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Rapid isolation of intrabody candidates by using an optimized non-immune phage antibody library

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Phage antibody library is a promising tool for rapidly creating *in vitro* single-chain Fv (scFv) antibodies to various antigens. The scFv can also act like a subcellularly-expressed antibody, known as intrabody, and can either be used as a novel research tool or used efficiently for targeted molecular therapy. However, there are only a few existing reports about the successful expression of scFvs as functional antibodies in the cell, mainly because poor quality scFv phage antibody libraries were used to isolate the intrabody clones. The aim of this study was to isolate intrabody-forming scFv clones from the non-immune scFv phage antibody library we have generated. Using this library, we isolated a scFv clone against the apoptosis-related intracellular protein Bid in two weeks. To evaluate the intrabody-forming quality of this anti-Bid scFv clone, we expressed it in cultured mammalian cells after fusing it with the fluorescent protein Venus. The expression of the soluble form of anti-Bid scFv-Venus fusion protein was confirmed by fluorescence microscopy analysis. These results show that our scFv phage library is not only optimized for antibody production but can also be used to efficiently generate intrabodies.

1. Introduction

Recently, it was suggested that most of the disease-related proteins or drug target proteins are located in the intracellular compartment. Therefore, it is important to elucidate the functions of these intracellular proteins to explore for novel drugs or clinical therapies and to develop ways for modulating their functions. In this respect, the intrabody technology, which is expressing an antibody in the cytoplasm, is considered to be a promising tool for analyzing the functions of subcellular proteins (Huston et al. 2001). Especially, intrabody can be useful for the analysis of post-translated modifications, because it can selectively inhibit the function of a protein. The intrabody-induced inhibition of selective protein function is different from the siRNA method, which acts by totally knocking down the protein expression. Intrabodies can also be of therapeutic use by neutralizing intracellular proteins (Miller et al. 2005; Alvarez et al. 2000). However, it has been difficult to establish the intrabody technology because the reducing cytoplasmic environment generally leads to low intrabody stability (Cattaneo et al. 1999).

Isolation of intrabodies using the phage or yeast antibody library can replace the hybridoma technique as the latter takes more time and effort (Popkov et al. 2005, 2003; Holler et al. 2000). However, only a few research groups have reported the creation of the intrabody from the non-immune phage antibody library (Rajpal et al. 2001; Colby et al. 2004), mainly because of the low quality of the antibody library.

We have already reported the construction of a high-quality non-immune phage antibody library and the optimized screening conditions to efficiently isolate a monoclonal antibody from this library (Imai et al. 2006). Thus, we believe that we can easily isolate subcellular protein-specific antibodies using this optimized non-immune phage library.

Using this phage library we first isolated a scFv clone against the apoptosis-related intracellular protein Bid, which is reported to be one of the candidate proteins relating to acute hepatitis (Yin et al. 1999). Next, we created a fusion protein between the anti-Bid scFv and the yellow fluorescence protein Venus, and then expressed this fusion protein as a soluble intrabody in cultured cells.

2. Investigations, results and discussion

We have previously reported methods to improve the quality of the non-immune murine scFv phage library and to use this phage library for the rapid isolation of monoclonal antibodies to various antigens (Imai et al. 2006). In this report, we isolated scFvs to an intracellular protein and then evaluated their intrabody formation quality. The scFv phage display library was prepared from the glycerol stock of *E. coli* TG1 containing the scFv phagemid library. The Bid-His-tag or native Bid protein was immobilized on the surface of the Biacore sensor chip and the affinity panning was performed for 3 to 5 cycles. The output/input ratio (titer of the recovered phage library after the panning/titer of phage library before the panning) was increased as the panning round was repeated (data not shown). This elevated output/input ratio indicated the en-

VL

	FR1	CDR1	FR2
scFv clone 1	DIVMTQSQKFMSTSVGDRVSVTC	KASQNVGTNVA	WYQQKPGQSPKALIY
scFv clone 2	--Q---TTSSL-A-L----TIS-	R---DISNYLN	------DGTV-L---

