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Characterization of Fv Fragments Expressed on Phage Surface

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We characterized a phage antibody in which an Fv fragment, namely, a free V_H fragment noncovalently associated with a V_L fragment that is fused with a truncated cpIII molecule (V_L-⊿cpIII), is expressed on the phage surface. D1.3 antibody specific for hen egg-white lysozyme was used as a model system. Both V_H and V_L-⊿cpIII fragments were stably expressed and associated with each other to form a faithful antigen-binding site. The results of Western blotting indicated that more than 5% of phages expressed the Fv fragment on their surface. Analysis of the kinetics of binding of the phage antibody to the antigen suggested the possibility of presence of phages having multiple-binding sites on a single phage particle.

Key words: Fv fragment, phage antibody, surface plasmon resonance, valency.

Phage antibody systems have the potential to replace conventional cell fusion technology for the preparation of monoclonal antibodies (*1-3*). Either Fab or a single-chain Fv (scFv) fragment has frequently been adopted as a molecular form that is expressed on the surface of a filamentous phage particle, for example, fd and M13, in such systems (*4, 5*). In the present study, we examined the characteristics of Fv molecules expressed on a phage surface. The questions to be addressed are as follows. How stably are Fv fragments expressed on the surface of phages? Does the Fv fragment have a faithful antigen-binding capability? What percentage of phages express Fv fragments on their surface? How many Fv fragments are expressed on a single phage particle?

MATERIALS AND METHODS

Construction of Plasmid DNAs—A monoclonal antibody, D1.3, specific for hen egg-white lysozyme (HEL) was used as a model system (*6, 7*). Plasmid pFv-PP, which encodes the V_H fragment of D1.3 and the V_L fragment of D1.3 fused with two Fc-binding domains of protein A from *Staphylococcus aureus*, was used as the starting DNA (*8*). Three primers were synthesized chemically: cpIII5′-1, 5′-GAgtcgacTCCATTCGTTTGTGAAT; cpIII5′-2, 5′-GAgtcgacGTCCATTCGTTTGTGAATATCAA; and cpIII3′, 5′-CTgtcgacAgctagcACCCAAAAGAACTGG (*Sal*I sites and an *Nhe*I site are indicated in small letters). Using M13mp18 DNA (*9*) as a template and the combinations of cpIII5′-1 and cpIII3′ and of cpIII5′-2 and cpIII3′ as primers, we amplified the DNAs that encoded the C-terminal half of cpIII by the polymerase chain reaction (PCR) (*10*). After digestion with *Sal*I, these DNAs were inserted into the *Sal*I site of pFv-PP. In this way we obtained plasmid pM13Fv, which encoded the V_H fragment and the V_L fragment fused with a truncated cpIII molecule (⊿cpIII; from proline at the 198th residue to serine at the 406th residue) (*9*). Another plasmid, pM13⊿Fv, encoded only the V_H and V_L fragments, since there was an insertion of a G residue after the *Sal*I site which resulted in a termination (opal) codon between the V_L gene and the ⊿cpIII gene.

Preparation of Phages—pM13Fv and pM13⊿Fv were transfected into *Escherichia coli* BMH71-18 (*11*), and transfectants were isolated from plates containing 50 μg/ml ampicillin. Portions of 4 ml of overnight cultures were inoculated into 12 ml of 2×TY medium (*11*) and incubated for 1 h at 30°C. After addition of 2 ml of a solution of M13K07 phage (10^{10} PFU/ml), the cultures were incubated further at 30°C for 1 h. Three-milliliter aliquots were inoculated into 500 ml of 2×TY medium containing 70 μg/ml kanamycin, and the cultures were incubated for 24 h at 30°C. A total of 3 liters of such cultures was prepared for pM13Fv- and pM13⊿Fv-harboring bacteria, respectively. For preparation of M13K07 phage, *E. coli* BMH71-18 without plasmid was used as a host. The procedure for purification of phages was essentially the same as that described previously (*12*). In brief, after incubation, the bacteria were removed by centrifugation. One liter of PEG/NaCl (20% polyethyleneglycol in 2.5 M NaCl) was added to the 3 liters of culture supernatants and the mixture was allowed to stand overnight at 4°C. Phages were pelleted by centrifugation. The pellets were divided into three equal portions, and each portion was resuspended by a different method: in 300 ml of TE (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) for 1 h at room temperature (R.T.); in 300 ml of TES (TE containing 0.1% Sarkosyl) for 1 h at R.T.; and, in 300 ml TES for 18 h at R.T. The phages were precipitated again by addition of 100 ml of PEG/NaCl. The precipitates were collected by centrifugation and dissolved in NET (10 mM Tris·HCl, pH 8.0, 0.1 M NaCl, and 1 mM EDTA) by rotation at 4°C overnight. The phages were collected by centrifugation at 170,000×*g* for 3 h at 4°C and dissolved in

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Abbreviations: CFU, colony-forming units; HEL, hen egg-white lysozyme; PCR, polymerase chain reaction; PEG, polyethyleneglycol; PFU, plaque-forming units; RU, resonance units; scFv, single-chain Fv; SPR, surface plasmon resonance.

