A simple method for displaying recalcitrant proteins on the surface of bacteriophage lambda

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ABSTRACT

Bacteriophage lambda (λ) permits the display of many foreign peptides and proteins on the gpD major coat protein. However, some recombinant derivatives of gpD are incompatible with the assembly of stable phage particles. This presents a limitation to current λ display systems. Here we describe a novel, plasmid-based expression system in which gpD deficient λ lysogens can be co-complemented with both wild-type and recombinant forms of gpD. This dual expression system permits the generation of mosaic phage particles that contain otherwise recalcitrant recombinant gpD fusion proteins. Overall, this improved gpD display system is expected to permit the expression of a wide variety of peptides and proteins on the surface of bacteriophage λ and to facilitate the use of modified λ phage vectors in mammalian gene transfer applications.

INTRODUCTION

Bacteriophage λ is a temperate phage with a double-stranded (ds) genome of $\sim$48 kb encapsulated in an icosahedral capsid ($\sim$50 nm in diameter) with a long and fibrous tail ($\sim$150 nm in length). The λ capsid is composed of two major coat proteins, gpE and gpD. Lambda capsid maturation begins with the formation of the prohead, composed solely of gpE (1). As the genomic DNA is packaged, the capsid expands in volume by $\sim$45% and $\sim$405 copies of gpD are then added to fully populate and stabilize the capsid (2).

gpD is a 109 amino acid protein (excluding the initial methionine which is removed from the mature protein) (3) and is required for the packaging of full-length genomes (4).

gpD-deficient λ mutants with genome sizes between 78–82% of wild-type are viable, although they are highly unstable in the presence of chelating agents such as EDTA. EDTA is thought to remove essential Mg^{2+} ions that serve to neutralize the negative charges associated with the phosphate backbone of the phage genome (2,4,5).

The discovery that foreign peptides or protein fragments can be displayed on the surface of filamentous bacteriophage (6,7) has led to the development of phage display technology, with applications that include antibacterial therapy, antibody discovery, cDNA screening and display and gene delivery to mammalian cells (8–40). Our laboratory is particularly interested in protein display on bacteriophage λ, since we believe that λ may have certain advantages over filamentous phages in gene transfer applications. These include the physical properties of λ phage, which is more similar in size and shape to mammalian viruses than M13, and the size and nature of the λ genome, which has a large dsDNA molecule versus the smaller single-stranded (ss) DNA genome of M13 (21).

Bacteriophage λ has been shown to be able to accommodate the display of foreign peptides and proteins on the gpD major coat protein (3,4,15,20,28,35,38). However, in the course of our studies, we have encountered certain proteins that are poorly tolerated when displayed on the bacteriophage λ capsid at high copy number (405–420 copies per capsid), resulting in an inability to generate recombinant phage particles that display the desired molecule. To solve this problem, we developed a novel dual expression system in which gpD deficient lysogens can be complemented with both wild-type gpD protein and gpD-fusion coat proteins—thereby generating mosaic phage particles that contain both wild-type and recombinant versions of gpD. In the present work, we demonstrate the application of this technology to the surface display of a genetically engineered derivative of the tenth fibronectin type III domain (3JCLI4), which has nanomolar

affinity for the cellular αvβ3 integrin receptor (41). The results show that the newly developed gpD co-expression system can permit the efficient display of an otherwise poorly tolerated protein on the surface of bacteriophage λ. This methodology is expected to prove useful in the future development of modified λ phage recombinants, including constructs that may have utility in human gene transfer applications.

