Making artificial antibodies: A format for phage display of combinatorial heterodimeric arrays

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ABSTRACT   The gene VII protein (pVII) and gene IX protein (pIX) are associated closely on the surface of filamentous bacteriophage that is opposite of the end harboring the widely exploited pIII protein. We developed a phagemid format wherein antibody heavy- and light-chain variable regions were fused to the amino termini of pVII and pIX, respectively. Significantly, the fusion proteins interacted to form a functional Fv-binding domain on the phage surface. Our approach will be applicable to the display of generic peptide and protein libraries that can form combinatorial heterodimeric arrays. Consequently, it represents a first step toward artificial antibodies and the selection of novel biological activities.

Now that combinatorial antibody libraries have been secured (1–9), a powerful next step is the evolution toward artificial antibody constructs. Artificial antibodies are defined here as protein motifs of large diversity that use the functional strategy of the antibody molecule, but can be free of loop and framework structural constraints. When reduced to its essence, the antibody molecule is a biological device for the presentation of a combinatorial array of peptide elements in three-dimensional space. The essential feature is that while CDRs (complementarity-determining regions) cooperate to form a binding site, their interaction is dynamic and functional with little structural association between the CDRs themselves. In this way, the full complement of amino acid residues is available for antigen recognition at a minimum energetic cost for binding. We propose that the ability to control the combinatorial design not only of sequence space but also of three-dimensional space would recapitulate and ultimately transcend the natural design of the immune repertoire.

Although phage display has been investigated intensively, many details of the phage particle itself have not been fully elucidated, and the possibility of alternative display formats also remain to be explored. The filamentous bacteriophage fd, and, similarly, M13, consists of a circular, single-stranded DNA molecule surrounded by a cylinder of coat proteins (Fig. 1). The molecular mass of a particle is about 1.6×10^7 Da, of which 88% is protein and 12% is DNA (10). There are about 2,700 molecules of the major coat protein pVIII that envelope the phage. At one end of the particle, there are five copies each of gene VII and VI proteins (pIII and pVI) that are involved in host–cell binding and in the termination of the assembly process. The other end contains five copies each of pVII and pIX that are actually hydrophobic peptides of 33 and 32 aa, respectively, required for the initiation of assembly and for maintenance of virion stability. Whereas pIII, pVI, and pVIII have been used to display biological molecules, pVII and pIX have not been utilized (1, 11).

Substantial information has been accumulated about the structural and the phage display characteristics of pIII and pVIII. Yet, the data concerning the minor proteins encoded by genes VII and IX are scanty (12, 13). Recently, on the basis of results from membrane-mimicking experiments, it was suggested that the principal conformational state of membrane-bound pIX *in vivo* is α-helical (13). Both pVII and pIX are synthesized without signal sequences, and the mechanism responsible for their insertion into the membrane is not known. In addition, both are presumed to span the membrane with their amino-terminal portion exposed to the periplasm. However, the proposed topology was based only on the observation that the molecules retained their amino-terminal formyl group after membrane insertion (14).

Herein, we describe the foundation of a combinatorial phage-display format for the construction of highly diverse heterodimeric polypeptide arrays. The approach used pVII and pIX for the display of fusion proteins that we hypothesized would be in close enough proximity to form dimeric motifs. In the process, the orientations of the pVII and pIX peptides in the phage coat were verified. The prototype for our methodology was the display of antibody heavy- and light-chain variable regions (V_H and V_L) individually expressed on pVII and pIX, respectively. By extending the approach, it should be possible to display and assay diverse libraries in which members can function as dimeric, artificial antibody species.

