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Highlighted paper selected by Editor-in-chief

Quality Enhancement of the Non-immune Phage scFv Library to Isolate Effective Antibodies

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The non-immune phage antibody library system is one of the most attractive technologies available to current therapeutic, diagnostic and basic scientific research. This system allows the rapid isolation of antibodies of interest that could subsequently be applied directly to drug delivery systems and antibody therapy. Previously, we reported the primer sets to encompass the antibody repertoire and thus improve library quality. However, a wide number of varying primer sets cause to decrease the amplification efficiency of antibody genes. In the present study, we re-generated the library primer sets newly and constructed an improved library from non-immune mice that was far superior in terms of variety and quality. This new library contained 2.4 billion independent clones. In addition, we optimized the selection step from this library to isolate high-affinity antibodies. The optimization of an affinity panning protocol by the incorporation of an automated Microfluidics instrument led to the successful isolation of three different monoclonal antibodies for human vascular endothelial growth factor receptor 2 (KDR). These antibodies were demonstrated to exhibit high specificity and were able to detect a mere 0.6 fmol of KDR by dot blot analysis. Previously reported antibodies for luciferase were also isolated successfully from this library. Our results clearly demonstrate the importance of the improved protocol for the library preparation of antibodies and the resulting isolation of antibodies for clinical and research applications.

Key words non-immune antibody library; phage display system; single-chain Fv; high-throughput screening; vascular endothelial growth factor receptor 2

Monoclonal antibodies are not only highly regarded as reagents for basic biochemical research but also for clinical diagnostic and therapeutic applications.[1—4] The production of a monoclonal antibody is laborious and time-consuming using established methodology involving the immunization of animals with large amounts of antigen, the isolation of B cells and the production of antibody-producing hybridomas. Therefore, the development of a rapid and easy method to produce monoclonal antibodies is highly desirable as an alternative to conventional hybridoma technology.[5,6]

Over recent years, phage display library technology has received a great deal of attention in terms of antibody production.[7—10] The phage display system is able to construct a large repertoire of protein or peptide libraries consisting of hundreds of millions of molecules. The phage antibody library which displays the single chain Fv fragment (scFv) of immunoglobulin is one of the most promising applications of the phage display system. Monoclonal antibodies against target antigens are obtained rapidly by the use of an affinity panning procedure *in vitro* which selects and amplifies the phage clones specific for the antigen required. There are two different types of phage antibody library system: the immune library made by using B cells isolated from patients or immunized animals as a gene source,[11] and the non-immune library made by using B cells isolated from healthy persons or non-immunized animals.[12,13] By using the non-immune library, antibodies for vast number of different kinds of antigens can be isolated without the need for *in vivo* immunization. However, several studies report that the common non-immune scFv libraries do not function effectively because of inefficiencies related to library preparation. The first problem associated with scFv library preparation is poor coverage of the PCR primer set used to amplify immunoglobulin genes. The second problem relates to the complicated procedure associated with the 3 fragment assembly which connects the VL and VH gene to a linker sequence. This assembly process is inefficient and often cause sequence frame shifts resulting in poor library quality.

In an attempt to overcome these problems, we previously attempted to construct a higher quality library by preparing an scFv library with improved PCR primer sets.[14] However, because the previous primer sets were constructed using mixed bases, the combinations of primer sets available to amplify the VL and VH fragments amounted to approximately two billion and six billion sets, respectively. Therefore, the ratio of one primer contained in primer sets was extremely low. In addition, because the amplification efficiency of PCR was extremely low, it was possible to incur bias in the amplification of antibody genes. Moreover, antibodies could not be isolated to some antigens using the previous library. In order to prepare a much better quality scFv gene library, it is necessary to re-design new primer sets for which the amplification efficiency of PCR is extremely high.

In the present study, we re-generated the original primer sets to remove any unnecessary primer variation whilst taking care to maintain sufficient diversity to encompass a wide variety of antibody genes. Using this new primer set, we were able to amplify antibody genes effectively and prepare a much better quality scFv gene library. Furthermore, we optimized the use of antibody selection methods with this new li-

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brary and successfully isolated several high affinity clones specific for a tumor endothelial marker, KDR.[15,16]

MATERIALS AND METHODS

Construction of scFv Genes Total RNA was extracted from spleen cells and femoral bone marrow cells prepared from non-immunized 6-week-old male C57BL/6, Balb/c, and C3H mice. The mRNA was purified using a mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) and first-strand cDNA was synthesized with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.). From each cDNA, immunoglobulin heavy-chain and light-chain genes were separately amplified and recombined by three subsequent PCR reactions as follows. In the first-step PCR, variable regions of the heavy and light chain genes (VH and VL) were amplified. The light chain 5′ primers were modified to include an *Nco* I site, and heavy chain 3′ primers to include a *Not* I site. Light chain 3′ primers were designed to assemble with heavy chain 5′ primers (Fig. 1). Light and heavy chain variable region genes were amplified by PCR reaction containing 1 μl of RT PCR products (cDNA) and 2 pmol of each primer set. The samples were cycled 35 times at 96 °C for 60 s, 50 °C for 60 s, and 68 °C for 60 s. The PCR products were then purified using a QIAquick PCR Purification Kit (QIAgen, Valencia, CA, U.S.A.). In the second PCR, equal amounts of the previously amplified VL and VH DNA were mixed and cycled 20 times at 96 °C for 60 s, 63 °C for 60 s, and 68 °C for 60 s without primers. The resulting PCR products, the scFv gene library, were then purified using a QIAquick PCR Purification Kit. In the third-step, the additional sequences were extended from *Nco* I and *Not* I sites upstream of the scFv gene library by overlap PCR using primer Y15 (5′-GCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATGCGGCCCAGCCGGCCATGGCC-3′) and primer Y16 (5′-TTAGTAAATGAATTTTCTGTATGAGGTTTTGCTAAACAACTTTCAACAGTCTATGCGGCACGCGGTTCCACGGATCCGGATACGGCACCGGCGCACCTGCGGCCGC-3′) by cycling 35 times at 96 °C for 60 s, 65 °C for 60 s, and 68 °C for 60 s.

Construction of Non-immune Mouse scFv Phage Libraries and DNA Sequencing The PCR amplified scFv genes and pCANTAB5E phagemid vector were digested with *Nco* I and *Not* I. The resultant scFv fragments were inserted into the pCANTAB5E vector to generate a scFv-gene III fusion library, using T4 ligase (Roche Diagnostics, Indianapolis, IN, U.S.A.) at 16 °C for 16 h. The ligated product, *E. coli* TG1 was transformed by electroporation and grown at 37 °C on culture plates containing 2YT medium supplemented with 100 μg/ml ampicillin and 2% glucose (2YTAG medium). Twelve clones were selected at random and insert plasmid DNA was sequenced and analyzed.

Phage Purification A scFv phage library containing 2.4×10^9 clones was used for the affinity selection. Glycerol stocks of the scFv library were grown in the log phase, rescued with M13KO7 helper phage (Invitrogen) and then amplified at 37 °C for 6 h in 2YT medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The phage was subsequently precipitated with PEG–NaCl (4% PEG, 0.5 M NaCl) and then resuspended in NTE buffer (100 mM NaCl, 10 mM Tris, and 1 mM EDTA).

Affinity Panning Affinity panning was performed by using the BIAcore 3000 system (BIAcore, Uppsala, Sweden). The sensor chip CM3 (BIAcore) was modified with 5 μg of antigen (KDR-Fc chimera; R&D systems Inc., Minneapolis, MN, U.S.A.) or luciferase (Promega Co., Madison, WI, U.S.A.). The scFv phage library was then incubated with 50% HBS-EP buffer (BIAcore) and loaded onto the antigen-immobilized sensor chip. After the binding step, the sensor chip was rinsed with HBS-EPT (HBS-EP containing 0.05% Tween 20). Bound phage was then eluted with Glycine–HCl buffer (pH 2) and Glycine–NaOH buffer (pH 11). These processes were regulated by BIAcore Control Software. The eluted phage was then incubated in log phase TG1 cells and glycerol stocks prepared for further repeat panning cycles. Phage titer was measured by counting the number of infected colony cells on Petrifilm (3M Co., St. Paul, MN, U.S.A.).