MATERIALS AND METHODS

Lambda D1180 (Luc) lysogens

Lambda D1180 (Luc) lysogens were provided as a kind gift from Dr Mahito Nakanishi and DNAVEC Corp. (15); these phage genomes contain a mammalian expression cassette that encodes for firefly luciferase under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter.

pTrc-gpD expression plasmids

An *Escherichia coli* codon-optimized derivative of the wild-type λ gpD gene was generated synthetically (GeneArt, Regensburg, Germany) and inserted into the pTrcHis plasmid (Invitrogen). The optimized sequence was selected on the basis of Entelechon's proprietary gene design software (http://www.entelechon.com/), which performs codon optimization for the species of interest, while avoiding specified restriction enzyme sites. The corresponding sequence is available via GenBank, with the accession no. DQ156943. The resulting construct, pTrc-gpD-fusion contained the codon-optimized gpD sequence (with its authentic ATG codon, but without a translational stop codon), followed by a short flexible linker sequence [G(SGGG)$_2$SGGT] and then by BamHI and KpnI restriction sites; desired fusion partners of interest were inserted between the KpnI site and a terminal HindIII site. Note that, during the process of cloning, the native, promoter-proximal NcoI site within pTrc was eliminated.

In order to derive a construct that expressed gpD alone, the gpD insert was subjected to PCR amplification using primers that added a translational stop codon at the 3′ end of the gpD gene. The oligonucleotide primers used for this amplification were: (i) gpD-StopFOR (5′-aagcttc**ATG**accagcaag-3′) and (ii) gpD-StopREV (5′-atctaagctt**CTA**tacaatactgattgcggtg-3′); underlined sequences denote the added BspHI and HindIII sites, respectively, while capitalized boldface letters denote the ATG codon and inserted translational stop codon, respectively. The resulting PCR product was restricted with BspHI and HindIII and then inserted into the parental pTrc vector using the NcoI and HindIII restriction sites, to create pTrc-gpD.

We next created a series of plasmid constructs in which specific sequences of interest were fused to gpD. The exogenous DNA sequences that we chose to add included an insert encoding for a high-affinity αvβ3 binding protein derived from the tenth fibronectin type III domain (3JCLI4) (41). This was PCR amplified from an available parental plasmid (41), using the following primers: 3JCLI4FOR (5′-atcggtacc-caggtttctgatgttccgcgt-3′) and 3JCLI4REV (5′-ggccaagctt-**CTA**ggtacggtagttaatcg-3′). The PCR product was then digested with KpnI and HindIII (these sites are underlined in the primer sequences) and inserted into the corresponding

restriction sites of our pTrc-gpD-fusion vector, so as to create pTrc-gpD-3JCLI4.

In order to create plasmid vectors that permit expression of two different forms of gpD within the same *E.coli* host cell, the pBR322-derived pMB1 origin of DNA replication in the pTrc-based expression plasmids [~20 copies/chromosome equivalent; (42,43)] was replaced with a compatible origin of DNA replication derived from the CloDF13 replicon [~20–40 copies/chromosome equivalent; (44,45)]. The CDF-derived origin and flanking antibiotic resistance marker were amplified by PCR from the pCDF-1b plasmid (Novagen), using CDFDUETFOR (5′-agctccatgggaagcacacggtcacactgct-3′) and CDFDUETREV (5′-agctgcatgcaagttagctcactcattaggga-3′), digested with SphI and NcoI (these sites are underlined in the primer sequences), and then inserted into the SphI and BspHI sites in pTrc and its derivatives. All gpD-encoding plasmids containing the pBR322 or CDF origins of replication also carry the ampicillin or spectinomycin antibiotic resistance genes, respectively.

