Gene cloning, purification, and stoichiometry quantification of phi29 anti-receptor gp12 with potential use as special ligand for gene delivery

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Abstract

Bacterial virus phi29 is the most efficient in vitro DNA packaging system, with which up to 90% of the added DNA can be packaged into purified recombinant procapsid in vitro. The findings that phi29 virions can be assembled with the exclusive use of cloned gene products have bred a thought that phi29 has a potential to be a gene delivery vector since it is a nonpathogenic virus. gp12 of bacterial virus phi29 has been reported to be the anti-receptor that is responsible for binding the virus particle to the host cell. We cloned the gene coding gp12, overexpressed it in *Escherichia coli*, and purified the gene product to study the properties and functions of gp12 in virus assembly. According to SDS PloyAcrylamide Gel Electrophoresis (SDS-PAGE) analysis and N-terminal sequencing, recombinant gp12 isolated from *E. coli* had a molecular mass of 80 kDa, and 24 amino acids at N-terminal were cleaved after expression. The purified recombinant gp12 was incorporated into phi29 particles and converted the gp12-lacking assembly intermediates of phi29 into infectious virions in vitro. This purified protein gp12 was able to compete with infectious phi29 virions for binding to the host cell, thus inhibiting the infection by phi29. Scanning Transmission Electron Microscopy (STEM) analysis and sedimentation studies revealed that recombinant gp12 products were assembled into biologically active dimers. Analysis of the dose–response curve showed that 12 dimeric gp12 complexes were assembled onto viral particles and that each virion contained 24 copies of gp12 molecules. The results provide a basis for future research into bacteriophage–host interaction by modifying the anti-receptor protein. The ultimate goal is to re-target the bacteriophage to new host cells for the purpose of gene delivery.
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1. Introduction

The viral assembly process has been gaining attention because of its fundamental importance to research on the development of synthetic viral particle vaccines, the construction of in vivo gene delivery systems, the design for intra-cellular immunization and antiviral drugs, the assembly of chimeric virus displaying antigenic determinant or ligands, and the study of macromolecular interactions. The assembly process for all linear double-stranded DNA viruses has been shown to be quite uniform, since in all cases the viral genomic DNA is inserted into a preformed procapsid. Bacterial virus phi29 of *Bacillus subtilis* is a well-studied double-stranded DNA virus, in part due to its simplicity in composition. The terminal protein gp3, which functions both as a primer in the initiation of DNA packaging and as an enhancer during packaging (Bjornsti et al., 1984; Blanco et al., 1994), is covalently linked to each 5′ end of the genomic DNA of phi29. With the aid of the DNA-packaging enzyme gp16, packaging RNA (pRNA), and ATP, the genomic DNA can be packaged into the phi29 procapsid, which consists of the major capsid protein gp8, head fiber protein gp8.5, scaffold protein gp7 (Camacho and Salas, 1977; Hagen et al., 1976), and connector protein gp10 (Guasch et al., 2002; Simpson et

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al., 2000). The packaged phi29 genomic DNA/procapsid complex can then be converted into infectious virions with the addition of tail protein gp9, upper collar protein gp11, anti-receptor gp12, and morphogenic factor gp13 (Lee and Guo, 1994, 1995).

A high efficiency (up to 90%) DNA packaging and viral assembly system for phi29 had been established, generating infectious virions in amounts up to 10^9 plaque forming units (pfu)/ml without a single background plaque (Guo et al., 1986; Lee and Guo, 1994, 1995). Since up to 19,000 base pairs of both specific (Guo et al., 1986, 1991) and nonspecific DNA (Grimes and Anderson, 1989) can be packaged into the procapsid efficiently, phi29 can be an ideal gene delivery system (Hoeprich et al., 2003) if the DNA-filled particle can target to specific receptor of the cell. The specific targeting and the change of viral tropism rely on the structure of the anti-receptor. gp12 of phi29 has been reported to be the anti-receptor that is displayed on the outer surface of the viral particles (Carrascosa et al., 1974). To construct a mutant gp12 either fused with a single chain antibody or combined with other anti-receptor, it is critical to know the stoichiometry and structure of gp12. The molecular basis of the structure and function of gp12 is not yet fully understood. Here we report the cloning, overproduction, and purification of gp12 and a detailed study of its features and stoichiometry using our in vitro assembly system (Guo et al., 1986; Lee and Guo, 1994, 1995; Guo, 2002a,b).

