Recombinant Technology

Isolation of monoclonal antibodies recognizing rare and dominant epitopes in plant vascular cell walls by phage display subtraction

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Abstract

A combination of phage display antibody technology and a subtraction method provides a powerful tool for the isolation of novel biomarkers. However, the dilemma that high stringency screening often reduces the diversity in the subtracted phage antibody repertoires and that it is difficult to isolate phage antibody against rare epitopes remain. Therefore, we carefully monitored the occupancy of differentiation-specific clones in a phage antibody library through an enrichment process, and succeeded in isolating monoclonal antibodies against rare and dominant epitopes in plant vascular cell walls. We also report that clones with stop and frameshift mutations significantly survived the enrichment process, owing to noncanonical translation mechanisms. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Phage display antibody technology mainly consists of the construction of a library of antibodies displayed on filamentous phage and the selection of phage antibodies (ΦAbs) by antigen binding. It is a powerful tool with which to generate monoclonal antibodies (mAbs), and has been applied to various molecular biological studies (for excellent reviews, see Hoogenboom et al., 1998; Hoogenboom and Chames, 2000; and for phage display applications, see Kay et al., 1996). One of the most important extensions of the technology is the subtraction method (Stausbøl-Grøn et al., 1996), which allows us to screen phenomenon-related mAbs without prior antigen purification, using complex antigens such as whole cells, cell extracts and fractions of cells. In this strategy, a complex antigen negative for a specific phenotype is used to remove ΦAbs against common epitopes, and a positive antigen is used to isolate ΦAbs against phenotype-specific epitopes. Phage display subtraction has been used successfully for the isolation of phenotype-related mAbs, e.g., blood cell-specific (Marks et al., 1993; de Kruif et al., 1995; Siegel et al., 1997), tissue-specific (Portolano et al., 1993; van Ewijk et al., 1997; Edwards et al., 2000; Stausbøl-Grøn et al., 2001) and tumor-related (Cai and Garen, 1995; Pereira et al., 1997;...
Noronha et al., 1998; Kupsch et al., 1999; Tordsson et al., 2000) mAbs. We have also recently applied phage display subtraction to the isolation of mAbs against ill-defined cell-wall components that mark specific developmental stages in plant vascular cells (Shinohara et al., 2000). Phage display subtraction has provided a number of such phenotype-related mAbs. The dilemma remains, however, that high stringency screening for the efficient isolation of phenomenon-related PhAbs often increases PhAbs against dominant epitopes in the subtracted library, reduces the diversity in the PhAb repertoire and makes it hard to isolate PhAbs against rare and sometimes unknown epitopes (Kristensen et al., 2000; Stausbøl-Grøn et al., 2001). If this dilemma can be resolved, the strategy will allow us to isolate phenomenon-related mAbs against antigens that are hard to purify by conventional methods, and will be of great value in identifying novel biomarkers. To overcome this difficulty and identify useful biomarkers, we carefully monitored the clonal occupancy of differentiation-specific PhAbs in a PhAb library subtracted using differentiation-induced and noninduced cell-wall fractions.

2. Materials and methods

2.1. Plant materials

_Zinnia_ mesophyll cells were cultured according to a previously described method (Sugiyama and Fukuda, 1995). Cell-wall fractions were isolated from _Zinnia_ mesophyll cells that had been cultured for 42 h in a differentiation-inductive medium which contained 0.1 mg/l 1-naphthaleneacetic acid and 0.2 mg/l benzyladene, or in a noninductive medium which contained only 0.1 mg/l 1-naphthaleneacetic acid. These fractions were designated D- and C-cell walls, respectively. Preparation procedures of these fractions were according to a previously described method (Shinohara et al., 2000).

2.2. Library construction

A single-chain antibody variable fragment (scFv) phage display library (4.6 × 10^6 independent transformants) was constructed as previously described (Shinohara et al., 2000). In brief, a female Balb/c mouse was hyperimmunized with D-cell walls. The murine spleen poly(A)^+ RNA was used for the construction of a scFv phage display library with the Recombinant Phage Antibody System (Amersham Pharmacia Biotech, Uppsala, Sweden) based on Hoogenboom et al. (1991).

