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Use of *hr3* enhancer and P74 TM domain in baculovirus surface display

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TABLE OF CONTENTS

1. ABSTRACT

Baculovirus surface display technique provides a new platform for novel vaccine research and production. Unfortunately, the low display efficiency in current methods and the waste of occlusion-derived virion (ODV) products limited its application. We investigated the use of two motifs, BmNPV *hr3* and transmembrane domain of P74 (P74TM), in display. Budding virus (BV) with *hr3* showed a 14.2-time enhanced display efficiency than the current recombinant BV. Hemagglutinin (HA) protein of H5N1 influenza virus was displayed by using 3 different vectors to ensure the improvement of display efficiency and the characters of displayed protein in recombinant baculovirus. Immunoassay demonstrated that the recombinant BV with TM/CTD of vsvG protein, and *hr3* could induce the highest level of neutralizing antibody against HA, suggesting that the optimized HA displaying BV could be a novel live virus-based vaccine candidate for influenza virus. In addition, GFP fused with the P74TM could be anchored to the ring zone in infected cell and the ODV envelope, which may luminate a new direction for ODV display and provide a promising strategy to use ODV products.

2. INTRODUCTION

Baculovirus is a kind of enveloped insect virus with large double-stranded circular DNA genomes (80-180kb) in rod-shaped nucleocapsids. Baculovirus produces two phenotypically distinct forms of infectious virions in the infection cycle: occlusion-derived virions (ODVs) and budded virions (BVs). ODVs are embedded in a crystalline matrix of proteins, and responsible for infecting insect midgut cells in the primary infection (1) and spreading between larvae. BVs are single virions with an envelope from plasma membrane and associated with cell-to-cell transmission in both infected insects and cultured cells. Baculovirus expression system (BES), widely used to express eukaryotic proteins (2, 3), has the following advantages: high-level expression of large soluble proteins with post-translational modification, and more security without the replication of baculovirus in mammalian cells.

Baculovirus surface display was a technique based on BES and first proposed as using GP64 protein or its signal peptide (SP) and transmembrane / cytoplasmic domain (TM/CTD) to anchor target proteins to BV's envelope (4-6). The proteins displayed on the surface of

recombinant BVs (rBVs) were proven effective to elicit antibody responses in mice model (7, 8). This technique was also used in gene delivery and eukaryotic library development (9). To improve the immune effect induced by rBVs, some methods have been employed to display more target proteins (10) and make the immune response more specific (11). But there are still some problems limiting the application of this technology, such as the impact of target proteins' low quantities and improper expressing phase to display and the waste caused by a large numbers of ODV products.

In this study, we used two motifs, *hr3* enhancer of Bombyx mori nuclear polyhedrosis virus (BmNPV) and TM of Autographa californica nuclear polyhedrosis virus (AcMNPV) P74 protein, to improve the baculovirus display system. *Hr3* could enhance the transcription of baculovirus early phase promoters (12), however, there has been no report on the impact of *hr3* to surface display. P74TM could anchor the GFP to the ring zone of the cells infected by baculovirus (13), however, it is unknown whether the fusion protein GFP-P74TM could be incorporated into the ODV. We compared the display efficiency of rBVs by using different promoters (Pgp64 and Pvp39) in the presence or absence of *hr3*, and the antibody levels induced by inoculating with the rBVs in mice model. In addition, in order to evaluate the potential of P74 on the ODV display, we purified the GFP-P74TM expressing ODV, and separated the envelope and nucleocapsid components to test whether the GFP-P74TM was incorporated into ODV.