Preparation of gpD-mosaic phage

Lysogens of TOP10 cells (Invitrogen) containing λ D1180 (Luc) (15) were transformed with pTrc plasmids encoding either wild-type gpD alone or gpD-3JCLI4 alone or the combination of wild-type gpD plus gpD-3JCLI4. Lysogens containing the coat protein plasmids were grown to mid-log phase at 32°C in the presence of antibiotics (ampicillin or spectinomycin, 50 μg/ml, Sigma). The lysogens, which contain a temperature-sensitive mutation in the cI repressor, were then induced by increasing the culture temperature to 45°C for ~15 min. After induction, cultures were incubated at 38°C for an additional 3 h to allow for phage replication and assembly. Cells were then collected by centrifugation and lysed with chloroform (Sigma). DNAse I (Worthington Biochemical Corp.) was added to a final concentration of 10 μg/ml to remove any contaminating nucleic acids, and lysed cultures were cleared of debris by centrifugation; phage were pelleted from the supernatant by ultracentrifugation. The resulting pellet was resuspended in phage suspension media [100 mM NaCl, 10 mM MgSO$_4$, 50 mM Tris–HCl (pH 7.5) and 0.1% gelatin] and further purified by cesium chloride density gradient ultracentrifugation. The resulting phage bands were pulled from the gradient using a syringe and 18G needle and dialyzed against 10 mM NaCl, 50 mM Tris–HCl (pH 7.5) and 10 mM MgCl$_2$. Phage preparations were titered on LE392 (*supE*, *supF*) *E.coli* host cells (Stratagene).

Production of polyclonal antiserum to gpD

The authentic (non-codon-optimized) λ phage gpD gene was amplified by PCR from pAT101 (kindly provided by Dr Andreas Plückthun and Dr Patrick Forrer, University of Zurich, Switzerland) (3,46) using the gpD forward primer (5′-ggtgcccatatggcgagcaaagaaaccttacc-3′) and the gpD reverse primer (5′-ccgacgggatcctcattaaacgatgctgattgc-3′) and then cloned into the pET15b plasmid (Novagen) using the NdeI and BamHI restriction sites (underlined). The resulting pET15b-gpD plasmid was transformed into BL21 cells (Invitrogen). Transformed bacteria were grown to mid-log phase and then induced for 3 h with isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration

of 1 mM. Cells were pelleted by centrifugation, lysed with BugBuster (Novagen), and treated with benzonase and lysozyme (Novagen) according to the manufacturer's recommendations. The resulting lysate was clarified by centrifugation and temporarily stored at $-20°C$. Thawed lysate was purified using a Co^{2+} column (BD Talon) according to the manufacturer's recommendations. The eluted fractions were analyzed by PAGE, followed by immunoblot analysis using an anti-His$_5$ antibody reactive with the His$_6$-tag that had been added to the gpD protein. Approximately 3 mg of PAGE purified gpD protein was used to raise a polyclonal antiserum in rabbits (Sigma Genosys). The reactivity of the resulting antiserum against gpD was confirmed by immunoblot analysis, and the antiserum was then preserved in 0.02% sodium merthiolate and stored in aliquots at $-80°C$ until use.

Immunoblot analysis of purified phage particles

A total of 1×10^9 plaque forming units (PFU) of CsCl-banded phage particles were combined with 2× SDS loading buffer and heated to 95°C for 5 min. Samples were separated on a 20% SDS–PAGE gel. Proteins were then transferred to a nitrocellulose membrane and incubated with the polyclonal anti-gpD anti-serum at a dilution of 1:1000 (in 1× phosphate-buffered saline (PBS)/0.1% Tween (PBST) containing 5% non-fat dry milk). After washing with PBST, the nitrocellulose membrane was incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (Amersham) at a dilution of 1:3000 (also in PBST containing 5% nonfat dry milk). HRP-conjugated antibody was detected using ECL-Plus substrate (Amersham). Blots were imaged using the ChemiDoc XRS chemiluminescence chamber and Quantity One software version 4.5.2 (BioRad).

RESULTS

Construction of gpD expression plasmids

In order to generate plasmids capable of efficiently expressing gpD in *E.coli* host cells, we elected to synthesize an *E.coli* codon-optimized (ECO) derivative of the wild-type λ gpD gene. We reasoned that codon optimization might lead to more efficient protein expression, as has been shown in other systems (47). In addition, the *E.coli* codon-optimized gene also has greatly reduced homology to its native λ counterpart (only 78% nucleotide identity). We anticipated that this would effectively eliminate the potential for homologous DNA recombination between the ECO-gpD sequence and its native λ phage counterpart—thereby reducing the possibility that a complementing gpD plasmid might recombine with a gpD-deficient λ lysogen.

