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A novel method for construction of gene fragment library to searching epitopes

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Abstract

Identification of the epitope sequence or the functional domain of proteins is a laborious process but a necessary one for biochemical and immunological research. To achieve intensive and effective screening of these functional peptides in various molecules, we established a novel screening method using a phage library system that displays various lengths and parts of peptides derived from target protein. Applying this library for epitope mapping, epitope peptide was more efficiently identified from gene fragment library than conventional random peptide library. Our system may be a most powerful method for identifying functional peptides.

Keywords: Phage display system; Gene fragment library; Random peptide library; Epitope mapping; TNF-α

The ability to identify active core or epitope peptides from bioactive proteins is of considerable interest to many researchers. Active-center peptide and binding domain peptide of protein have been expected for target peptide, biological tool, and more reasonable medicine, such as RGD peptide [1], Tat peptide [2], and Angiostatin/Endostatin [3,4]. On the other hand, applications of epitope peptide for anti-viral, cancer, and allergy immunotherapy have been extensively tried [5–8]. One of the most effective and frequently used methods for searching and identifying these functional peptides is phage display technology. Phage library which involves the expression of random peptides on its envelope as a fusion protein has been commonly used for this purpose [9–11]. But screening of target peptide from random peptide library is not effective, because theoretical diversity of random peptide library is enormous. For example, while the theoretical diversity of 10 mer random peptide library is 10 trillion (20^{10}), the maximum diversity is actually 10 million (1/1000 of theoretical size). Thus construction of gene fragment library which expresses random fragments of cDNA on phage particles has been tried [12–14]. Unlike random peptide library, gene fragment library is usually constructed for each target protein and supposed to be quite effective at much lower library sizes. If the length of target protein is 200 amino acids, the theoretical diversity is 2 million. However, conventional method for gene fragment library has the following limitations: (1) the gene fragmentation process with DNase is incomplete, resulting in poor variety of the fragment library; (2) with the use of blunt-ended insert DNA,

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unidirectional cloning cannot execute; (3) translational frame shift cannot be prevented. Therefore, this conventional phage library method is extremely limited for isolating functional peptide fragments. We therefore improved the technique and established a novel library system which enabled construction of a gene fragment library covering all regions and various lengths of the target protein.

Materials and methods

Reagents. Reagents for transcription were from Promega (Madison, WI), and T7 RNA polymerase was from TAKARA BIO (Shiga, Japan). Smart Race cDNA Amplification Kit was from Clontech Laboratories (Mountain View, CA). Other reagents for reverse transcription were from Invitrogen (Tokyo, Japan). 5′-RACE PCR was performed by Advantage-HF2 PCR kit (Clontech Laboratories, Inc.). Accu Taq LA DNA polymerase (Sigma–Aldrich Japan, Tokyo, Japan) was used for nested PCR. DNA and RNA were purified with QIAquick PCR Purification Kit and RNeasy mini kit (QIAGEN, Valencia, CA), respectively. *Escherichia coli* TG1 was purchased from STRATAGENE (Tokyo, Japan). Anti-FLAG monoclonal antibody was from Sigma–Aldrich. Rabbit anti-human TNF-α polyclonal antibody was from CALBIOCHEM (Darmstadt, Germany). Mouse anti-M13 phage-horseradish peroxidase (HRP) conjugate and pCANTAB5E were from Amersham–Pharmacia Biotech (Uppsala, Sweden).

