Establishment of insect cell lines expressing green fluorescent protein on cell surface based on AcMNPV GP64 membrane fusion characteristic

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Abstract Displaying a protein on the surface of cells has been provided a very successful strategy to function research of exogenous proteins. Based on the membrane fusion characteristic of *Autographa californica* multiple nucleopolyhedrovirus envelope protein GP64, we amplified and cloned N-terminal signal peptide and C-terminal transmembrane domain as well as cytoplasmic tail domain of *gp64* gene into vector pIZ/V5-His with multi-cloning sites to construct the cell surface expression vector pIZ/V5-gp64. To verify that the vector can be used to express proteins on the membrane of insect cells, a recombinant plasmid pIZ/V5-gp64-GFP was constructed by introducing the PCR amplified green fluorescent protein (GFP) gene and transfected into insect cell lines Sf9 and H5. The transected cells were screened with zeocin and cell cloning. PCR verification results showed that the GFP gene was successfully integrated into these cells. Green fluorescence in Sf9-GFP and H5-GFP cells was observed by using confocal laser scanning microscopy and immunofluorescence detection indicated that GFP protein was located on the cell

membrane. Western blot results showed that a fusion protein GP64-GFP of about 40 kDa was expressed on the membrane of Sf9-GFP and H5-GFP cells. The expression system constructed in this paper can be used for localization and continuous expression of exogenous proteins on insect cell membrane.

Keywords *gp64* gene · GFP · Insect cell line · Expression vector · Cell surface display

Introduction

The expression of proteins or polypeptides on cell surfaces is of great value for ligand selection, protein–protein interaction and bioreactor development. Cell surface display system was first proposed in 1985 and has achieved rapid development in recent years (Smith 1985). The glycoprotein 64 (*gp64*) gene is the most commonly used gene in baculovirus surface display system. It is widely present in insect baculovirus and is a class III viral fusion protein (Rohrmann 2013; Backovic and Jardetzky 2011), which is transported and fused to the cell membrane during the viral infection cycle to form envelope fusion proteins of budded viruses (BVs) and assists BVs to fuse to the infected cell membrane (Blissard and Rohrmann 1989), thus completing the virus infection process (Blissard and Wenz 1992; Li and Blissard 2011). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) GP64 protein consists of a total of 512 amino acid residues. Its N-terminus is a

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secretory signal peptide (SP) sequence to guide the correct processing of protein synthesis and transportation to cell membrane (Oomens and Blissard 1999; Zhou and Blissard 2008). Its C-terminus contains a cytoplasmic tail domain (CTD) and a hydrophobic transmembrane domain (TMD) that anchor viral envelope proteins to the cell membrane (Kadlecek et al. 2008). Since expression of proteins on the cell surface requires a domain to anchor the target proteins to the cell surface, one can insert an exogenous protein into the region between N-terminal SP and C-terminal CTD of *gp64* to make a fusion protein that could be expressed together with the envelope protein and displayed on the infected cell surface. For example, Xu et al. (2011) inserted E-envelope protein of Japanese encephalitis virus (JEV) between the TMD and CTD of *gp64* into a baculovirus expression vector and successfully expressed the protein on the surface of Sf9 cells. They inoculated the recombinant baculovirus vector into mice and pigs and successfully made them achieve immunogenicity to JEV in vivo. Peralta et al. (2013) fused VP1 protein of foot-and-mouth disease virus (FMDV) with *gp64* or G glycoprotein from the vesicular stomatitis virus (VSV-G) and found that different recombinant baculovirus vectors all could massively express VP1 fusion protein in cells. The baculovirus is a transient gene transfer vector. Insect cells infected with baculovirus will die and lyse continuously, thus the exogenous protein could not be expressed continuously.

Therefore, in this study, we intend to use the characteristic that baculovirus GP64 protein can be used for membrane surface expression by inserting the N-terminal SP, C-terminal TMD and CTD into the insect cell expression vector pIZ/V5-His to construct a recombinant vector pIZ/V5-gp64, which can express foreign proteins on cell surface. We then inserted green fluorescent protein (GFP) reporter gene into the expression vector and transformed it into insect cell lines Sf9 and H5. The expression of GP64-GFP on the surface of transgenic insect cells was detected by immunofluorescence and western blotting. The results will provide support for the localization and continuous expression of exogenous proteins on insect cell membrane.