The ECO-gpD gene was inserted into pTrc to create pTrc-gpD (Figure 1A), and a sequence cassette corresponding to the
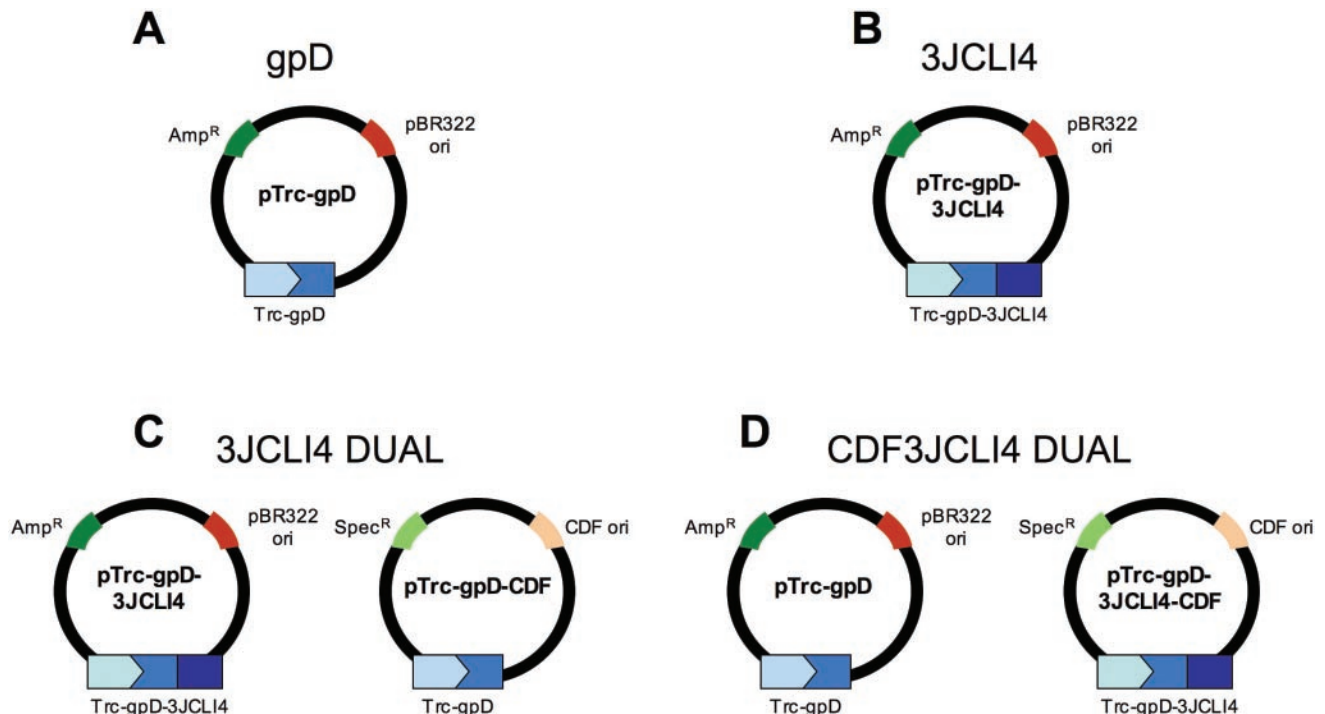
Figure 1. gpD Expression constructs. Plasmid vectors for prokaryotic expression of wild-type and recombinant forms of gpD are shown. (**A**) An ECO derivative of gpD (blue) was inserted into an ampicillin-selectable, pBR322-based pTrc expression plasmid, where it was placed under the transcriptional control of the high-level, regulatable Trc promoter (light blue). (**B**) A sequence coding for a high-affinity αvβ3 binding protein derived from the tenth fibronectin type III domain (3JCLI4; dark blue) was inserted at the 3′ end of the gpD insert in the plasmid shown in (A), resulting in the generation of a construct that encoded a gpD-3JCLI4 fusion protein. (**C**) A matched dual expression plasmid set, capable of co-expressing wild-type gpD and the gpD-3JCLI4 fusion protein is shown. The ampicillin selection marker (dark green) and pBR322-derived (dark red) origin of replication in the plasmid shown in (A) were replaced with a spectinomycin resistance gene (light green) and a pCDF1-derived origin of replication (light red), to generate the gpD-encoding plasmid shown on the left. (**D**) A second matched dual expression plasmid set, capable of co-expressing wild-type gpD and the gpD-3JCLI4 fusion protein is shown. In this case, the ampicillin selection marker and pBR322-derived origin of replication in the plasmid shown in (B) were replaced with a spectinomycin resistance gene and a pCDF1-derived origin of replication, to generate the gpD-3JCLI4-encoding plasmid shown on the right.
