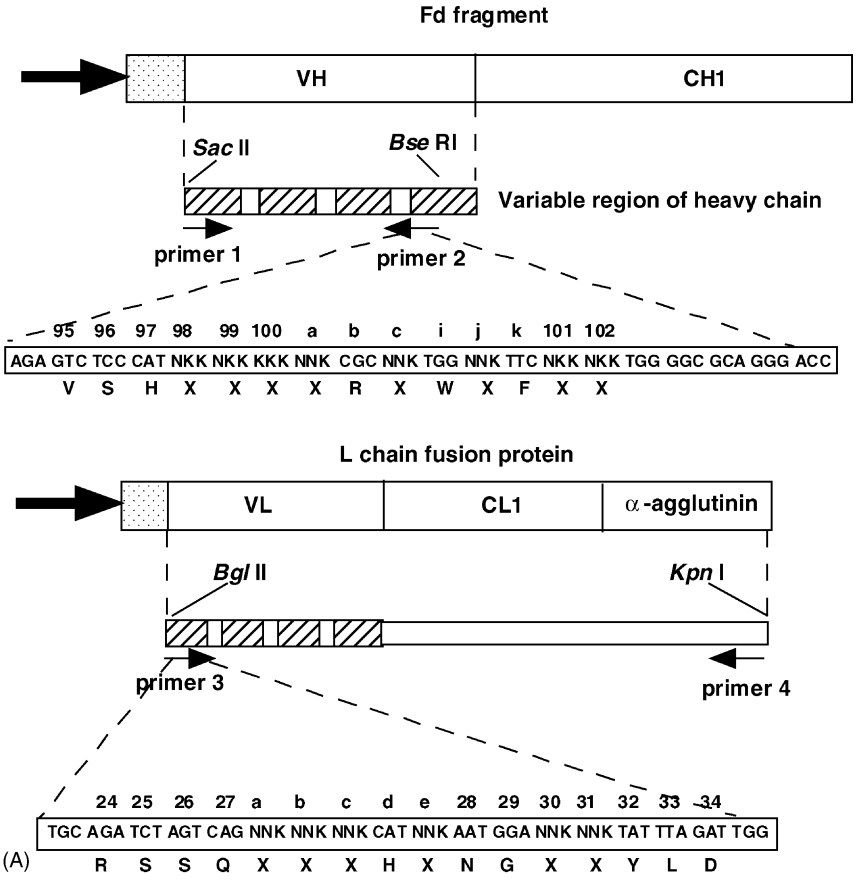

Fig. 2. Strategy for construction of a combinatorial library of catalytic antibodies on yeast-cell surface. (A) Generation of HCDR3 and LCDR1 libraries by primers designed with randomized codons. (B) Construction of a combinatorial library of catalytic antibodies on yeast-cell surface and selection with FACS.