Phage ELISA Following the panning process, individual TG1 clones were picked, grown at 37 °C in 96-well plates and then rescued *via* M13KO7 helper phage infection. The amplified phage preparation was then blocked with 2% Block Ace (Dainippon Sumitomo Pharma Co., Osaka, Japan) at 4 °C for 1 h and then added to an immunoassay plate

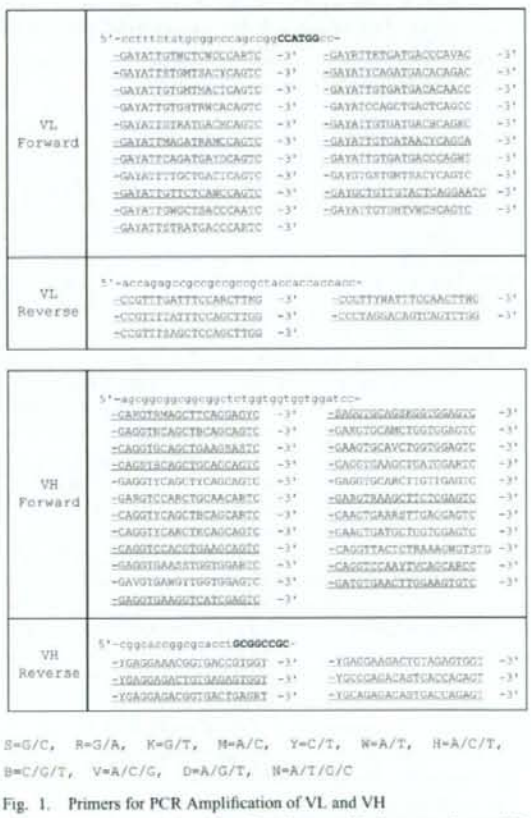


Fig. 1. Primers for PCR Amplification of VL and VH

New primer sets were designed that allowed immunoglobulin genes to be amplified as efficiently as possible by referring previous reports and the Kabat Database. Linker sequences were added downstream of the VL gene primers and upstream of the VH gene primers so that the amplified VL and VH genes could be easily connected.

coated with the antigens. Plates were then incubated for 2—3 h, with agitation at 250 rpm, and were then washed three times with phosphate buffered saline/0.1% Tween 20 (PBST), and finally incubated with HRP-conjugated anti-M13 monoclonal antibody (Amersham Bioscience). Plates were then washed once more with PBST and then TMB peroxidase substrate (MOSS, INC.) was added. Absorbance was then measured at 450 nm and 655 nm as a reference.

Dot Blot Analysis Antigens were immobilized on nitrocellulose membranes using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The membrane was incubated with blocking solution (10% skimmed milk, 25% glycerol) for 2 h and then washed twice with TBST (Tris buffered saline containing 0.1% Tween 20). The phage preparation was pre-incubated with 90% blocking solution at 4 °C for 1 h and then applied to each well. After 2—3 h incubation, the apparatus was washed five times with TBST and then incubated with HRP-conjugated anti-M13 monoclonal antibody. After three further washes with TBST and TBS, the membrane was treated with ECL-Plus reagent (Amersham Bioscience) and detected by use of a CCD image analyzer (LAS-3000, Fuji Photo Film Co. Ltd., Kanagawa, Japan).

RESULT AND DISCUSSION

In this study, we aimed to isolate a scFv monoclonal antibody from a non-immune antibody phage display library constructed using our improved PCR primer set. We specifically designed the primer sets to produce a non-immune antibody library that was superior in variety and accuracy than the past libraries (Fig. 1). To begin with, we re-designed primers which allowed immunoglobulin genes to be amplified as efficiently as possible by excluding an unnecessary mixture of bases from the previous primer sets. The combinations of primers to amplify the VL and VH genes were approximately four thousand and three thousand sets, respectively. Furthermore, the combination of primer sets to construct a scFv gene was more than ten million. The diversity of this primer set increased by approximately 10000-fold in comparison with that of the past reports. In addition, by using these primer sets randomly, we confirmed that the PCR products were gotten from all reactions (data not shown). In addition, in accordance with our previous report, linker sequences were added downstream of the VL gene primers and upstream of the VH gene primers such that the amplified VL and VH genes were easily connected, thereby reducing the possibility of frame shift in the sequence. Using the modified primer sets, the non-immune murine scFv phage library was prepared by the method described in our previous report.[14]

The titer of the resultant scFv phage library was 2.4×10^9 cfu. The repertoire of this library was as diverse as that of the library previously reported. DNA sequence analysis of twelve clones picked randomly from the library demonstrated no evidence of frame shift in the scFv genes (Fig. 2). Because the diversity of the CDR3 domain that is important for antigen binding is estimated to be in the region of twenty million, we suggest that our new scFv library has almost equal potential as the murine or human immune system.[18,19] Additionally, because the pIII protein of phage is generally toxic against *E. coli*, it was likely that contamination of the plasmid coding for the frame-shifted scFv gene promotes the production of the wild type phage that does not display antibody (data not shown). This means that we could not isolate

VL

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	Linker
1	DIVMTQSHKFMSTSVGDRVSITC	KASQDVSTAVA	WYQQKPGQSPKLLIY	SASYRYS	GVPDRFTGSGSGTDFTFTISSVQAEDLAVYYC	QQHYSTPFT	FGGGTKLEIKR	GGGSGGGGSGGGGS
2	--------FSS-YA-L-S--T---	------IRSYLS	-Y-----K---T---	S-NRLVD	---S---S------Q-YS-----SLEV--MGI---	LLSTVSS--	----------	----------
3	-------TFLTL-VTI-QPA--S-	-S--KLLSSDGKTYLN	-LL-R------K---	LV-KLD-	----------------K--R-E-----G-----	M-GTNF-R-	----------	----------
4	--Q---AATSVFVTP-EG---S-	RS-RSLLHSDGKTYLY	-FL-R------Q----	KM-NLA-	-------S------A---R--R-E---YS----	M-HLE--L-	--A--------	----------
5	--------Q----------V---	----M-C-G-R--	---------A----	--------	----------S-----E-F-	----------	----------	----------
6	--LI----------	----------	----------	W--T-HT	----------Y-----S-------L---	--HY-T-L-	--A-----LE-	----------
7	--E----Q----------	----M-G-R--	---------A----	--------	----------S----E-F-	--------L-	--------L-	----------
8	E-------Q----------	----M-C-R--	---------A----	VT-KLM-	----------S----E-F-	--------L-	--------L-	----------
9	--------FAI-A--P-EK-TM--	S-SS-SYMH	-----S-T---RW--	IT-KLA-	---A--S------SIS----R-E---A-T---	--WS-S-F-	--A--------	----------
10	----------	----------	----------	M--TRHT	----------S----E-F-	---------	--------L-	----------
11	---LE----------	----------	----------	------T	----------F---S----------	--HY-T---	--------L-	----------
12	----------	------G----	----------	W--T-HT	----------Y-----S-------L---	--HY-T---	----------	----------