MATERIALS AND METHODS

Construction of Flag and pVII/pIX Fusion Proteins. The fusion proteins were constructed by fusing a Flag peptide to the N or C terminus of pVII and pIX. The constructs were amplified by PCR with single-stranded VCSM13 DNA as template. The primers used for the four permutations were as follows: pVII-Flag, VII-FOR (5′-CTATCCATGGCAATGG-AGCAGGTCGCGGATTTC-3′) and VII-fBW (5′-ATTTA-GCTAGCTTATTTGTCATCGTCATCTTTGTAGTCTCT-TTGACCCCCAGCGATTAT-3′); Flag-pVII, VII-fFOR (5′-CTATCCATGGCAGACTACAAAGATGACGATGAC-AAAATGGAGCAGGTCGCGGATTTC-3′) and VII-BW (5′-GATTTAGCTAGCTTATTATCTTTGACCCCCAGC-GATTAT-3′); pIX-Flag, IX-FOR (5′-CTATCCATGGCAA-TGAGTGTTTTAGTGTATTCT-3′) and IX-fBW (5′-ATT-TAGCTAGCTTATTTGTCATCGTCATCTTTGTAGTCT-GAGGAAGTTTCCATTAAACG-3′); Flag-pIX, IX-fFOR (5′-CTATCCATGGCAGACTACAAAGATGACGATGA-CAAAATGAGTGTTTTAGTGTATTCT-3′) and IX-BW (5′-GATTTAGCTAGCTTATTATGAGGAAGTTTCCAT-TAAACG-3′).
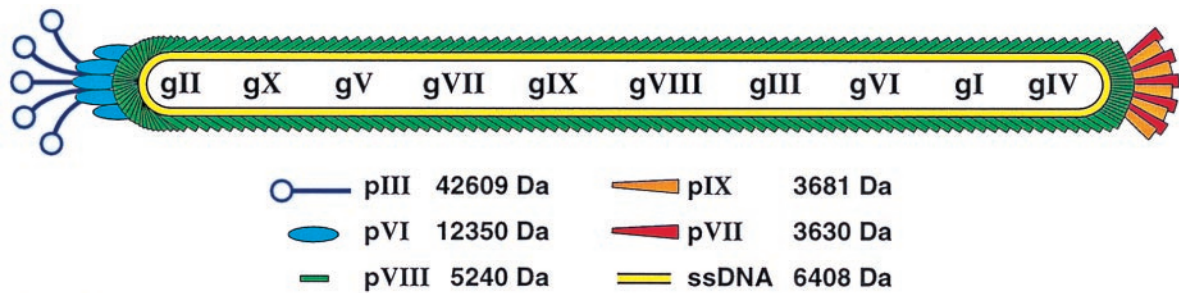

Fig. 1.   Filamentous phage fd architecture.

The PCR products were digested by restriction enzymes *Nco*I and *Nhe*I and inserted into the phagemid vector pCGMT (15).

Construction of V$_H$-(Gly$_4$Ser)-pVII and V$_L$-(Gly$_4$Ser)-pIX. Two murine catalytic antibodies, 21H3 and 2H6 (15–18), and one murine anti-cocaine antibody, 92H2 (generated by our laboratory), were used to construct V$_H$-(Gly$_4$Ser)-pVII and V$_L$-(Gly$_4$Ser)-pIX fusion proteins. The V$_H$ sequences in each case were fused to the N terminus of pVII, and the V$_L$ sequences were fused to the N terminus of pIX. The constructs were engineered by inserting a linker sequence, Gly$_4$Ser, between V$_H$ and pVII and between V$_L$ and pIX. The Fab genes were available from our previous work and were the most readily manipulated sequences for amplification of variable regions. First, the V$_H$-linker fragments were amplified by PCR by using primers H1 and H2 for Fab 21H3, H1 and H3 for Fab 2H6, and H1 and H4 for Fab 92H2. The V$_L$-linker fragments were amplified by using primers L1 and L2 for Fab 21H3, L1 and L3 for Fab 2H6, and L1 and L4 for Fab 92H2. Then, linker-pVII and linker-pIX were amplified with VCSM13 as template. The primers VII-F and VII-BW were used for linker-pVII, and primers IX-F and IX-BW were used for the linker-pIX construct. Finally, V$_H$-(Gly$_4$Ser)-pVII and V$_L$-(Gly$_4$Ser)-pIX were assembled through overlap-extension PCR by mixing equimolar amounts of V$_H$-linker and linker-pVII, or V$_L$-linker and linker-pIX. The primers H1 and VII-BW were used for V$_H$-(Gly$_4$Ser)-pVII, and the primers L1 and IX-BW were used for V$_L$-(Gly$_4$Ser)-pIX. The sequences of primers used were as follows and as shown above: H1 (5′-GCCTACGGCAGCCGCTGGATTGTTATTACT-3′), H2 (5′-GCCAGAACCACCACCAGAGACAGTGACCAG-3′), H3 (5′-GCCAGAACCACCACCAGAGACGGTGACT-GAGGTTCC-3′), H4 (5′-GCCAGAACCACCACCAGAT-GAGGAAACGGTGACCGT-3′), L1 (5′-GCTATCGCGAT-TGCTGTGGCACTGGCTGGT-3′), L2 (5′-GGAGCCGC-CGCCGCCAGAATCAGCCCGTTTGATTTC-3′), L3 (5′-GGAGCCGCCGCCGCCAGAATCAGTCCGTTTCAAC-TC-3′), L4 (5′-GGAGCCGCCGCCGCCAGAGACCAGGC-CCCCGAGGCC-3′), VIIF (5′-TCTGGTGGTGGTTCTG-GCATGGAGCAGGTCGCGATTTC-3′), IXF (5′-TCTG-GCGGCGGCGGCTCCATGAGTGTTTTAGTGTATTCT-3′).