3. MATERIALS AND METHODS

3.1. Materials

Sf9 *Spodoptera frugiperda* insect cells were cultured in TNM-FH Insect Medium (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) (pH 6.2) at 27 degree centigrade. MDCK cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS (pH 7.4) at 37 degree centigrade with 5% CO_2. The H5N1 influenza virus (A/Zhejiang/16/06) was kindly provided by the Institute of Virology, Zhejiang Provincial Center for Disease Prevention and Control,China (14, 15). Rabbit anti H5N1 avian influenza virus HA protein polyclonal antibody was produced by Abcam (Cambridge, UK). Rabbit anti-GFP polyclonal antibody and mouse anti-VP39 polyclonal antibody were prepared by our laboratory. Rabbit anti-P74 polyclone antibody was kindly presented by Prof. Chuanxi Zhang from Zhejiang University, China.

3.2. Cloning of Pgp64, Pvp39, hr3 and P74TM

The Pgp64, Pvp39 and P74TM sequences were amplified from AcMNPV genome by PCR using *Pfu* DNA polymerase with primers Fpgp64 (5'-TACGTA (*SnaBI*) TATTTAAATA AACCAAACAC-3') / Rpgp64 (5'-GGATCC (*BamHI*) CTTGCTT GTGTGTTCCT-3'), Fpvp39 (5'- TACGTA (*SnaBI*) GAAGCGTCCC CATTTTCCAA-3') / Rpvp39 (5'- GGATCC (*BamHI*) ATTGTTGCCG TTATAAATAT-3') and Fp74tm (5'-gca tgc (*SphI*) tctatgaacaaag-3')/ Rp74tm (5'- ggt acc (*KpnI*)ttaaaataacaaatcaattg-3'). The G at site -52 of Pgp64 was mutated to T to destroy the ATG at site -54. The *hr3* sequence was amplified from BmNPV genome by PCR using *Pfu* DNA polymerase with primers F*hr3* (5'-CCTAGG (*AvrII*) AGAC AACAAAGATT TATTTTATTC ATGCCACTAC TCGGTTCCGT-3') / R*hr3* (5'- CCTAGG (*AvrII*) ACGTTCGTGC CAGAAATTAA TTTCTCCGCG TCGTATTATA CGAT-3'). The PCR products were sequenced and inserted into pFastBac1 and pFastBac Dual vectors.

3.3. Construction of the display vectors

Recombinant AcMNPVs were produced according to the manual of Bac-to-Bac Baculovirus Expression Systems (Invitrogen, Carlsbad, CA). The pFastBac1 vector was used to construct BV display vectors. Pph promoter, between *SnaBI* and *BamHI*, was replaced by Pgp64 or Pvp39, respectively. Then, the SP and TM/CTD of GP64 were inserted into *BamHI* and *KpnI/HindIII* sites in multiple cloning sites (MCS) to construct the BV display vectors, pPph-GP64, pPgp64-GP64 and pPvp39-GP64. The BmNPV *hr3* sequence was inserted into the *AvrII* site on pPph-GP64, to get another BV display vector, pPph-GP64-*hr3* (Figure 1A). Luciferase gene was introduced into these vectors as a reporter gene. The pFastBac Dual vector was used to construct ODV displaying vectors. The polyhedrin protein gene was inserted into the pFastBac Dual MCS I under the control of Pph promoter, to get the pFBD-PH vector. Then P74TM was inserted into *SphI/KpnI* sites on the pFBD-PH MCS II under the control of P10 promoter. GFP gene was introduced into the MCS II before P74TM to produce the GFP-P74TM fusion protein.

3.4. Construction of HA gene displaying vector

The BV display vectors were applied to produce the recombinant BVs with HA. The ectodomain of HA protein (HAect codon 17–530) was introduced into Pph-GP64 (TM/CTD of GP64 was inserted between *NspI* and *HindIII* here) between *BamHI* and *NspI* to construct the pPph-HA-GP64. Then TM/CTD of GP64 was replaced by TM/CTD of vsvG (codon 441–511) to get the pPph-HA-vsvG. Finally, the BmNPV *hr3* sequence was introduced into pPph-HA-vsvG at *AvrII* site to construct pPph-HA-vsvG-*hr3* vector (Figure 2A).

