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Baculovirus display of functional antibody Fab fragments

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Abstract The generation of a recombinant baculovirus that displays antibody Fab fragments on the surface was investigated. A recombinant baculovirus was engineered so that the heavy chain (Hc; Fd fragment) of a mouse Fab fragment was expressed as a fusion to the N-terminus of baculovirus gp64, while the light chain of the Fab fragment was simultaneously expressed as a secretory protein. Following infection of Sf9 insect cells with the recombinant baculovirus, the culture supernatant was analyzed by enzyme-linked immunosorbent assay using antigen-coated microplates and either an anti-mouse IgG or an anti-gp64 antibody. A relatively strong signal was obtained in each case, showing antigen-binding activity in the culture supernatant. In western blot analysis of the culture supernatant using the anti-gp64 antibody, specific protein bands were detected at an electrophoretic mobility that coincided with the molecular weight of the Hc-gp64 fusion protein as well as that of gp64. Flow cytometry using a fluorescein isothiocyanate-conjugated antibody specific to mouse IgG successfully detected the Fab fragments on the surface of the Sf9 cells. These results suggest that immunologically functional antibody Fab fragments can be displayed on the surface of baculovirus particles, and that a fluorescence-activated cell sorter with a fluorescence-labeled antigen can isolate baculoviruses displaying specific Fab fragments. This successful baculovirus display of antibody Fab fragments may offer a novel approach for the efficient selection of specific antibodies.

Keywords Baculovirus · Surface display · Insect cell · Sf9 · Antibody · Fab fragment

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Introduction

A display of heterologous peptides or proteins on the surface of virus particles serves as a powerful tool for the selection of genes encoding desired properties from diverse libraries based on protein interactions. A successful example of such display technology includes phage display for isolating antibodies that recognize specific antigens from large combinatorial libraries (Bradbury and Marks 2004; McCafferty et al. 1990). In this system, filamentous bacteriophages that infect *Escherichia coli* are engineered to display antibody fragments on their surface by fusing the DNA that encodes antibody fragments with the gene encoding one of the phage coat proteins (Smith 1985;

Smith and Petrenko 1997). Based on interactions between expressed antibody fragments and target antigens, bacteriophages displaying antibody fragments that bind specifically to the target can be isolated from a large library of different expression clones, and specific phage clones can then be amplified via the infection of host *E. coli* cells. Single-chain Fv (scFv) fragments, which join the VH and VL domains of an immunoglobulin with a flexible peptide linker, and Fab fragments, which consist of two chains, the VH + CH1 (Fd fragment) and the VL + CL (light chain), have often been expressed on the surface of filamentous bacteriophage. When displayed on the phage surface, Fab fragments tend to be more functional than the corresponding scFv fragments; some scFv fragments show a lower affinity than the corresponding Fab fragments (Bird and Walker 1991; Bradbury and Marks 2004). However, Fab fragments are often produced at significantly lower levels in *E. coli* than scFv fragments, because the former is twice the size of the latter and requires the assembly of two polypeptide chains with a disulphide bond. In addition, phage display has limitations to the successful presentation of eukaryotic proteins that require complex folding and extensive post-translational processing and modifications due to the use of the prokaryotic host.

Recently, baculoviruses such as the *Autographa californica* nucleopolyhedrovirus (AcNPV) have been successfully used for the display of foreign proteins on the surface of viral particles by fusing the protein to the major baculoviral envelope glycoprotein, gp64 (Boublik et al. 1995; Grabherr et al. 2001; Mäkelä and Oker-Blom 2006; Yamaji 2011). After the infection of insect cells with such a recombinant baculovirus, the gp64-fusion proteins are expressed and transported to the cell membrane, where they are picked up by progeny viruses during the budding process, thereby displaying the gp64-fusion protein on the surface of baculovirus particles. Baculovirus display allows the presentation of complex proteins following the eukaryotic posttranslational processing and modification of insect cells. Reportedly, scFv fragments have been successfully displayed in a functional form on the AcNPV surface by fusion to gp64 (Mottershead et al. 2000; Ojala et al. 2001). However, there would be little advantage to the use of baculoviruses displaying scFv fragments for the selection of specific antibodies, because scFv phage displays have been successfully used. In the present study, the generation of a recombinant baculovirus displaying an antibody Fab fragment on its surface was investigated. Recombinant baculoviruses were designed so that either the Fd fragment or the light chain of an Fab fragment was expressed as a gp64-fusion protein, while the other chain of the Fab fragment was simultaneously expressed as a secretion protein. The results obtained in the present study suggest that antibody Fab fragments can be displayed on the surface of baculovirus particles in an immunologically active form.