CDR2	FR3	CDR3	FR4
SASYRYS	GVPDRFTGSGSGTDFTLTISNVQSEDLAEYFC	QQYNSYPYT	FGGGTKLEIKR
YT-RLH-	---S--S-------YS-----LEQ--I-T---	--G-TL-L-	--A--------

VH

	FR1	CDR1	FR2
scFv clone 1	EVQLVESGGGLVKPGGSLKLSCAASGFTFS	SYAMS	WVRQTPEKRLEWVA
scFv clone 2	Q---QQ--AE-A---A-V----K---Y--T	--W-H	--K-R-GQG---IG

CDR2	FR3	CDR3	FR4
TISDGGSYTYYPDNVKG	RFTISRDNAKNNLYLQMSHLKSEDTAMYYCAR	RVGPPHYYAMDY	WGQGTSVTVSS
Y-NPSSG--K-NQKF--	KA-LTA-KSSSTA-MELRS-T---S-V---T-	WDY	-----TLQS--

Fig. 1: Amino acid sequence of the antibodies isolated from the non-immune scFv phage library by affinity panning
Amino acid sequences of the scFvs for His-Bid or native Bid, isolated from the library, were determined by DNA sequence analysis. VL; light chain variable domain of an antibody. VH; heavy chain variable domain of an antibody. FR; framework region. CDR; complementarity determining region. "–" in Fig. 1 means that the same amino acid occurs at that position in clone 2 as the clone 1

richment of the antigen-binding scFv clones. A total 96 clones were randomly picked from the post-panning output phage library and their bindings to each antigen were tested by phage ELISA. Among them, several phage clones showed higher binding to the target antigens, Bid-His or native Bid (data not shown). Analysis of the DNA sequences of these positive clones led to the identification of one Bid-His binding scFv clone and another native Bid binding scFv clone (Fig. 1). The antigenic specificity of these scFvs was investigated by phage ELISA using various proteins as antigens. The scFv clone isolated by using the Bid-His (scFv clone 1) as antigen bound not only to the Bid-His but also to the other His-tagged proteins (data not shown). Thus, scFv clone 1 may be an anti-His-tag scFv antibody. On the other hand, the scFv clone isolated by using the native Bid as antigen (scFv clone 2) bound specifically to the native Bid, but not to the His-tagged caspase-8, His-tagged importin-β, luciferase, tumor necrosis factor receptor 2 (TNFR2)-Fc-chimera, His-tagged Venus and KDR-Fc-chimera (Fig. 2). This result suggested that the scFv clone 2 was an anti-Bid specific scFv antibody.

Very often a His-tag is added to a recombinant protein for purification purposes. However, we have found that when a His-tagged protein was used as an antigen for panning, the clones of binding to the His-tag concentrated rapidly. Therefore, a modified method, which includes a step to eliminate the tag-binding clones, should be developed when having to use the His-tagged protein as antigen for panning.

In order for scFv to act as an intrabody, the most important criteria is that the scFv is expressed in the soluble form in an environment that is not suitable for antibodies (Worn and Pluckthin 2001). To evaluate the characteristics of the scFvs as intrabodies, the fluorescent protein Venus was fused to the C-terminus of the scFv and each fusion construct was subsequently analyzed for subcellular expression. The expression vector harboring the scFv clone 1 (anti-His tag scFv) or clone 2 (anti-Bid scFv) was transfected into the HEp2 cells and the expression of the fluorescent fusion protein was observed using the Olympus IX-81 fluorescence microscope. The fluorescent images 24 h after transfection revealed that both the scFv-Venus fusions were expressed in the cytoplasm (Fig. 3). Although