Materials and methods

Materials

Test cell lines

The insect cell line Sf9 of *Spodoptera frugiperda* and the cell line BTI-Tn5B1-4 (H5) of *Trichoplusia ni* were kindly provided by Dr. Granados at the BTI Institute of Cornell University.

Plasmid and strains

Plasmid pGEM3ZGP64 containing AcMNPV *gp64* gene was kindly provided by Dr. Zhaoifei Li from the Northwest A&F University (Li and Blissard 2011) and vector pHF315-gfp-clwt containing full-length of GFP gene was kindly provided by Dr. Fuping Song of the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences. The vector pIZ/V5-His was purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* Trans109 competent cells were purchased from TransBionovo Co., Ltd. (Beijing, China).

Reagents

TNM-FH insect cell culture medium was purchased from Thermo Scientific HyClone (Logan, UT, USA). Fetal bovine serum (FBS), *Taq* DNA polymerase, dNTPs, DNA marker, EasyPure quick gel extraction kit and EasyPure plasmid miniprep kit were from TransBionovo Co., Ltd. BenchMarkTM Prestained Protein Ladder, Transfection reagent Cellfectin[®] II and Zeocin were purchased from Invitrogen. Genomic DNA purification kit MagExtractor[®]-Genome was from TOYOBO Co., Ltd (Osaka, Japan). Anti-GFP tag rabbit polyclonal antibody, anti- β -actin mouse monoclonal antibody, AP conjugated goat anti-rabbit IgG (H + L), AP conjugated goat anti-mouse IgG(H + L) and Cy3 conjugated goat anti-rabbit IgG(H + L) were from Abbkine (Redlands, CA, USA). Mem-PERTM Plus Membrane Protein Extraction Kit was purchased from Pierce (Rockford, IL, USA). BCIP/NBT Color Development Substrate was purchased from Promega (Madison, WI, USA).

Primers and DNA sequencing

Primers were designed using Primer Premier 5.0 based on the sequences of AcMNPV *gp64* gene and GFP gene, respectively. Primers synthesis and recombinant vectors sequencing were conducted by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Methods

Construction of recombinant vectors

Figure 1 shows the flowchart of construction of recombinant vectors. Using plasmid pGEM3ZGP64 as the template, the *gp64* N-terminal signal peptide (*gp64sp*) fragment was amplified using upstream primer (CCCAAGCTTATGCTACTAGTAAATCAGTCACAC containing a *HindIII* site) and downstream primer (GGAATTCGCATGCGGATCCGGTACC-CATTTGCGCGTTGCAAGTGCT containing one site of *EcoRI*, *SphI*, *BamHI* and *KpnI* each). The *gp64* C-terminal transmembrane domain and cytoplasmic tail domain (*gp64ctd*) were amplified using upstream primer (GGAATTCGAGCTCGCGGCCGCTAAATTGACTTC containing one site of *EcoRI*, *SacI*, and *NotI* each), and downstream primer (GCTCTAGAT-TAATATTGTCTATTACGGTTTCT containing a *XbaI* site) in a 50 µL system containing 0.5 µL of pGEM3ZGP64, 1.0 µL of each primer, 5.0 µL of 10 × buffer, 4.0 µL of 2.5 mM dNTPs and 0.5 µL of *Taq* DNA polymerase at the conditions of 5 min denature at 94 °C followed by 30 cycles of 40 s at 94 °C, 40 s at 55 °C, and 30 s at 72 °C and final extension at 72 °C for 10 min. The amplified *gp64sp* and *gp64ctd* fragments were digested using *EcoRI* and ligated together. The ligation product was further amplified using the upstream *gp64sp* primer and downstream *gp64ctd* primer in a 50 µL system containing 1.0 µL of ligation product, 1.0 µL of each primer, 5.0 µL of 10 × buffer, 4.0 µL of 2.5 mM dNTPs and 0.5 µL of *Taq* DNA polymerase at the conditions described above. The product and plasmid pIZ/V5-His were digested using *HindI* and *XbaI*, ligated together, and transformed into *E. coli* Trans109 competent cells to obtain the recombinant vector pIZ/V5-gp64.