After digestion with *Nco*I and *Nhe*I, the V$_H$-(Gly$_4$Ser)-pVII fusion proteins of 21H3, 2H6, and 92H2 were ligated into phage display vector pCGMT-1b that was derived from pCGMT and contained two ribosome-binding sites and two leader sequences of *ompA* and *pelB* (Fig. 2). V$_L$-(Gly$_4$Ser-pIX) was digested with *Sac*I and *Nhe*I and ligated into pCGMT-1b that had the V$_H$-(Gly$_4$Ser)-pVII insert.

Electron Microscopy. Stable complexes of 5-nm gold particles bound to PCP-BSA were prepared according to known methodology (19, 20). The designation PCP referred to the phosphonate hapten originally used to obtain the antibodies 21H3 and 2H6 (16). To ensure the removal of any unbound protein, the complexes were centrifuged through a 7% glycerol cushion as described (20). The pelleted complexes were resuspended in PBS containing 0.1% BSA and stored at 4°C. Bacteriophage were prepared by 1 mM isopropyl β-D-thiogalactopyranoside induction at 30°C overnight, precipitated by polyethylene glycol, and diluted into PBS containing 0.01% BSA to a final concentration of 5×10^{10} colony-forming units (cfu)/ml. A 2-μl aliquot of the diluted phage was applied to Formvar-coated nickel grids (200 mesh) for 5 min. Unbound phage were removed by washing with PBS and then 1% BSA in PBS for 10 min. PCP-BSA-gold complexes were applied to the grids undiluted. After 30 min, the grids were washed with PBS and then stained with 1% uranyl acetate for visualization by electron microscopy. Randomly selected areas on the grids were photographed to quantify the number of gold particles associated with phage.

Phage Preparation and Phage ELISA. A single clone of XL1-Blue cells carrying the phagemid pCGMT (containing Flag-pVII or pVII-Flag or Flag-pIX or pIX-Flag) or pCGMT-1b [containing V$_H$-(Gly$_4$Ser)-pVII and V$_L$-(Gly$_4$Ser)-pIX] was picked up and grown at 37°C in superbroth medium containing 1% glucose, 10 μg/ml tetracycline, and 100 μg/ml carbenicillin until OD$_{600}$ ≈ 0.1. Helper phage VCSM13 were added, and the cells were grown at 37°C for another 2 h, and then kanamycin and isopropyl β-D-thiogalactopyranoside were added to a final concentration of 70 μg/ml and 1 mM, respectively. The cells were grown at 28–30°C overnight. The supernatant of the phage was used directly for ELISA. The microtiter plates (Corning) were coated with 25 μl per well of 10 μg/ml mouse anti-Flag antibody to assay Flag constructs with pVII/pIX, PCP-BSA to assay 21H3 and 2H6 constructs, and the cocaine conjugate GNC-BSA (21) to assay the 92H2 construct. The phage ELISA was performed as described (15, 22).