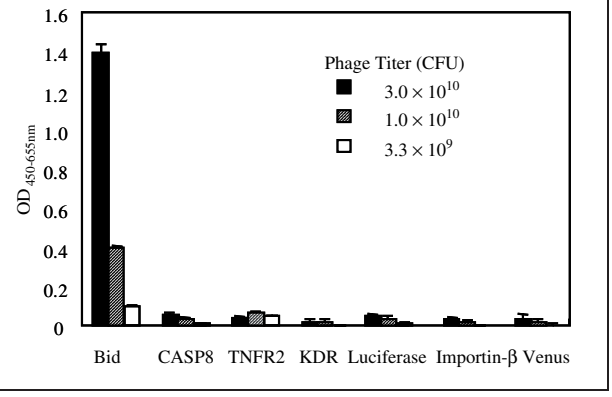

Fig. 2: Binding specificity of the isolated anti-Bid scFv antibody
Binding specificity of the anti-Bid scFv was determinded by phage ELISA. Native human Bid, His-tagged human caspase-8, human TNFR2-Fc-chimera, human KDR-Fc-chimera, luciferase, His-tagged importin-β, His-tagged Venus (50 ng each) were immobilized on the immunoassy plate and then the purified anti-Bid scFv phage (3.0×10^{10}, 1.0×10^{10} and 3.3×10^{9} CFU each) was applied to the wells. Each bar represents the mean OD450-655 nm ± SD (error bars) in three wells

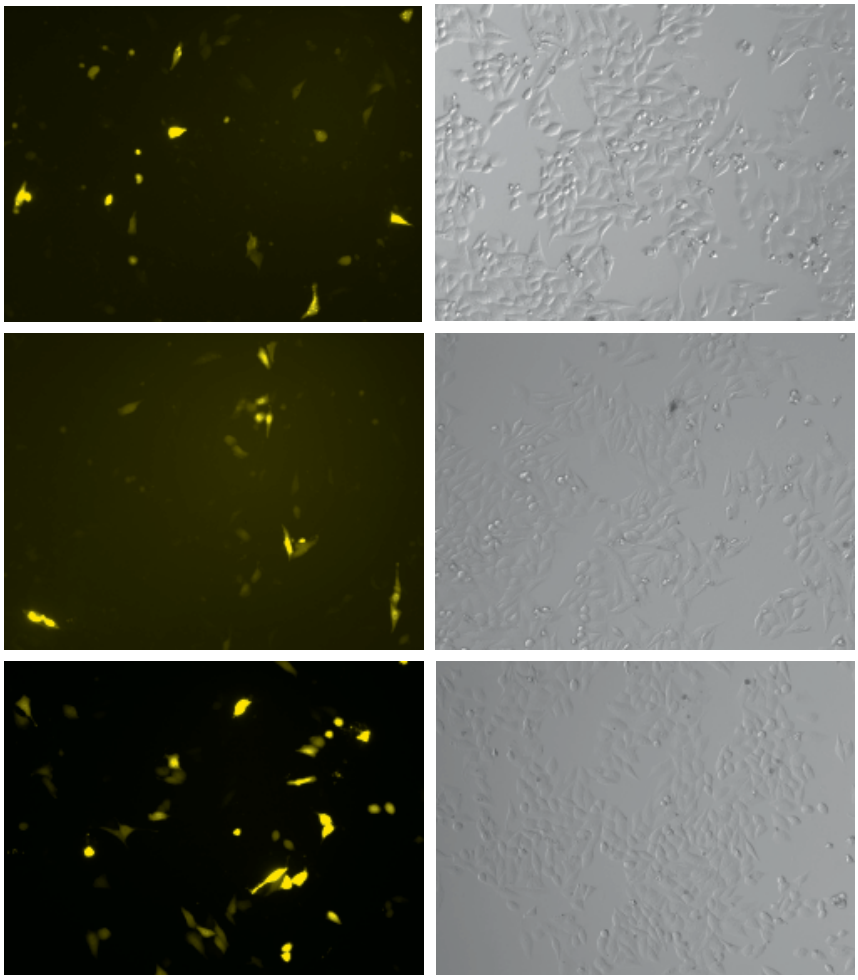

Fig. 3:
Intracellular expression of scFv-Venus fusion protein
The left panels decipt the fluorescence in the Venus (YFP) channel. The right panels show the bright-field images. The expression plasmids containing the scFv-Venus gene fusions were transiently transfected into the HEp2 cells by lipofection. A. Cells transfeted with the scFv clone1-Venus fusion construct. B. Cells transfected with the scFv clone 2-Venus fusion construct. C. Cells transfected with the pTriEx vector containing the Venus gene (control)

