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Effect of protein properties on display efficiency using the M13 phage display system

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The M13 phage display system is a powerful technology for engineering proteins such as functional mutant proteins and peptides. In this system, it is necessary that the protein is displayed on the phage surface. Therefore, its application is often limited when a protein is poorly displayed. In this study, we attempted to understand the relationship between a protein's properties and its display efficiency using the well-known pIII and pVIII type phage display system. The display of positively charged SV40 NLS and HIV-1 Tat peptides on pIII was less efficient than that of the neutrally charged RGDS peptide. When different molecular weight proteins (1.5–58 kDa) were displayed on pIII and pVIII, their display efficiencies were directly influenced by their molecular weights. These results indicate the usefulness in predicting a desired protein's compatibility with protein and peptide engineering using the phage display system.

1. Introduction

Phage display systems have attracted much attention as the best technology to create functional mutant proteins and peptides ever since Smith et al. reported that random peptides could be displayed on the surface of filamentous M13 phage (Smith 1985). Many researchers have applied this system in attempts to create human antibodies and tissue-specific peptides (Schier et al. 1996; Maruta et al. 2003; Imai et al. 2006). Indeed, we have been successful in creating a useful mutant TNF to be used as a drug (Shibata et al. 2004; Yamamoto et al. 2003). Thus, the phage display system has a wide range of applications (Stich et al. 2003; Gourdine et al. 2005; Takashima et al. 2000).

Filamentous M13 phage has a circular single stranded DNA and takes the form of a long tube that consists of eleven kinds of proteins. This virus effectively proliferates upon infection of *E. coli* (Sidhu 2001; Bayer and Feigenson 1985; Kuhn 1987). In the phage display system, a fusion protein composed of target-molecule and coat protein is derived from a phagemid vector, and wild-type phage composition proteins (pI–pXI) are derived from a helper phage genome. These components can make phage libraries that display target-molecules by assembling within the periplasm of *E. coli*. The most useful characteristic of this system is that protein libraries can be displayed easily on the phage surface by inserting gene libraries within the phage genome. Target-molecules are obtained rapidly by the use of an *in vitro* affinity panning procedure that selects and amplifies specific phage clones (Smith 1985).

In the phage display system, target-molecules can be displayed on coat proteins (pIII, pVI, pVII, pVIII, pIX), though generally they are displayed on pIII or pVIII. Displaying 0–1 molecule per phage in the pIII type phage display system is suitable for isolating high-affinity molecules (Chasteen et al. 2006; Keresztessy et al. 2006). Alternatively, ten molecules can be displayed on a phage particle in the pVIII type phage display system to select low-affinity molecules (Verhaert et al. 1999; Kneissel et al. 1999; Lowman 1997).

As described, the phage display system is the most useful tool to create bioactive peptides and functional mutant proteins. However, because the efficiency of display is influenced by the properties of the target protein (molecular weight, electric charge, etc.), poor display often limits its application. Despite this problem, there is little research examining the relationship between display efficiency and a protein's properties. Thus, studies are warranted in order to apply the phage display system effectively. In this report, we prepared phages that displayed proteins of different molecular weights and electric charges to ascertain the relationship between display efficiency and protein properties.