2. Materials and methods

2.1. In vitro assembly of phi29

The synthesis and purification of pRNA (Hoeprich and Guo, 2002; Zhang et al., 1994, 2001), procapsid (Guo et al., 1986; Guo et al., 1991; Lee and Guo, 1994, 1995), DNA-gp3 (Guo et al., 1987a; Lee and Guo, 1994), gp16 (Guo et al., 1986, 1987b; Huang and Guo, 2003a,b), and the preparation of the gp9, gp11, and gp13 (Lee and Guo, 1995) have been described previously. Briefly, 5 μl of purified procapsids (0.4 mg/ml) was dialyzed on a 0.025-μm membrane type VS filter (Millipore) against TBE (89 mM Tris–borate, pH 8.3, 20 mM EDTA) for 15 min at ambient temperature. One microliter of pRNA (100 ng/μl) was added, and the mixture was transferred for dialysis against TMS (50 mM Tris–Cl, pH 7.8, 10 mM $MgCl_2$, 100 mM NaCl) for 30 min at ambient temperature. The presence of Mg^{2+} in the TMS buffer promotes the binding of pRNA to the procapsid. These pRNA-enriched procapsids were mixed with 3 μl of reaction buffer (10 mM ATP, 6 mM spermidine, 3 mM β-mercaptoethanol, 50 mM Tris–Cl, pH 7.8, 10 mM $MgCl_2$, 100 mM NaCl) DNA-gp3 that had been dialyzed against TMS for 40 min at ambient temperature, 6 μl of DNA-packaging protein gp16 that had been dialyzed against 0.01 M Tris–Cl, pH 7.5, and 0.04 M KCl for 40 min on ice.

For the assay of incorporation of gp12 into phi29 particles, the DNA-filled capsids were incubated with purified tail protein gp9, along with purified gp12, gp11, and gp13 for 2 h at ambient temperature as reported previously (Lee and Guo, 1995). The assembly mixtures were plated on *B. subtilis* $SU^{+44}(sup^+)$ (Camacho and Salas, 1977; Mellado et al., 1976).

2.2. Construction of plasmid for the expression of the gp12 gene

An *Eco*RI fragment (nt 9862 to 15,806 of phi29 genome) containing the gene coding for gp11 (upper collar), gp12, and gp13 (enzyme needed for neck and tail assembly) of phage phi29 was isolated from the phi29 genome and inserted into the *Eco*RI site of pBluescript SK(+) (Stratagene), generating a plasmid pBlue11-12-13. A 6.0 kb *Xba*I/*Eco*RV fragment was isolated from this plasmid and inserted into the *Xba*I/*Eco*RV sites of plasmid pET3c/EV, resulting in plasmid pAR11-12-13. The *Xba*I/*Dra*I fragment of plasmid pAR11-12-13 was isolated and inserted into the *Xba*I/*Eco*RV sites of plasmid pET3c/EV, generating a plasmid pAR11-12. In order to remove the gp11 gene and to construct the plasmid pAR12d11, the *Xba*I/*Hin*dIII fragment was deleted from pAR11-12 by re-ligation after filling the *Xba*I/*Hin*dIII-digested fragment with T4 DNA polymerase. The resulting plasmids were transformed into the expression host *Escherichia coli* HMS174(DE3).

2.3. Expression and purification of gp12 from E. coli

An overnight culture (10 ml) of *E. coli* HMS174(DE3) containing plasmid pAR12d11 was inoculated into 1 liter of LB broth with 50 μg/ml ampicillin and incubated at 37 °C for 3 h. After adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, the *E. coli* culture was incubated for an additional 3 h at 37 °C. Cells were collected by centrifugation. The cell pellet was resuspended in 20 ml of TMS and lysed by passage through a French press cell. The cell debris was removed by centrifugation at 10,000 rpm in a JA20 rotor (Beckman) for 2 h, and the supernatant was precipitated with ammonium sulfate. The precipitate between 30% and 50% saturation was collected by pelleting via centrifugation at 10,000 rpm for 20 min at 4 °C. The pellet was resuspended in 20 ml TMS. After being dialyzed against TMS overnight at 4 °C, the protein solution was loaded into a DEAE-cellulose column (25×3 cm) equilibrated with TMS. The flow-through solution was collected. gp12 was further purified by phosphocellulose and eluted with a NaCl gradient of 0.1–1.0 M.