Materials and methods

Insect cells and media

The insect cell line used in the present study was Sf9 (BD Biosciences, San Jose, CA, USA) derived from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda*. Cell density was determined by microscopically counting the number of cells with a Bürker-Türk hemocytometer, while cell viability was judged by trypan blue dye exclusion (Yamaji et al. 1999). The cells were maintained at 27 °C in T-flasks in a non-humidified incubator. The culture medium used for routine maintenance was TNM-FH, consisting of 51.19 g L^{-1} TNM-FH (Sigma-Aldrich, St. Louis, MO, USA) and 0.35 g L^{-1} $NaHCO_3$, with the addition of 10 mg L^{-1} gentamicin sulfate and 1 g L^{-1} Pluronic F-68 [block copolymer glycol of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene), Sigma-Aldrich] supplemented with 10 % fetal bovine serum (FBS) (BioWest, Nuaillé, France) (Yamaji et al. 1999). TNM-FH is a modification of a synthetic basal medium, Grace's medium, by supplementation with 3.33 g L^{-1} lactalbumin hydrolysate and 3.33 g L^{-1} yeast extract. Unsupplemented Grace's medium (Life Technologies, Carlsbad, CA, USA) was used when Sf9 cells were transfected with recombinant bacmid DNA.

Plasmid construction

The catalytic antibody 6D9, which was generated by immunization of mice with a transition state analog, catalyzes the hydrolysis of a non-bioactive chloramphenicol monoester derivative to produce chloramphenicol (Miyashita et al. 1997; Takahashi et al. 2001). In the present study, for the expression of the

Fab fragment of this antibody on the baculovirus surface, recombinant baculoviruses were constructed that contain both the genes encoding the heavy chain (Hc; Fd fragment) and light chain (Lc) of the Fab fragment, one of which was fused to the 5′ end of a second copy of the AcNPV gp64 gene. Recombinant baculoviruses were generated using the Bac-to-Bac baculovirus expression system (Life Technologies), which was based on site-specific transposon-mediated insertion of the foreign gene of interest from a donor plasmid pFastBac into a baculovirus genome called a bacmid in *E. coli* (Luckow et al. 1993), as described below. Recombinant pFastBac vectors were constructed as follows.

The DNA encoding the Hc gene of the 6D9 Fab was PCR amplified from the plasmid pARA7-6D9Fab (Miyashita et al. 1997) using primers 1 and 2 (Table 1). The amplified fragment was cloned between the AcNPV gp64 signal sequence and the gp64 mature domain in the plasmid pBACsurf-1 (Merck, Tokyo, Japan) at the *Kpn*I-*Sma*I site. The resultant plasmid was designated pBACsurf-1-6D9Hc. The DNA encoding the Lc gene of the 6D9 Fab was amplified from pARA7-6D9Fab using primers 3 and 4, and was cloned at the *Kpn*I-*Sma*I site of pBACsurf-1 to give pBACsurf-1-6D9Lc.

The DNA encoding the *Drosophila* immunoglobulin heavy chain binding protein (BiP) signal sequence and the 6D9 Fab Hc gene was PCR amplified from the plasmid pXINSECT-6D9Hc (Yamaji et al. 2008) using primers 5 and 6 (Table 1). The plasmid pFastBac Dual (Life Technologies) contained two strong AcNPV promoters, the polyhedrin promoter and the p10 promoter, to allow the simultaneous expression of two proteins (Fig. 1). The amplified fragment was cloned downstream of the polyhedrin promoter in the pFastBac Dual at the *Eco*RI-*Hin*dIII site. The resultant plasmid was designated pFastBac -/Hc. The DNA encoding the BiP signal sequence and the 6D9 Fab Hc gene was amplified from pXINSECT-6D9Hc using primers 7 and 8, and was cloned downstream of the p10 promoter in the pFastBac Dual at the *Sma*I-*Kpn*I site to give pFastBac Hc/-.