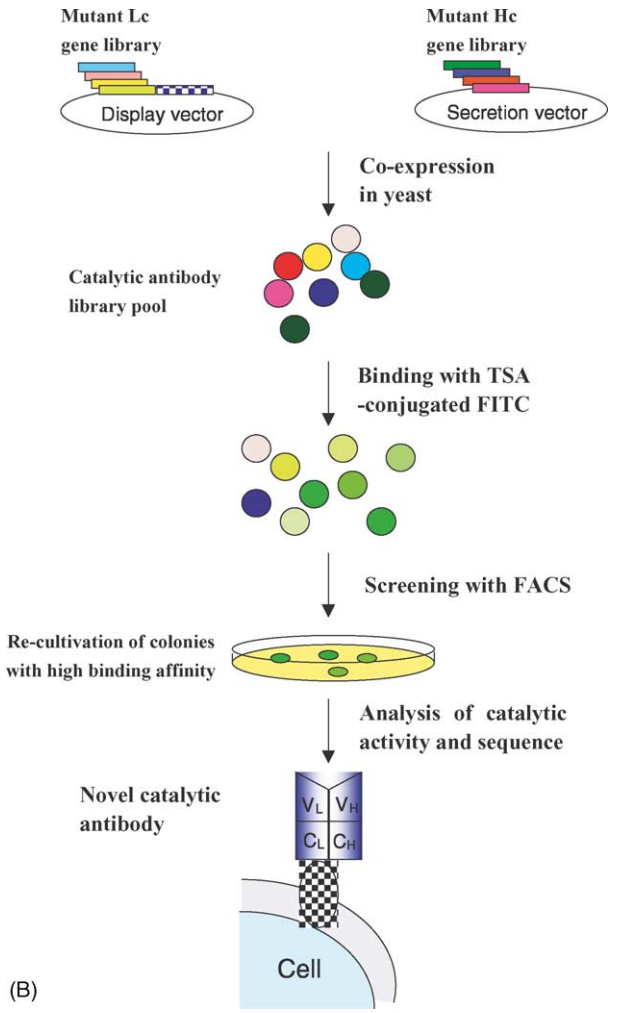

Fig. 2. (*Continued*).

3. Results and discussion

3.1. Strategy for construction of combinatorial library of catalytic antibodies

In order to display the Fab fragment of 6D9 on yeast-cell surface, the 6D9 light chain (Lc), fused to the C-terminal half of α-agglutinin with a Gly_4Ser linker at the C-terminal end, was anchored to yeast-cell wall and the Fd fragment (Fd) of the heavy chain joined to the light chain with a disulfide linkage (as described in Section 2). As a result, the catalytic antibody 6D9 in heterodimeric form was active on yeast-cell surface and was able to bind with TSA to catalyze the hydrolysis of a chloramphenicol monoester derivative and produce chloramphenicol [14]. This system allows the construction of a combinatorial library of catalytic antibodies on double chains (heavy and light chains) through simultaneous mutation and evolution of the catalytic antibody, and selection from a mutagenized protein pool using a fluorescent-labeled hapten.

On the basis of determination of the interaction between TSA3 and the specific amino-acid residues [5,18], we prepared libraries of the 6D9 catalytic antibody with heavy-chain CDR3 mutation, and in double chains (heavy chain CDR3 and light chain CDR1), by simultaneous mutations with PCR using site-directed mutagenic primers containing degenerated codons at positions Asp^{H98},