2.4. Determining the shape and size of gp12 by STEM

Scanning Transmission Electron Microscopy (STEM) can be used to examine unstained isolated biological molecules and to determine their masses (Wall et al., 1998; Wall and

Simon, 2001). A 2.3 mm titanium grid covered with holey film was dropped onto a fresh thin (2 nm) carbon film floated on water to remove the salt. The grid was washed, and tobacco mosaic virus (TMV) was applied for 1 min. After more washes, 3 µl of purified gp12 at a concentration of 50 µg/ml was applied for 1 min. The grid was thoroughly washed with ammonium acetate, blotted to a thin layer of liquid, and plunged into a liquid N_2 slush to rapidly freeze it. The sample was freeze-dried overnight and transferred to the microscope under vacuum.

2.5. Determining molecular mass by STEM

The STEM is operated in a dark field at 40 kV. The microscope operation and data acquisition are under computer control. A scan is 512×512 pixels. The pixel size is 1 nm for mass measurements. The number of electrons scattered into an annular detector from a pixel is directly proportional to the mass contained within its area. By summing the number of scattered electrons over the particle of interest and subtracting the contribution of the supporting film, the mass of the particle can be determined. TMV is a control, for both the microscope, and also, importantly, for the sample preparation. The STEM analysis has been reported before (Cerritelli et al., 1996).

2.6. Molecular mass of gp12 oligomer determined by sedimentation

The molecular mass of the purified gp12 was determined by velocity sedimentation. Purified gp12 (100 µl, along with molecular weight markers, bovine serum albumin (BSA), lactic dehydrogenase, Immunoglobulin G (IgG) and catalase) was centrifuged in a 15–35% (volume/volume in TMS) linear glycerol gradient in a Beckman sw55 rotor for 22 h at 35,000 rpm at 4 °C.

2.7. Stoichiometry of gp12 assembled into viral particles determined by log/log plot

Concentration dependence was determined with the in vitro phi29 assembly system mentioned above in the presence of all components in optimal or excess concentration except for gp12. gp12 was prepared with a serial, two-fold dilution. Each diluted gp12 was added into the assembly mixture as described (Lee and Guo, 1994, 1995). A log scale was used on both the x-axis and the y-axis to determine the slopes.

2.8. Inhibition of phi29 infection by purified gp12

B. subtilis SU[+44] (Camacho and Salas, 1977) (100 µl of log phase culture) was mixed with a varied amount of gp12 in a two-fold serial dilution and pre-incubated for 30 min at ambient temperature. A 100-µl solution containing 1000 pfu of infectious phi29 was added into the mixture and onto LB plates.

3. Results

3.1. Identification of a processed gp12 purified from E. coli

A plasmid pAR12d11 was constructed to carry the gp12 gene driven by a T7 promoter (Fig. 1), and transformed into a host cell HMS174 (DE3)(Rosenberg et al., 1987; Studier et al., 1990). gp12 was overproduced after IPTG induction and the purified protein was analyzed by SDS PloyAcrylamide Gel Electrophoresis (SDS-PAGE) as shown in Fig. 2A.

The predicted molecular weight of intact gp12 is 94 kDa. Previous analysis found that gp12 was cleaved into a smaller protein after expression in vivo (Carrascosa et al., 1974; Tosi et al., 1975). The molecular weight of recombinant gp12 purified from E. coli is approximately 80 kDa, which is very close to the size of native gp12 from infectious phi29 on the SDS-gel (Fig. 2). gp12 purified from both the E. coli cell and the virus was subjected to N-terminal protein sequencing. It was revealed that gp12 purified from the virus has the sequence N-SerThrLysPro-GluLeuLys, which matches the amino acids 2–8 of gp12 ORF, Met[1]Ser[2]ThrLysProGluLeuLysArgPheGluGlnPhe-GlyGluMetMetValGlnLeuTyrGluArgTyrLeu[25]Pro[26]Thr[27]Ala[28]Phe[29]Asp[30]Glu[31]Ser[32], indicating that the N terminus of gp12 from the infected virus was not cleaved. This result agreed with the finding by Anderson et al. that the cleavage is at the C terminus (Peterson et al., 2001). However, N-terminal sequence of the recombinant gp12 purified from E. coli was N-LeuProThrAlaPheAspGluSer, which perfectly matches amino acids 25–32 of the gp12 ORF. This indicates that 24 amino acids at the N terminus of gp12 were cleaved in the E. coli cell after expression. The recombinant gp12 protein is similar in size to the native gp12, suggesting that its C terminus may be processed in the similar way when expressed in E. coli. Since recombinant gp12 is active in in vitro assembly (see Section 3.3), this suggested that neither terminus was essential.