NET by rotation at 4°C overnight. Finally, the phages were purified by centrifugation at 36,000 rpm for 1 h with a stepwise gradient of CsCl. The bands of phages were collected and dialyzed against NET. The phages were quantitated by measuring the absorbance at 260 nm.

Measurement of the Infectivity of Phages and Phagemids—One hundred microliters of an appropriately diluted solution of phages or phagemids and a 500-μl culture of *E. coli* XL1-Blue at the logarithmic phase ($A_{600}=0.8$) were mixed and allowed to stand at R.T. for 45 min. For assays of colony formation, 100 μl of solution was spread directly on plates containing 50 μg/ml ampicillin. For assays of plaque formation, 4 ml of soft agar (0.65% Bacto agar, 2× TY medium) was mixed with samples and poured onto plates. Both sets of plates were incubated at 37°C overnight, then colonies or plaques were counted.

Analysis of the Dissociation of the V_H and V_L Fragments of Fv Molecules by HPLC—Two columns, G3000SW and G2000SW (TOSO), were connected in tandem. All chromatography was carried out at R.T. with NETS (NET containing 0.1% Sarkosyl) as running buffer. Ten micromolar Fv, Fv-P, and Fv-PP (*8*) were suspended in NETS and 10 μl of each sample was immediately injected onto the column. Another 10 μl of each sample was injected onto the column after standing at R.T. for 24 h. Absorbance of eluates at 280 nm was measured.

Analysis of Fv Fragments Expressed on the Surface of Phages—The procedure for preparing proteins from phage particles was essentially the same as that described previously (*13*). In brief, phages in NET were collected by centrifugation at 170,000×*g* for 3 h at 4°C. The phages were suspended at a concentration of 11.2 OD_{260} units (DU)/ml in 10 mM Tris·acetate, pH 5.4, 5% SDS with 1% β-mercaptoethanol, with rotation at R.T. for 24 h. After neutralization of the suspension with 1 N NaOH, $MgCl_2$ was added to a final concentration of 0.1 M. The complex of DNA and Mg^{2+} ions was removed by centrifugation at 150,000×*g* for 12 h at 20°C. The supernatant was saved and boiled for 2 min. An equal volume of loading buffer (*14*) was added, and 15-μl aliquots were subjected to SDS-PAGE on a 16% gel. The conditions for blotting were the same as those described previously (*14*). Antibodies raised in rabbits and specific for D1.3 were used as the first antibody. For detection, horseradish peroxidase-conjugated antibodies raised in donkey against rabbit IgG were used. To quantitate Fv fragments expressed on phage surfaces, authentic Fv fragments were also analyzed by Western blotting.

Detection of Fv Fragments Expressed on the Phage Surface by Surface Plasmon Resonance (SPR) in the BIAcore System—The molecular nature of Fv-derived portions expressed on the phage surface was characterized by the BIAcore system (*15-17*). Affinity-purified antibodies against D1.3 were chemically coupled to the support on a biosensor chip, in accordance with the manufacturer's instructions, by the amine coupling method. The immobilized antibodies on the sensor chips were adjusted to the amounts giving 5,200±300 resonance units (RU). All the experiments were carried out at a flow rate of 5 μl/min with HBS (10 mM HEPES, pH 7.4, 3.4 mM EDTA, 150 mM NaCl, and 0.5% Tween 20) at 30°C. All the samples of phages were dialyzed against HBS before use. Twenty-microliter aliquots of phage solution (3.7 DU/ml) were injected into the system. Chips were regenerated with 0.1 N HCl.

Analysis of Antigen-Binding Activity of Fv Fragments Expressed on the Phage Surface by the BIAcore System—Dissociation kinetics for the reaction with HEL bound to a solid support were characterized by the BIAcore system. HEL was chemically coupled to the support on a biosensor chip. The immobilized HEL on the sensor chips was adjusted to the amounts giving 1,800±200 RU. The other conditions were the same as above. In the first experiment, 20-μl aliquots of suspensions of M13Fv, M13ΔFv, and M13K07 phages, before and after treatment with Sarkosyl for 1 or 18 h, were injected. Five concentrations, namely, 3.7, 1.8, 0.9, 0.45, and 0.23 DU/ml, of phages were used for each analysis. In the second experiment, after 20-μl aliquots of phage solution (3.7 DU/ml) had been injected, 20 μl of 10-fold diluted antiserum raised against M13 phage was injected into the system. In this experiment, additional washing was carried out with 20% formic acid after washing with 0.1 M HCl for the regeneration of chips.