2. Investigations, results and discussion

In this study we examined the relationship between protein properties (molecular weight, electric charge etc.) and the efficiency of display with pIII and pVIII coat proteins of the filamentous M13 phage display system (Fig. 1). To begin with, we prepared phages that displayed different electrically charged peptides on pIII (Fig. 2B) and evalu-
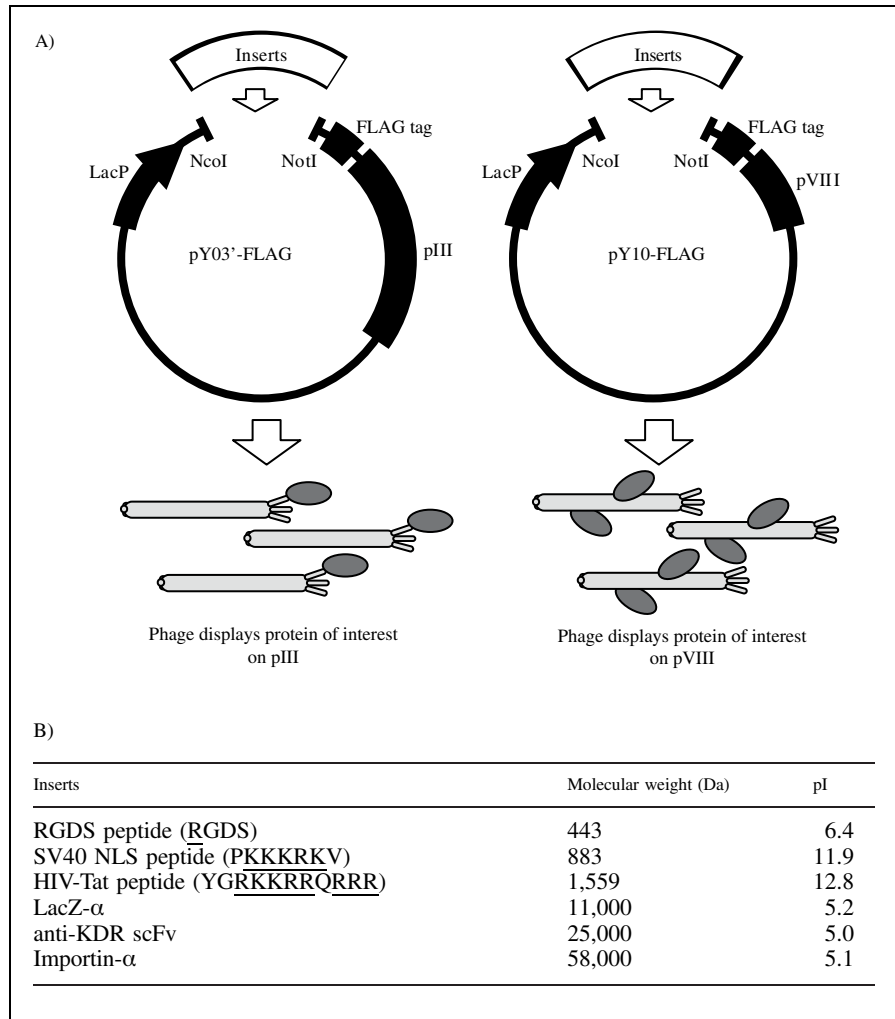
Fig. 1:
Construction of phagemid vectors encoding different proteins or peptides.
A) Different inserts were cloned into pY03′-FLAG and pY10-FLAG phagemid vectors. Phage particles displaying proteins fused to pIII and pVIII were prepared from pY03′-FLAG and pY10-FLAG, respectively. B) Different inserts and their molecular weights

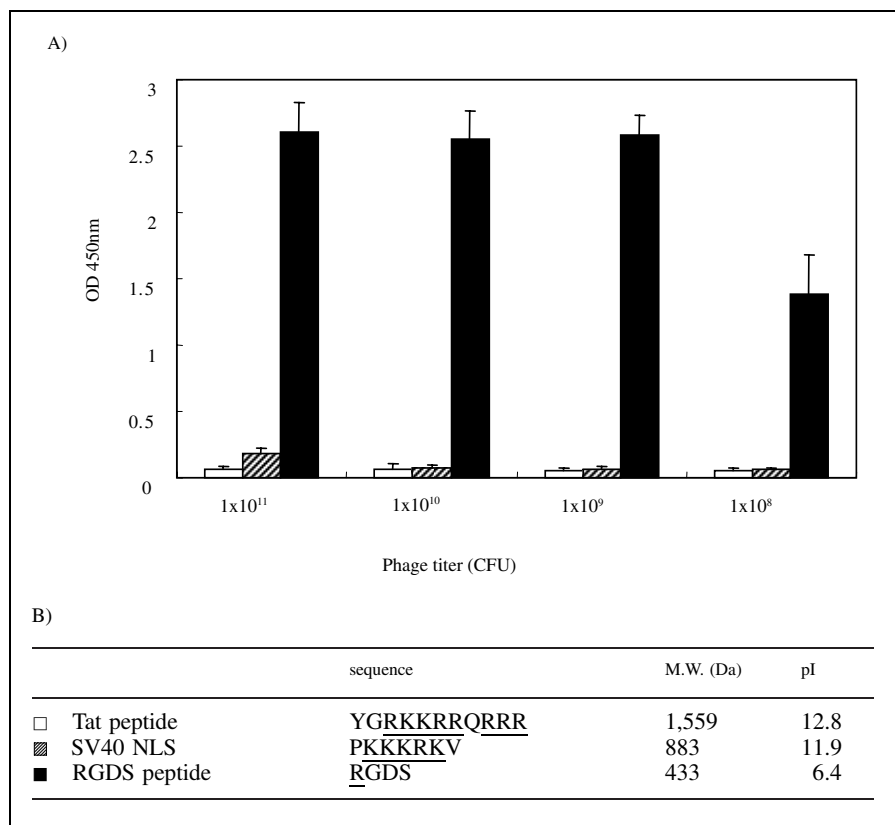| Inserts	Molecular weight (Da)	pI
RGDS peptide (RGDS)	443	6.4
SV40 NLS peptide (PKKKRKV)	883	11.9
HIV-Tat peptide (YGRKKRRQRRR)	1,559	12.8
LacZ-α	11,000	5.2
anti-KDR scFv	25,000	5.0
Importin-α	58,000	5.1