Construction of gene fragment library. Fig. 1 is a flow diagram that shows the construction of gene fragment library. TNF-α coding target region, domain 1, 2, and 3 were amplified and T7 promoter was added at the 5′ end by PCR. PCR products were transcribed with T7 RNA polymerase at 37 °C for 2 h, yielding sense RNA of only the target region. The RNA samples were reverse transcribed with the Smart Race cDNA Amplification Kit using random nonamer primers that contained *Mro*I site at the 5′ end. In this reaction, after reverse transcriptase reaches the ends of the mRNA template, it adds several dC residues to synthesized cDNA. The adaptor oligonucleotide anneals to the tail of the cDNA and serves as an extended template for reverse transcriptase. Following reverse transcription, the first-strand cDNA was used directly in 5′-RACE PCR using synthetic primers, which anneal to the adaptor oligonucleotides and *Mro*I site, respectively. The condition of 5′-RACE PCR was cycled 5 times at 94 °C for 30 s, 72 °C for 3 180 s, 5 times at 94 °C for 30 s, at 70 °C for 30 s, and at 72 °C for 180 s, and 20 times at 94 °C for 30 s, at 68 °C for 30 s, at 72 °C for 180 s. Consequently, dsDNA was obtained, which contains T7 promoter and *Mro*I site, and begins randomly at the 5′ end. After the cDNA was transcribed with T7 RNA polymerase, mRNA was reverse transcribed by Super Script III using random nonamer containing the *Nco*I site to yield single strand DNA that began randomly at the 3′ end of the sense strand. The gene library was amplified by PCR and constructed with *Nco*I site at the 5′ end and *Mro*I site at the 3′ end, and coded various range of TNF-α. PCR was cycled 35 times at 96 °C for 60 s, at 59 °C for 60 s, and at 68 °C for 60 s. The gene library was then digested with *Nco*I and *Mro*I was ligated with the phagemid vector pY03-FLAG (*Mro*I) to display TNF-α fragments on the phage surface as fusion proteins with g3p. pY03-FLAG (*Mro*I) was constructed by inserting the *Mro*I and FLAG sequence between E tag and g3p gene of pCANTAB 5E. The phage library was prepared as described [15].

Selection of phages displaying FLAG tag. Ten micrograms per milliliter of Anti-FLAG monoclonal antibody was coated onto Maxisorb immunotubes (NUNC). After blocking, TNF-α gene fragment phage library was then added into the anti-FLAG antibody-coated immunotubes and incubated for 1 h at 4 °C. Random 18 mer peptide phage library was constructed by almost the same method as previously described [16] and used as a control. After washing the tubes with PBS containing 0.05% Tween 20, the bound phages were eluted by incubating the tubes with 100 mM HCl. Eluted phages were immediately neutralized with 1 M Tris–HCl and then added to log phase *E. coli* TG1 cells. For panning of the anti-TNF-α antibody, the infected TG1 cells were grown to log phase, rescued with M13KO7 helper phage, and purified by polyethylene glycol (PEG) 6000/NaCl precipitation.

Selection of phages displaying peptide bound to anti-TNF-α antibody. Ten micrograms per milliliter of rabbit anti-TNF-α polyclonal antibody was coated onto 96-well immune plate (NUNC). The procedures were followed as mentioned above (the section of "*Selection of phages displaying FLAG tag*"). After the third round of panning, eluted phages in each round of panning were used for phage ELISA to estimate the number.

Phage ELISA. For measurement of output/input ratio, the eluted phages were added to 96-well immune plate coated with each antibody and incubated at RT for 2 h. The plates were washed three times with PBS and 0.05% Tween PBS, and incubated with anti-M13 phage-horseradish peroxidase (HRP) conjugate for 1 h. After incubation, the plates were washed three times, TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan) was added, and the absorbance was read at 450 nm using a microplate reader. To assess affinities of individual phage clones, infected TG1 cells were isolated, grown at 37 °C in 96-well plate, and rescued with M13KO7 helper phage. Amplified phage particles were added to anti-TNF antibody coated plate and following the above procedure.

Peptide ELISA. Biotinylated epitope peptide was used for binding analysis. Mab1-peptide, Mab4-peptide, and 3D6-peptide were used for control peptides. Each peptide corresponds in position to a.a. 127–137, a.a. 34–45, and a.a. 22–33 of TNF-α, respectively. Peptides were added to 96-well immune plate coated with the anti-TNF antibody and detected by Streptavidin HRP conjugate. The following procedure was performed as described in the above section.