we will have to assess the binding of scFv to antigen in the cell and the anti-apoptotic property of the anti-Bid intrabody (clone 2) in the near future, our results suggest that we were able to isolate intrabodies from our non-immune phage antibody library.

We believe that intrabody-induced functional inhibition of Bid could be a novel therapeutic method for treating diseases, such as acute hepatitis, for which there are no treatment available (Yin et al. 1999).

It is not known which antibody can be expressed in the cytoplasm. Such fundamentally useful information can be achieved by isolating various antigen specific intrabody candidate clones using our phage library and analyzing their amino acid sequences.

3. Experimental

3.1. Cell

Human pharynx cancer cell line HEp2 cells (TKG0403, Cell Resource Center for Biomedical Research, Tohoku University) were sub-cultured in RPMI-1640 medium containing 10% fetal calf serum, 0.1% 2-mercaptoethanol and 1% sodium pyruvate. TNF-α treatment induces apoptosis in HEp2 cells (Berkova et al. 1999).

3.2. Non-immune phage scFv antibody library

Construction of the non-immune murine scFv phage library was described previously (Imai et al. 2006). The phage display library was prepared from the glycerol stock of *E. coli* TG1 containing the scFv gene library, and was immediately used for affinity panning.

3.3. Affinity panning using BIAcore

Affinity panning of antigens was performed using the microfluidic flow system based BIAcore 3000 instrument (BIAcore International AB., Uppsala, Sweden) as described previously (Imai et al. 2006). Briefly, 5 μg His-tagged Bid (Alexis Biochemicals Co., Taejon, Korea) or non-tagged Bid (Sigma-Aldrich Co., St. Louis, MO) was immobilized on the surface of the sensor chip CM3 (BIAcore). The phage library solution was injected onto the sensor chip at flow rate of 3 μL/min. After injection, the sensor chip was rinsed 10 times with the HBS-EPT (HBS-EP running buffer containing 0.05% Tween 20, Biacore). The bound phages were eluted with glycine-HCl (pH 2.0), and were treated first with glycine-NaOH (pH 11.0) and then with tris-HCl (pH 8.0) to neutralize. Recovered phages were amplified by infecting log phase TG1 cells for the next round of panning.

3.4. Phage ELISA

Single clones of affinity-panned phages were prepared from the culture supernatant of TG1 in 96 well format plate. These phages were blocked with 2% Block Ace (Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) at 4 °C for 1 h, and then applied to the antigen-immobilized immuno-assay plate. His-tagged human caspase-8, His-tagged human-importin-β, luciferase, tumor necrosis factor receptor 2 (TNFR2)-Fc-chimera, His-tagged Venus and KDR-Fc-chimera were used as antigens. After shaking for 2∼3 h at 250 rpm, the bound phage was detected using the HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare Biosciences AB, Uppsala, Sweden).

3.5. Intracellular expression of scFv-Venus fusion protein

The cDNA of the fluorescent protein Venus was kindly provided by Dr. Miyawaki (Riken Brain Science Institute, Saitama, Japan). The isolated scFv genes were inserted into the NcoI and NotI digested mammalian expression vector pTriEx (Novagen), which contained the Venus gene. The resulted plasmid containing the scFv-Venus fusion protein gene was transfected into the HEp2 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 24 h incubation at 37 °C, the expression of the scFv-Venus fusion protein in the cells was observed using the Olympus IX-81 fluorescence microscope (Tokyo, Japan).

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