The GFP fragment was amplified using pHT315-gfp-clwt as template and a primer set of

GGGGTACCATGAGTAAAGGAGAAG containing a *KpnI* site and CGGAATTCCTTTGTATAGTTCATCCA containing a *EcoRI* site in a 50 µL system as described above at the conditions of 5 min denature at 94 °C followed by 30 cycles of 40 s at 94 °C, 40 s at 56 °C, and 60 s at 72 °C and final extension at 72 °C for 10 min. The amplicon and plasmid pIZ/V5-gp64 were digested using *KpnI* and *EcoRI*, ligated together and transformed *E. coli* Trans109 competent cells to obtain the recombinant vector pIZ/V5-gp64-GFP.

Insect cell line transfection and screening

The constructed vector pIZ/V5-gp64-GFP was transfected into insect Sf9 and H5 cells using Cellfectin®II liposomal transfection reagent following the protocol provided by the manufacturer. The cells were then cultured in TNM-FH medium containing 400 µg/mL of zeocin and cloned using the cloning cylinders method following the protocol for InsectSelect™ pIZ/V5-His. The transgenic cell lines Sf9-GFP and H5-GFP were obtained and sub-cultured.

Identification of transgenic cell lines by PCR

The genomic DNAs of insect cells Sf9-GFP and H5-GFP were extracted using a genomic DNA purification kit. A set of GFP primers were used for PCR amplification in the above mentioned reaction system and conditions to examine whether GFP gene was present in the transgenic cell lines.

Immunofluorescence detection of transgenic cell lines

One microliter of 2×10^5 cells/mL transgenic insect cell lines Sf9-GFP and H5-GFP in logarithmic growth phase were inoculated, respectively, in a 15 mm glass bottom cell culture dish for laser confocal microscope (Nest Biotechnology Co., Ltd., Wuxi, China). After incubation at 27 °C for 3 days, cells were stained with anti-GFP tag rabbit polyclonal antibody and Cy3 conjugated goat anti-rabbit IgG(H + L) for 1 h, respectively, 1 mL (1 µg/mL) of 4',6-diamidino-2-phenylindole (DAPI) for 5 min, washed twice with PBS, observed under a confocal laser scanning microscopy and photographed.

Fig. 1 The flowchart of construction of recombinant vectors pIZ/V5-gp64 and pIZ/V5-gp64-GFP

Western blot detection of GFP protein

10^6 cells of the transgenic insect cell lines Sf9-GFP and H5-GFP in the logarithmic growth phase were collected and lysed using sodium dodecyl sulfate (SDS) sample buffer as the total cellular proteins. Meanwhile, cell membrane proteins were extracted using the Mem-PERTM Plus Membrane Protein Extraction Kit and stored at $-80\text{ }^{\circ}\text{C}$. Meanwhile, cells were broken by ultrasonication. The homogenate was centrifuged at $760\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$ to remove nuclei and unbroken cells. Then supernatant was further centrifuged at $32,000\times g$ for 1 h at $4\text{ }^{\circ}\text{C}$, yielding a supernatant of the cytosol fraction. The protein samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. The PVDF membranes were then immersed in the anti-GFP tag rabbit polyclonal antibody and anti- β -actin mouse monoclonal antibody solution and incubated for 1 h on a vertical shaker, respectively. After having washed with PBS, the membranes were immersed in the AP conjugated goat anti-rabbit IgG (H + L) antibody and AP conjugated goat anti-mouse IgG(H + L) solution and incubated for 1 h on a vertical shaker, respectively. After having washed with PBS, the membranes were incubated in BCIP/NBT Color Development Substrate at dark to visualize the expressed protein bands.