VH

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
1	EVQLVESGGGLVKPGGSLKLSCAASGFTFS	SYGMS	WVRQTPEKRLEWVA	TISDGGSYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCAR	DGGVYFDY	WGQGTTLTVSS
2	Q---QQ-AE-------A-V-I--K---YA--	--M-N	--K-S-G-----IG	Q-YP-SGD-S-NGRT--	EA-LTA-KSSS-A-MQL---T---S-V-F--G	GTYYFDY	----------
3	D-A---------------P----------	---I---	---------K-----	-------DN---------	---------R---------R----------	DRY	----------
4	--MK----------	D---H	----A---------	-----S--I--A-T---	----------F---Y--R----------	LTTVVDY	----------
5	--K---------Q----------	------	------S-R--I--	T-S-N-SS---------	----------	DDHLYLLAY	-----LV---
6	---R----------	--T--	---------R-----	-------DN---------	---------R---------R----------	RSNYIYDGIYDN	--A---V----
7	----------Q----------	D---H	----A---------	-----S--I--A-T---	--------F---Y---------T-	GTNWIYDY	----------
8	--E-----E----------	--A--	---------R-----	------DYI--A-T---	---------R---------T-	EERTYDGY	----------
9	---M----D----Q---S--H----T-------	D-Y-A	----------	N-NYD---S---L---L-S	--I---------I----------T-----	DLYIYDY	----------
10	---M----------	--A--	---------M-----	T---D---Y---------	----------G----------	HGSSSYGFAY	-----LV---
11	--K----E----Q---S--H----T-------	D-Y-A	----V---------	N-NYD---S---L---L-S	--I---------I----------T----T-	EGCTYCFDY	----------
12	Q---QQ-AE-------A-V-I--K---YA--	DSMH-N	--K-S-G-----IG	S-YF-DGD-N-NGRT--	EA-LTA-KSSS-A-MQL---T---S-V-F---	DDD	----------

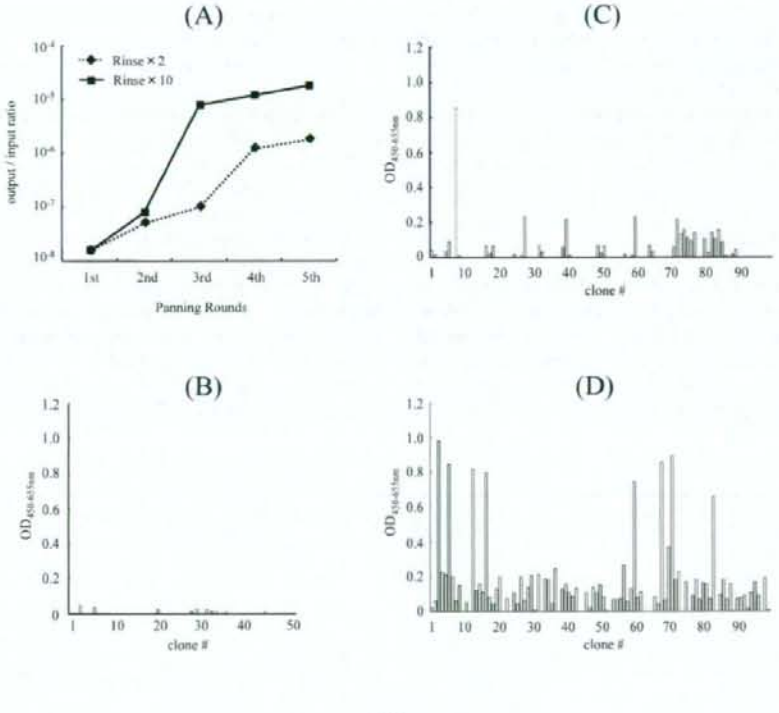
Fig. 2. Predicted Amino Acid Sequences of Non-immune Library

Amino acid sequences of 12 clones which were randomly picked from the library and analyzed by DNA sequence.

antibodies by pannig because the wild type phage is amplified faster than the antibody displaying phage. However, we strongly believe that this library was of high quality because all randomly-picked clones maintained the scFv sequence.

To obtain an antibody for a target antigen by using the phage display system, it is important to effectively increase the phage clones interact with the target antigen by repeated affinity panning. Although an immunoplate or immunotube is commonly used for the affinity panning,[20,21] it is commonly known that these techniques are inefficient in terms of antibody enrichment, are difficult to automate and exhibit difficulties in controlling the precise settings for panning conditions. Therefore, our present study utilized an automated microfludics system with a surface plasmon resonance analyzer (BIAcore 3000, BIAcore International AB, Uppsala, Sweden) to ensure that the panning procedure was both easy and optimized.

Generally, either acidic or basic solutions are required for the elution step during panning.[12,22] In our study, both acidic (pH 2) and basic (pH 11) buffers, containing a surfactant, were used for the elution step to completely dissociate the entrapped antibodies at high affinity. To evaluate the usefulness of the library, affinity panning against the human KDR, as a model antigen, was performed (Fig. 3). The experiment involving two rinse procedures exhibited an increase of approximately 100 fold in the ratio of phage titer after the fifth panning, while the experiment involving ten rinses exhibited a 1000-fold increase (Fig. 3A). Additionally, phage ELISA



(E)

VL

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	Linker
anti-KDR-1	DIVMTQSPATLSVTPGDRVSLSC RASQSISDYLH	WYQQKSHESPRLLIK YASQSIS	GIPSRFSGSGSGS-TTLSINSVEPEDVGVYYC	QNGHSFPT	FGGGTKLEIKR	GGGGSGGGTGGGGS		
anti-KDR-2	--------QKPH-TSV------VT- W----VGTWVA	------PGQ--RA---Y	S--YRY- -V-D--T------TD---T-SN-QS--LAE-F---QYR-Y-W-	--------L--	----------			
anti-KDR-3	--------RKTH-TSV------VT- W----VGTWVA	------PGQ--RA---Y	S--YRY- -V-D--T------TD---T-SN-QS--LAE-F---QYR-Y-LL-	--A-----I--	----------			

VH

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
anti-KDR-1	EVKLVESGGALVKPGGSLKLSCAASGFTFT SYAMS	WVRQTPEQRLEWVA	AISYGGSDTSYRQRVKGRFTISRDNAKNTLYLQMSSLRSEDTAMYYCTR	HNGYYAMDY	WGQGTSVTVSS		
anti-KDR-2	Q-QF-F---GG---F---S-S-----A---F---S	--A-S	--R-T-ERS----VA	T-DS-G-Y-Y-PSSV--RFTISRDNAKN-L-LQM----RS---T-M----A-	QRAIGSIWYTSV	---A---T------		
anti-KDR-3	E-QL-E---GG---Q---S-S-----A---F---S	--G-S	--R-T-ERS----VA	T-DS-G-Y-Y-PSSV--RFTISRDNAKN-L-LQM----RS---T-M----A-	HYIGSYYFDY	------TL------		

Fig. 3. Enrichment and Cloning of Antibodies to Human KDR from Non-immune scFv Phage Library by Affinity Panning

Enrichment of the desired clones was performed by affinity panning on the immobilized human KDR using the BIAcore 3000 system. (A) The ratio of phage titer at each panning round was plotted. The ratio was calculated as follows: (titer of the output phage)/(titer of the input phage). The closed line represents the data from the procedure involving ten rinses and the dashed line represents the data involving two rinses (B). After the fifth panning on human KDR, the binding properties of the selected phage clones were analyzed by ELISA. The data represent the results of measurement of clones before panning (B), after fifth panning involving two rinses (C) and after fifth panning with ten rinses (D). (E) Amino acid sequences of 8 clones high affinity clones for human KDR analyzed by DNA sequence.