Results

Library construction

We used human tumor necrosis factor-α (TNF-α) as a model protein to confirm the usefulness of our method. One area of improvement was that we could generate gene fragments with the *Sfi*I site at the 5′ end and *Mro*I site at the 3′ end, in the same orientation as the original gene, by using unidirectional reverse transcription and amplification of mRNA by T7 RNA polymerase [17]. Three TNF-α gene fragment libraries were constructed using TNF-α cDNA divided into 3 domains (domain 1, a.a. 1–85; domain 2, a.a. 40–123; and domain 3, a.a. 75–157) as a template. This library theoretically contains all TNF-α peptide sequences of less than 46 a.a. The TNF-α fragment library was produced by the procedure shown in Fig. 1. The number of the independent clones was 2.0×10^7 CFU, containing from domain 1, 7.1×10^6 CFU; domain 2, 5.6×10^6 CFU; and, domain 3, 7.3×10^6 CFU. The repertoire of the library sufficiently exceeded the theoretical variety for a fragment peptide library from 3 domains (8.2×10^3 CFU). The sequences of clones from this library were randomly analyzed (Fig. 2). Although gene fragments from domain 2 and 3 library tended to be located nearer the 5′ end of each domain, gene fragments from domain 1 were originated from various lengths and parts of the TNF-α sequence. All of the gene fragments had the assumed orientation. Thus we have some success in the creation of a library composed of fragments of various lengths and parts of TNF-α. However, the library was initially contaminated by unexpected clones whose lengths of the insert gene that were not multiples of 3, resulting in