3.2. Molecular mass, shape, and size of gp12 complex observed by STEM

Scanning Transmission Electron Microscopy (STEM) was used to determine the mass, shape, and size of the
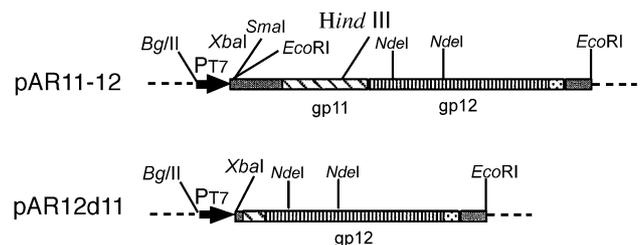

Fig. 1. Construction of plasmid for the expression of gp12 gene. The expression plasmid was derived from pAR11-12 which encodes both gp11 and gp12. The XbaI/HindIII fragment was deleted from pAR11-12 by re-ligation after filling the XbaI/HindIII-digested fragment.
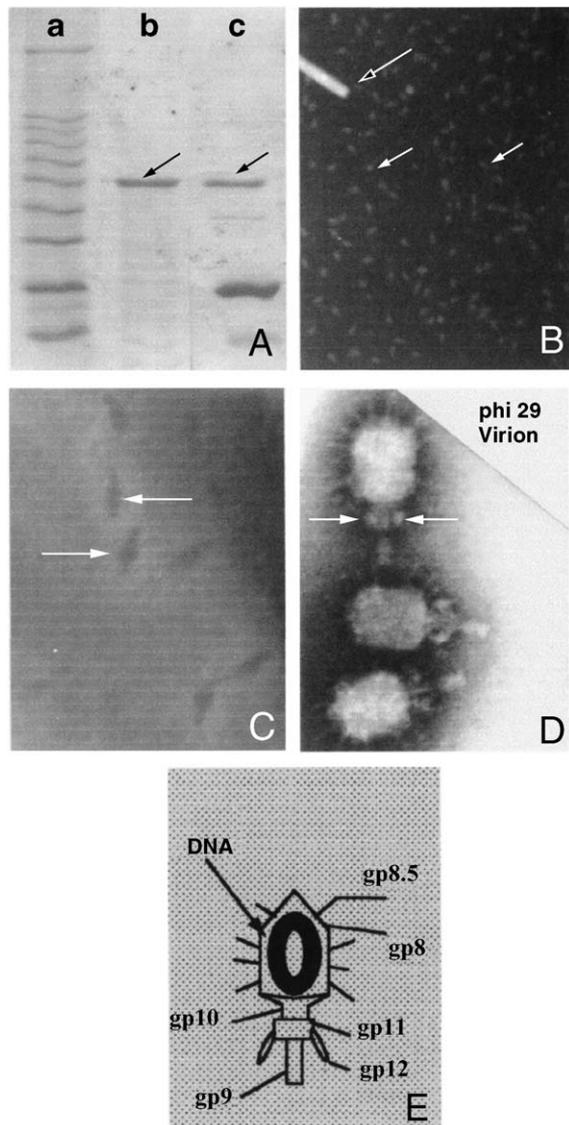
Table 1
Production of infectious virus with gp12

Experiments	Components			Virus produced (pfu/ml)
	gp12 extract	Pure gp12	ATP (1.4 mM)	
1	+	−	+	10^8
2	−	+	+	10^8
3	−	+	−	0
4	−	−	+	0

The listed components were added to the mixture containing purified recombinant procapsids, pRNA, gp16, DNA-gp3, gp9, gp11, and gp13 in a 20-µl reaction with 10 mM MgCl₂, 100 mM NaCl, and 50 mM Tris, pH 8.0.

3.3. Incorporation of gp12 into phi29 particles

To examine whether recombinant gp12 can be assembled onto the DNA/procapsid complex and be incorporated into the phi29 virion, purified gp12 was incubated with the DNA-filled capsids in the presence of pRNA, the DNA-packaging protein gp16, neck protein gp11, tail protein gp9, and the morphogenetic factor gp13 (see Materials and methods). It was found that infectious virus was produced to a concentration of 10^8 pfu/ml, but only when in the presence of purified gp12. When gp12 was absent, not a single plaque was detected (Table 1). This result indicates that recombinant gp12 from E. coli is functional in phi29 assembly in vitro.