Selective Enrichment. The 2H6 phage were diluted into 92H2 phage at a ratio of 1:100 or 1:10^8, and the mixture was used for selection against PCP-BSA. In another experiment, the 92H2 phage were mixed with 2H6 at a ratio of 1:100 and the mixture was used for selection against GNC-BSA. Microtiter plates were coated overnight at room temperature with 25 μl per well of the PCP-BSA or GNC-BSA conjugate (10 μg/ml in PBS). After washing five times with water, the plates were blocked with Blotto (PBS/4% nonfat dry milk) at 37°C for 1 h. Then, 50 μl (10^{12} cfu per well) of phage mixture (prepared as
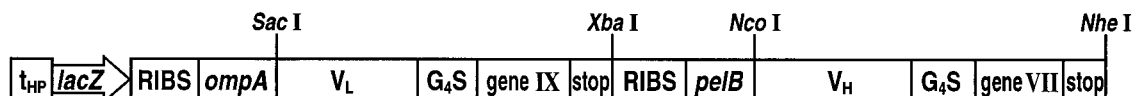

Fig. 2.   Gene construct of the phagemid pCGMT-1b used for Fv display.

described above) was added and incubated for 1 h at 37°C. The plates were washed 20 times with PBS containing 0.1% Tween-20 and 10 times with PBS. The bound phage then were eluted with 50 μl per well of elution buffer (0.1 M HCl/glycine/0.1% BSA, pH 2.2). After 10 min, the elution buffer was removed and neutralized with 3 μl of 2 M Tris base. Phage eluted from the first round of selection were used to infect *Escherichia coli* XL1-Blue cells. Phage particles were rescued from the cells and used for the subsequent round of antigen selections.

Catalytic Activity Analysis. The reactions were carried out in 100 mM Bicine (*N,N*-bis(2-hydroxyethyl)glycine), pH 8.5, containing 10% DMSO as cosolvent. Approximately 150 nM 21H3 phage (1×10^{14} cfu/ml) in 100 mM Bicine, pH 8.5, was mixed with 5 mM (*S*)-(−)-*sec*-phenethyl alcohol and 16 mM vinyl 4-acetamidophenylacetate prepared as stock solutions in DMSO. A 50-μl aliquot was removed every 30 min and quenched by adding 4 μl of 10% HClO$_4$. The formation of the product (*S*)-(−)-*sec*-phenethyl 4-acetamidophenylacetate was monitored by using reversed-phase HPLC [C18-Vydac 201TP54 column; isocratic mobile phase (62% water/0.1% trifluoroacetic acid/38% acetonitrile); 1.75 ml/min; 254 nm].

Analysis of the Dissociation of the V$_H$ and V$_L$ Fragments on the Surface of Phage. Phage-Fv antibody (5×10^{13} cfu) in 1.5 ml was treated with 0.1% Sarkosyl (*N*-lauroylsarcosine sodium salt; Sigma) in PBS for 20 h and then loaded onto a HIPrep 26/60 Sephacryl S-100HR gel-filtration column (Pharmacia) preequilibrated with PBS containing 0.1% Sarkosyl. The phage fraction was collected, polyethylene glycol-precipitated, and dialyzed into PBS for assay by phage ELISA.

RESULTS

Orientation of pVII and pIX on the Phage Particle. It was essential to know the orientation of pVII or pIX on the surface of phage to correctly display peptides and proteins. We fused a Flag tag, an octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) that has been used widely in protein detection and purification, to the N or C terminus of pVII and pIX and inserted the constructs into a phagemid vector, pCGMT. Phage particles carrying the four different fusion proteins, Flag-pVII and Flag-IX (Flag fused to the N terminus of pVII or pIX, respectively) and pVII-Flag and pIX-Flag (Flag fused to the C terminus of pVII or pIX, respectively), were rescued from XL1-Blue cells containing those phagemid. The binding capacities of the phage particles to the mAb anti-Flag M2 that recognized the Flag tag were tested by ELISA. It was shown that only the Flag fused to the N terminus of pVII or pIX could be detected by phage ELISA (Fig. 3). The results indicated that the Flag peptide in these constructs was exposed on the outside of the phage coat, and, therefore, the N termini of pVII and pIX also must be on the exterior of the phage particle.