3.5. Generation of the recombinant baculovirus

Display vectors were transformed and transpositioned with bacmids in DH10Bac *E. coli* competent cells. Recombinant bacmid DNA were extracted and transfected Sf9 cells. Recombinant BVs were harvested from the supernatant of transfected cells. Bac (non-recombinant virus) BVs were used as control in future experiments.

3.6. Purification of BVs

The supernatant of Sf9 cells infected by recombinant BVs was collected at 24 and 48 hpi, and centrifuged at 5,000 *g* and 4 degree centigrade for 30 min to exclude cell debris. Recombinant BVs were precipitated after centrifuging at 100,000 *g* and 4 degree centigrade for 2 h. The BV pellets were resuspended in PBS (pH 7.2), then loaded on a 5-ml 25–56% discontinuous sucrose

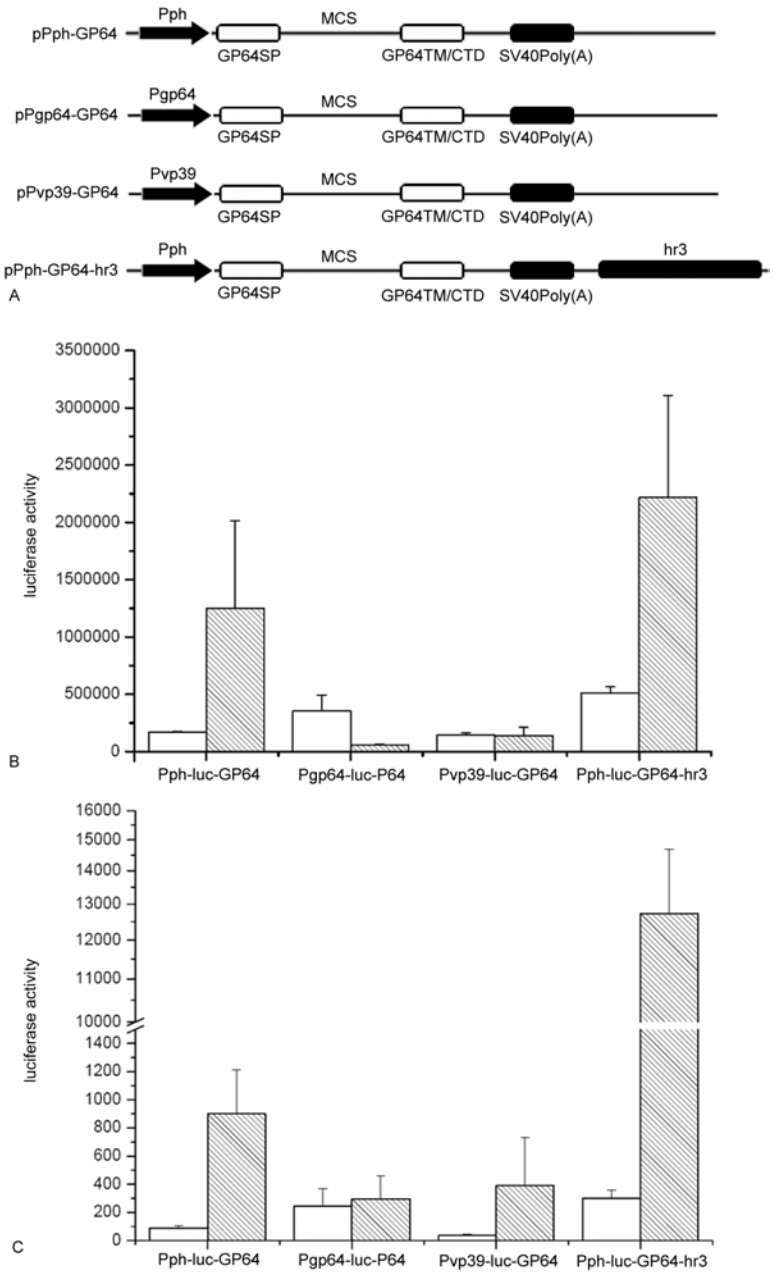

Figure 1. The displaying efficiency of rBVs with luciferase A. Profiles of the recombinant baculovirus display vectors. Serial elements were introduced into the pFastBac1 vector in sequence as shown. The luciferase gene was inserted in MCS. B. Luciferase activity of infected cells at 24 and 48 hpi. C. Luciferase activity of recombinant viruses at 24 and 48 hpi. Blank bars represented samples of 24 hpi, and filled bars represented samples of 48 hpi; error bars represented the SDs.