RESULTS

A Molecular Form of Phage Antibody—In the present study, we constructed a gene, as shown in Fig. 1, using the V_H and V_L genes encoding a monoclonal antibody, D1.3, which is specific for HEL. The V_L gene was fused with ΔcpIII, which encodes the C-terminal half of the cpIII molecule from the 198th to the 406th amino acid residue (*9*). The ΔcpIII fragment can be anchored on the surface of M13 phage, as shown by Barbas *et al.* (*18*). The structure of the protein A-derived portion was the same as that described in a previous paper (*8*). pM13Fv encodes the V_H fragment and the V_L fragment fused with ΔcpIII (V_L-ΔcpIII). As a control, another plasmid, pM13ΔFv, was constructed that encodes only the V_H and V_L fragments.

Dissociation of the Complexes between V_H and V_L Fragments by Treatment with a Solution of Sarkosyl—To characterize the molecular forms of the antibody-derived fragments expressed on the surface of phages, as well as their stability, conditions for the dissociation of the complex between V_H and V_L fragments were investigated. Although an 8 M solution of urea has conventionally been utilized for such dissociation, this treatment would result in the destruction of phage particles. A relatively mild detergent, namely, Sarkosyl, has frequently been used for dissolving precipitates of phages (*11*). The effects of the treatment with Sarkosyl on the infectivity of phages and phagemids were examined. Using helper phages, we grew pM13Fv and pM13ΔFv in *E. coli* BMH71-18, and the resultant phage particles were extensively purified. M13K07, which is a helper phage, was also prepared. It has been shown that the fd phage DNA in the virion has a molar extinction coefficient per phosphate group at 260 nm of 6750 (*19*). Since the length of the DNA of M13K07 is 6.4 kb and that of M13Fv and M13ΔFv is 4.8 kb, one DU of phage (defined in "MATERIALS AND METHODS") should correspond to approximately 1.3×10^{13} phage particles for M13K07 and 1.8×10^{13} phage particles for M13Fv and M13ΔFv. Plaque-forming units (PFU) and colony-forming units (CFU) on ampicillin-containing plates were counted and are indicated in Table I. The results for the samples without treatment with Sarkosyl showed that our preparation of

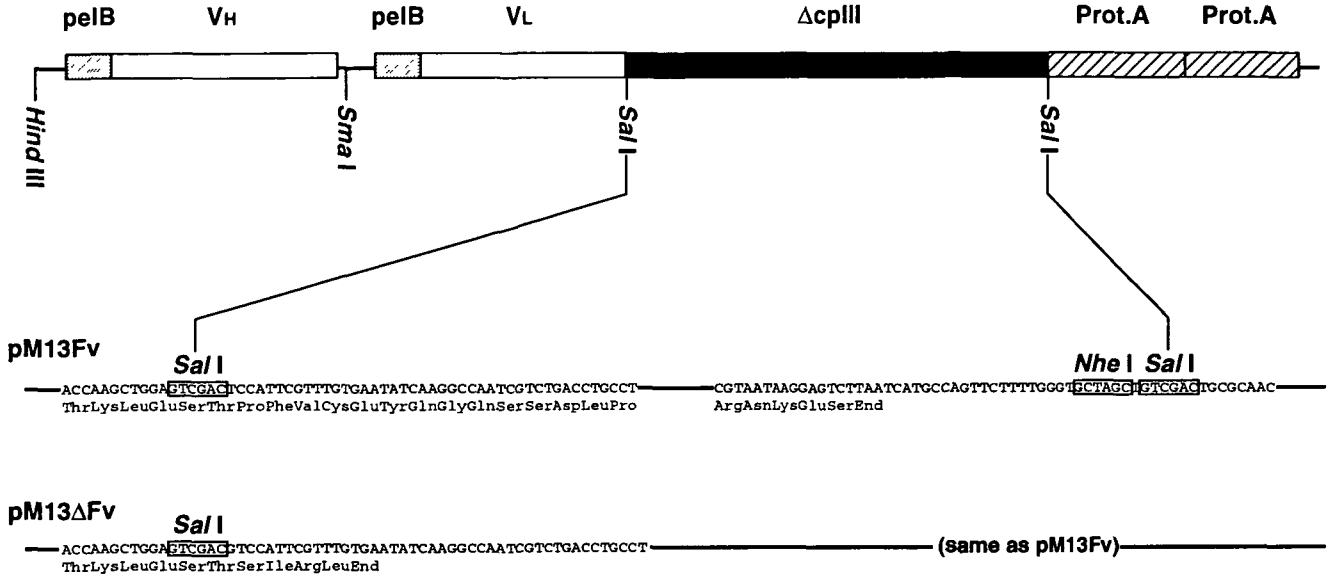

Fig. 1. **Molecular scheme for synthesis of the phage antibody.** Structures of V_H, V_L, and the two domains of protein A are the same as those described in a previous paper (*8*). Between the genes for V_L and protein A, a *Sal*I fragment encoding a truncated cpIII (⊿cpIII) with a termination codon at the 3′ end was inserted. pM13Fv encodes the V_H fragment and the V_L fragment fused with ⊿cpIII (V_L-⊿cpIII). When pM13Fv is digested with *Sal*I and ligated, the resulting plasmid encodes Fv-PP (*8*). In the case of pM13⊿Fv, one extra nucleotide was inserted just after the *Sal*I site. Although the difference between pM13Fv and pM13⊿Fv is only a single nucleotide, pM13⊿Fv-harboring cells produce only the Fv portion.