analysis of 250 randomly picked clones showed that the number of positive clones binding to the KDR in the experiment involving ten rinses was higher than that involving two rinses (Figs. 3B—D). These results demonstrated that an optimized panning procedure with ten rinses effectively enriched the antibody clones specific for the target antigen. DNA sequence analysis of the eight clones of high affinity for human KDR demonstrated that these clones consisted of three different arrangements of antibodies (Fig. 3E). And then, we also confirmed that soluble formed scFvs of them could bind to KDR (data not shown). ELISA also demonstrated that these anti-KDR antibodies reacted not only with human KDR but also with murine KDR at similar affinity (Fig. 4). Because of natural tolerance, it is generally difficult to obtain an antibody to not only an antigen which has high homology but also an allogeneic antigen of the immunized animal. However, in our experiment, anti-KDR scFvs isolated from a non-immune mouse antibody library were able to bind murine KDR (allogeneic antigen). Therefore, it is suggested that these antibodies are of great importance to both mouse models and human research. This is because the non-immune scfv phage library, constructed by connecting VL and VH genes *in vitro*, included a nonexistent repertory naturally. This result shows that our non-immune scFv phage library is a useful technological resource for producing antibodies to autoantigens

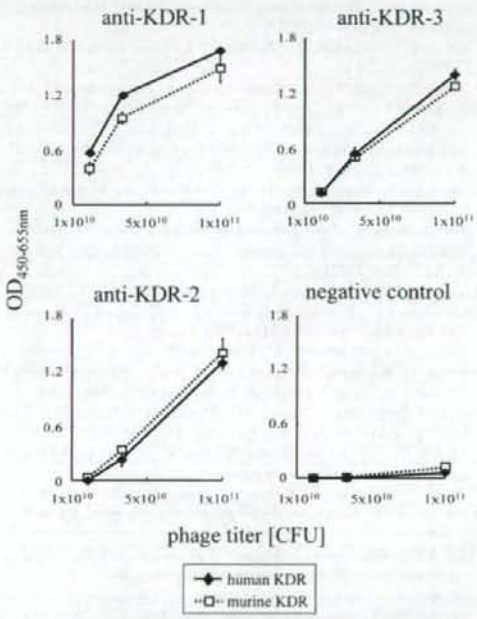


Fig. 4. Binding Properties of the Isolated Anti-KDR scFv Antibodies against Human or Murine KDR

The binding properties to human KDR (◆) and murine KDR (□) of anti-KDR scFvs displayed on the phage surface were measured by ELISA as described in the Materials and Methods. Three phage clones, anti-KDR-1, anti-KDR-2, and anti-KDR-3 were analyzed. A clone displaying scFv to luciferase was used as a negative control.

We then investigated the binding specificity of the three anti-KDR scFv antibodies by dot blot assay. These antibodies could bind native and denatured KDR, but not other antigens, tumor necrosis factor receptor 2 (TNFR2), luciferase, bovine serum albumin (BSA), importin-α, and importin-β (Fig. 5). The fact that these scFvs did not react to the TNFR2-Fc chimera indicates that these reacted not with the Fc domain but rather the KDR domain. It was also revealed that the sensitivity of dot blot analysis using the three scFvs as primary antibodies was as high as the level of detection of 100 pg (0.6 fmol) of immobilized antigen by anti-KDR-1 and anti-KDR-3 and 10 ng (60 fmol) by anti-KDR-2 (Fig. 6). The detection limit of general antibodies of the IgG type is approximately 1 ng (data not shown). In contrast, anti-KDR-1 and anti-KDR-3 antibodies could detect 100 pg of KDR. The data therefore suggests that the affinity of anti-KDR scFv antibodies is better than antibodies of the IgG type. This shows that the anti-KDR antibodies obtained from our library were of high quality and could recognize very small amounts of antigen.

To examine the usefulness of the library, we tried to isolate an antibody to a previously reported antigen, luciferase (Fig. 7). As panning to the luciferase was repeated, the output/input ratio was gradually elevated and the enrichment of the library reached approximately 5000-fold after the fifth pan-

	KDR (10ng)	KDR (1ng)	KDR (100pg)	KDR (10pg)	none
anti-KDR-1	●	●	●		
anti-KDR-2	●				
anti-KDR-3	●	●	●		

Fig. 6. Sensitivity of Antigen Detection by Anti-KDR scFvs

Anti-KDR scFv phages were reacted with KDR-Fc chimera (10 ng, 1 ng, 100 pg and 10 pg in each spot) immobilized on a nitrocellulose membrane using a dot blot manifold.

	hu-KDR-Fc-chimera	hu-TNFR2-Fc-chimera	luciferase	BSA	hu-importin-α	hu-importin-β	denatured KDR	none
anti-KDR-1	●						●	
anti-KDR-2	●						●	
anti-KDR-3	●						●	

Fig. 5. Binding Specificity of Anti-KDR scFv Antibodies

Binding specificity of anti-KDR scFvs was examined by dot blot analysis. Native hu-KDR-Fc-chimera, hu-TNFR2-Fc-chimera, luciferase, BSA, hu-importin-α, hu-importin-β and denatured hu-KDR (100 ng each) were dot blotted onto a nitrocellulose membrane and then the purified anti-KDR scFvs phage was reacted in the wells.

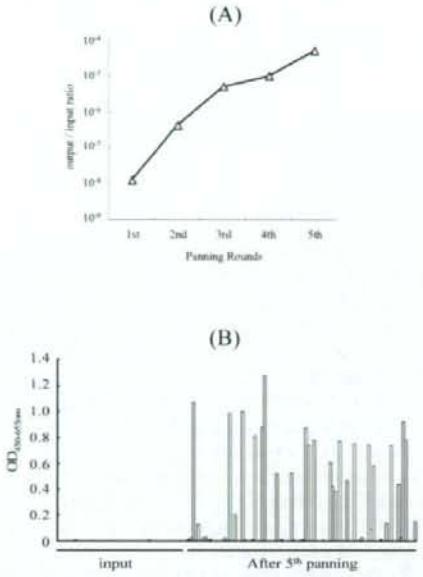


Fig. 7. Enrichment and Cloning of scFv Antibodies to Luciferase

Anti-luciferase scFv phages were selected from the non-immune phage library. Five rounds of affinity panning to the luciferase were performed using the BIAcore 3000 system. The ratio of the titer of output phage/input phage at each panning round was plotted (A). After fifth panning, the scFv phage clones of affinity with luciferase were analyzed by ELISA (B).

ning (Fig. 7A). A total of 150 phage clones were randomly picked and their binding to luciferase tested by ELISA (Fig. 7B). The number of positive clones was increased by affinity panning to luciferase and a high number of clones were successfully isolated. Because the variety of previous primer sets was very excessive, the amplification efficiency of PCR was extremely low. On the other hand, we confirmed that all combinations of these primer sets could amplify effectively. Moreover, antibodies to some antigens could not be isolated from the previous library. Using the present library, however, antibodies to all ten kinds of antigens tested were successfully isolated (data not shown). In addition, anti-KDR scFvs isolated from the present library were far superior in terms of antigen specificity and sensitivity and were able to bind an allogeneic antigen. Collectively, these results suggest that the present library was far superior to the previous one.

Over recent years it has become highly expected that antibodies should be able to be applied not only as a biochemical reagent for basic research but also as diagnostic tools and antibody-based medicine.[23—27] It is therefore vital to be able to obtain the desired antibody for various antigens rapidly. Because the non-immune scFv antibody phage library reported here can isolate antibodies for various antigens *in vitro*, we suggest that this resource will prove highly beneficial for future research and clinical applications.

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Creation of Novel Protein Transduction Domain (PTD) Mutants by a Phage Display-Based High-Throughput Screening System

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Significant research effort is currently focused on Protein Transduction Domains (PTDs) as potential intracellular drug delivery carriers. However, the application of this technology is limited because the transduction efficiencies are often insufficient for therapeutic purposes, even using HIV-1 Tat peptide. Here we describe a high-throughput screening method based on a phage display system for isolating novel PTDs with improved cell penetration activity. The screening method involves using protein synthesis inhibitory factor (PSIF) as cargo of PTD. Using this method, several Tat-PTD mutants of superior cell-penetrating activity were isolated. Interestingly, the amino acid sequence of the PTD mutants contained some characteristic residues, such as proline. Thus, our screening method may prove useful in determining the relationship between protein transduction and amino acid sequence.