Display of Fv Fragments by Interaction of pVII and pIX Constructs. Our principal aim was to test the hypothesis of whether a functional heterodimeric protein could be displayed via the pVII and pIX format. Hence, in three separate examples, the heavy chains of the murine mAbs 21H3, 2H6, and 92H2 were constructed as fusion proteins with pVII and the light chains were constructed as fusion proteins with pIX, and the constructs were displayed on the surface of phage particles. For negative controls, we fused only the V$_H$ of these three antibodies to pVII or the V$_L$ to pIX and omitted the other chain. The correct construction of phagemid pCGMT-1b-21H3, pCGMT-1b-2H6, and pCGMT-1b-92H2 was confirmed by DNA sequencing. The phage particles that displayed antibody 21H3, 2H6, or 92H2 were rescued from XL1-Blue cells with helper phage VCSM13.

Phage ELISA showed that 21H3 and 2H6 phage antibodies specifically bound PCP-BSA and 92H2 phage antibody specifically bound GNC-BSA. A typical result for the 21H3
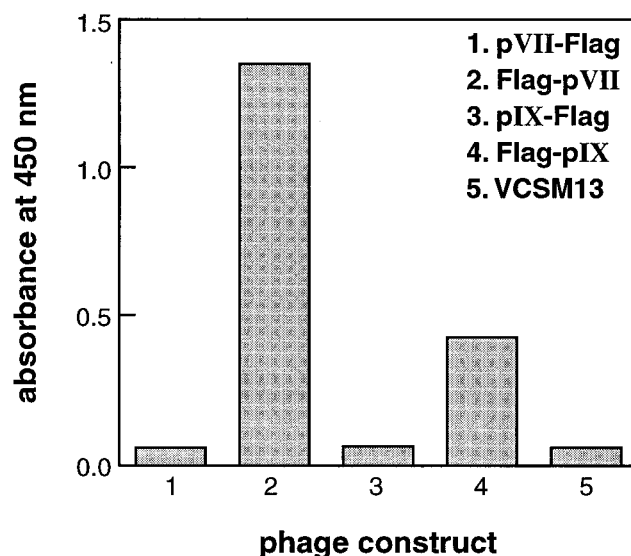

FIG. 3. Phage ELISA of the different Flag and pVII or pIX fusion proteins.

construct is shown (Fig. 4). For the former two antibodies, the binding activity could be inhibited by free hapten PCP, and for 92H2, it could be inhibited by cocaine. There was no binding to BSA itself. No binding activities were detected for the phage displaying only the V$_H$ or V$_L$ chains (Fig. 4). Consequently, it was concluded that when both the V$_H$ and V$_L$ chains were displayed on the surface of phage as pVII and pIX fusion proteins, respectively, interaction of the chains occurred to form a functional Fv antibody motif.

In the case of the 21H3 phage Fv, the catalytic activity also was investigated and compared with our previously studied 21H3 IgG antibody (18). When 150 nM of the phage antibody (1×10^{14} cfu/ml) was mixed with 5 mM alcohol and 16 mM vinyl ester, the product ester could be detected within 30 min (data not shown). A comparison of time-course curves showed that the catalytic activity from 150 nM phage Fv was roughly equal to 50 nM of the IgG.