The DNA encoding the BiP signal sequence and the 6D9 Lc gene was amplified from the plasmid pXINSECT-6D9Lc (Yamaji et al. 2008) using primers 5 and 9 (Table 1), and was cloned downstream of the polyhedrin promoter in the pFastBac Dual at the *Eco*RI-*Hin*dIII site. The resultant plasmid was designated pFastBac -/Lc. The DNA encoding the BiP signal sequence and the 6D9 Lc gene was amplified from pXINSECT-6D9Lc using primers 7 and 10, and was cloned downstream of the p10 promoter in the pFastBac Dual at the *Sma*I-*Kpn*I site to give pFastBac Lc/-.

The DNA encoding the gp64 signal sequence, the 6D9 Fab Hc gene, and the gp64 mature domain was amplified from pBACsurf-1-6D9Hc by using primers 11 and 12 (Table 1). The amplified fragment was cloned downstream of the polyhedrin promoter at the *Eco*RI-*Hin*dIII site of pFastBac Lc/-. The resultant

Table 1 List of oligonucleotides synthesized for plasmid vector construction

Primer	Sequence (5′–3′)	Restriction site
1	CGGGGTACCACTCGAGTCTGGGGGAGG	*Kpn*I
2	TCCCCCGGGAACTAGTACAATCCCTGGGCAC	*Sma*I
3	CGGGGTACCAGAGCTCGTGATGACCCAGAC	*Kpn*I
4	TCCCCCGGGATCTAGAATTAACACTCATTCC	*Sma*I
5	CCGGAATTCATGAAGTTATGCATATTACTGGCCGTCGTG	*Eco*RI
6	CCCAAGCTTACTAGTACAATCCCTGGGCACAATTTTCTT	*Hind*III
7	TCCCCCGGGATGAAGTTATGCATATTACTGGCC	*Sma*I
8	CGGGGTACCACTAGTACAATCCCTGGGCACAATTTTC	*Kpn*I
9	CCCAAGCTTTCTAGAATTAACACTCATTCCTGTTG	*Hind*III
10	CCGGGTACCTCTAGAATTAACACTCATTCCTGTTG	*Kpn*I
11	CCGGAATTCATGGTAAGCGCTATTGTTTTATATG	*Eco*RI
12	CCCAAGCTTTTAATATTGTCTATTACGGTTTC	*Hind*III
13	CGGGCTAGCATGGTAAGCGCTATTGTTTTATATGTGC	*Nhe*I
14	CGGGCTAGCTTAATATTGTCTATTACGGTTTCTAATC	*Nhe*I

Restriction sites are underlined

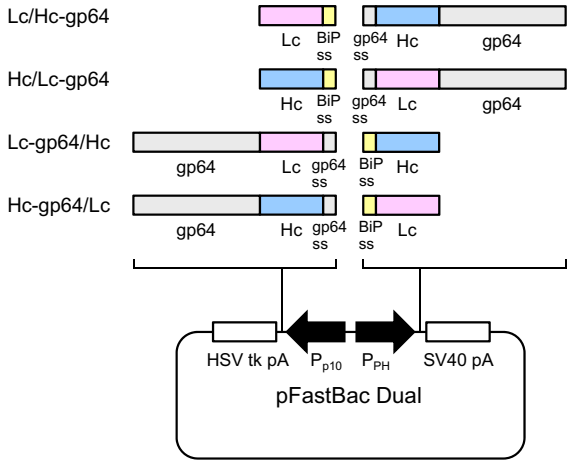

Fig. 1 Schematic representation of the donor plasmid vectors for the generation of recombinant baculoviruses displaying an Fab fragment on the surface. *Hc* heavy chain (Fd fragment), *Lc* light chain, *ss* signal sequence, *BiP Drosophila* immunoglobulin heavy chain binding protein, P_{PH} *Autographa californica* nucleopolyhedrovirus (AcNPV) polyhedrin promoter, *pA* polyadenylation signal, *HSV tk* herpes simplex virus thymidine kinase, P_{p10} AcNPV p10 promoter