Key words phage display system; protein transduction domain; high-throughput screening; HIV-1 Tat; intracellular drug delivery

Recent advances in proteomics have allowed a number of refractory diseases, such as cancer and neurodegenerative disorders, to be studied at the molecular level. The main causative factor of such disease states is often associated with intracellular organelles or particular subcellular proteins. Thus, the intracellular organelles, proteins or genes might constitute the therapeutic target. Recently, it was discovered that certain peptides, referred to as protein transduction domains (PTDs), can penetrate cells accompanied by a large molecular cargo. Considerable research effort is currently focused on utilizing PTDs as peptide-based carriers for intracellular drug delivery.[1—3]

Tat peptide, derived from the HIV-1, and Antennapedia peptide, derived from Drosophila Antennapedia homeotic transcription factor, are well known PTDs that have been tested as drug delivery carriers for various disease models.[4—9] PTDs can even deliver bulky molecular cargos (>100 kDa) into a wide variety of cell types.[10—13] However, to use PTDs as effective intracellular drug delivery carriers with clinical applications, it is necessary to create novel PTDs with greater protein-transduction potency than exists naturally.

An attempt to create a novel PTD by modification of the peptide structure has already been reported.[14,15] However, because it is difficult to predict the transduction activity of the peptide based on structural information alone, novel peptides must be generated by introducing amino acid substitutions and then the effects determined by trial and error. Recently, we have successfully generated a technology for creating novel muteins (mutant proteins) that have non-native functions using a phage display system.[16] This prompted us to apply phage display technology to screen for novel PTDs.

The phage display system is a protein selection methodology in which a library of mutant proteins or peptides can be screened and the desired molecules easily identified by linking DNA information (genotype) with phenotype (protein expression).[16—20] By applying this methodology, novel PTDs can be selected such as those transduced into the cell by a different mechanism or those with tissue/cell specificity. In general, the phage display system is used to isolate antibody and peptide ligands using an affinity selection step to target the desired molecules. However, for the discovery of PTDs it is necessary to construct a screening method to select clones that are transduced into the cell rather than simply selecting those that bind to the cell surface. We designed a high-throughput screening method to isolate effective PTDs by fusing PTD with Protein Synthesis Inhibitory Factor (PSIF).[21] Here, we used our methodology to identify novel Tat mutants with greater transduction potency than wild-type Tat PTD.

MATERIALS AND METHODS

Library Construction A gene library of Tat mutant peptides was constructed by randomization of codons (except arginine codons) of Tat [47—57] using PCR primers containing NNS sequences (N; A/T/G/C, S; G/C). Two primer sequences were used in this PCR. Forward primer, Y-oligo22 3′ ex (5′-TCA CAC AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA GCT TTG GAG CC-3′) contained a *Hind*III site and annealed on pCANTAB phagemid vector. Reverse primer, Tat[47—57] R (5′-TC ATC CTT GTA GTC TGC GGC CGC ACG ACG ACG SNN ACG ACG SNN SNN ACG SNN SNN GGC CAT GGC CGG CTG GGC CGC ATA GA AAG-3′) contained five NNS codons and a *Not*I site. After amplification of the Tat[47—57] mutant gene, the PCR fragments were digested with *Hind*III and *Not*I and cloned into the pCANTAB phagemid vector (Invitrogen Corp., Carlsbad, CA, U.S.A.). *E. coli* TG1 cells (Stratagene, La Jolla, CA, U.S.A.) were transformed with the phagemid by electroporation and then phage displaying Tat mutant peptide library were produced by infection of M13KO7 helper phage (Invitrogen Corp.).

Cell Panning The human keratinocyte cell line, HaCaT, was seeded in 6 well tissue culture plates at 5×10^5 cells/well

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and cultured overnight. The culture medium was changed to Opti-Mem I medium (Invitrogen Corp.) containing 2% BSA for blocking and incubated for 2 h at 37 °C. Purified phage library was pre-incubated with the same medium at 4 °C for 1 h. The phage solution was then applied to the HaCaT cells and incubated for 2 h at 37 °C. Unbound phage was removed by extensive washing (20×) with PBS (pH 7.2). Phage particles bound or internalized with the HaCaT cells were subsequently rescued by adding ice cold 50 mM HCl to each well and incubating for 10 min at 4 °C. The solution containing lysed cells and phage library was collected and neutralized by adding 1.0 M Tris–HCl pH 8.0. The phage clones contained in the solution were propagated by infecting *E. coli* TG1 and applied for the next round of panning. The cell panning was repeated two more times (*i.e.* 3 panning rounds in total).

Expression of PTD-PSIF Proteins Protein synthesis inhibitory factor (PSIF, PE fragment) is an approximately 40 kDa fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa*[22] (ATCC strain No.29260). PSIF lacks its cell binding domain, and has been successfully used as a cytotoxic portion of a recombinant immunotoxin.[23] We cloned the cDNA for PSIF from *Pseudomonas aeruginosa*, Migula by PCR using the primer set: 5′-GAT GAT CGA TCG CGG CCG CAG GTG CGC CGG TGC CGT ATC CGG ATC CGC TGG AAC CGC GTG CCG CAG ACT ACA AAG ACG ACG ACG ACA AAC CCG AGG GCG GCA GCC TGG CCG CGC TGA CC-3′ and 5′-GAT CGA TCG ATC ACT AGT CTA CAG TTC GTC TTT CTT CAG GTC CTC GCG CGG CGG TTT GCC GGG-3′. The PCR product was cloned into modified pCANTAB phagemid vector. After 3 rounds of cell panning, the enriched library of PTD candidate cDNA clones were purified from phage-infected TG1 cells and inserted into the PSIF-fusion expression vector derived from phagemid pCANTAB5E. TG1 cells were transformed with the PTD-PSIF fusion library and monocloned. Transformed TG1 clones were picked, transferred to a 96 well plate format and cultured in 2-YT medium (Invitrogen Corp.) containing 2% glucose and 100 μg/ml ampicillin until the OD_{600} reached 0.5. PTD-PSIF protein was expressed in the supernatant by culturing the cells for 12 h at 37 °C in 2-YT growth medium with no glucose in the presence of 100 mM IPTG. These supernatants were harvested and used for the cellular cytotoxicity assay.

Cytotoxicity Assay of PTD-PSIF Fusion Protein against HaCaT Cells HaCaT cells were seeded on 96 well tissue culture plates at 1.5×10^4 cells/well in Opti-Mem I medium containing 50 μg/ml cycloheximide. Each culture supernatant from the PTD-PSIF clones was then added to an individual well. After incubation at 37 °C for 24 h, viability of HaCaT cells was assessed using the MTT assay.

Flow Cytometry Analysis of FITC-Labeled PTDs on Live Cells HaCaT cells were seeded on 24 well tissue culture plates at 1.0×10^5 cells/well. After incubation for 24 h at 37 °C, the cell monolayer was treated with FITC-labeled PTDs diluted in growth medium at a final concentration 10 μM for 3 h. Cells were then washed and any PTDs adsorbed to the cell surface digested using 2.5% trypsin. Cellular fluorescence was then measured by flow cytometry (Becton Dickinson, Oxford, U.K.).

***In Vitro* Safety Assessment** HaCaT cells were seeded on 96 well tissue culture plates at 1.6×10^4 cells/well. After incubation for 24 h at 37 °C, FITC-labeled PTDs were added to the cell monolayer at three different concentrations (3 μM, 10 μM or 30 μM). After additional incubation for 24 h at 37 °C, cell viability was assessed by the WST-8 assay (Dojindo Lab., Kumamoto, Japan).