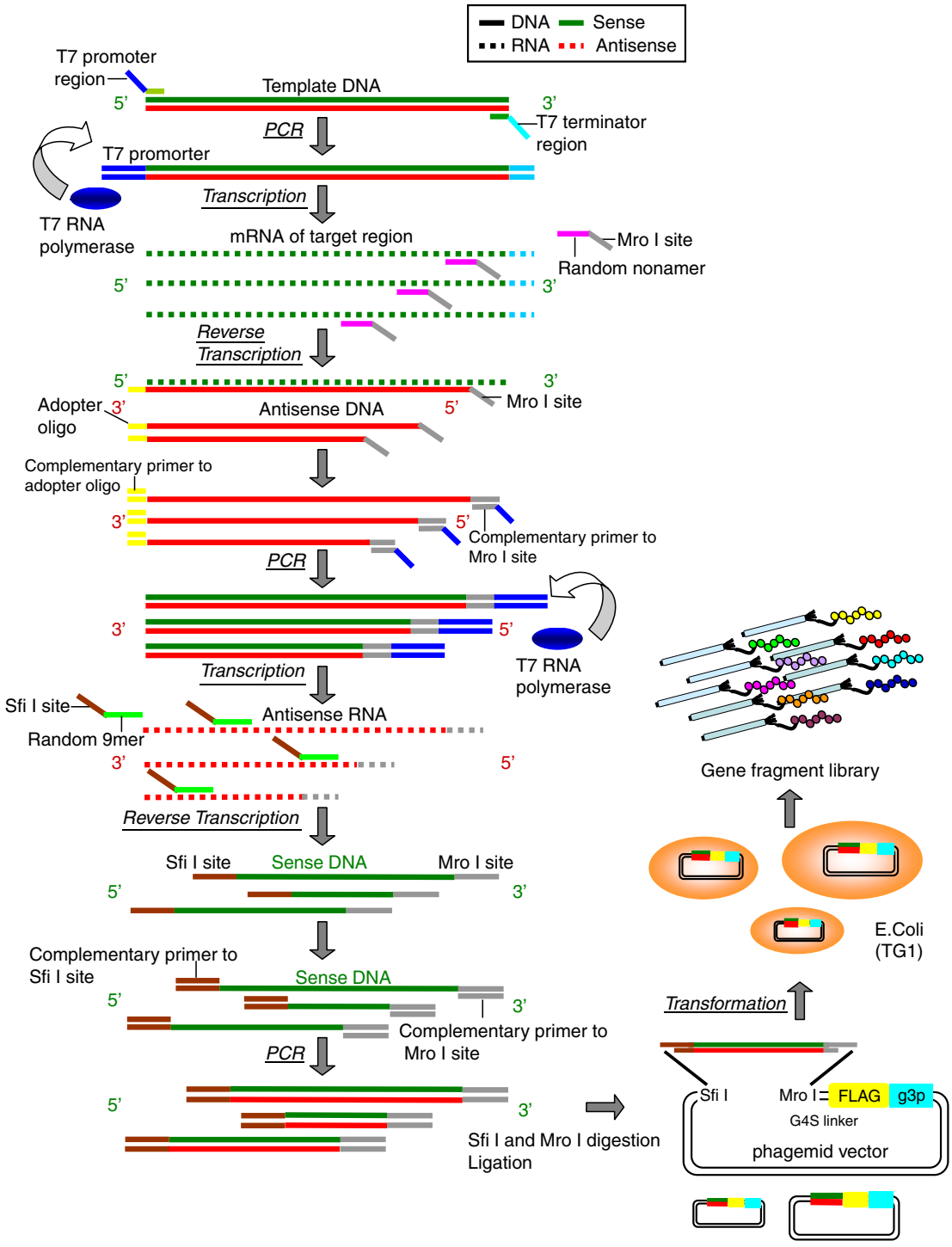

Fig. 1. Scheme for construction of a novel gene fragment library.

frame shifts. These clones cannot express target peptide as a fusion protein with envelope g3p and FLAG peptide, which develops downstream. Phage clones that did not express fragments of TNF-α and g3p as a fusion protein were removed with FLAG tag, which was inserted between the DNA coding fragment peptide by using anti-FLAG antibody. We were thus able to create a library that covered TNF-α fragments of various lengths and regions.

Affinity selection with anti-TNF-α antibody

To assess whether a specific peptide could be selected from this library, epitope mapping of a rabbit anti-TNF-α polyclonal antibody was performed. The number of phage clones expressing peptides that bind to anti-TNF-α antibody was estimated by measuring the output phages after each panning round using anti-TNF-α and

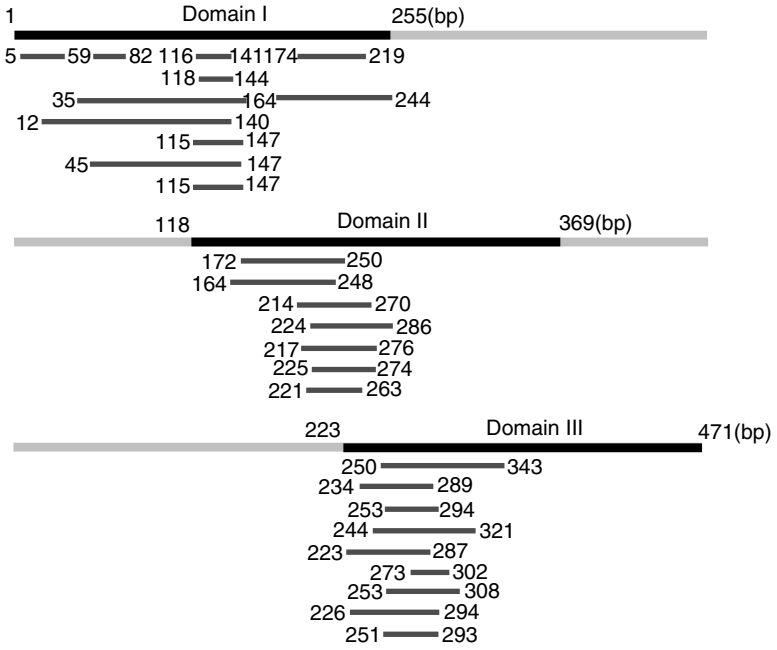

Fig. 2. Schematic representation of nucleotide sequences of peptides selected from the TNF-α gene fragment library.

anti-FLAG antibody (Fig. 3). Consequently, the output/input ratio of phage clones bound to anti-TNF-α antibody increased with each panning round, suggesting that the phage which expresses the peptide bound to the antibody was enriched. In contrast, when a random peptide library was used as a control, the number of control phage clones did not increase even after the second panning round. These results suggested that target peptides can be selected more effectively using our gene fragment library than with a conventional random peptide library.