TABLE I. **Quantification of plaque/colony forming units of prepared phage & phagemids.**

Clone	Washing conditions	Plaque no./1 OD_{260}	Colony no./1 OD_{260}
		10^{12}	10^{12}
M13K07	no wash	3.3	nd
	mild wash	3.1	nd
	ex. wash	3.2	nd
M13Fv	no wash	0.27	0.91
	mild wash	0.37	1.2
	ex. wash	0.32	1.3
M13⊿Fv	no wash	0.30	0.96
	mild wash	0.27	1.3
	ex. wash	0.23	1.2

M13K07 had 0.25 PFU per phage particle, while M13Fv and M13⊿Fv had roughly 0.06 CFU per phage particle. Plaques formed by M13Fv- and M13⊿Fv-infected bacteria should be derived from helper phages. Thus it appears that around 10% of the phages in the preparations were helper phages. The infectivity of these three kinds of M13 phage particles was not affected at all by treatment with a 0.1% solution of Sarkosyl for either 1 h (mild washing) or 18 h (extensive washing).

The effects of the treatment with Sarkosyl on the stability of Fv complexes were examined by HPLC. The artificial forms of antibodies, that is, Fv, Fv-P, and Fv-PP (*8*), were dissolved in NETS and immediately injected onto the column. Elution profiles are indicated in Fig. 2. Major parts of each sample showed a single peak. Minor parts of the samples ran faster through the column. When each samples were allowed to stand at R.T. for 24 h, V_H and V_L, V_L-P, and V_L-PP were dissociated and, moreover, probably denatured. Such coupling of the dissociation of Fv into V_H and V_L fragments with the denaturation of each fragment has also been observed in the thermal denaturation (*20*).

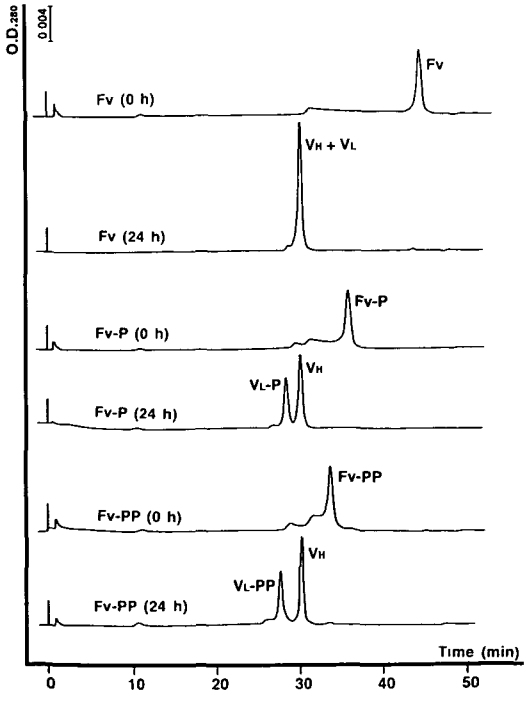

Fig. 2. **Dissociation of the Fv structure into V_H and V_L fragments by treatment with Sarkosyl.** Details of the characterization of Fv, Fv-P, and Fv-PP molecules are described in a previous paper (*8*). These three artificial molecules were suspended in NETS containing 0.1% Sarkosyl and immediately injected onto the column. Another sample was injected onto the column after standing at R.T. for 24 h with the Sarkosyl solution.

The V_H and V_L fragments in the denatured form ran much faster through the column. This observation is consistent