3.4. Inhibition of phi29 infection by purified gp12

Further experiments tested whether the recombinant gp12 assembled and folded into the appropriate conformation as the authentic phi29 anti-receptor is by competitive inhibition in plaque formation. A fixed amount of host cell B. subtilis Su^{+44} was mixed with an increasing amount of purified gp12. After incubation, the above host cells were
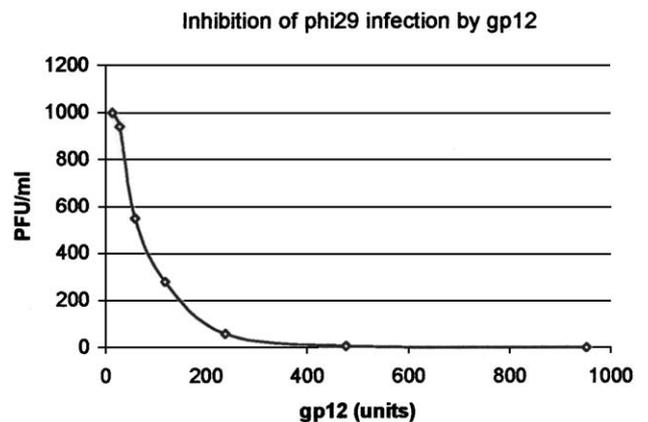
Fig. 2. Images of the purified gp12. (Panel A) SDS-PAGE showing the purity of gp12 (lane b). Lane a is the molecular weight marker and lane c is the purified phi29 virion, showing two major bands representing gp12 (arrow) and the capsid proteins gp8. (Panel B) Images of purified gp12 oligomers (short arrow) prepared by Scanning Transmission Electronic Microscopy (STEM). The white bar (long arrow) is a tobacco mosaic virus as a size marker. (Panel C) gp12 images with higher magnification. (Panel D) Purified phi29 virion with assembled recombinant gp12 oligomers (arrow). (Panel E) Illustration of the location of gp12 and DNA in a phi29 viral particle.

Fig. 3. Inhibition of phi29 infection by gp12. A fixed amount (100 µl) of host cell Su^{+44} was mixed with an increasing units of purified gp12. After incubation, the above mixture was incubated with a fixed amount of infectious phi29 virions for 30 min and then plated. One unit is defined as the amount of recombinant gp12 needed to produce 10^4 pfu/µl of infectious virus in a 20-µl reaction with the standard in vitro assembly procedure.

recombinant gp12 protein. The purified samples were found to contain homogenous particles with a rhombus shape (Fig. 2B–C). The size of the particles was approximately 15×8 nm. The size and shape of recombinant gp12 were identical to the native gp12 on the phi29 infectious virion (Fig. 2D). Mass measurements on the freeze-dried recombinant gp12 using STEM (Cerritelli et al., 1996) yielded masses around 200±20 kDa, slightly more than twice that of the gp12 monomer (about 80 kDa). This suggests that the purified gp12 exists as dimer.
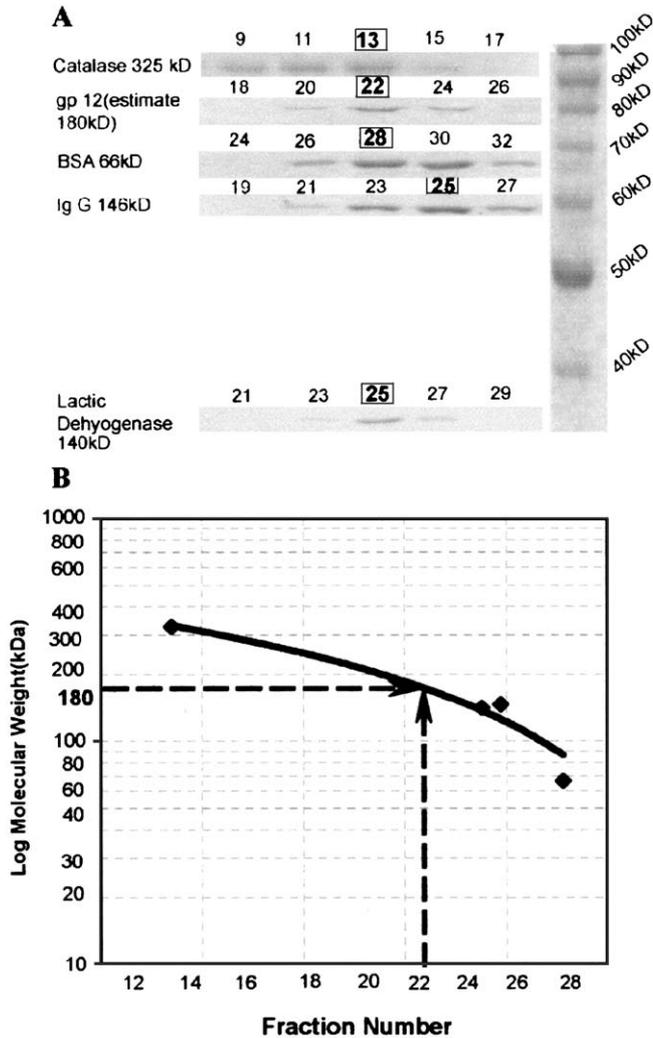
Fig. 4. Determination of the stoichiometry of purified gp12 complex by sedimentation. Sedimentation is from right to left, with catalase (325 kDa, centered at fraction 13), BSA (66 kDa, center at fraction 28), IgG (146 kDa, centered at fraction 25), and lactic dehydrogenase (140 kDa, centered at fraction 25) as the molecular weight standard (A). Plotting of molecular weight against the sedimentation rate reveals that the complex of gp12 is close to 180 kDa (B).