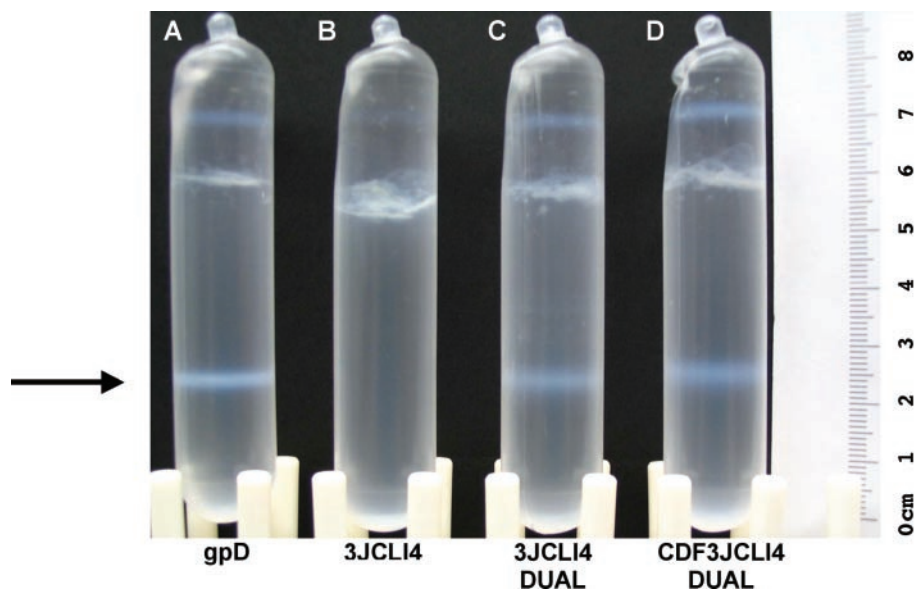
Figure 2. Co-expression of 3JCLI4-gpD and wild-type gpD yields intact phage particles. Lysogens of TOP10 cells containing gpD-deficient λ D1180 (Luc) were transformed with single plasmid vectors encoding either wild-type gpD (gpD; see Figure 1A) or the recombinant gpD-3JCLI4 fusion protein (3JCLI4; see Figure 1B). Alternatively, the cells were co-transformed with two plasmids, corresponding to the constructs shown in Figure 1C (3JCLI4 DUAL) or Figure 1D (CDF3JCLI4 DUAL); these paired constructs permitted the co-expression of wild-type and recombinant gpD in the same *E.coli* host cell. Following lysogen induction and cell lysis, phage particles were pelleted and subjected to cesium chloride density gradient purification. The results are shown; the large arrow denotes the characteristic λ phage band (the upper bands correspond largely to non-infectious protein debris; data not shown). It can be readily appreciated that stable phage particles were recovered from all of the preparations, except for the phage that were exclusively complemented by the recombinant gpD-3JCLI4 protein. This suggests that the gpD-3JCLI4 protein failed to support the assembly of stable phage particles and that this deficiency could be overcome by co-expression of the wild-type gpD protein (as in the 3JCLI4 DUAL and CDF3JCLI4 DUAL preparations).