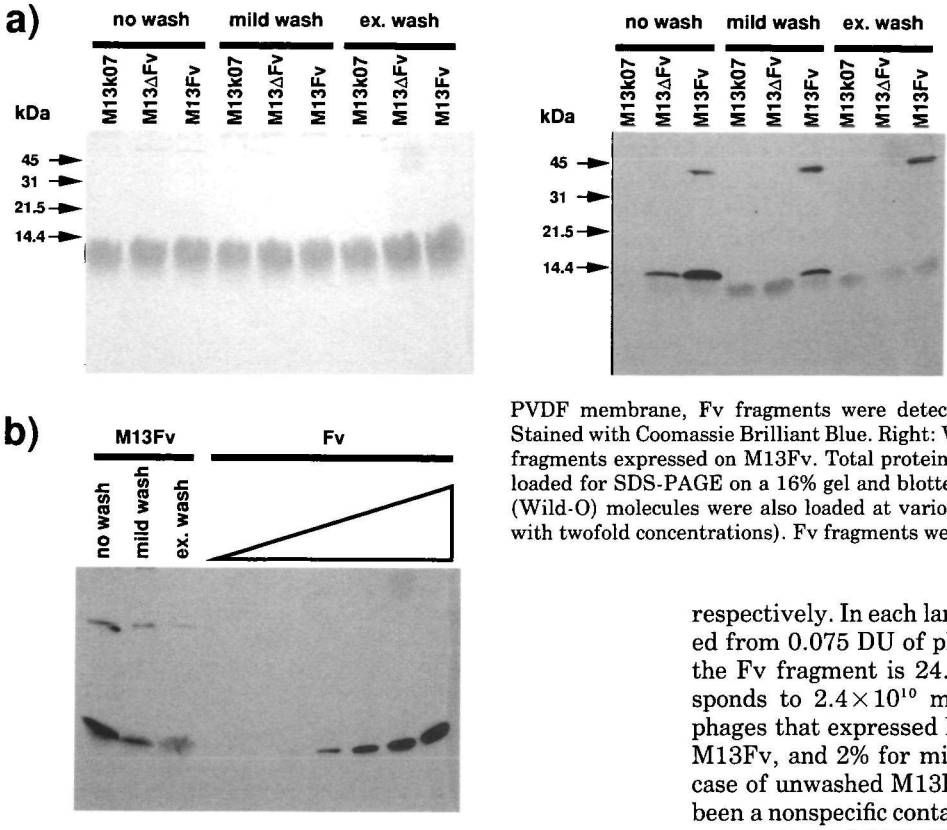

Fig. 3. **Detection and quantification of Fv fragments expressed on the surface of phage.** (a) Three kinds of phage, M13K07, M13⊿Fv, and M13Fv, were purified. Each clone was treated in three different ways: no treatment; treatment with 0.1% Sarkosyl for 1 h; and treatment with 0.1% Sarkosyl for 18 h. The protein fractions were prepared from the purified phages by removal of DNA as described in "MATERIALS AND METHODS." In each lane, total proteins derived from 0.075 DU of phages were loaded for SDS-PAGE on a 16% gel. After blotting onto a PVDF membrane, Fv fragments were detected with D1.3-specific antibodies. Left: Stained with Coomassie Brilliant Blue. Right: Western blottings. (b) Quantification of Fv fragments expressed on M13Fv. Total proteins derived from 0.075 DU of phages were loaded for SDS-PAGE on a 16% gel and blotted onto a PVDF membrane. Authentic Fv (Wild-O) molecules were also loaded at various concentrations (from 0.125 ng to 8 ng with twofold concentrations). Fv fragments were detected with D1.3-specific antibodies.

with the results shown in Fig. 3 in our previous paper (*8*). These results indicated that the extent of Fv forms expressed on the surface of phage can be regulated by treatment with Sarkosyl without distrupting the phage particles.

Expression of Fv Molecules Fused with Truncated cpIII on the Surface of M13 Phages—The expression of the V_H fragment and V_L-⊿cpIII on the surface of phages was examined by Western blotting with D1.3-specific antibodies (Fig. 3a). While Fv fragments in M13⊿Fv should not be anchored on the surface of M13 phages, a band whose mobility seemed to correspond to that of either the V_H or V_L fragment appeared in the Western blot. This band of V_H or V_L should be due to contaminants, since it disappeared when the phages were washed with 0.1% Sarkosyl for 1 h. In the case of M13Fv, by contrast, two bands, one thick band that corresponded to the V_H fragment and another band that corresponded to V_L-⊿cpIII, were clearly detected. Even when the phages were washed with 0.1% Sarkosyl for 1 h, both bands remained, although the intensity of the band that corresponded to V_H became weaker. When the phages were extensively washed for 18 h, the band corresponding to V_H disappeared completely, while the band of V_L-⊿cpIII remained without any decrease in intensity. From these observations, we concluded that the V_H and the V_L fragments were both expressed on the phage surface in an associated form, and that only the V_H fragment was removed from the phage by treatment with Sarkosyl.

To quantitate the Fv fragments expressed on the surface of M13Fv, another Western blotting experiment was carried out, as shown in Fig. 3b. The intensity of the bands of V_H expressed on unwashed M13Fv and on mildly washed M13Fv seemed to correspond to 4 and 1 ng of Fv fragments, respectively. In each lane, we applied total proteins prepared from 0.075 DU of phages. Since the molecular mass of the Fv fragment is 24.7 kDa, 1 ng of Fv fragment corresponds to 2.4×10^{10} molecules. Thus, the percentage of phages that expressed Fv molecules was 7% for unwashed M13Fv, and 2% for mildly washed M13Fv phages. In the case of unwashed M13Fv, the free V_H fragment may have been a nonspecific contaminant, similar to that found in the preparation of M13⊿Fv. In the case of mildly washed M13Fv, some of the V_H fragment associated with V_L-⊿cpIII may have been removed from the phage surface by washing with Sarkosyl. Moreover, a substantial amount of protein should have been lost during removal of DNA from the phage particles by centrifugation. Therefore, the actual percentage of M13Fv phages that expressed Fv molecules, although difficult to estimate, should be much higher than the above estimate for mildly washed M13Fv. Thus, we can conclude that M13Fv does express Fv molecules on its surface and that such phages probably represent more than 5% of the population of M13Fv phage.