mixed with a fixed amount of infectious phi29 virions and plated (see Materials and methods). It was found that as the amount of gp12 increased, the plaque forming units decreased (Fig. 3). This indicates that the purified gp12 could compete with infectious phi29 for binding to the receptors of the host cell and thus inhibit phi29 infection.

3.5. Stoichiometric quantification of the purified gp12 complex by sedimentation

The mass of the purified gp12 in oligomeric form was determined by velocity sedimentation. Recombinant gp12 was analyzed by ultracentrifugation in a 15–35% glycerol gradient along with molecular weight standards. Fig. 4 shows that gp12 migrated faster than bovine serum

albumin (BSA), which has a molecular weight of 66 kDa, and its final position was close to that of dehydrogenase (141 kDa), a tetramer composed of four 35 kDa monomers. A plot of the log molecular weight against the migration rate of the molecular weight standards revealed that the molar mass for gp12 is about 180 kDa, which suggests that gp12 exists in solution mostly as a dimer (predicted to be 160 kDa). This result supports the finding by STEM that the gp12 exist as dimers rather than monomers in solution.

3.6. Stoichiometric quantification of the assembly of gp12 into infectious phi29 virion

A method was developed to determine the stoichiometry of viral components by comparing the slopes of dilution factor curves of assembly components with the yield of virions assembled in vitro. Components with known stoichiometry served as standard controls. A more dramatic influence of the dilution factor on the reaction indicates a
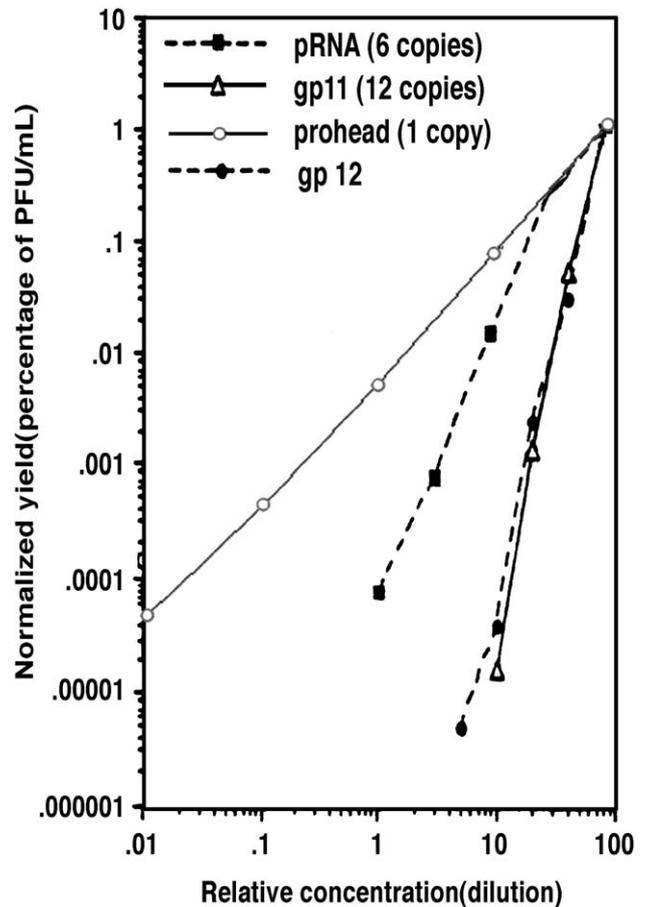