gradient and centrifuged at 100,000 *g* and 4 degree centigrade for 2 h. The BV band was collected and mixed with three-volume PBS (pH 7.2), then centrifuged at 100,000 *g* and 4 degree centigrade for an additional 2 h. The pellet was resuspended in PBS (pH 7.2).

3.7. Purification of ODVs

Sf9 cells infected by PH and PH-GFP-P74TM BVs for 5 days were collected by centrifuging at 2,000 *g* for 30 min. After washed by water once, the infected cells were lysed for 1 hour by adding lysis buffer (20mmol/L Tris, 1mmol/L EDTA, 100nmol/L NaCl, 0.5% Tween 20 pH8.0). The cell lysate was centrifuged at 2,000 *g* for 30 min. The pellet was washed by 40% sucrose once and water twice, then dissolved in an alkaline solution (100mmol/L Na_2CO_3, 100mmol/L NaCl) for 20 min followed by adding 1mol/L Tris-HCl (pH 7.6 to stop the action. The solution was centrifuged at 2,000 *g* for 30 min. The supernatant was overlayed on a 5-ml 25–56% discontinuous sucrose gradient, and centrifuged at 90,000 *g*

and 4 degree centigrade for 1 h. The band between 25% and 56% sucrose was collected and mixed with three-volume PBS (pH 7.2), then centrifuged at 100,000 *g* and 4 degree centigrade for 2 h. The pellet was resuspended in PBS (pH 7.2).

3.8. Separation of the ODV components

The ODV solution dissolved in a detergent-contained solution (1% NP40, 10mmol/L Tris-HCl, pH8.5) for 30 min, was loaded on 30% glycerin and centrifuged at 120,000 *g* for 1 h to separate the soluble envelope and unsoluble capsid. Saturated ammonium sulfate solution was added to supernatant and the pellet to precipitated proteins. The protein precipitations were collected by centrifuged at 100,000 *g* for 30 min and dissolved in PBS (pH 7.2).

3.9. Measurement of the recombinant baculovirus titer

BV titers were represented as $TCID_{50}$. 10^4 Sf9 cells and 100 µl BV dilutions (diluted from 10^{-2} to 10^{-9}) were added into each well of a 96-well plate and incubated for 10 days. $TCID_{50}$ was calculated from the number of wells in which Sf9 cells had cytopathic effect observed under the microscope with Reed-Muench method (16).

3.10. Western blot

Virus samples were analyzed by SDS-PAGE gel and electroblotted to PVDF membranes (Osmonics, Pittsburgh, PA). The membranes were blocked with 5% milk in TTBS (TBS containing 0.0625% Tween 20, pH 7.4) for 30 min at 37 degree centigrade, and incubated in primary antibodies for 1 h at 37 degree centigrade. After washing 3 times with TTBS, the membranes were incubated in alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG or horse anti-mouse IgG (Vector, Burlingame, CA, diluted 1:200) for 1 h at 37 degree centigrade. After washing another 3 times with TTBS, the blots were developed with BCIP/NBT substrates (Promega, Madison, WI) at room temperature.

3.11. Luciferase assay

Luciferase assay was performed according to the manual of Luciferase Assay System (Promega). For each sample, 5×10^6 Sf9 cells were infected by 3×10^7 $TCID_{50}$ rBVs with luciferase displayed on the surface. The new produced rBVs were collected from the culture supernatant by ultracentrifugating at 24 and 48 hours post-infection (hpi), and the infected cells were also prepared for test. The luciferase activities of rBV and infected cell samples were tested by using Luciferase Assay Kit (Promega, USA).