Fig. 2:
Influence of the efficiency of peptide-display by the ionic charge of peptides.
The efficiency of peptide-display on pIII was assessed by phage ELISA. Displayed peptides were fused to FLAG-tag – pIII on the phage particle and captured by immobilized anti-FLAG antibody. After washing, the number of captured phage was assessed by anti-M13 HRP conjugate. Two positively charged peptides (Tat peptide; □ and SV40 NLS; ▨) and a neutral peptide (RGDS; ■) were used in this experiment (n = 3). Each data value represents the mean ± S.D. B) Sequences of displayed peptides and their pIs. Cationic amino acids are underlined. All pI values were calculated by Expasy Compute pI/Mw tool (http://au.expasy.org)

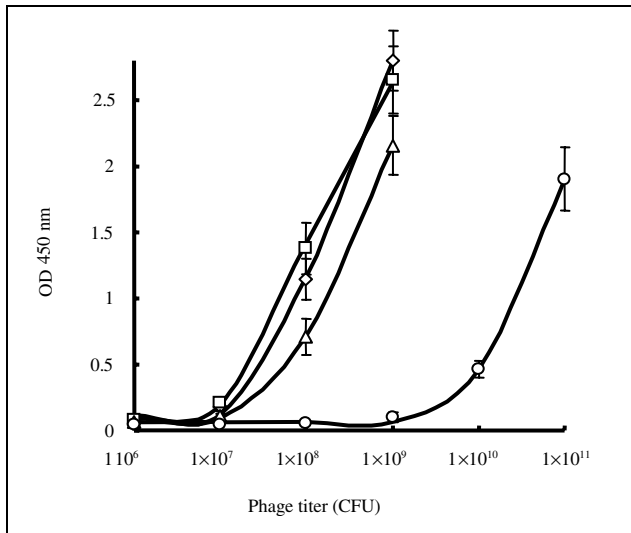|		sequence	M.W. (Da)	pI
□	Tat peptide	YGRKKRRQRRR	1,559	12.8
▨	SV40 NLS	PKKKRKV	883	11.9
■	RGDS peptide	RGDS	433	6.4

Fig. 3: Comparison of the efficiency of protein-display using pIII type phage display.

The efficiency of protein-display on pIII was assessed by phage ELISA. Proteins with different molecular weights (approximately 400–58,000 Da) were displayed on phage particle as pIII fusion proteins. This experiment was performed using the same method as Fig. 2 (n = 3). Each data value represents the mean ± S.D. □, RGDS-pIII phage; ◇, LacZ-pIII phage; △, scFv-pIII phage; ○, Importin-α-pIII phage

weight RGDS peptide bound to anti-FLAG antibody at a concentration of 10^6–10^9 CFU. The higher molecular weight importin-α (58 kDa) displayed on the phage surface could not bind at the same concentration, needing 10^9–10^{11} CFU. In general, the amount of phage prepared by following the standard protocol was approximately 10^{12}–10^{13} CFU (Imai 2006). To create functional mutants using a phage library, it is desirable to use an amount of phage in excess (more than 100-fold) of the phage library (approximately 10^6–10^9 CFU). When proteins display on the phage surface efficiently, the experiment can proceed without bias. However, our result suggests that a phage library displaying high molecular weight proteins may be of low quality simply because the levels of the desired proteins are not sufficiently expressed for screening. This introduces a selection bias for those proteins that can be expressed at the proper level.