Individual clones were isolated from output phages after each panning round and ELISA was performed to select clones that bound to anti-TNF-α antibody. Many clones had strong affinity for the antibody after the second panning, whereas almost none of the clones did prior to panning (Fig. 4). In addition, similar results were observed using other clones of anti-TNF-α antibodies (data not shown). In order to identify the peptide containing the epitope, we analyzed the insert sequences of phage clones which bound strongly to the antibody. Unexpectedly, we obtained phage clones which displayed peptides that contained amino acid 15–33 sequence of TNF-α (Fig. 5). Thus, this TNF-α fragment peptide was chemically synthesized as an epitope peptide and assessed its affinity for anti-TNF-α

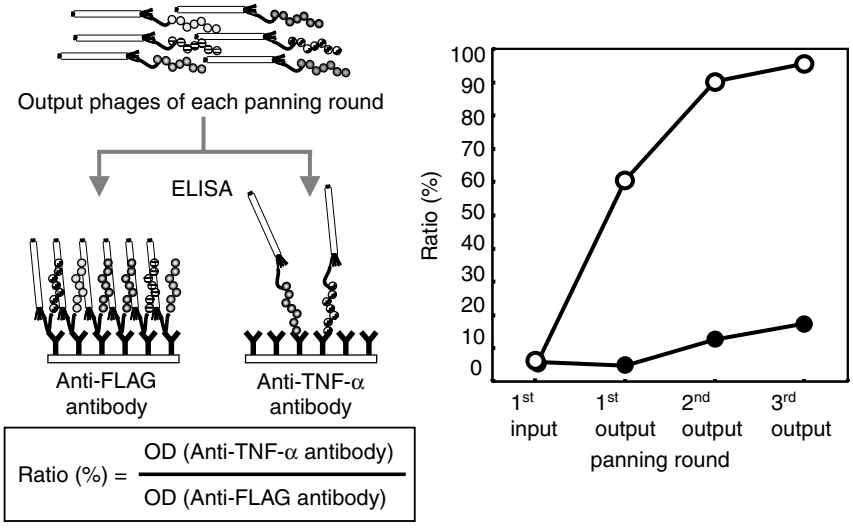

Fig. 3. Selection of phage clones expressing peptides binding to anti-TNF-α antibody. TNF-α gene fragment library (○) and the random 18 mer peptide library (●) were applied to immunotubes with immobilized anti-FLAG antibody or anti-TNF-α polyclonal antibody. Phage clones bound to each antibody were then selected as described in Materials and methods.

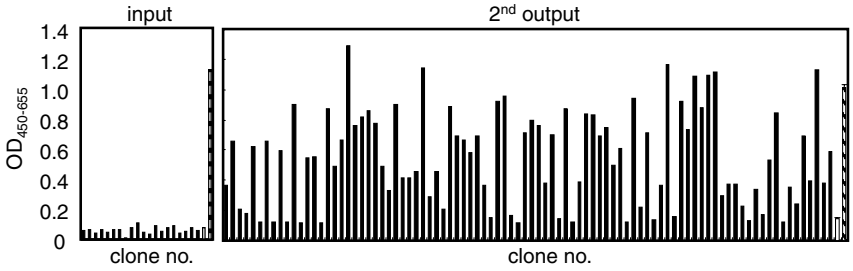

Fig. 4. Affinities of monoclonal phages for anti-TNF-α antibody. Ninety clones were selected randomly from input or second output phage clones and their affinities for the antibody were estimated by phage ELISA. Phage clone expressing TNF-α was used as a positive control (striped column), and IFN-α was used as a negative control (open column).

	1 10 20 30 40
TNF-α:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANG
Clone 16/19/26/72:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRP
Clone 20:	LVVANPQAEGQLQWLNRQ
Clone 21:	YVVANPQAEGQLQWLNRRD
Clone 22/47/51:	YVVANPQAEGQLQWLNRP
Clone 30/36/70:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNQ
Clone 34:	HVVANPQAEGQLQWLNRRE
Clone 35:	NVVANPQAEGQLQWLNRRE
Clone 38:	YVVANPQAEGQLQWLNRH
Clone 42:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRP
Clone 46:	VHVVANPQAEGQLQWLNRRE
Clone 49:	LVVANPQAEGQLQWLNRRD
Clone 57:	TAHVVANPQAEGQLQWLNRRG
Clone 61:	HFVANPQAEGQLQWLNRQR
Clone 66:	LVVANPQAEGQLQWLNRR
Clone 68/82:	HVVANPQAEGQLQWLNRRE
Clone 71:	HVVANPQAEGQLQWLNHQ
Clone 86:	FRSSSRTPSDKPVAHVVANPQAEGQLQWLNRL
Clone 88:	FVVANPQAEGQLQWLNRK

Fig. 5. Amino acid alignment of peptides presented by phage clones bound to anti-TNF-α antibody. The amino sequences of fragments which strongly bound to the anti-TNF-α antibody in Fig. 4 and their sequence alignment with TNF-α are shown.