Results and analysis

Construction of recombinant vectors pIZ/V5-gp64 and pIZ/V5-gp64-GFP

The recombinant expression vector pIZ/V5-gp64 was obtained by ligating the N-terminal SP and C-terminal CTD of AcMNPV gp64 gene with vector pIZ/V5-His. Identification of the pIZ/V5-gp64 using *Hind*III and *Xba*I digestion showed a vector fragment of 2.9 kb and an insert *gp64* fragment of 301 bp (Fig. 2). In addition, PCR amplification using the upstream N-terminal *gp64sp* primer and downstream C-terminal *gp64ctd* primer also produced a fragment of about

Fig. 2 Identification of recombinant vector pIZ/V5-gp64 by restriction enzyme digestion and PCR. *M* DNA marker DL 5000; 1 *Hind*III and *Xba*I double digestion; 2 PCR amplification

300 bp (Fig. 2). Moreover, Sanger sequencing showed that pIZ/V5-gp64 vector was successfully constructed.

The GFP fragment was inserted into the vector pIZ/V5-gp64 to obtain the recombinant plasmid pIZ/V5-gp64-GFP. The recombinant plasmid was identified by *Eco*RI digestion, as well as *Eco*RI and *Kpn*I dual digestion. *Eco*RI digestion showed a single fragment of 3.8 kb and *Eco*RI and *Kpn*I dual digestion showed a fragment of about 3.0 kb and a fragment of 720 bp. In addition, PCR amplification using GFP primers also produced a fragment of 720 bp (Fig. 3). Sanger sequencing confirmed that the recombinant plasmid pIZ/V5-gp64-GFP was successfully constructed.

Screening of GFP transgenic cell lines

The recombinant vector pIZ/V5-gp64-GFP was transfected into cell lines Sf9 and H5 using liposome-mediated method, respectively. The transgenic cell lines Sf9-GFP and H5-GFP were selected by zeocin and cloned using cloning cylinders. Three cell colonies of Sf9-GFP and five cell colonies of H5-GFP were acquired. Green fluorescence could be observed in these cell colonies. Comparison of their morphology and size with their original cell lines showed no significant differences. One of the Sf9-GFP and H5-GFP cell colonies was selected for

Fig. 3 Identification of recombinant vector pIZ/V5-gp64-GFP by restriction enzyme digestion and PCR. *M* DNA marker DL 5000; 1 *Kpn*I and *Eco*RI double digestion vector; 2 *Eco*RI digestion vector; 3 PCR amplification

subculturing and testing. The two transgenic cell lines have been passed more than 20 times so far.

Identification of transgenic cell lines by PCR

Genomic DNAs of Sf9, H5, Sf9-GFP and H5-GFP cells were amplified by PCR using a set of GFP primers and a 720 bp band was amplified from the genomic DNA of the transgenic cell lines Sf9-GFP and H5-GFP, but not the original Sf9 and H5 cell lines (Fig. 4), demonstrating that the target GFP gene was successfully introduced into the transgenic cells.

Fig. 4 Identification of transgenic cell line Sf9-GFP and H5-GFP by PCR. *M* DNA marker DL 5000; 1 H5-GFP; 2 H5; 3 Sf9-GFP; 4 Sf9

Immunofluorescence detection of transgenic cell lines

Fluorescent signals were observed in some of Sf9 and H5 cells at 48 h post transfection of the recombinant plasmid and can be observed obviously in cells after subculture. Green fluorescence in Sf9-GFP and H5-GFP cells was observed by confocal laser scanning microscopy. Immunofluorescence detection indicated that GFP protein was successfully expressed on the cell membrane. The red signal was the membrane stained with Cy3 and the blue signal was the nuclei stained with DAPI (Fig. 5).

Detection of GFP in transgenic cell lines by Western blot

The total protein, cytosolic protein, and membrane protein of transgenic cell lines Sf9-GFP and H5-GFP were extracted and analyzed by Western blot. The results showed that a fusion protein of about 40 kDa was detected in both total protein and membrane protein samples (Fig. 6). The band is greater than 27 kDa of the theoretical molecular weight of GFP and probably resulted from fusion of GP64 and GFP. The theoretical molecular weight of the fusion protein is 36.9 kDa. It indicated that GP64-GFP protein is successfully localized and expressed on the cell membrane.