desired fusion partner (3JCLI4) was then inserted as a C-terminal fusion to gpD, to create pTrc-gpD-3JCLI4 (Figure 1B). Additionally, constructs were generated in which the pBR322 origin of replication in the pTrc-based vectors was replaced with the CDF origin of replication, to yield pTrc-gpD-CDF (Figure 1C) and pTrc-gpD-3JCLI4-CDF (Figure 1D). Protein expression from each of these constructs was confirmed by immunoblot analysis of IPTG-induced bacterial cell lysates using a gpD-specific rabbit antiserum (data not shown); the constructs were then transformed into λ D1180 lysogens, in order to examine their ability to complement this gpD-deficient phage strain and thereby permit recovery of infectious phage particles.

Co-expression of wild-type and recombinant gpD permits surface display of otherwise recalcitrant fusion proteins

Lysogens of TOP10 cells containing λ D1180 (Luc) were transformed with either the pTrc-gpD-3JCLI4 or pTrc-gpD expression plasmid, encoding the gpD-3JCLI4 fusion protein or wild-type gpD, respectively. Following heat induction, cell lysis and cesium chloride density gradient ultracentrifugation of a 1 1 preparation, no phage particles could be recovered from the lysogens that had been transformed with the plasmid vector encoding the recombinant gpD-3JCLI4 coat protein fusion—as reflected by the absence of the characteristic λ phage band in the cesium chloride density gradient (Figure 2B). In contrast, when the λ D1180 (Luc) lysogens were transformed with a plasmid encoding wild-type gpD, infectious phage particles were readily recovered (Figure 2A).

These results suggested that the gpD-3JCLI4 fusion protein may either interfere with phage assembly or prevent the formation of stable phage particles. In considering how we might resolve this unexpected difficulty, we decided to try to express the gpD-3JCLI4 fusion protein on the surface of phage λ through the use of a mosaic approach, in which the final virion would contain a mixture of both wild-type and recombinant gpD subunits.

Lysogens of TOP10 cells containing λ D1180 (Luc) were co-transformed with plasmids encoding both recombinant and wild-type forms of gpD. This was achieved using a set of gpD expression vectors that contained one of two origins of replication—either the pBR322-derived pMB1 origin (pBR-ori) or a second compatible, low-copy origin derived from pCDF-1b (CDF-ori). Lysogens were then cotransformed with either (i) a pBR-ori based plasmid encoding a recombinant form of gpD (pTrc-gpD-3JCLI4) plus a CDF-ori based plasmid encoding wild-type gpD (pTrc-gpD-CDF), or (ii) a CDF-ori based plasmid encoding a recombinant form of gpD (pTrc-gpD-3JCLI4-CDF) plus a pBR-ori based plasmid encoding wild-type gpD (pTrc-gpD). Following heat induction of the lysogen, cell lysis, and cesium chloride density gradient ultracentrifugation, we were able to efficiently recover phage containing the gpD-3JCLI4 fusion protein that had previously proven recalcitrant—as reflected by the presence of a characteristic phage band in the CsCl gradient (Figure 2C and D).