3.12. Immunization

Six-week-old Balb/c female mice were subcutaneously injected with 2×10^8 $TCID_{50}$ purified rBVs displaying HA on the surface without adjuvant, boosted at 10th day and 20th day after first inoculation, and killed at 27th day. Blood was collected and the sera were separated for test.

3.13. Neutralization assays

Neutralization assays were performed according to the PRNT (plaque reduction neutralization test) procedure (17). In brief, after inactivated at 56 degree centigrade for 30 min, all serum samples were two-fold diluted from 1/10 to 1/320 in DMEM to 50µl. 100 PFU (plaque forming unit)/50ul influenza virus dilution was also prepared in DMEM. Each 50µl serum dilution was mixed with 50µl influenza virus dilution and the final dilution of the testing serum was from 1/20 to 1/640. The virus/serum dilution mixtures were incubated at 37 degree centigrade for 1 h, then added to 35 mm dishes with a monolayer of MDCK (Madin-Darby canine kidney) cells and incubated for another 1.5 h at 37 degree centigrade. Then the mixtures were removed and DMEM with 1% LMP and 2 mg/ml BSA was added. The virus/DMEM mixture was inoculated as a virus control to determine the average plaque count. After 3 days incubation at 37 degree centigrade, neutral red was added to dye the live cells for 4 h, then the medium was removed. The dishes with monolayer cells were dried by air and the plaques were counted.

4. RESULTS

4.1. Comparison of baculovirus displaying efficiency

To evaluate the impact of different promoters and *hr3* enhancer on the BV display, we compared the display efficiency of rBVs with luciferase displayed on the surface. The luciferase activity of infected cells and rBVs samples could represent the expression and display levels, respectively. At 24 hpi, without *hr3*, Pgp64 exerted the highest transcription activity among the three promoters, while with *hr3*, the activity of Pph increased by 3 times and exceeded Pgp64 (Figure 1B, blank bars). The displayed luciferase increased with the increase of its expression, so that both luciferase expression and display of Pph-luc-GP64-*hr3* were higher than the other three kinds of rBVs (Figure 1B and 1C, blank bars). At 48 hpi, Pph had the highest activity among three promoters, which was different from the situation at 24 hpi. In addition, *hr3* enhanced the activity of Pph as at 24 hpi. The luciferase displayed on the Pph-luc-GP64-*hr3* virus was 14.2 times higher than Pph-luc-GP64 (Figure 1C, filled bars), although the activity of Pph was just increased by 1.8 times by *hr3* (Figure 1B, filled bars). The BV surface display was enhanced by adding *hr3* to Pph.

4.2. Surface display of HA protein

Based on the comparison results of BV displaying efficiency, we constructed three HA displaying vectors (Figure 2A). Bac, a virus without any recombinations, was used as a negative control. Purified rBVs were examined by Western blot (Figure 2B). There are two HA protein bands located at about 80 kDa (HA0 in Figure 2B) and 45 kDa (HA1 in Figure 2B) in Pph-HA-GP64, Pph-HA-vsvG and Pph-HA-vsvG-*hr3*. Furthermore, there were more HA fusion proteins displayed on Pph-HA-vsvG-*hr3* than Pph-HA-vsvG and Pph-HA-GP64. No HA was detected in Bac. Compared with HA displayed on the Pph-HA-vsvG-*hr3*, HAect expressed in prokaryotic was at 60kDa, and HA expressed in MDCK cells infected by H5N1 influenza virus was at about 50 kDa instead of 45 kDa, since 45 kDa band was not detected in the MDCK cells (Figure 2C).

4.3. Immune effect of rBVs displaying HA protein

To test the immune effect of the rBVs displaying HA protein, neutralization assay was performed.