Discussion

In this study, a recombinant vector pIZ/V5-gp64 for expression of foreign genes on cell surface was successfully constructed. The multi-cloning site was introduced to facilitate the study on expression and function of the target proteins on cell surface. It was also confirmed that the constructed vector pIZ/V5-gp64 could express the target gene on the surface of insect cells through the successful expression of reporter gene GFP in insect cells Sf9 and H5.

Since the first report on phage display (Smith 1985), surface display technology has rapidly advanced and a number of prokaryotic and eukaryotic surface display systems have been established (Boulain et al. 1986; Roy et al. 1991; Smith 1985). In recent years, the surface display technology has been widely used in baculovirus, *E. coli*, yeast and mammalian cells. For

Fig. 5 Green fluorescence detection and Immunofluorescence detection of transgenic cell lines Sf9-GFP and H5-GFP. **a** GFP distributed in Sf9-GFP; **b** Cell nuclei of Sf9-GFP stained with DAPI; **c** GFP immunofluorescence distributed in Sf9-GFP;

d Merge of **b** and **c**; **e** GFP distributed in H5-GFP; **f** Cell nuclei of H5-GFP stained with DAPI; **g** GFP immunofluorescence distributed in H5-GFP; **h** Merge of **f** and **g**

Fig. 6 Western blot analysis of transgenic cell lines Sf9-GFP and H5-GFP. **M** Protein molecular weight marker; **1** Total protein of Sf9-GFP; **2** Total protein of H5-GFP; **3** Cytosolic protein of Sf9-GFP; **4** Cytosolic protein of H5-GFP; **5** Membrane protein of Sf9-GFP; **6** Membrane protein of H5-GFP

examples, the surface display system constructed by the expression of outer membrane porin SmoA using *Synechococcus elongatus* can be used as a continuous bioproduct platform (Fedeson and Ducat 2016), the surface display systems constructed based on *Bacillus thuringiensis* (Bt) strain BMB171 using two N-terminal domains of autolysin as anchoring motifs can be used to improve catalytic and antibacterial activity of chitinase Chi9602ΔSP (Tang et al. 2017), and the surface expression system VSV-Ebola based on vesicular stomatitis virus can be used to screen

antibodies and vaccines against Ebola viruses (Khurana et al. 2016). However, studies on cell display technology are mainly focused on bacteria and fungi, there are also some applications on insect cells. For examples, rabies virus glycoprotein has been expressed in *Drosophila* S2 cell (Santos et al. 2016) and rice stripe virus (RSV) Nsvc2 protein has been expressed on the Sf9 surface as the membrane glycoproteins of the enveloped viruses to study the membrane fusion activity of Nsvc2 (Zhao et al. 2012). In this study, we utilized cell membrane specific

anchoring characteristic of baculovirus envelope protein GP64 and ligated its N-terminal signal peptide and C-terminal transmembrane domain as well as cytoplasmic tail domain with the vector pIZ/V5-His to construct a recombinant vector for insect cell surface expression. Unlike other baculovirus expression vector system constructed with *gp64* (Ikonomou et al. 2003; Grabherr and Ernst 2010), in which the target protein is displayed on the viral envelope and the cell membrane after transfection and cannot be continuously expressed as the virus-infected cells are ruptured, the vector constructed in this study can be used to continuously express and display the target proteins on the surface of insect cell lines without being affected by cell passaging. Thus, it overcomes the shortcomings of transient expression of the baculovirus expression vector system.

The recombinant expression vector can be used to express the receptor protein on the cell surface to study the function of the receptor protein and the interaction mechanism between proteins or to screen the specific antibody. Meanwhile, the system can be also used to express reporter genes to detect target proteins' localization and expression in the transgenic cell clones, thus providing a simple, intuitive and efficient research tool for the expression of various target genes.

However, expression of different genes may differ significantly in different cells. Their transcriptional pathway may be also different in different cells (Mann and King 1989). There is no guarantee that each foreign target protein can be translated and folded correctly and expressed on the cell surface. Therefore, the expression and application of the expression vectors constructed in this study in insect cell lines other than Sf9 and H5, or other mammalian cells need further exploration.

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