The above conclusion, that when the phage was treated with Sarkosyl, only the V_H fragment was removed from the phage and the V_L-⊿cpIII was still anchored on the surface of phage, was further supported by the experiment using the BIAcore system (*7-9*) as shown in Fig. 4. In this experiment, D1.3-specific antibodies were chemically coupled to the solid support on a biosensor chip. In this case, washing with Sarkosyl did not have a major effect on the binding of M13Fv to the D1.3-specific antibodies. Since D1.3-specific antibodies bind not only the V_H fragment but also the V_L fragment, these results indicate that only the V_H fragment was removed from the surface of phages by washing with Sarkosyl and that V_L-⊿cpIII remained anchored on the phage surface.

Antigen-Binding Capability of Antibodies Expressed on the Surface of Phages—The capacity for binding of antigen of Fv fragments expressed on the surface of phages was examined by the BIAcore system. HEL was chemically coupled to the solid support on a biosensor chip. In the first experiment, unwashed preparations of phage were analyzed, as shown in Fig. 5a. Neither M13K07 nor M13⊿Fv bound to HEL. The shift in base lines was probably derived

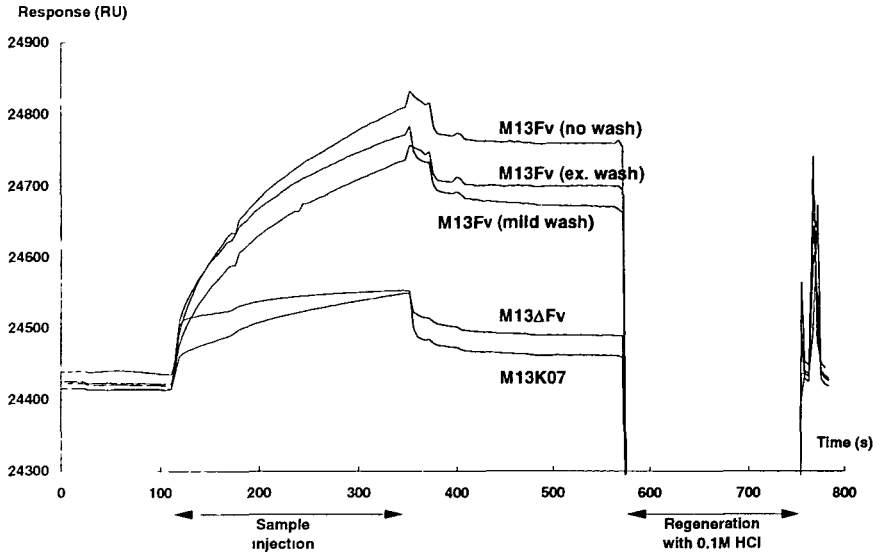

Fig. 4. **Detection of V_H and V_L fragments expressed as phage antibody by the BIAcore system.** D1.3-specific antibodies were coupled to the solid support on the biosensor chip. The experimental conditions are described in "MATERIALS AND METHODS."