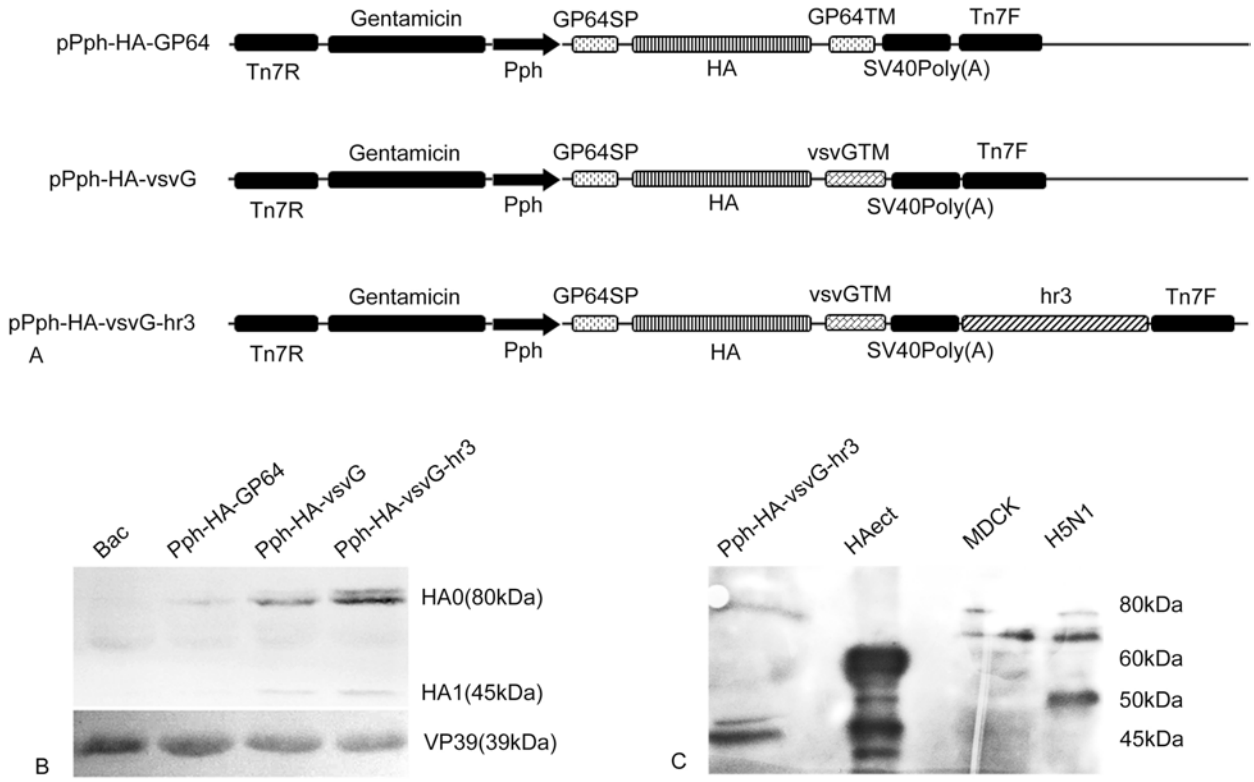

Figure 2. The HA protein displayed on rBVs. A. Profiles of the recombinant baculovirus display vector for displaying HA. B. Detection of HA protein in recombinant baculoviruses by Western blot. C. The comparison of HA expressed in baculovirus, *E.coli* and mammal cells.

Neutralization assay was used to determine the titer of neutralizing antibody, which represented the antivirus capability. The results were shown in Table 1. When the plaque number was decreased to 50%, the mice sera dilution was determined as positive. The Pph-HA-GP64 rBV immunized mice sera showed neutralizing activity to H5N1, but its activity to H5N1 was much lower than the Pph-HA-vsvG and Pph-HA-vsvG-*hr3*. To inhibit 50% of the H5N1 infectivity, Pph-HA-vsvG rBV immunized mouse sera could be diluted by 200 times, and Pph-HA-vsvG-*hr3* rBV immunized mouse sera up to 300 times. There was no neutralizing activity detected in Bac immunized mouse sera.