To examine the efficiency of pIII-display in greater detail, we quantified the number of molecules displayed on the phage surface by electrophoresis analysis using CsCl purified phage (Fig. 4). These results (Fig. 3, 4) demonstrate that the efficiency of RGDS peptide-display on pIII was the best (approximately 2 molecules/phage). The display efficiency decreased as the molecular weight of the target protein increased. Because the titer of all phages prepared in this experiment was determined, we suggested that the display of different molecular weight proteins did not affect the efficiency of phage-preparation (data not shown). Additionally, the proteins used in this experiment (RGDS, LacZ, scFv and importin-α) were expressed efficiently in *E. coli*. Therefore, we suggest that the efficiency with which a protein is displayed on pIII is directly related to its molecular weight.

Finally, we examined the efficiency of pVIII-display by Western blot and confirmed that it also decreased as the molecular weight increased (Fig. 5). Interestingly, this result shows that scFv (25 kDa) could be displayed on pVIII efficiently. Because the pVIII phage display system is generally believed to be limited in its application precisely by the molecular weight of displayed protein, many used it only for display of peptide libraries (Verhaert et al. 1999; Kneissel et al. 1999; Lowman 1997; Gaillard et al. 1999). However, our result suggests that the pVIII system could be applied to larger molecules. This could provide useful
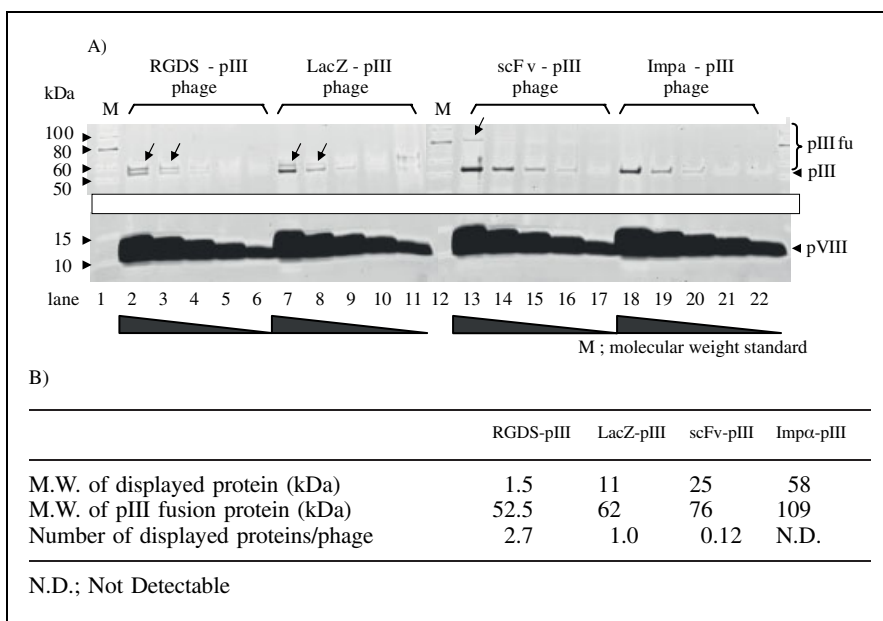
ated the relationship between electric charge and display efficiency using FLAG tagged ELISA (Fig. 2A). The display of positively charged SV40 NLS and HIV-1 Tat peptides were less efficient than that of the neutrally charged RGDS peptide. Generally, positively charged peptides are easy to adsorb onto various surfaces (Gaillard et al. 1999), and they repulse each other. Therefore, positively charged peptides may interfere with phage assembly in the periplasm.