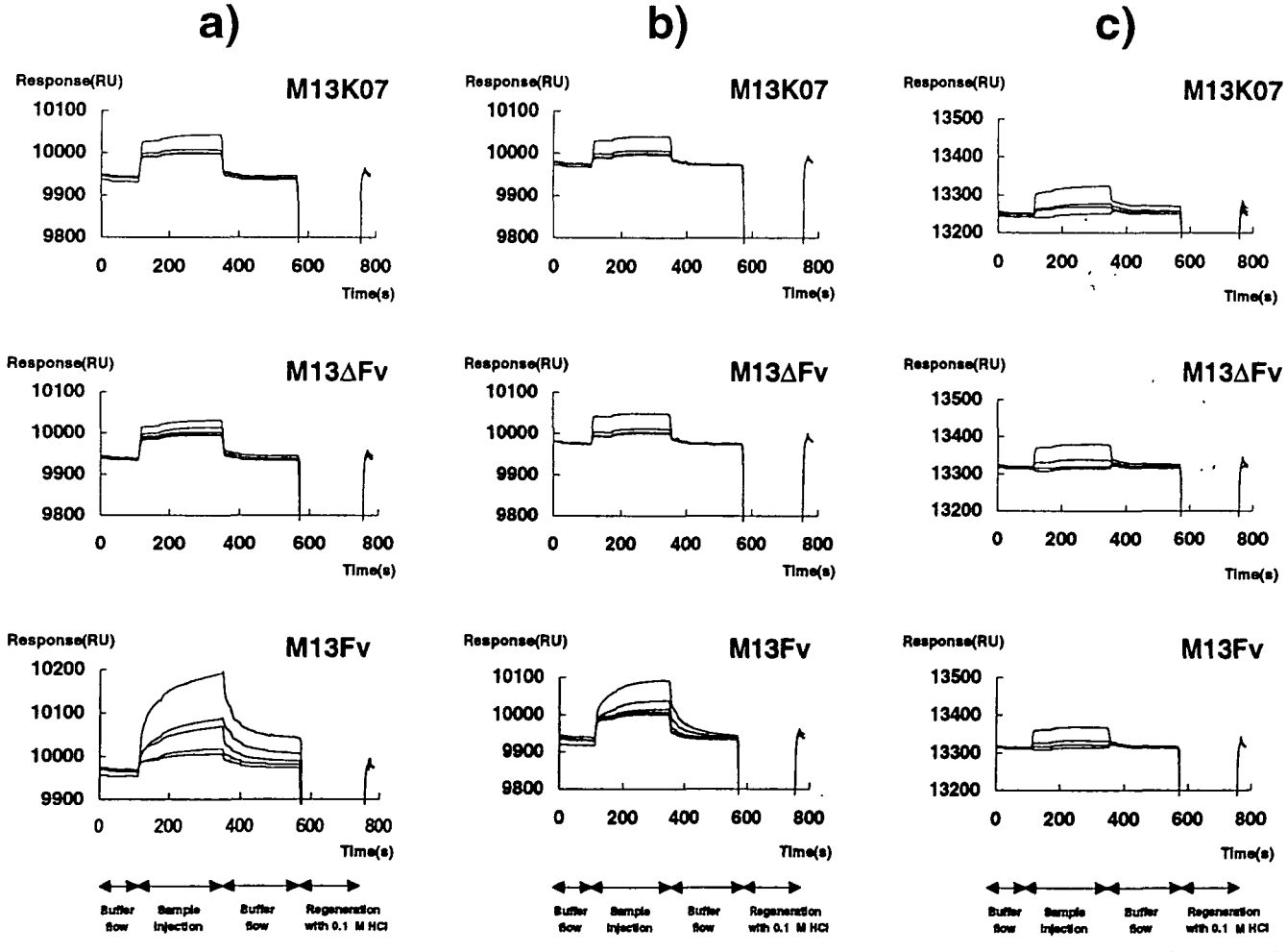

Fig. 5. **Analysis of the antigen-binding ability of the phage antibody by the BIAcore system.** Three kinds of phage were prepared and each phage was treated in three different ways: (a) no treatment; (b) treatment with Sarkosyl for 1 h; (c) treatment with Sarkosyl for 18 h. Phage solutions were serially diluted to 3.7, 1.8, 0.9, 0.45, and 0.23 DU/ml. Conditions for analysis with the BIAcore system are described in "MATERIALS AND METHODS."

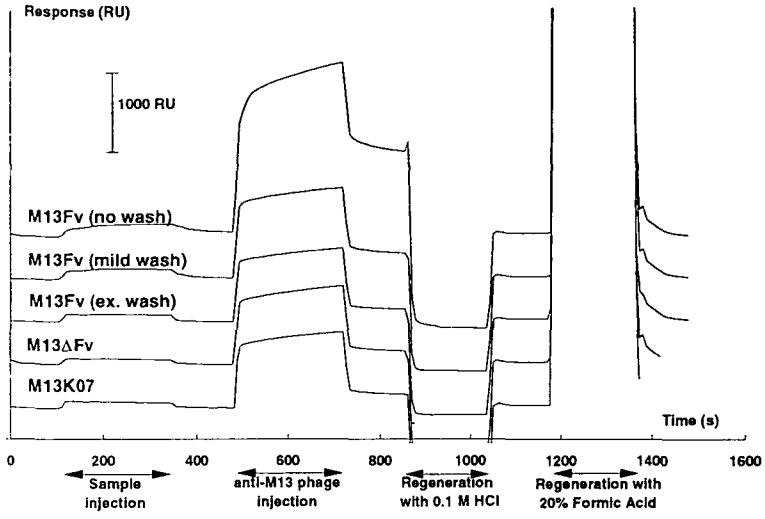

Fig. 6. **Detection of phages bound to antigens by the BIAcore system.** After phages had been injected into the system, antibodies specific for M13 phage were also injected. The number of RU, which reflects the amount of protein bound to the antigen, was greatly enhanced in the case of M13Fv (no washing) and slightly enhanced in the case of M13Fv (mild washing). The details of experimental conditions are given in "MATERIALS AND METHODS."