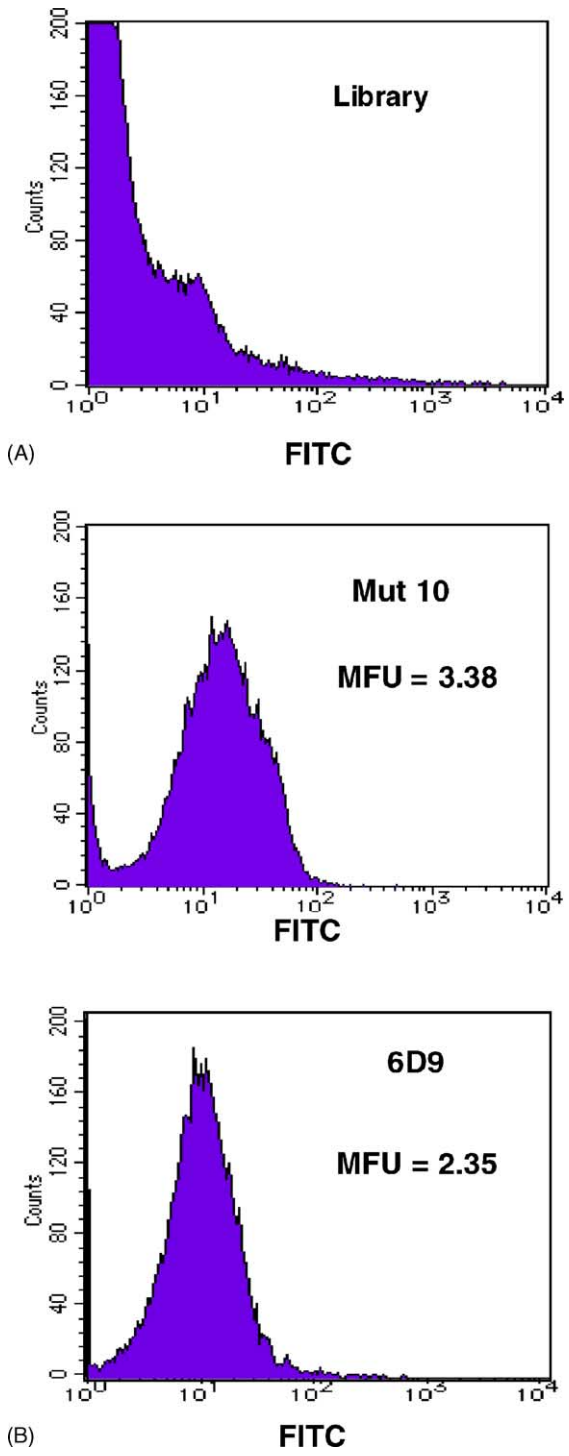

Fig. 3. Flow-cytometric analysis. (A) Flow-cytometric analysis for the library of catalytic antibodies labeled with FITC-conjugated TSA3. (B) Flow-cytometric analysis for a mutant displayed on the yeast-cell surface and comparison to 6D9. MFU: mean of fluorescence unit.

Table 1
Amino acid sequence and binding affinity of mutants generated in both chains of 6D9

Fab	LCDR1	HCDR3	Mean of fluorescene (unit/10^5 cells)	Hydrolysis product (μM)
6D9 (wild)	RSSQTIVHSNGDTYLD	VSHYDGSRDWYFDV	2.35	4.35
Mut5	RSSQTIVHSNGDTYLD	VSHWDGSREWQFDG	2.11	3.20
Mut6	RSSQTIVHSNGDTYLD	VSHRHGIREWYFRW	2.23	2.33
Mut9	RSSQTIVHSNGDTYLD	VSHCEGMREWYFEG	2.97	5.27
Mut10	RSSQTIVHSNGDTYLD	VSHCDGSREWLFEW	3.38	3.63
Mut11	RSSQTFRHVNGCLYLD	VSHYDGIRDWYFNV	0.765	3.84
Mut13	RSSQVNSHWNGTMYLD	VSHYHGSRDWYFDV	0.432	3.99
Mut15	RSSQVPIHGNGTSYLD	VSHYHWRRDWCFDV	0.428	5.02
Mut17	RSSQVPIHGNGHLYLD	VSHYHGSRTWYFDV	0.555	1.84
Mut19	RSSQVPIHVNGMGYLD	VSHYDGSRDWYFDV	0.608	3.70

Gly^{H99}, Ser^{H100a}, Asp^{H100c}, Tyr^{H100j}, Asp^{H101}, Val^{H102}, Thr^{L27a}, Ile^{L27}, Val^{27c}, Ser^{L27e}, Asp^{L30}, Thr^{L31}, and Tyr^{H97} (Fig. 2A). LCDR1 and HCDR3 libraries were constructed with the pIRS-Lc and pICAS-Fd vector, respectively, and co-transformed into MT8-1 to obtain a library of $>10^4$ colonies (Fig. 2B).

3.2. Evolution and selection of catalytic antibody on yeast-cell surface

The evolution of catalytic antibody on yeast-cell surface should be performed to optimize the differential affinity for the transition state of a reaction relative to the ground state. The library of yeast surface-displayed antibodies was therefore sorted after labeling with FITC-conjugated TSA (Fig. 3A). The top 0.25% of the yeast population was collected in each sort and cells directly plated on selective media.

3.3. Catalytic antibody analysis of isolated variants

Colonies isolated from the HCDR3 libraries and the double-chain (heavy chain CDR3 and light chain CDR1) mutagenic library of the 6D9 catalytic antibody were analyzed with flow cytometry and compared with 6D9 (Fig. 3B). Nine colonies from the plates were subjected to further analysis of catalytic activity and sequence (Table 1). All nine mutants had binding affinity to TSA3 and catalyzed the hydrolysis of chloramphenicol monoester 4, with two colonies displaying higher catalytic activity than wild type. High catalytic activity did not however correspond to high binding affinity.

The results demonstrate that a combinatorial library of catalytic antibody can be efficiently constructed on yeast-cell surface using a yeast-displayed Fab fragment of the catalytic antibody. Comparison between the original and the evolved catalytic antibodies showed that high catalytic activity did not necessarily correspond to high TSA3-binding affinity. The relationship between structure and activity requires further investigation.

It is important nevertheless that it was possible with FACS to select several novel catalytic antibodies with enhanced activity from the library. Because in vitro evolution allows us to perform repeated library selection with an antigen corresponding to the TSA structures, the yeast-cell surface engineering system allows us to evolve catalytic antibodies in in vitro combinatorial libraries in a manner similar to the natural evolution of enzymes.

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