Electron Microscopy. The specific binding of phage Fvs to their antigen labeled with 5-nm colloidal gold was visualized directly by electron microscopy. Examination of filamentous
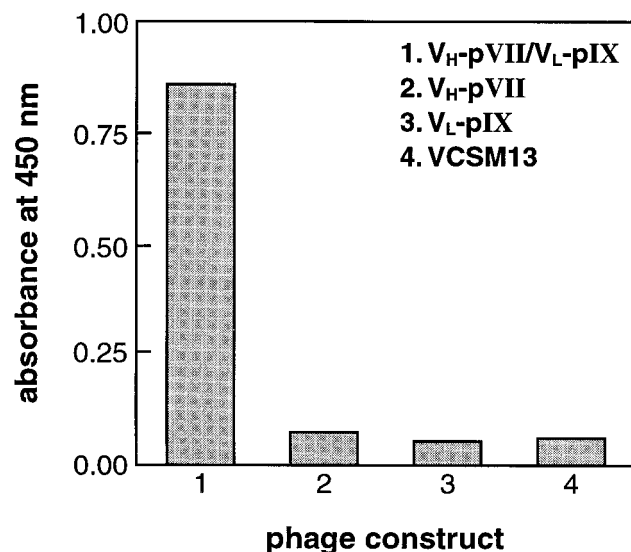

FIG. 4. Phage ELISA of the antigen (PCP-BSA)-binding capacities of the different fusion constructs of the 21H3 V$_H$ and V$_L$ with pVII and pIX.

phage 21H3 and 2H6 revealed specific labeling by the PCP-BSA-gold complex at one end of the phage (Fig. 5). It was observed that some of the phage were labeled by more than one gold particle (Fig. 5B). The specificity of the labeling to 21H3 and 2H6 phage was indicated by the absence of labeling by using a BSA-gold complex and also by the fact that 92H2 phage could not be labeled by the PCP-BSA-gold complex (data not shown).

Selective Enrichment of a Target Antibody. We demonstrated the potential for pVII- and pIX-displayed Fv fragments in selection and evolution experiments by enrichment of a mixture of two antibodies based on function. The 2H6 and 92H2 phage Fvs were used for the experiment because they had similar cell densities under the same growth conditions. The 21H3 gene was more toxic to XL1-Blue cells and showed a much lower cell density.

In our first experiment, the enrichment was tested by mixing 2H6 phage Fv and 92H2 phage Fv at a ratio of 1:100 or 100:1 followed by selection against PCP-BSA or GNC-BSA. After one round of panning, 9 of 10 randomly selected clones were 2H6 phage Fv when mixed at a ratio of 1:100 and selected against PCP-BSA, and 6 of 10 clones were found to be 92H2 phage Fv when mixed at a ratio of 1:100 and selected against GNC-BSA. The results showed that at least a 100-fold enrichment could be achieved per round of panning when a functional Fv fragment was displayed on the phage surface as a result of the interaction of pVII and pIX constructs.

In a second experiment, a selection from a highly dilute mixture was examined. The 2H6 phage Fv were mixed with 92H2 phage Fv at a ratio of $1:10^8$ and panned against immobilized PCP-BSA. The panning was performed for a total of two rounds. After each round of panning, the phage were pooled and tested by ELISA for their ability to bind PCP-BSA and GNC-BSA. The phage mixture before panning showed no binding activity to PCP-BSA and strong binding to GNC-BSA. However, after two rounds of selection, phage showed greatly enhanced binding to PCP-BSA and weak binding to GNC-BSA (Fig. 6). To verify the ratio of 2H6 and 92H2 clones before and after each round of panning, phage were rescued from 15 randomly selected clones and tested by ELISA for their ability to bind PCP-BSA and GNC-BSA. Of the 15 clones, the number
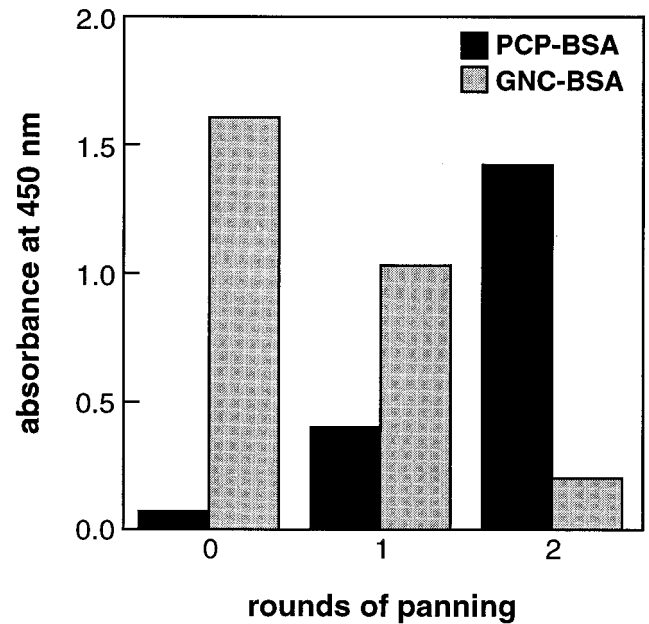