Fluorescence Microscopic Analysis HeLa cells were seeded on a chamber coverglass at 3.0×10^4 cells/well in culture medium (MEM 10% fetal calf serum) and incubated for 24 h. A 2 μM aliquot of streptavidin modified Qdot525 (Quantum Dot Co., Hayward, CA U.S.A.) was incubated with 200 μM of synthesized biotinylated PTDs at room temperature for 5 min and diluted in culture medium containing 10% fetal calf serum (FCS) and 5 nM PTD-conjugated Qdot. HeLa cells were then treated with the culture medium containing PTD-Qdot and 100 ng/ml Hoechst 33342 (Invitrogen Corp.) and incubated at 37 °C for 1 h. The medium was then changed for Qdot-free medium and the cells observed by fluorescence microscopy using an Olympus IX-81 microscope (Olympus Co., Tokyo, Japan) at various time points.

RESULTS AND DISCUSSION

In this study, a screening method for Tat PTD mutants with efficient cell penetrating activity was established and novel peptide sequences were identified (Fig. 1). Mutagenic PCR, using primers Y-oligo22 3′ex and Tat[47—57]R), was used to prepare a mutant peptide gene library of Tat in which 5 codons were randomized within the Tat[47—57] peptide. All the natural arginine codons of this peptide were retained because arginine was reported to have an important roll for penetrating into the cells.[24] The PCR product was then ligated into the phagemid vector. Approximately 16 million colony forming units (cfu) were obtained after transformation of *E. coli* TG1 with the phagemid. DNA sequence analysis of the library confirmed it to be derived from independent clones (Table 1). Our results established that the library had an enormous repertoire covering the 3.2 million theoretical combinations of 5 amino acids. From this, a 1.0×10^{12}—10^{13} cfu phage library displaying Tat mutant was prepared. In order to enrich the phage clones which bound or internalized to the cells, 3 rounds of cell panning using the HaCaT cell line was performed. The enriched phage clones included not only PTDs capable of penetrating the cell but also those peptides which simply bind to the cell surface.

To allow the differential selection of PTDs capable of penetrating the cell we designed a high-throughput screening method by fusing PTD with PSIF. PSIF from *Pseudomonas aeruginosa*, was not by itself cytotoxic because the cell-binding domain was truncated. However, PSIF shows cytotoxicity when it is fused to a carrier, such as PTD, because it can then enter the cytoplasm.[21] PSIF-fusion is a simple and effective screening method for novel PTDs because the penetrating ability of the peptide can be evaluated from the cytotoxic effects of the fused protein. Figure 2A shows the cellular uptake of PTD-PSIF fusion from the Tat mutant library before cell panning. No clones displaying stronger cytotoxicity than wild-type Tat-PSIF fusion could be detected. However, after 3 rounds of cell panning, over 80% of the analyzed 800 clones showed stronger cytotoxicity than wild-type Tat-PSIF.

(Fig. 2B). Using this rapid PSIF screening method, we isolated superior PTD candidates in only 2—3 weeks. Clones showing enhanced cytotoxicity over wild-type Tat peptide were isolated and the DNA sequences analyzed.

Next, FITC labeled PTD mutant candidates were synthesized and cellular uptake was determined by flow cytometry (Fig. 3). Each of the PTD candidates displayed similar or increased uptake compared with wild-type Tat[47—57] or Tat[48—60]. In particular, cellular uptake efficiency of YM2 or YM3 was 2.5 to 3 fold greater than wild-type Tat. Table 2 shows the amino acid sequences of clones YM2 and YM3. Some clones, including YM2 or YM3, have an increased number of arginine residues (clones 1, 6 and 7, Table 1). Moreover, all the clones shown in Tables 1 and 2 have almost the same isoelectric point (pI) of *ca* 13. In general the transduction ability of PTDs is associated with cationic amino acid residues, such as arginine. However, our data indicates that the transduction ability of PTDs is not wholly dependent on the total number of arginine residues or the overall pI. Interestingly, YM2 and YM3 include some characteristic amino acid residues, such as proline. In addition, these PTD candidates have arginine at the same position as Tat 54, which is thought to be important for transduction. In this way, our phage display system can correlate the amino acid sequence of the peptides with transduction ability. Thus, for the first time, it may be possible to experimentally determine the factors that influence intracellular transduction other than cationic amino acids or pI.

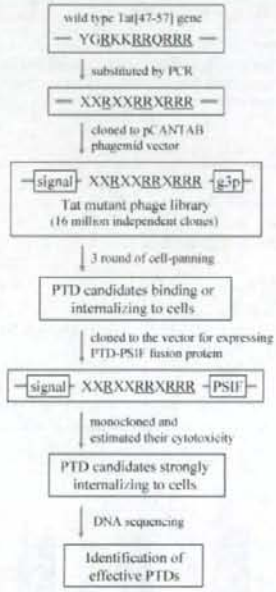

Fig. 1. Overview of the Creation of the Novel PTDs Using a Phage Display System Based High-Throughput Screening Method

The Tat mutant peptide gene library was constructed by randomization of the Tat[47—57] sequence, except for the arginine codons. Fixed arginine residues are underlined. Substituted amino acids are shown as X. After amplification, mutant Tat genes were cloned into pCANTAB phagemid vector. The Tat mutant phage library was produced from phagemid transformed TG1. The phage library was then subjected to 3 rounds of cell panning as described in Materials and Methods. Mutant Tat clones binding or internalizing to the cell were initially concentrated from the library. PTD candidates were then purified and cloned into PSIF expression vector. Monoclonal TG1s containing individual PTD-PSIF encoding phagemid were picked up separately into a 96 well format. The cytotoxicity of the PTD-PSIF proteins was assessed in order to isolate Tat peptide mutants that are strongly internalized within the cell. Approximately 1000 clones can be simultaneously assayed for cytotoxicity by this procedure. The amino acid sequence of effective Tat mutants were readily obtained from their DNA sequence.

To utilize PTD as an effective intracellular drug delivery carrier, the peptide must be nontoxic to the cells. Using the assay for HaCaT cells, no cytotoxicity was observed with peptides YM1, YM2 and YM3 (Fig. 4). Polyarginine is one of the representative artificial PTDs and, like Tat peptide, is highly efficient at transducing cargo into the cell.[24] However, polyarginine (Arg 11) displayed more cytotoxicity than Tat peptide.[25] Our initial assessment, conducted on a specific cell line, indicates that all 3 novel PTDs are safe drug carriers.

Another research group has also reported the generation of novel PTDs with enhanced transduction potential compared to that of Tat peptide.[14] However, it was never demonstrated whether these PTDs actually introduced cargos into the cell. Therefore, we examined whether our PTDs candidates were able to deliver macromolecules. Qdots, a fluorescent semiconductor nanocrystal, was used as a model macro drug molecule. Qdots streptavidin conjugate was modified with either biotinylated Tat[47—57] or YM3 peptide and then applied to cultured HeLa cells. After 1 h, Tat[47—57] or YM3 labeled Qdot were localized near the cell membrane (Figs. 5a, d). For these observations, Hela cell was used in spite of HaCaT cell because the localization analysis of Qdots in HaCaT cell was difficult due to its small cytoplasmic area. Upon further incubation, the location of the Qdot-PTD conjugates changed (Figs. 5b, e) until after 20 h the Qdot was observed at the perinuclear region (Figs. 5c, f). However, Qdots was not ob-

Table 1. Amino Acid Sequences and pI Values of Tat Mutants from the Library

Clone	Position											pI
	47	48	49	50	51	52	53	54	55	56	57	
Tat[47—57]	Y	G	R	K	K	R	R	Q	R	R	R	12.8
Clone1	T	L	R	T	R	R	R	N	R	R	R	13.3
Clone2	N	Y	R	T	G	R	R	K	R	R	R	12.8
Clone3	L	T	R	Q	T	R	R	M	R	R	R	13.2
Clone4	S	K	R	T	W	R	R	N	R	R	R	13.2
Clone5	K	E	R	H	L	R	R	H	R	R	R	12.8
Clone6	D	R	R	N	S	R	R	N	R	R	R	12.9
Clone7	H	R	R	P	V	R	R	F	R	R	R	13.3
Clone8	A	P	R	D	W	R	R	A	R	R	R	12.8

Sequence analysis of random phage clones isolated from the library. The library confirmed it to be derived from independent clones.