4.4. The application of P74TM in ODV display

Sf9 cells were infected by PH and PH-GFP-P74TM rBVs for 72h and fixed for observation under a fluorescence microscope. There were occlusion bodies observed in both kinds of infected cell nucleus, particularly in baculovirus ring zone. Fluorescence was observed in cytoplasm near the nucleus and in the ring zone of cells infected by PH-GFP-P74TM rBV, rather than PH rBV (Figure 3A). The purified ODVs and separated components were tested by Western blot to detect where GFP-P74TM was distributed. P74 was detected in both purified ODVs and in both envelope components, and VP39 was detected in both purified ODVs and in both capsid components, which showed the ODVs purification and components separation were successful. GFP-P74TM was found to be distributed in purified PH-GFP-P74TM ODV and its envelope component (Figure 3B). The results suggested that P74TM could localize GFP to the ring zone and package GFP to the ODV envelope.

5. DISCUSSION

Baculovirus surface display has been widely used in many fields, although there are still some problems in use. Here, we did some improvements to optimize the system and make it applied better.

Previous reports showed that the homologous region 3 (*hr3*) sequence of BmNPV could enhance the transcription of baculovirus early phase promoters and non-virus promoters (18). *Hrs* are composed of repeats with a core of 30 bp imperfect palindrome, and are origins of DNA replication and transcriptional enhancers in many kinds of baculoviruses (19-22). The cis-linked *hrs* enhanced transcription of early promoters by binding to IE1, a baculovirus immediate-early protein (23). In this study, we chose two late phase promoters, promoters of GP64 and VP39, which were the main envelope and capsid proteins of baculovirus, and compared their effects on display with Pph. The results showed that *hr3* enhanced the activity of Pph, and obviously improved the display. However, Pph was a very late phase promoter and its

Table 1. Neutralizing antibody levels of mice sera immunized by HA-displayed baculoviruses.

50%-virus-inhibited sera dilution	Bac	Pph-HA-GP64	Pph-HA-vsvG	Pph-HA-vsvG-*hr3*
Less than 20	10			
20-50		9		
50-100		1		
100-150			2	
150-200			3	
200-250			4	4
250-300				1
More than 300			1	5
Total	10	10	10	10

The serum dilutions which inhibited 50% influenza virus infection were calculated and sorted.

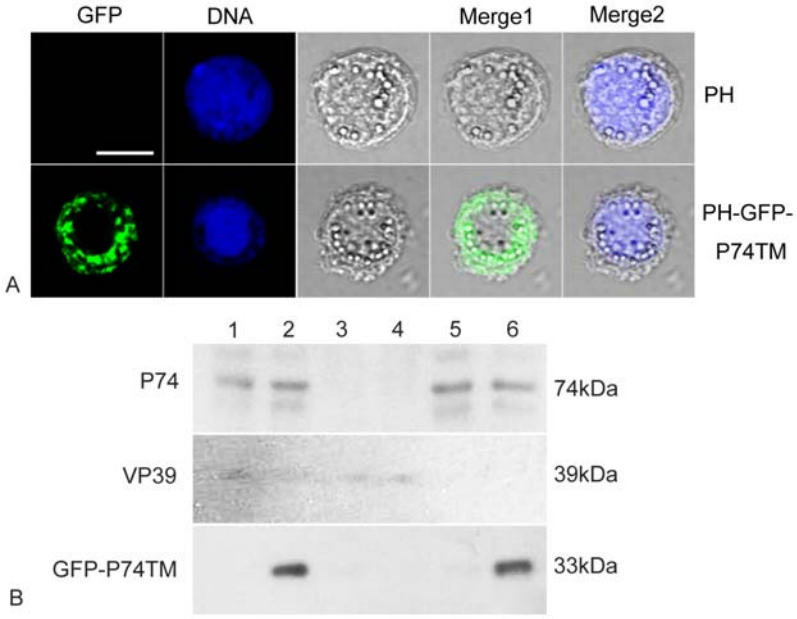

Figure 3. Distribution of GFP-P74TM fusion protein in infected cells and ODV. A. The fluorescence showed the localization of GFP-P74TM in infected cells. Horizontal bars represented 10 μm. B. GFP-P74TM in ODV was tested by Western blot.

transcription was not affected by IE1, therefore, there may be another mechanism to relate *hr3* to Pph.