FIG. 6.   Enrichment of anti-PCP 2H6 phage Fv during panning. Pooled phage eluted after each round were amplified, and 10^{10} cfu were added to each well of a microtiter plate precoated with PCP-BSA or GNC-BSA. Phage were detected by using an anti-M13 antibody as described (*Materials and Methods*).

of 2H6 clones increased from 7 after first round of panning to 15 after the second round of panning. In contrast, of 15 clones randomly selected from the unpanned phage mixture, none were 2H6. The results showed that a 10^8-fold enrichment was accomplished after only two rounds of panning.

Dissociation Assay of the Displayed Fv Fragment. V_H and V_L domains were found to associate and form an antigen-binding site on the phage surface (23). Though unlikely in our experiments, the possibility existed that while one variable domain was displayed on the phage surface as the intended pVII or pIX fusion protein, the other domain existed as a free
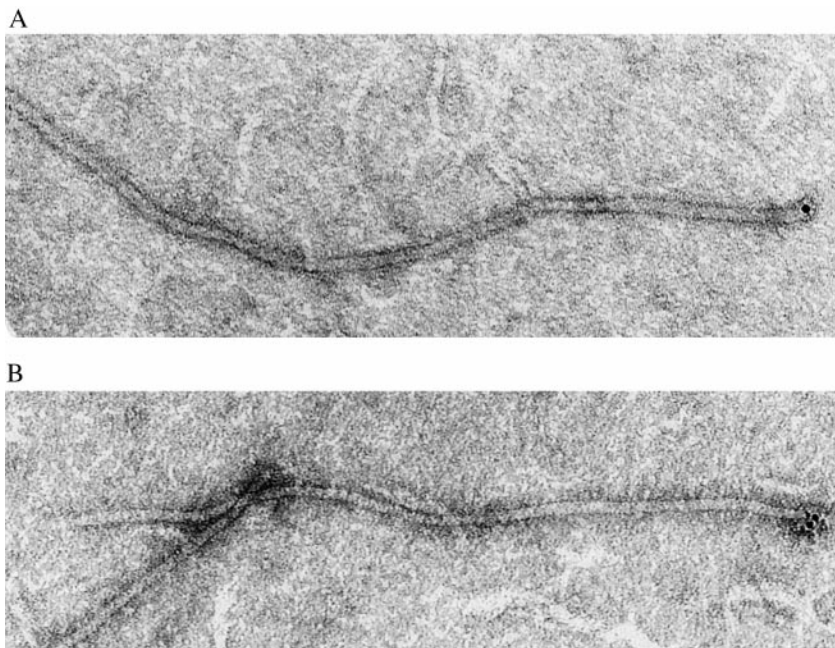

FIG. 5.   Electron micrographs showing antigen-specific labeling of filamentous phage displaying the 2H6 Fv heterodimer. Similar results were obtained for the 21H3 phage Fv; however, the micrographs were not as distinct. (*A*) A phage specifically labeled with a 5-nm colloidal gold particle adhered to the PCP-BSA antigen (×105,000). (*B*) A phage on the same grid as in *A* labeled with two gold particles (×105,000).

chain and associated with the first in the periplasmic space. To verify that the Fv on the phage surface resulted through interaction of both pVII and pIX fusion proteins, the 21H3 phage Fv was treated with 0.1% Sarkosyl in PBS at room temperature for 20 h and purified by gel filtration. Sarkosyl, a relatively mild detergent, has been used frequently for dissolving precipitates of phage particles and was used successfully to dissociate Fv fragments (23). The phage fraction was collected and dialyzed against PBS to remove the Sarkosyl. The antigen-binding activity of 21H3 was tested by phage ELISA before and after the Sarkosyl treatment, and no difference was observed (data not shown).