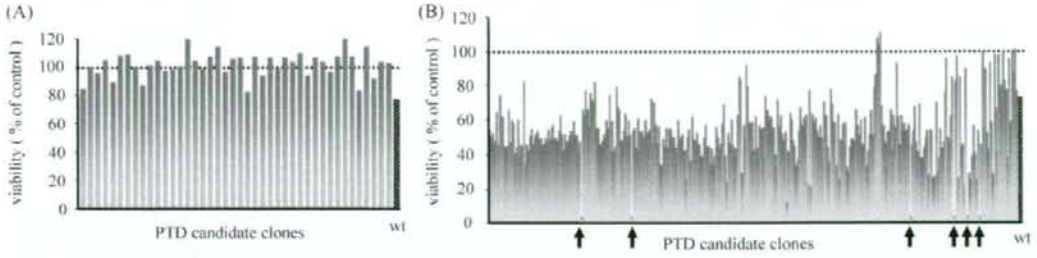

Fig. 2. Cellular Cytotoxicity Assay of the Monoclonal PTD Candidate-PSIF Fusion Proteins to HaCaT Cells

These figures show the cellular uptake of individual clones-PSIF fusion proteins from (A) Tat mutant library before cell panning and (B) concentrated novel PTD candidates after 3 rounds of cell panning. Cellular cytotoxicy was assessed using the MTT assay. The dose of PTD-PSIF fusion clones was adjusted to retain *ca.* 80% viability when using wild-type Tat-PSIF fusion protein (left stripy column). Clones in the arrowed columns showed greater cytotoxicity over wild-type Tat-PSIF fusion protein.

Table 2. Nucleotide and Amino Acid Sequences and pI Values of Novel PTDs

Clone	Position											pI
	47	48	49	50	51	52	53	54	55	56	57	
Tat[47—57]	Y	G	R	K	K	R	R	Q	R	R	R	12.8
	TAC	GGT	CGT	AAA	AAA	CGT	CGT	CAG	CGT	CGT	CGT	
YM1	R	N	R	A	R	R	R	Q	R	R	R	13.4
	AGG	AAC	CGT	GCC	CGC	CGT	CGT	CAG	CGT	CGT	CGT	
YM2	P	V	R	R	P	R	R	R	R	R	R	13.4
	CCC	GTG	CGT	CGC	CCC	CGT	CGT	CGG	CGT	CGT	CGT	
YM3	T	H	R	L	P	R	R	R	R	R	R	13.3
	ACC	CAC	CGT	TTG	CCC	CGT	CGT	CGC	CGT	CGT	CGT	

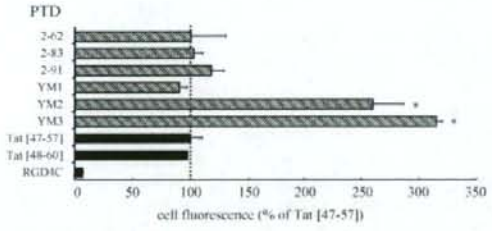

Fig. 3. Cellular Uptake of PTD-FITC Conjugates into HaCaT Cells

FITC labeled PTDs were incubated with HaCaT cell monolayer for 3 h and their cellular uptakes were estimated by flow cytometry analysis. Stripy columns show uptakes of PTD candidates from the Tat mutant library. Black columns show uptake of control PTDs. Control PTD sequences are as follows; Tat[47—57] (wild type Tat PTD): YGRKKRRQRRR, Tat[48—60]: GRKKRRQRRRPPQ, RGD4C: CDCRGDCFC. This experiment was performed at $n=3$. Each data value represents the mean±S.D. * $p<0.005$, compared with Tat[47—57].

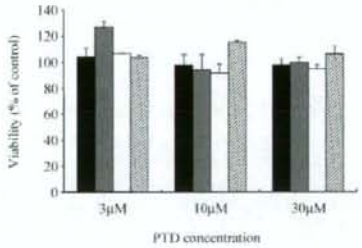

Fig. 4. *In Vitro* Safety Assessment of Tat Mutants

FITC labeled Tat[47—57] (■), YM1 (■), YM2 (□) or YM3 (■) were incubated with HaCaT cell monolayer for 24 h and their cytotoxicity was estimated using the MTT assay. Non-treated cells were arbitrarily given a value of 100%.

served in the cell nucleus. Recently, Tat peptides were reported to enter the cell by macropinocytosis.[26,27] By analogy, a large proportion of the incorporated Qdots may become trapped in the macropinosome and thus fail to transfer into the nucleus. Therefore, to achieve efficient drug delivery into the cytosol or organelles, the cargo must be released from the macropinosome. One possible strategy would be to incorporate the HA2 peptide to enhance the liberation of carrier and cargo protein from the endosome.[26,28]

It is reported that PTDs are able to deliver various bioactive molecules into cells. However their transduction efficiencies are not sufficient to achieve effective protein-based therapy. In this report, we used a high throughput screening method to successfully identify novel PTD mutants with improved cell penetrating activity over wild-type Tat peptide. The PTD mutants were found to contain some characteristic amino acids. These findings indicate that there may be many factors to account for cell penetration other than the presence of cationic amino acids. Using our high-throughput screening method, it should be possible to formulate some generic rules concerning the mechanism of cell penetration and subcellular transport. In conclusion, our high-throughput screening system is expected to contribute to the development of protein-based therapies.

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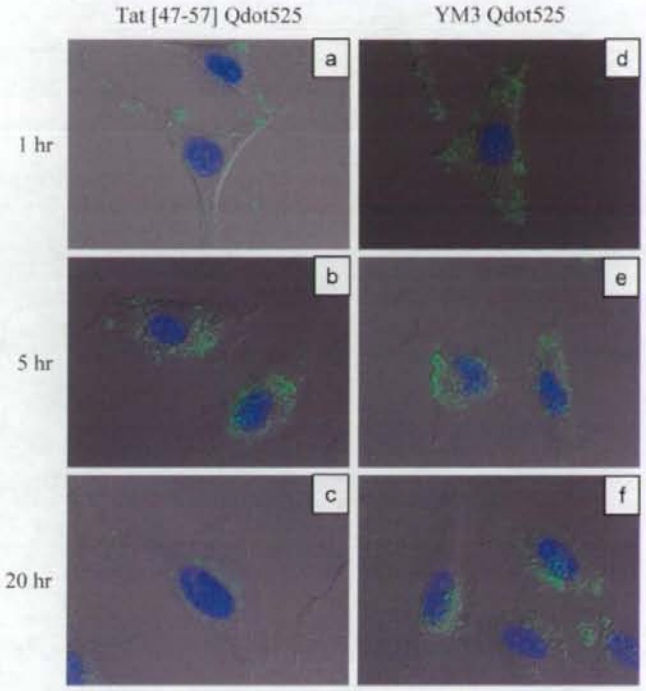

Fig. 5. Cellular Uptake and Intracellular Behavior of PTD-Qdot Complex in HeLa Cells

5 nM Tat[47—57] (a, b and c) or YM3 (d, e and f) labeled Qdots were incubated with HeLa cells. The cells were observed using fluorescence microscopy after 1 h (a and d), 5 h (b and e) or 20 h (c and f). The cell nucleus was stained with Hoechst 33342.