from differences in sample conditions with respect, for example, to solute, pH, and/or ionic strength. M13Fv was clearly bound to HEL although the number of RU due to the binding was not very large, being about 200 RU for a phage solution of 3.7 DU/ml. Since the number of RU exhibited in the experiment in Fig. 4 was 300-350 RU for interaction between anti-D1.3 antibodies and M13Fv (3.7 DU/ml), this value, 200 RU, was high enough to demonstrate the antigen-binding capability of M13Fv. Since anti-D1.3 antibodies bound not only to the Fv but also to the V_L-⊿cpIII fragment without V_H fragment, and since the efficiency of interaction between anti-D1.3 antibodies and the phage antibody should be higher than that between HEL and the phage antibody, these results suggested that the majority of the phages that anchored the V_L-⊿cpIII fragment formed the Fv structure with the V_H fragment. The shape of the dissociation curve suggested that some of the phages dissociated rapidly from HEL ($k_{off} = 3 \times 10^{-2}\ s^{-1}$) and the remainder dissociated slowly ($k_{off} \leq 1 \times 10^{-3}\ s^{-1}$). This result suggested the possibility that different types of phage may have been included in the unwashed preparation of M13Fv. Values of k_{on} and k_{off} of authentic Fv molecules, as measured by the BIAcore system in a previous study (*8*), were $1.71 \times 10^5\ M^{-1} \cdot s^{-1}$ and $1.98 \times 10^{-3}\ s^{-1}$. We suspected that some of the phages expressed only a single Fv molecule, while others expressed multiple Fv molecules on a single phage particle. This interpretation was consistent with the results with the preparation of mildly washed M13Fv. The curve for the dissociation from HEL was no longer biphasic, as we expected for a phage antibody with a single antigen-binding site ($k_{off} = 3 \times 10^{-2}\ s^{-1}$; Fig. 5b). Extensively washed M13Fv lost all HEL-binding activity (Fig. 5c). The apparent discrepancy in the value of k_{off} between monovalent Fv fragments and monovalent phage antibody (2×10^{-3} and $3 \times 10^{-2}\ s^{-1}$, respectively) is probably due to the difference in molecular size. In the BIAcore system, larger molecules give larger values of k_{off}. This tendency was observed in the analysis of the association of Fv, Fv-P, and Fv-PP with HEL in the previous paper (*8*). As an alternative interpretation, it could be argued that the contaminated Fv fragment in M13Fv affected the dissociation, since the V_H fragment of D1.3 had a HEL-binding capability (*21*).

The phage antibodies bound to HEL on the sensor chips were detected directly by injection of M13-specific antibodies. Figure 6 indicates that injection of antibodies specific for M13 phage greatly enhanced the RU in the case of unwashed M13Fv. The washing with Sarkosyl decreased the RU, and upon extensive washing, the signal due to phages bound to HEL fell to the background level. These results prove that phages expressing Fv fragments on their surface could bind to HEL.

DISCUSSION

In the present study, we addressed the following issues: 1, infectivity of the phage; 2, percentage of phages expressing antibodies in the total population of phages; 3, proper formation of antigen-binding sites on the phage surface and the ability to bind the antigen; 4, valency of antibodies expressed on a single phage particle. First, we constructed a gene, namely, genes for a V_H domain, a V_L domain fused with truncated cpIII, and two domains of protein A, arranged in tandem. When helper phages were infected into bacteria that harbored plasmid pM13Fv, the resultant phage containing pM13Fv DNA was found to have sufficient infectivity for practical use. The percentage of phages expressing Fv molecules in the total phage population was estimated by Western blotting, which indicated that more than 5% of phages did express Fv molecules. Bass *et al.* (*22*) reported that about 10% of the phagemid particles contain one copy of the fused protein on the phage surface in which human growth hormone was fused with a truncated cpIII of M13 phage. Garrard *et al.* (*23*) estimated that there is an average of 0.4 Fab molecule per phage particle in which a light chain and an Fd fragment fused with a truncated cpIII of M13 were expressed. Although our estimation was rather smaller than these observations, the system and the method of estimation are different from each other and our estimation should be considered to show a minimum value, as described in "RESULTS." The analysis with the BIAcore system proved directly that Fv fragments expressed on the surface of phages could bind to the antigen that had been chemically coupled to the solid support.

The analysis of kinetics using the BIAcore system showed a biphasic feature of the dissociation curve, fractions with a larger and a smaller k_{off}. We argued that these fractions could correspond to a monovalent form and a multivalent form, respectively, in "RESULTS." Although all of our data seemed to be consistent with our interpretation, it has been generally argued that the phage antibody should be monovalent in the case of the phagemid system (*22, 23*). Other than our interpretation, several possibilities could be pointed out. This may reflect the characteristics of the biosensor chips in the BIAcore system. Accessibility of the ligand and receptor on the chips could be heterogeneous. The biphasic nature of the dissociation curve could be an aggregation of multiple phages. Since significant amounts of the V_H fragment contaminated the purified phage fractions, they might disturb the analysis by the BIAcore system. We can argue against these possibilities. The dissociation curve of mildly washed M13Fv was not biphasic. Aggregation of phages, if it occurred, might have happened in the process of pelleting by centrifugation and/or precipitation with PEG/NaCl solution. We washed phages with Sarkosyl solution at an early stage of their purification. M13⊿Fv contaminated with V_H fragment did not bind to the antigen. However, the interpretation of the data showing the biphasic feature of the dissociation curve may still be debatable.

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