2.3. Enrichment of differentiation-specific phage antibodies through panning

PhAb (2 × 10^10 transducing units in 400 μl 2% [w/v] dry milk–PBS) from the library was incubated with 100 μl packed volume (PV) of the C-cell walls (blocked with 5% dry milk–PBS at 4 °C for 30 min) at room temperature for 2 h with slow rotation and then centrifuged (5000 × g, 2 min). The supernatant was incubated with 20 μl PV of the D-cell walls (blocked with 5% dry milk–PBS at 4 °C for 30 min) as described above. The D-cell walls were washed twice with 2% dry milk–PBS, 10 times with 0.1% Triton X-100–PBS (PBS/T) and 10 times with PBS. PhAbs bound to the washed D-cell walls were rescued as ampicillin-resistant colonies by the direct addition of exponentially growing _Escherichia coli_ cells (TG1, supE strain) to the washed D-cell walls. The subtracted library was used for further rounds of subtractive panning. The second to fourth rounds of subtractive panning were carried out under higher stringency, in which PhAbs (2 × 10^10 transducing units) were suspended in 5% dry milk–PBS instead of 2% dry milk–PBS, and PhAbs bound to D-cell walls were washed 20 times with PBS/T instead of 10 times.

2.4. Finger printing assay, sequencing, site-directed mutagenesis

scFv DNA fragments from randomly picked colonies of the library were amplified by colony PCR using S1 5'-d(CAACGTGAAAAATTATTATTCGC)-3' and S6 5'-d(GTAAATGAATTTTCTGTATGAGG)-3' oligonucleotide primers, which anneal just outside each end of the scFv genes. The amplified scFv DNAs were restricted with Cfr13I (Toyobo, Tokyo, Japan) and then separated by agarose gel electrophoresis. Once clones were identified by the fingerprinting assay, their scFv DNAs were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using a commercially available kit (Applied Biosystems, Fos-
ter City, CA, USA). Site-directed mutagenesis was performed by overlap extension, as described previously (Sambrook and Russell, 2001).

2.5. ELISAs

To perform monoclonal ΦAb ELISA, ΦAbs from single clones were quantified using an ELISA with anti-M13 antibody–horseradish peroxidase (HRP) conjugates (Amersham Pharmacia Biotech) on the basis of a standard curve prepared using M13 phage (M13KO7). Each monoclonal ΦAb (10^{11} plaque-forming units as an interpolated value) were incubated overnight at 4 °C with 20 μL PV of the D- and C-cell walls that had been treated with 1% (v/v) H2O2–PBS for 3 h at 4 °C and then blocked with 5% dry milk–PBS for 1 h at 4 °C. ΦAbs bound to the cell walls were detected with anti-M13 antibody–HRP conjugate (1:5000). The signals were developed using 1717 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution (Bio-Rad Laboratories, Hercules, CA, USA) and the A415 value. The A415 value for solution without ΦAb treatment was subtracted from the A415 of each sample to control for background absorbance. M13 phage were used instead of ΦAb as a negative control.

An ELISA using fixed whole plant cells was performed as reported previously (Shinohara et al., 2000) with some modifications. Because of the different stabilities of the epitopes, all procedures in the ELISA (except the colorimetric reaction) were performed at room temperature for the scFv of clone #3, but at 4 °C for the scFv of clone #27. The colorimetric reaction took 5 min for the scFv of clone #3 and 10 min for the scFv of clone #27.

Every ELISA experiment shown in this report was performed twice or more, and each result showed similar reproducibility.

2.6. ScFv protein expression

Soluble scFv proteins were prepared from non-suppressor E. coli cells (HB2151) infected with monoclonal ΦAbs according to a method described previously (Kipriyanov et al., 1997). Induced soluble scFv proteins were purified from periplasmic fractions or culture media by an anti-E-tag antibody affinity column (Amersham Pharmacia Biotech).