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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

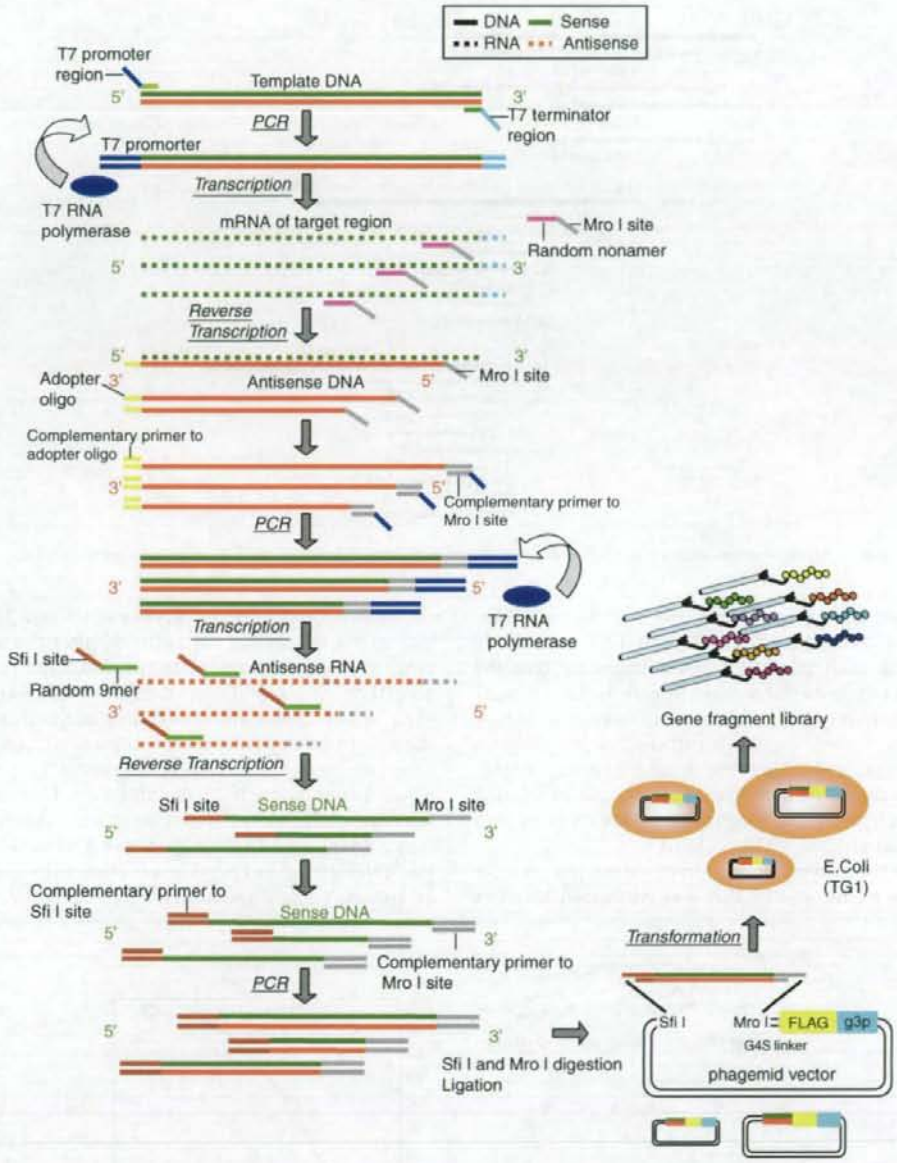

Fig. 1. Scheme for construction of a novel gene fragment library.

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

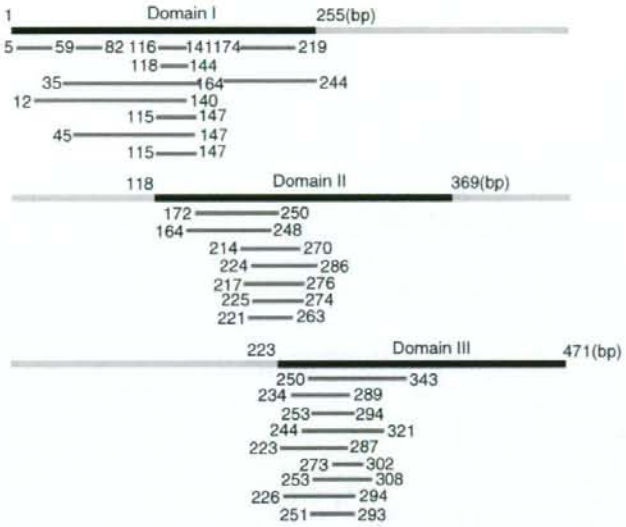

Fig. 2. Schematic representation of nucleotide sequences of peptides selected from the TNF-α gene fragment library.

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

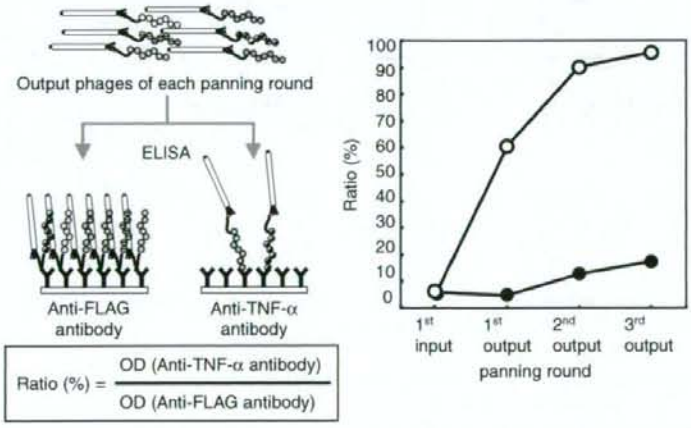

Fig. 3. Selection of phage clones expressing peptides binding to anti-TNF-α antibody. TNF-α gene fragment library (○) and the random 18 mer peptide library (●) were applied to immunotubes with immobilized anti-FLAG antibody or anti-TNF-α polyclonal antibody. Phage clones bound to each antibody were then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

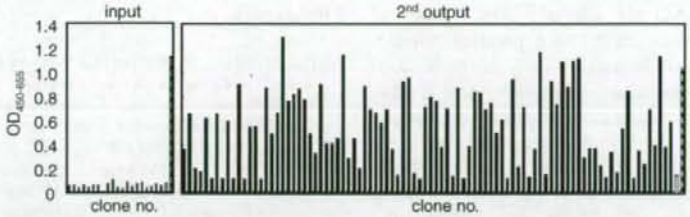

Fig. 4. Affinities of monoclonal phages for anti-TNF-α antibody. Ninety clones were selected randomly from input or second output phage clones and their affinities for the antibody were estimated by phage ELISA. Phage clone expressing TNF-α was used as a positive control (striped column), and IFN-α was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

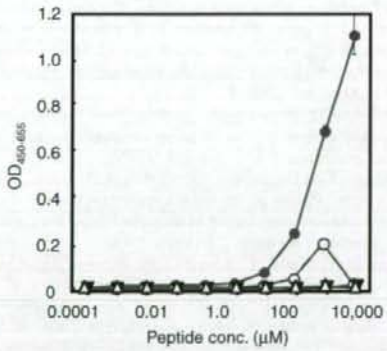

Fig. 6. Binding analysis of synthetic peptide to anti-TNF-α antibody by ELISA. Biotinylated epitope peptide (HVVANPQAEGQLQWLNRRA :●) and biotinylated control peptides (Mab1-peptide; EKGDRLSAEIN:▼), Mab4-peptide (NALLANGVELRD:△), and 3D6-peptide (AEGQLQWL NRRA:○) were applied to solid-phase anti-TNF-α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