DISCUSSION

Despite the enormous attention focused on pIII- and pVIII-mediated phage display, no published reports for pVII and pIX existed. As described herein, it was demonstrated that pVII and pIX could be used for displaying the Flag peptide when fused to the N termini of the two coat proteins. Then, of greater significance, we showed that antibody variable regions fused to pVII and pIX engaged in a dynamic interaction to display a functional Fv antibody, a representative heterodimeric motif.

The pIII, pVI, pVIII, pVII, and pIX coat proteins are integral inner membrane proteins before assembly (24). With the advent of our results, all five now have been used for displaying proteins on the phage surface. Other workers showed that pVII and pIX were present as five molecules located at the same end of the phage particle that emerged first from the cell and were required for the initiation of assembly through interaction with the packaging signal of the phage genome (11). The absence of pVII and pIX resulted in an extremely low production of phage. Although it had been suggested previously that pVII and pIX were not functional with another protein fused to their N termini (24), our investigation showed that such fusions were viable. The possible reasons for our success were that (*i*) a leader sequence was incorporated that targeted the fusion proteins to the inner membrane and prevented accumulation in the cytoplasm and (*ii*) the wild-type pVII and pIX from helper phage were used to compete with the pVII and pIX fusion proteins. With the success of our work, pVII and pIX should be readily applicable to combinatorial phage-display protocols utilizing highly diverse protein sequences.

The specific gold labeling of phage Fv determined from electron microscopy clearly showed the presence of gold at one end of the phage particle. Interestingly, phage that harbored either one or two gold labels were observed. We presume that the latter resulted from the bivalent display of Fv fragments, rather than multiple gold labeling of PCP-BSA. Approximately 20% of the phage were labeled by gold and, therefore, displayed a functional Fv antibody. However, a kinetic analysis showed that the activity of 150 nM 21H3 phage Fv was equal to the activity of 50 nM 21H3 IgG. The data, taken together, along with the assumption that the specific activities of the Fv and IgG were comparable suggested some phage particles simultaneously displayed more than one Fv fragment. A multivalent display of Fv fragments at the C terminus of pIII was shown previously (23). Furthermore, the modulation of growth conditions resulted in altered valencies of antibody display (25), and, therefore, our induction with 1 mM isopropyl β-D-thiogalactopyranoside might well increase the possibility of multivalency.

Fv fragments are heterodimers made up of V_H and V_L domains and are the smallest antibody fragments that contain all the information necessary for specific antigen binding. However, the noncovalently associated chains in an isolated Fv fragment are not highly stable and tend to dissociate (26). Known methods for stabilizing Fv fragments are as single-chain Fvs (scFvs) (1, 27, 28) and as disulfide-stabilized Fvs (dsFvs) (29–31). Yet, scFvs still are often unstable (29, 32, 33) and can have lower affinities compared with Fabs and whole IgG because the linker interferes with binding or does not sufficiently stabilize the heterodimer (30, 34, 35). Although the dsFvs generally are more stable and do not have a linker, they require the incorporation of a disulfide into the library construction. In addition, it is likely that dsFv libraries cover a biased antibody subset in which the interchain disulfide does not interfere with antigen binding (35). The Fv antibody displayed by pVII and pIX in our format can be viewed as a phage-stabilized Fv (psFv) that mimics the natural antibody structure without the disadvantages of scFvs and dsFvs. Our Fvs retain affinity and are robust, in that each chain is anchored independently to the phage coat.

Most important, our new format would be particularly useful for the combinatorial display of heterodimeric arrays. Furthermore, although the reasons are not yet clear, this format appears to yield a particularly powerful enrichment during panning protocols. The pVII and pIX apparently are in close enough proximity for fusion proteins with the V_H and V_L of an antibody to form a functional heterodimer. We fully anticipate that the approach can be extended to the display of diverse polypeptides for the creation of artificial antibodies. The ability to display a large repertoire of novel dimeric binding domains unconstrained by the specific programming of antibody structure will increase our understanding of protein—protein interactions and potentially lead to the discovery of unique biological activities.

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