These recombinant phage preparations were collected from the CsCl gradient, dialyzed, and titered on LE392 *E.coli* host cells. All of the phage preparations, including the mosaic phages were found to be infectious (Figure 3). The titers of the mosaic phages were generally similar to those of
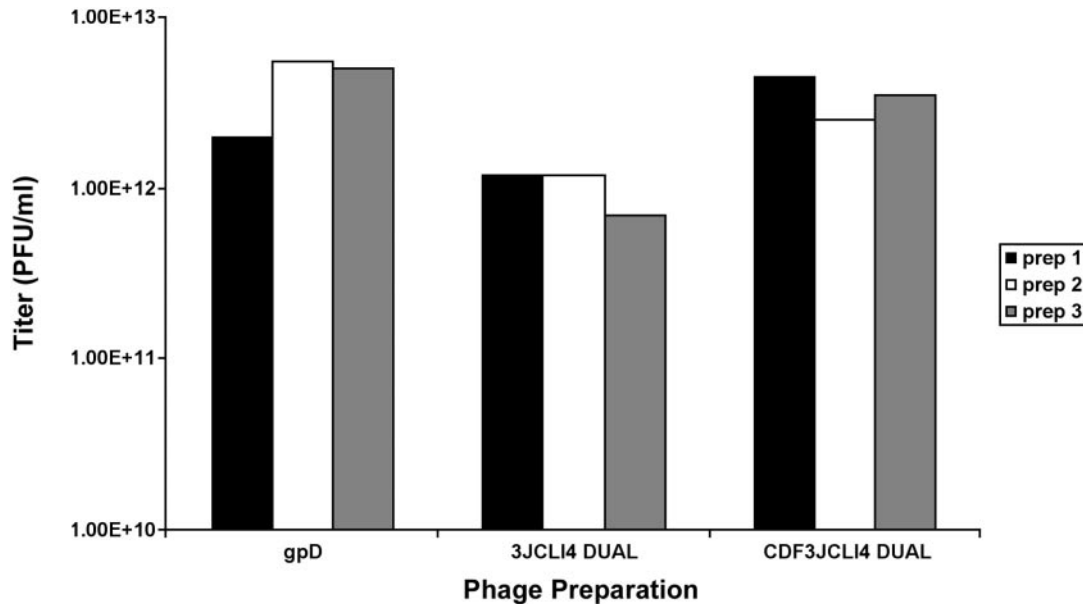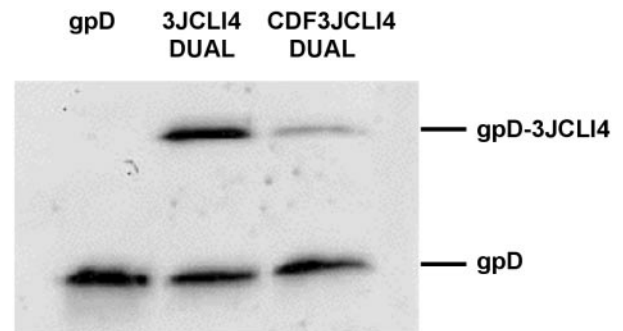
Figure 3. Co-expression of 3JCLI4-gpD and wild-type gpD yields infectious phage particles. Lysogens of TOP10 cells containing gpD-deficient λ D1180 (Luc) were transformed with plasmid vectors encoding either wild-type gpD alone (gpD; see Figure 1A) or with two plasmids, corresponding to the constructs shown in Figure 1C (3JCLI4 DUAL) or Figure 1D (CDF3JCLI4 DUAL); these paired constructs permitted the co-expression of wild-type and recombinant gpD in the same *E.coli* host cell. Following lysogen induction, CsCl-banding and dialysis, purified phage particles were titered on LE392 *E.coli* host cells. Results shown represent phage titers from three separate phage preparations; no appreciable amounts of infectious phage were recovered from lysogens that expressed only the gpD-3JCLI4 fusion protein (Figure 1B).

wild-type phage preparations (i.e. gpD-deficient phage that were complemented using wild-type gpD alone), although it was noted that the use of the pBR-based gpD fusion constructs resulted in somewhat lower titers of the mosaic phage, as compared to their CDF-based counterparts (compare titers for 3JCLI4 DUAL with those for CDF3JCLI4 DUAL; Figure 3). The reasons for this are uncertain, but may relate to differences in plasmid copy number and/or efficiency of protein expression from the different plasmid backbones.