Soluble scFv expression was estimated using whole-cell extracts, periplasmic fractions and culture medium. On the other hand, scFv displayed on phage particles was estimated using ΦAbs purified by polyethylene glycol precipitation (Sambrook and Russell, 2001). These samples were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and blotting onto nitrocellulose membrane (Towbin et al., 1979). Blotted scFv proteins were detected using anti-E-tag antibody and alkaline phosphatase-labeled antimouse IgG antibody (Dako, Carpinteria, CA, USA).

3. Results and discussion

To isolate mAbs against differentiation-specific wall components, specific ΦAbs in a ΦAb library were enriched by three or four rounds of subtractive panning in which nonspecific ΦAbs were removed by coprecipitation with an excess of C-cell walls, and specific ΦAbs were then selected with D-cell walls. The ratio of selected ΦAbs to applied ΦAbs reached saturation after three rounds (Fig. 1).

To monitor the enrichment process, clonal redundancies in the third and fourth round libraries were checked by PCR-restriction fragment length polymorphism (RFLP) analysis of the scFv genes. We found that 93 ΦAbs randomly picked from the third round library were ascribed to 27 clones and 94 ΦAbs randomly picked from the fourth round library were ascribed to nine clones. This means that clonal occupancy became more biased toward increasing clonal redundancy through the enrichment process. As a
result, a total of 28 clones (numbered #1–28) were identified from 187 ΦAbs (Fig. 2A).

To identify the cause of the increasing clonal redundancy, the 28 isolated clones were analyzed in detail. An ELISA against D- and C-cell walls revealed that 10 ΦAbs (derived from clones #1–7, #9, #25 and #27) of the 28 monoclonal ΦAbs bound more strongly to D-cell walls than to C-cell walls (Fig. 2A). Sequencing the 28 scFv genes revealed that (i) scFv sequences of clones #1–7 and #9 had significant homology, even in the complementarity-determining regions (Fig. 3), and (ii) 21 clones contained stop and/or frameshift mutations (Fig. 2B). The expression of soluble scFv protein in nonsuppressor bacterial cells was tested for the monoclonal ΦAbs that gave high absorbance values in the ELISA against D-cell walls by immunoblotting using anti-E-tag antibody (Fig. 2B). Clones #3, #7, #9 and #27, which had no stop or frameshift mutations, expressed soluble scFv proteins at high levels. Clones #2 and #6, with opal mutations, expressed soluble scFv protein slightly but significantly. Clones #1, #4 and #5, with amber mutations, and clone #25, with a frameshift mutation, expressed soluble scFv proteins at levels below detection.

The significant homology among clones #1–7 and #9 (Fig. 3) indicates that their scFvs recognize the same epitope. However, the apparent binding activity to D- and C-cell walls varied among the ΦAbs from these clones (Fig. 2A). Although no opal suppressor strains were used for ΦAb production, tryptophanyl-tRNA decodes an opal codon with low efficiency even in wild-type cells (Hirsh and Gold, 1971). Therefore, clones #2 and #6, containing opal mutations, may produce slight but substantial amounts of scFv-displaying phage particles. Indeed, the low decoding efficiency of the opal codons in clones #2