plasmid was verified by sequencing and designated pFastBac Lc/Hc-gp64 (Fig. 1). In a similar manner, the DNA encoding the gp64 signal sequence, the 6D9 Lc gene, and the gp64 mature domain from pBACsurf-1-6D9Lc was cloned downstream of the polyhedrin promoter at the *Eco*RI-*Hin*dIII site of pFastBac Hc/- to give pFastBac Hc/Lc-gp64. The sequence was verified.

The DNA encoding the gp64 signal sequence, the 6D9 Lc gene, and the gp64 mature domain was amplified from pBACsurf-1-6D9Lc using primers 13 and 14 (Table 1). The amplified fragment was cloned downstream of the p10 promoter at the *Nhe*I site of pFastBac -/Hc. The resultant plasmid was verified by sequencing and designated pFastBac Lc-gp64/Hc (Fig. 1). In a similar manner, the DNA encoding the gp64 signal sequence, the 6D9 Fab Hc gene, and the gp64 mature domain from pBACsurf-1-6D9Hc was cloned downstream of the p10 promoter at the *Nhe*I site of pFastBac -/Lc to give pFastBac Hc-gp64/Lc. The sequence was verified.

Generation of recombinant baculoviruses

MAX Efficiency DH10Bac competent *E. coli* cells (Life Technologies) were transformed with the plasmid pFastBac Lc/Hc-gp64, pFastBac Hc/Lc-gp64, pFastBac Lc-gp64/Hc, or pFastBac Hc-gp64/Lc to generate respective recombinant bacmids according to a protocol recommended by the manufacturer. Recombinant bacmid DNA was isolated and used to transfect Sf9 cells with Cellfectin reagent (Life Technologies) in Grace's medium, resulting in the release of recombinant baculovirus into the medium. High-titer stock of each recombinant baculovirus was prepared via multiple rounds of amplification, and the titer was determined using a FastPlax titer kit (Merck).

Cell culture and infection with recombinant baculovirus

Sf9 cells were inoculated at a density of 1 or 2 $\times$ 10^6 cells cm^{-3} in 6-well plates with 2 mL of fresh culture medium. High-titer recombinant baculovirus stock solution was added to each well for a multiplicity of infection (MOI) of 1 or 5 plaque-forming units (pfu) per cell. The time of viral solution addition was designated post-infection time zero. The cells in the plates were statically cultivated at 27 °C in an incubator. At appropriate times, the infected cell suspensions were removed from the wells, and the cells and culture supernatants were separated via centrifugation.

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants containing recombinant baculovirus were analyzed via ELISA, as previously described (Yamaji et al. 2008). ELISA plates were coated with a conjugate of hapten (a transition-state analog for the hydrolysis of a chloramphenicol monoester derivative) and bovine serum albumin (BSA) (Takahashi et al. 2001) as the antigen, and horseradish peroxidase-conjugated goat anti-mouse IgG (Exalpha Biologicals, Watertown, MA, USA) was used.

Western blot analysis

Culture supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5 % gel under reducing conditions, followed by transfer to a poly(vinylidene difluoride) (PVDF) membrane. Western blot analysis was carried out using mouse anti-AcNPV gp64 (AcV5) monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and alkaline phosphatase-conjugated goat

anti-mouse IgG (H&L) (Rockland Immunochemicals, Gilbertsville, PA, USA). Immunoreactive bands were detected using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega, Madison, WI, USA) (Yamaji et al. 2008).

Flow cytometry

The cell suspension removed from the 6-well plates was incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Life Technologies) for 30 min at room temperature. After washing with phosphate-buffered saline (PBS) twice, the cells were suspended in 1 mL of PBS. Fluorescent cells were analyzed using a flow cytometer equipped with an argon laser with excitation at 488 nm (FACSCalibur, Becton, Dickinson & Company, Sparks, MD, USA). Fluorescence emission was collected using a 530/30 band-pass filter.