Second, we examined the relationship between molecular weight and display efficiency again using FLAG tagged ELISA (Fig. 3). Because the display of positively charged sample was less efficient (Fig. 2), we used the neutrally charged proteins (pI 5.0–6.4) (MW 1.5–58 kDa) displayed on pIII to examine the influence of molecular weight on display. Phage displaying the low molecular

Fig. 4:
Calculated quantity of pIII displayed proteins using Sypro® Ruby staining.

A) The efficiency of display on pIII was quantified using CsCl purifed phages. RGDS-pIII (lanes 2–6), LacZ-pIII (lanes 7–11), scFv-pIII (lanes 13–17) and Impα-pIII phage (lanes 18–22) were used in this experiment. Molecular weight standard was loaded in lanes 1 and 12. Starting from the left, 1×10^{13} vp, 3.3×10^{12} vp, 1.1×10^{12} vp, 3.7×10^{11} vp and 1.2×10^{11} vp were loaded. B) The number of displayed proteins per one phage particle was calculated by fluorescence image analysis. Fluorescence intensity was quantified by Typhoon image analyzer

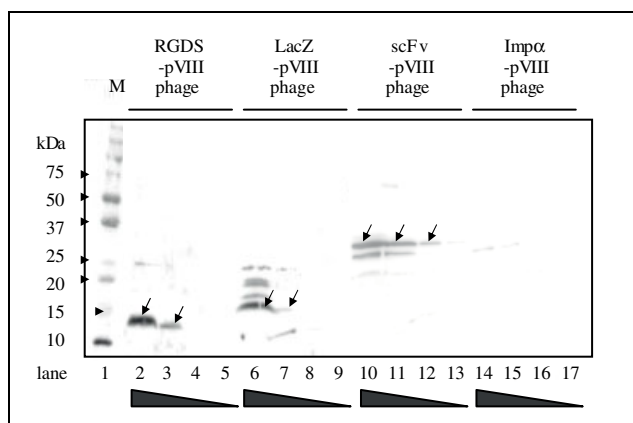|	RGDS-pIII	LacZ-pIII	scFv-pIII	Impα-pIII
M.W. of displayed protein (kDa)	1.5	11	25	58
M.W. of pIII fusion protein (kDa)	52.5	62	76	109
Number of displayed proteins/phage	2.7	1.0	0.12	N.D.

N.D.; Not Detectable

Fig. 5: Comparison of the efficiency of pVIII display protein on phage particles.
The efficiency of display on pVIII was assessed by anti-FLAG western blot. PEG-purified RGDS-pVIII (lanes 2–5), LacZ-pVIII (lanes 6–9), scFv-pVIII (lanes 10–13) and Impα-pVIII phage (lanes 14–17) were used in this experiment. Molecular weight standard was loaded in lane 1. Starting from the left, 1.5×10^{11} cfu, 5×10^{10} cfu, 1.7×10^{10} cfu and 5.5×10^9 cfu were loaded

additional information by expanding the application of phage display systems to create various mutant proteins.

In this study, different kinds of sample peptides (SV40 NLS, HIV-1 Tat, RGDS) and proteins (RGDS, LacZ, scFv, importin-α) that could be readily expressed in *E. coli* were used as model molecules. The display of positively charged SV40 NLS and HIV-1 Tat peptides on pIII was less efficient than that of the neutrally charged RGDS peptide. When different molecular weight proteins (1.5–58 kDa) were displayed on pIII and pVIII, their display efficiencies were directly related to their molecular weights.

When comparing the efficiency of display between the four model proteins, additional factors (i.e. refolding efficiency, etc.) may account for the differences. These results show at least that the electric charge affected the efficiency of phage display and that high molecular weight proteins could not be displayed on the phage surface successfully. Recently, it was reported that improving the phagemid vector provided better efficiency of protein refolding in *E. coli* and enhanced protein display on the phage surface (Guo et al. 2003). Consequently many hope that the display efficiency of various molecules could be improved using this methodology. However, while this method improves the quality of fusion protein expression, it does not take into account the efficiency of protein assembly for the construction of phage particles. Therefore, it is still important to be able to predict the molecules that will be compatible for protein and peptide engineering using phage display by understanding the properties of this system as they were described in this report.