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**Fig. 2.** Characterization of clones in the ΦAb library. (A, left) Frequency of clones in 93 colonies from the third-round library (open column) and in 94 colonies from the fourth-round library (closed column). (A, right) Monoclonal ΦAb ELISA against D (shaded column) and C (dotted column) cell walls, in which (-) indicates the negative control using M13 phage instead of ΦAb. The ELISA data are depicted as one representative of two independent experiments. (B) Stop and frameshift mutations and soluble scFv expression in clones. scFv genes of all the clones were sequenced in both directions, and each of the stop and frameshift mutations was certified by multiple sequencing using independently amplified PCR products. Ochre, amber and opal indicate stop mutations coded by UAA, UAG and UGA, respectively. “−1” denotes single-base deletions. * The VH sequence of clone #19 was abnormally short (169 bp) and had no homology to any given VH sequence except primer annealing sites. ++: detected strongly; +: detected weakly; −: below detectable levels; N.D.: not determined.
and #6 was verified by their expression of soluble
scFv in nonsuppressor bacterial cells (Fig. 2B). On the
other hand, clones #1, #4 and #5, with the amber
mutation, probably produced scFv-displaying phage
particles at high levels because AAbs were produced
in supE bacterial cells and their amber codons were
decoded by suppressor glutaminyl-tRNA, although
soluble scFvs from clones #1, #4 and #5 were not
expressed in nonsuppressor bacterial cells (Fig. 2B).
In fact, clones #1 and #6, which may produce small
amounts of scFv-displaying ΔAbs, exhibited strong
binding activity to D-cell walls but not to C-cell walls,
whereas clones #1, #3–5, #7 and #9, which produce
medium levels of scFv-displaying ΔAbs, showed
strong binding activity to both D- and C-cell walls
(Fig. 2A). Therefore, it is obvious that the apparent
binding activity seen in the ELISA correlates with the
levels of scFv-displaying ΔAbs. To check the satu-
ration of binding activity, the ELISA was performed
using dilution series of ΔAbs of clones #1–4, #6 and
#7. As a results, the binding activity to D-cell walls
were two to six times larger than that to C-cell walls in
a certain ΔAb concentration of each clone (data not
shown). Accordingly, the results can be interpreted to
indicate that the epitope recognized by scFvs from
clones #1–7 and #9 was differentiation-specific.

Fifteen clones had frameshift mutations (Fig. 2B).
Interestingly, almost all clones, except clone #19, had
sequences that could be restored to the open reading
frame of the scFv gene by a single base insertion at a
certain position. It is known that frameshift mutations
are often leaky (Atkins et al., 1972), and several
noncanonical translation mechanisms responsible for
frameshift decoding have been reported (reviewed by
Gesteland and Atkins, 1996; Baranov et al., 2001).
Therefore, we searched for known frameshift decod-
ing signals (i.e., tandem slippery sequences in the –1
frame relative to the translation start site, pseudoknot
structures located downstream from a tandem slippery
sequence, Shine–Dalgarno-like sequences, ribosomal
hopping sites) through their frameshift windows,
which were defined at the 3′ border by a nonsup-
pressed stop codon in zero frame and at the 5′ border
by a nonsuppressed stop codon in the shifted frame.
Although none of the known signals responsible for
programmed frameshifting were found, there were
two possible sites (AAAUAAAA in an scFv gene of
clone #12 and GUGUGA in an scFv gene of clone
#20, the 3′ border underlined) of short-range riboso-
mal hopping. There were no distinct ribosomal hop-
sing sites at the other 3′ borders, but it seems plausible
that ribosomal hopping or other noncanonical trans-
lation mechanisms occasionally decoded the frame-
shifts in the extended frameshift windows.

To confirm that scFvs from the clones with stop and
frameshift mutations can bind to D-cell walls, the
mutations in the scFv genes were inverted by site-
directed mutagenesis. The reversed mutation restored
scFv expression on phage particles in all clones tested
(Fig. 4A), and ΔAbs from the revertants showed
binding activity to D-cell walls (Fig. 4B). The reverse
mutation increased ELISA absorbance values in all
clones except clone #25 (Fig. 4).

Taken together with the fact that these clones
survived three or four rounds of subtractive panning,
it is probable that many of the clones with stop and
frameshift mutations produced functional scFvs on
phage particles by noncanonical translation mecha-
nisms. Noncanonical translation mechanisms, includ-
ing ribosomal hopping, are often minor events (Gesteland and Atkins, 1996). Although scFvs were not detected on AAbs with frameshift mutations, it seems plausible that rarely displayed scFvs were sufficient to bind the phage particles to antigens. The production of functional scFvs in mutant clones is supported by a previous report that clones containing stop and frameshift mutations are found at nonnegligible levels during screening despite affinity-based enrichment in a phage display system (Goldman et al., 2000). An important but unsolved question is: what is the underlying cause of stop and frameshift mutations? Because some of these mutations occur within the primer annealing site, it is likely that the mutations were caused artificially during library construction. Other mutations may occur naturally in mice during affinity maturation of antibodies or artificially during the PCR amplification of scFv genes.