Results and discussion

Display of Fab fragments on the baculovirus surface

Gp64 is the major envelope glycoprotein of AcNPV and plays an essential role in viral entry into insect cells via baculovirus-cell fusion (Jarvis and Garcia 1994). During the infection cycle, gp64 is abundantly expressed and transported to the surface of infected insect cells for incorporation into budding baculovirus particles. In the present study, to display an antibody Fab fragment on a baculovirus surface, a recombinant baculovirus was designed so that either the Hc (Fd fragment) or the Lc of an Fab fragment was expressed as a fusion protein to the N-terminus of AcNPV gp64 to anchor the fusion protein in the envelope of the baculovirus via the transmembrane region of gp64, while the other chain of the Fab fragment was simultaneously expressed as a secretory protein. For expression as a gp64-fusion protein, the cDNA fragment encoding the Hc or Lc gene of the 6D9 Fab fragment was inserted between the signal sequence and the mature protein domain of a second copy of the AcNPV gp64 gene (Fig. 1). On the other hand, the *Drosophila* BiP signal sequence (Furuta et al. 2010; Yamaji et al. 2008) was employed upstream of the Lc or Hc gene for secretory expression. The resultant DNA fragments were PCR-cloned into the donor plasmid, pFastBac Dual, which contained two multiple cloning sites downstream of either the AcNPV polyhedrin promoter or the AcNPV p10 promoter (Fig. 1). Four kinds of recombinant baculoviruses were then constructed using the Bac-to-Bac baculovirus expression system, and each recombinant baculovirus was successfully amplified to a high titer of more than 10^8 pfu mL^{-1} via multiple rounds of infection of Sf9 cells. This result suggests that the use of a second copy of gp64 for the display of foreign proteins significantly disturbs neither the production nor the infectivity of recombinant baculoviruses (Boublik et al. 1995; Grabherr et al. 2001).

After the infection of Sf9 cells with respective recombinant baculoviruses, the culture supernatants were analyzed via ELISA using antigen-coated 96-well plates and anti-mouse IgG. A strong signal was obtained from each culture supernatant, which showed antigen-binding activity in the culture supernatant (Fig. 2a). In the ELISA with anti-AcNPV gp64 antibody instead of anti-mouse IgG, a positive signal was also observed in the culture supernatant of Sf9 cells infected with the recombinant baculovirus generated with pFastBac Dual Hc-gp64/Lc (data not shown). In western blot analysis of the culture supernatant using the anti-AcNPV gp64, specific protein bands were detected at an electrophoretic

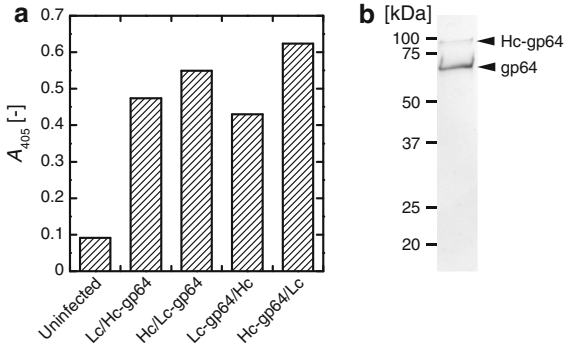

Fig. 2 Analyses of the culture supernatant of Sf9 cells infected with recombinant baculoviruses. **a** Enzyme-linked immunosorbent assay (ELISA) using anti-mouse IgG. A_{405}, absorbance at 405 nm in ELISA. The values represent means of duplicate determinations. Cells were infected with recombinant baculovirus generated with pFastBac Dual Lc/Hc-gp64, Hc/Lc-gp64, Lc-gp64/Hc, or Hc-gp64/Lc at a multiplicity of infection (MOI) of 5 plaque-forming units (pfu) per cell, and were incubated for 3 days in static culture. **b** Western blot analysis using anti-AcNPV gp64. Cells were infected with recombinant baculovirus Hc-gp64/Lc at an MOI of 1 pfu per cell and incubated for 3 days