Here, we used the HA protein of H5N1 influenza virus to test the immune effect of rBVs. The outbreaks of H5N1 have caused great concern throughout the world as the mortality rate is nearly 60% (24). HA is a primary antigenic protein of influenza A virus, and inoculation with HA protein is known to induce neutralizing antibody and prevent influenza virus infection (25). The results of Western blot showed the HA0 band was larger than both the calculated molecular weight and the HAect produced in *E. coli*, which indicated a possible post-translational modification on HA. The HA1 expressed in baculovirus system was a little smaller than the HA1 expressed in H5N1-infected MDCK cells. One possible reason is that the reported defect in the N-linked glycosylation of baculovirus system (26). HA displayed on Pph-HA-vsvG was more than Pph-HA-GP64, which was consistent with the previous results (27), and HA displayed on Pph-HA-vsvG-*hr3* was more than Pph-HA-vsvG, which confirmed that *hr3* could enhance the display of Pph vector.

The results of immune assay indicated that Pph-HA-vsvG-*hr3* could induce the highest level neutralizing antibodies against HA among the three rBVs, which suggested that a higher percent of HA could induce more effective immune response against HA, and meant that less rBVs were needed to get the same immune effect. The purified rBVs were particles with 0.1μm in diameter, and could induce immune response without an adjuvant. Immunization with fewer antigens and without adjuvant would be less harmful to animals. It has been reported that BV inoculation could introduce non-special immune reaction to protect animals from the influenza virus attack (28). The recombinant BmNPVs produced from pPph-HA-GP64 have been proven to be a safe and effective H5N1 vaccine candidate (29). Here, the results showed that Pph-HA-vsvG-*hr3* was more effective than Pph-HA-GP64 in inducing immune response against HA. In summary, Pph-HA-vsvG-*hr3* rBV appeared to be a good candidate for effective vaccine against influenza virus.

Almost 80% of baculovirus products are embedded in occlusion bodies. However, in existing methods, these

products are all discarded, which causes the low yield of baculovirus surface display. As there was more and more research on ODV, the possibility of ODV display was discussed (30). In this study, we proposed a method to display GFP on ODV by using the TM of P74. P74, an ODV envelope protein of AcMNPV, was essential for primary infection process (31). The IgG-binding Z domains could be displayed on ODV by fused with P74 (32). We displayed GFP on ODV by fusing it with P74TM, and found that GFP-P74TM proteins were localized in both ring zone and ODV envelope component. These results suggested that P74TM, instead of the whole P74, could be an ideal tool for ODV display, and pointed out a direction to use the large amount ODV products.

In summary, we developed baculovirus surface display system in two points: *hr3* could enhance the display of vectors with Pph, and TM of P74 could display GFP on ODV. The optimized rBVs with HA would be a good vaccine candidate for influenza virus prevention. These results could be the complements and extensions for baculovirus surface display technique.

6. ACKNOWLEDGMENT

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Abbreviations: ODV: occlusion-derived virion; TM: transmembrane domain; BV: budding virus; HA: hemagglutinin; BES: baculovirus expression system; SP: signal peptide; CTD: cytoplasmic domain; GFP: green fluorescent protein; FBS: fetal bovine serum; PFU: plaque forming unit; *hr3*: homologous region 3; MCS: multiple cloning sites

Key Words: Baculovirus Surface Display, *hr3*, P74, GP64, H5N1, Transmembrane Domain, BV, ODV, Hemagglutinin

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