The relative ratio of AAbs with low scFv-displaying efficiency may decrease in a AAb library through the enrichment process despite the scFv specificities. Indeed, most clones with stop and frameshift mutations were not found in the fourth round library. Therefore, the reversion of stop or frameshift mutations in scFv genes of screened clones may be a way to increase the isolation of various mAbs. On the other hand, the preclusion of clones with stop and frameshift mutations via affinity-selection for antibody fragments is a good way to improve the performance of the phage display library (Hoogenboom et al., 1998).

Soluble scFvs of clone #3 (as a representative of the high-homology cluster clones #1–7 and #9) and clone #27 were expressed and purified using an anti-E-tag affinity column. The soluble scFvs were used for whole-cell ELISA against cultured plant cells. This revealed that both epitopes increased specifically on cells cultured in a differentiation-inductive medium but not on those cultured in a noninductive medium.

**Fig. 4.** Reverse mutations of stop and frameshift mutations in scFv genes restored the expression of scFvs on phage particles (A, immunoblotting assay of AAbs purified by polyethylene glycol precipitation) and cell wall-binding activity (B, monoclonal AAb ELISA against cell walls). Arrows indicate the position of the 70-kDa scFv protein fused with gIIIp, a minor capsid protein of M13 phage. “–1” and “ochre” indicate −1 frameshift and ochre stop mutations, respectively. The ELISA data are depicted as one representative of two independent experiments.

**Fig. 5.** Changes in epitopes of clones #3 and #27 in isolated Zinnia mesophyll cells during culture in a differentiation-inductive (●) or a noninductive medium (○). Each point represents the mean of three samples, and vertical lines indicate standard deviations.
(Fig. 5). These results demonstrate that both the scFvs are differentiation-specific. The epitopes of clones #3 and #27 are obviously different from each other because they showed different increments through the culture period (Fig. 5), different tissue specificities and different subcellular localization (data not shown). Soluble scFv of clone #3 gave higher absorbance values (Fig. 5) with a shorter reaction time in the ELISA than clone #27, indicating that an epitope recognized by the scFv of clone #3 is more dominant than one recognized by the scFv of clone #27.

Clonal occupancy of the high-homology cluster clones (#1–7 and #9), which probably recognize the same epitope, reached 74% through three rounds of panning and 99% through four rounds of panning (cf. Fig. 2A). This result indicates that clonal occupancy of the FabAbs recognizing the dominant epitope increased steadily through the enrichment process. In contrast, although clone #27 was found in the third round library (Fig. 2A), clone #27 was not found in the fourth round library despite its obvious differentiation specificity (Fig. 5). Therefore, affinity-based enrichment using complex antigens may not only remove nonspecific FabAbs but also reduce the relative ratio of FabAbs against rare epitopes by increasing the relative ratio of FabAbs against dominant epitopes.

In phage display subtraction using complex antigens, feasible screening processes vary from case to case, and different parameters (e.g., concentration and nature of the antigen, source and size of the library, blocking and washing during selection, the use of subtraction strategies) are involved in the outcomes of selections (Hoogenboom et al., 1999; Mutuberria et al., 1999). Of parameters related to selections, the number of panning is quite important to attain the sensitivity with which FabAbs against rare epitopes are isolated. Our results clearly indicate that FabAbs against rarer epitopes are more easily screened from an earlier round library and that a later round library mainly consists of FabAbs against dominant epitopes. Thus, we conclude that using the least number of rounds of subtractive panning necessary to achieve both sufficient enrichment of specific FabAbs and maximum clonal diversity is the key to isolating FabAbs against rare epitopes, and our findings may contribute to the isolation of useful biomarkers.

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