Fig. 5. Log/log plot of concentration dependence on gp12 for phi29 assembly. Concentration dependence of gp12 was determined with the in vitro phi29 assembly system in the presence of all components in optimal or excess concentration except for gp12. gp12 was prepared with a serial, two-fold, dilution. Prohead, gp11, pRNA with known stoichiometry were tested with same method and used as control.
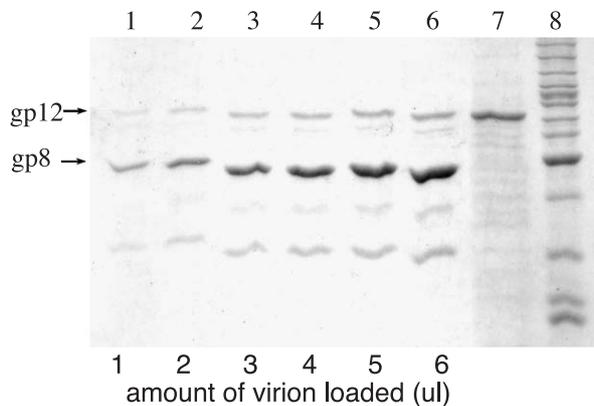
Fig. 6. SDS-PAGE to estimate the copy number of gp12 on phi29 virion. Lane 1–6 contain different amount of purified wildtype phi29 virions. Lane 7 contains recombinant gp12 purified from *E. coli*. Lane 8 is molecular weight ladder.

larger stoichiometry. A slope of one (as is the case with phi29 DNA) indicates that one copy of the component is involved in the assembly of one virion. A slope larger than one indicates multiple-copy involvement. By this method, the stoichiometry of DNA-gp3, gp9, and pRNA have been investigated (Chen et al., 1997; Lee and Guo, 1994, 1995; Trottier and Guo, 1997).

The concentration dependence of the phage assembly on gp12 was determined by this method. A log/log plot (Fig. 5) of gp12 concentration versus phage assembly showed that the slope of the response curve was high, close to the slope for gp11, which has been shown to have a stoichiometry of 12 (Peterson et al., 2001; Trottier and Guo, 1997). Since gp12 forms dimers in solution as described above, 24 copies of gp12 are incorporated into the phi29 virion.

The stoichiometry gp12 on the infectious wild type phi29 virion was also estimated by Coomassie brilliant blue stained SDS-PAGE gel (Fig. 6). The dye density of gp12 from 6 μl of virion (Lane 6) is similar to the dye density of gp8 from 1 μl of virion (Lane 1). As previous reported, the copy number of gp8 is 219 and the molecular weight of gp8 and gp12 is 50 (Peterson et al., 2001) and 80 kDa, respectively. The copy number of gp12 equals to [(219

copies $\times$ 50 kDa)/80 kDa]/6 (the ratio of six to one μl) = 23. The result agrees with that from STEM and sedimentation studies.

4. Discussion

We cloned the gp12 gene and over-expressed it in *E. coli*, and then the recombinant protein was purified by ammonium sulfate precipitation and ion-exchange columns. Two pieces of evidence indicated that the purified gp12 protein is biologically active. In vitro assembly assay showed that recombinant gp12 could be incorporated into infectious phi29 virion, with a high titer of 10^8 pfu/ml. On the contrary, not a single plaque was produced when gp12 was absent. Furthermore, purified gp12 was able to inhibit virus infection in a dose-dependent manner, as shown in Fig. 3.