An aliquot (1×10^9 PFU) of each of these phage preparations was extracted, separated on a 20% SDS–PAGE gel, and subjected to immunoblot analysis using the rabbit polyclonal antiserum against gpD. As expected, the phage preparations that were derived from lysogens that had been cotransformed with wild-type and recombinant gpD expression plasmids contained two distinct forms of gpD—consistent with their being gpD-mosaic phages (compare results for gpD wild-type to those for the other constructs shown in Figure 4). It was also noted that use of the pBR-based gpD fusion constructs resulted in slightly greater levels of recombinant gpD into the phage particles, as compared to the CDF-based plasmids (compare results for 3JCLI4 DUAL with those for CDF3JCLI4 DUAL; Figure 4).

DISCUSSION

Phage display technology allows for the presentation of foreign peptides and proteins on the surface of bacteriophage (6,21,51). This technology was developed and explored for the most part with filamentous phage and it has been shown that surface expression of receptor-binding peptides or proteins can result in phage binding to mammalian cells and,

Figure 4. Co-expression of 3JCLI4-gpD and wild-type gpD results in the generation of mosaic phage particles. Lysogens of TOP10 cells containing gpD-deficient λ D1180 (Luc) were transformed with plasmid vectors encoding either wild-type gpD alone (gpD) or with two plasmids that permitted the co-expression of wild-type and recombinant gpD in the same *E.coli* host cell (3JCLI4 DUAL and CDF3JCLI4 DUAL; see Figures 1–3). Following lysogen induction, CsCl-banding and dialysis, purified phage particles were titered on LE392 *E.coli* host cells. A total of 1×10^9 PFU of each preparation was then loaded on a 20% SDS–PAGE gel and phage protein content was examined by immunoblot analysis, using a rabbit polyclonal antiserum directed against gpD. The results show incorporation of wild-type gpD ($\sim$12 kDa) into all of the phage preparations (as expected), as well as inclusion of recombinant gpD-3JCLI4 ($\sim$23 kDa) in those preparations that were generated from lysogens that had been co-transformed with both wild-type and recombinant gpD expression vectors. It is also apparent that lower levels of recombinant gpD-3JCLI4 were incorporated when the recombinant protein was expressed from a CDF-origin containing construct, as compared to a pBR322-based plasmid.

in some cases, in phage internalization (14–16,23–25,35, 36,52,53). When such phage contain mammalian expression cassettes, this can result in expression of encoded genes within the target cells of interest (14,15,23–25,35,37). These

advances have led to the proposed use of bacteriophage as gene delivery vehicles.

The development of recombinant λ phage vectors capable of efficiently transducing mammalian cells is also likely to require the expression of specific cell-targeting ligands on the phage surface, as well as additional modifications to the phage coat that may facilitate cell uptake or phage uncoating. However, a number of technical limitations must be circumvented before this, will be possible. Among these is the fact that some recombinant gpD proteins cannot support phage assembly/stability, as shown here for gpD-3JCLI4. In an effort to address this problem, we have developed a plasmid-based dual expression system that permits the generation of mosaic phage particles that incorporate a mixture of both wild-type and recombinant gpD. This approach is conceptually analogous to the mosaic gene VIII vectors that have been developed for the surface display of large proteins on the major capsid protein of filamentous phage (7,48–50). Creation of these gpD-mosaic lambda phage particles is possible through the use of *E.coli* host cells that contain (i) an inducible, gpD-deficient λ lysogen and (ii) two independently selectable plasmid vectors that express wild-type gpD and the recombinant gpD protein of interest, respectively.

The development of this flexible gpD co-expression system is expected to substantially facilitate future development of lambda phage display vectors, as well as gene transfer vectors based on this bacteriophage.

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Conflict of interest statement. None declared.

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