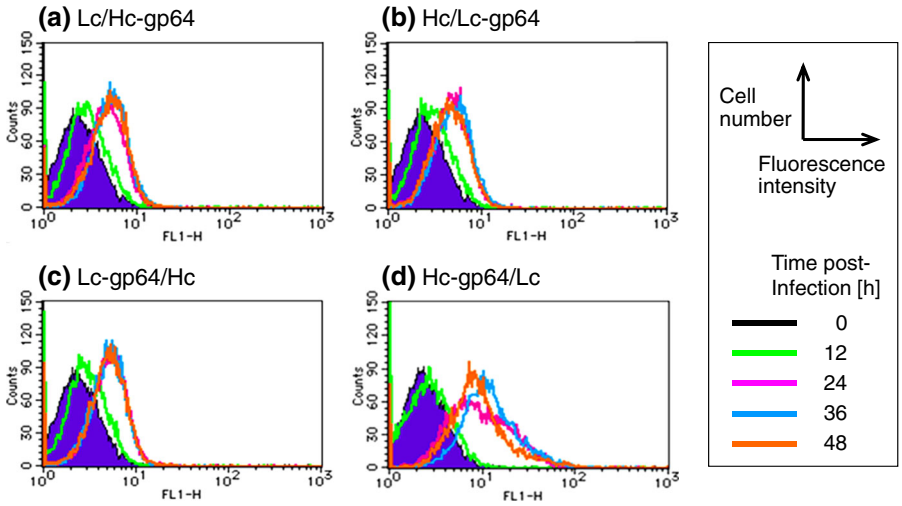

Fig. 3 Flow cytometric analysis of cells infected with recombinant baculovirus. Sf9 cells were infected at an MOI of 5 pfu per cell with recombinant baculovirus Lc/Hc-gp64 (**a**), Hc/Lc-gp64 (**b**), Lc-gp64/Hc (**c**), or Hc-gp64/Lc (**d**)

mobility that coincided with the molecular weight of the Hc-gp64 fusion protein as well as with that of gp64 (Fig. 2b), indicating the presence of Hc-gp64 fusion protein in the culture supernatant. These results suggest that the recombinant baculovirus directed the expression of the Fab fragment with antigen-binding activity, and that the Fab fragments were successfully displayed on the surface of baculovirus particles via fusion to gp64.

Expression on the surface of infected insect cells

Sf9 cells infected with the recombinant baculoviruses were analyzed by flow cytometry using FITC-labeled anti-mouse IgG. Figure 3 shows the distribution of the fluorescence intensity of baculovirus-infected Sf9 cells obtained 12–48 h post-infection. The flow cytometric histograms for cells infected with each recombinant baculovirus shifted to the right 24–48 h after infection, particularly the cells infected with the recombinant baculovirus generated with pFastBac Dual Hc-gp64/Lc (Fig. 3d). Fluorescent microscopy was also performed with Sf9 cells infected with the recombinant baculovirus Hc-gp64/Lc using FITC-labeled anti-mouse IgG. A strong green fluorescence was observed around the periphery of cells (data not shown). These results indicate that Sf9 cells infected with the recombinant baculovirus expressed Fab fragments on their surface for incorporation into budding virions. The above results also suggest that baculovirus-infected insect cells that display specific Fab fragments, and thereby the recombinant baculovirus that contains the DNA encoding the Fab fragment, can be efficiently isolated using a fluorescence-activated cell sorter (FACS) with a fluorescence-labeled antigen. The baculovirus display technologies were successfully used for the generation and screening of cDNA expression libraries (Crawford et al. 2004, 2006; Ernst et al. 1998; Harel et al. 2008; Wang et al. 2004), though they have not yet been applied to the selection of antibodies. While further developments are needed, the baculovirus display of antibody molecules, including Fab fragments, may provide an opportunity for the selection of functional antibodies with high affinity and specificity.

In conclusion, simultaneous expression of the Hc of an Fab fragment as a gp64-fusion protein, and of the Lc as a secretory protein, in baculovirus-infected insect cells allows the display of the Fab fragment in an immunologically functional form on the baculovirus particles. The baculovirus display of antibody Fab fragments might be a useful tool for the generation of monoclonal antibodies with high affinity and specificity.

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