We employed two methods to investigate the oligomerization of gp12, and both suggested that gp12 exists as dimer in solution. STEM is a useful tool to directly observe the size and shape of biological molecules, and it can also measure their molecular masses by calculating the number of electrons scattered by the sample. The molecular mass measured by STEM was around 200 kDa, which is slightly more than twice that of a monomer. Velocity sedimentation carried out in a glycerol gradient yielded a molecular mass of about 160 kDa, also indicating that recombinant gp12 is a dimer. Since the size and shape of recombinant gp12 in solution were identical to the native gp12 on the phi29 infectious virion (as revealed by STEM), we suggest that native gp12 also forms a dimer, the active form in the phi29 virion.

gp12 is displayed on the outer surface of the viral particles and has been reported to be essential for infection of phi29. In our competition assay (Fig. 3), a significant decrease in phi29 infection was observed when increasing amounts of purified gp12 were pre-incubated with host cell Su^{+44}. This saturation curve suggests that there are viral receptors on the surface of host cells, which bind to anti-receptor gp12 on the virus and could also be competitively
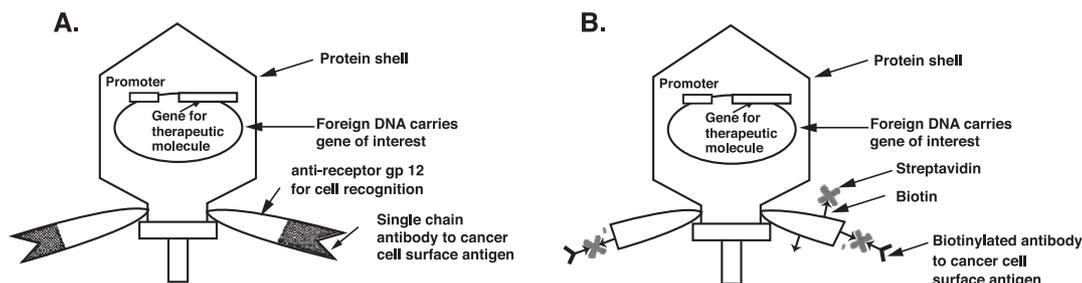

Fig. 7. Hypothetical model using phi29 as a gene delivery vector to destroy cancer cell. A DNA harboring the gene with therapeutic value will be packaged into procapsid of phi29 by the efficient in vitro phi29 DNA packaging system (Guo et al., 1986, 1991; Lee and Guo, 1995). gp12 protein is fused with single chain antibody (A), or linked to biotinylated antibody via streptavidin after biotinylation of the purified (B). This modification would target the recombinant phi29 particles carrying therapeutic gene to specific cancer cells expressing the surface cancer antigens.

occupied by free recombinant gp12. Thus, there are methods to change the tropism of phi29 infection though modifying gp12, such as through the construction of a gp12 fusion protein carrying another anti-receptor of the species of interest. Recombinant fusion gp12 with a single chain antibody could change phi29 tropism (Fig. 7).

phi29 is a virus that infects *B. subtilis*, a bacterium that is not a pathogen of humans. It is simple in structure and quite small in size (Anderson et al., 1966; Anderson and Reilly, 1993; Carrascosa et al., 1973; Meifer et al., 2001), 42 nm in comparison with 600 nm for pox virus and 70–90 nm for adenovirus. We are able to assemble infectious phi29 virions in vitro with all proteins produced from cloned genes and both DNA and RNA synthesized in vitro (Lee and Guo, 1994, 1995). The system components, with the exception of ATP, are entirely produced by cloned genes in an *E. coli* expression system. Using this system, we are able to produce up to 10^9 virions per milliliter with zero background. In this highly efficient in vitro DNA packaging system, up to 90% of the added DNA can be incorporated into the protein shell (Guo et al., 1986; Lee and Guo, 1995). Although the DNA-packaging signal of phi29 was identified to be located at the 5′ end of the genomic DNA and is inserted first during DNA packaging (Bjornsti et al., 1983; Grimes and Anderson, 1989), DNA without the phi29 DNA-packaging signal can be packaged into the procapsid directly (Grimes and Anderson, 1989), suggesting that there is the potential to use the in vitro assembled virus to deliver genes (Hoeprich et al., 2003). For gene therapy, the current stumbling block is the lack of delivery systems that can target specific cells. Here we characterize gp12 as antireceptor, thus providing the possibility of constructing a specific targeting gene delivery vehicle by modifying gp12. As shown in Fig. 7, a fusion gp12 protein is designed to carry antibody to a cancer cell. This fusion protein can be expressed and purified from *E. coli* and be incorporated onto phi29 by in vitro assembly system. Recently, we were able to incorporate the biotinylated gp12 into the DNA-filled particle by in vitro assembly (unpublished results). Biotinylated antibody could also be linked via streptavidin to the biotinylated gp12. After binding, phi29 particles containing the therapeutic gene packaged in vitro could enter the cancer cell by endocytosis. Subsequently, the therapeutic gene will be expressed, thereby destroying the cancer cell (Fig. 7).

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