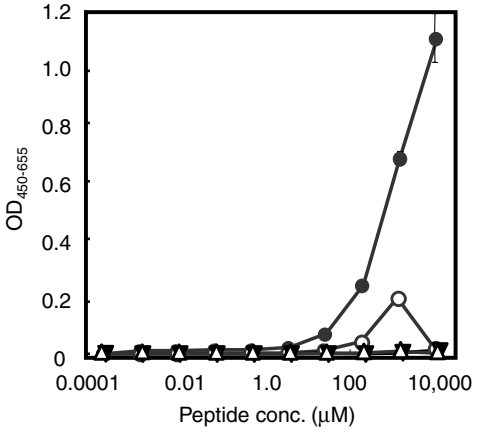

Fig. 6. Binding analysis of synthetic peptide to anti-TNF-α antibody by ELISA. Biotinylated epitope peptide (HVVANPQAEGQLQWLNRRA :●) and biotinylated control peptides (Mab1-peptide; EKGDRLSAEIN:▼), Mab4-peptide (NALLANGVELRD:△), and 3D6-peptide (AEGQLQWL NRRA:○) were applied to solid-phase anti-TNF-α antibody. Binding peptides were detected by avidin-HRP.

antibody by ELISA. Although control peptides did not bind to anti-TNF-α antibody, this synthetic peptide containing TNF-α fragment peptide dose-dependently bound to the antibody. These results indicated that the displayed peptides on the phage surface behaved similarly to free peptides and amino acids 15–33 were actually epitope of the antibody (Fig. 6).

Discussion

In this study, we improved the method for construction of gene fragment phage library and applied this library to epitope mapping. Although gene fragment libraries have been expected to be superior in availability [18,19], they are constructed from cDNA fragments generated by digestion with a non-specific endonuclease, resulting in blunt-end ligation (very low efficiency) and contamination of reversely oriented fragments [12–14]. Thus it is inefficient to identify functional peptides and epitope peptides from gene fragment library constructed by this conventional method. Therefore, focusing on unidirectionality of reverse transcription reaction, we created gene fragments using reverse transcription following transcription of mRNA by T7 RNA polymerase (Fig. 1). This process made it possible to insert gene fragments retaining proper orientation into phagemid vector and ligate each protruding ends.

Additionally, using FLAG tag selection which removed phage clones causing frame shifts and expressing nonspecific peptides, we successfully ameliorated the method of gene fragment library to be superior in quality and diversity (Fig. 2). However, gene fragments from domain 2 and 3 tended to be deflected to the 5′ end of template. We think this problem will be resolved by appropriately changing a temperature of annealing in RT-PCR for each template. In consideration of this point, we are now constructing gene fragment library of some virus envelope proteins for searching functional peptides.

To assess the availability of our strategy, we tried epitope mapping of anti-TNF-α antibody from TNF-α fragment library. After selection of anti-TNF-α antibody, all amino acid sequences of peptides which strongly bound to the antibody contained amino acids 15–33 sequence of TNF-α (Fig. 5). There are very few reports to confirm that selected phage clones almost encode convergent sequence like this. We predicted there are two reasons: rabbit anti-human TNF-α antibody is easy to recognize the epitope containing an amino acid sequence that differs between human and rabbit TNF-α; and TNF-α fragment library constructed in this study is of dramatically higher quality and diversity than conventional phage libraries. In fact, residues 20–32 of TNF-α have low homology among species and the peptides obtained after the panning contained residues 20, 22, 30, and 31, residues which differ between human and rabbit TNF-α [20]; thus, the peptide was recognized as an epitopic region. Additionally, compared to random peptide library, phage clones bound to the antibody were quite efficiently concentrated from our TNF-α fragment library (Fig. 3). Our system provided a useful strategy for comprehensively searching and identifying functional peptides from various proteins, such as cytokines, extracellular matrix, and coat proteins of viruses. This novel method is likely to be useful for the development of pharmaceuticals, targeting peptides, molecular biological tools, and vaccines.

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