3. Experimental

3.1. Phagemid vectors and inserts

The pY03′-FLAG phagemid vector was modified from pCANTAB-5E (GE Healthcare Ltd.). To create this vector, the E-tag from the original vector was changed to a FLAG tag (DYKDDDDK). The pY10-FLAG phagemid vector was constructed by replacing the pIII gene in pY03′-FLAG with the pVIII gene. Genes encoding peptides (RGDS, HIV-Tat, SV40 NLS) were synthesized by Operon Biotechnologies Inc., USA. The lacZ-α gene had already been cloned into pY03′-FLAG and pY10-FLAG. The anti-KDR scFv gene was isolated from an optimized non-immune phage antibody library previously described (Imai et al. 2006). The human importin-α gene was amplified from a human bone marrow cDNA library (TAKARA Bio. Inc.). These inserts were digested and cloned into each phagemid vector.

3.2. Phage preparation

Phage was prepared by following a standard protocol. Briefly, phage particles were prepared from *Escherichia coli* (TG1 strain, Stratagene corporation) by co-infection with M13KO7 helper phage (Invitrogen Corporation). Amplified phage in culture media was roughly purified by PEG precipitation. Part of the purified phage was added to the TG1 bacteria, and the phage titer (cfu) was calculated by counting infected TG1 colonies. If necessary, additional purification using a CsCl gradient was performed as described below.

3.3. Phage ELISA

Immunoplates (Nalge Nunc International) were immobilized with anti-FLAG M2 antibody (Sigma-Aldrich Corporation) diluted to 5 μg/ml in bicarbonate buffer (Sigma-Aldrich Corporation). Plates were blocked with 2% block ace (Nakarai Tesque Inc.) for 2 h at 37 °C. Phage solution (PEG-purified) in 0.4% block ace was serially diluted and applied to the wells. After a 1 h incubation at room temperature, the binding phage was detected by anti-M13 HRP conjugate (GE Healthcare Ltd.).

3.4. Purification of phage particles under CsCl gradient

Amplified phage was purified by PEG precipitation. Phage pellets were resuspended in TBS buffer. CsCl powder (IWAI chemicals company) and additional TBS buffer were added to the phage solution up to 31%. After CsCl gradient ultracentrifugation at $400,000 \times g$ at 5 °C for 20 h, the concentrated phage band was isolated. TBS (five volumes) was added to the purified phage and centrifuged again at $400,000 \times g$ at 5 °C for 4 h to remove the CsCl. The obtained phage was resuspended in TBS and used for experiments.

3.5. Sypro Ruby staining

After purifying the phage under a CsCl gradient, the number of phage particles (vp/ml) was estimated from its absorbance according to the standard protocol. Serially diluted phage samples were resolved by SDS – poly acrylamide electrophoresis (SDS-PAGE). Gels were incubated in SYPRO® Ruby protein gel stain reagent (Pearce Biotechnology, Inc., USA) overnight at room temperature. After washing with wash buffer (10% methanol and 7% acetic acid) for 30 min, fluorescence was detected using the Typhoon Variable Image Analyzer (GE Healthcare Ltd.). The number of surface-displayed proteins was calculated from fluorescence intensity using ImageQuant TL software (GE Healthcare Ltd.) assuming that one phage particle contained five pIII coat proteins on its surface.

3.6. Anti-FLAG western blotting

SDS-PAGE was performed using serially diluted phage purified by PEG precipitation. Phage protein in the gel was transferred to PVDF membrane (GE Healthcare Ltd.) using the Hoefer TE 70 semi dry transfer unit (GE Healthcare Ltd.). Membranes were blocked in 4% block ace for 1 h. FLAG-tagged pVIII fusion protein was detected with anti-FLAG M2 antibody (Sigma-Aldrich Corporation) and anti-mouse IgG HRP conjugate (Sigma-Aldrich Corporation). After detection by ECL plus reagent (GE Healthcare Ltd.), its luminescence was quantitated using the LAS-3000 Lumi Imager (Fujifilm Corporation).

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