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A minimum fragment of polyhedrin for higher expression of foreign proteins in a baculovirus expression system

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ABSTRACT

Previously, we found that baculovirus polyhedrin fragments can be successfully used as a fusion partner for the hyper-expression of target proteins. Here, we minimized the fusion fragment of polyhedrin for higher expression of target proteins in a baculovirus expression vector system (BEVS). Recombinant viruses were generated to express enhanced green fluorescent protein (EGFP) fused with various shortened polyhedrin fragments based on the previously reported region of polyhedrin, amino acids 19 to 110. A dramatic increase in the production of EGFP was observed when polyhedrin amino acids 32 to 59 were fused with it. Additionally, this result was confirmed through the fusion of amino acids 32 to 59 with firefly luciferase and with gD of porcine Aujeszky's disease virus. However, fusion expression with ORF2 of porcine circovirus did not increase the expression efficiency and indicated that the efficiency of fusion expression with polyhedrin fragments may depend on the target protein. In conclusion, we suggest that polyhedrin amino acids 32 to 59 are a new fusion partner for hyper-enhanced production without affecting the bioactivity of target proteins in the baculovirus expression system.

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Introduction

The baculovirus expression vector system (BEVS) is well known as a foreign protein production platform for producing complex proteins and providing rapid access to biologically active proteins in insect cells or larvae. The proteins produced in the BEVS are used for research and industry purposes. Recently, many of the developments approved for use as animal and human drugs, such as several vaccines for porcine circovirus (Blanchard et al., 2003), human papillomavirus (Harper, 2009), cervical cancer (Harper et al., 2006) and influenza (Cox and Hollister, 2009; Wen et al., 2009), have accelerated the use of BEVS and increased its importance in the medicine field (Cox, 2012). However, low yields of recombinant proteins during baculovirus-based production are frequently encountered. Research efforts over the last decade have been challenged to increase the productivity of the BEVS (Hitchman et al., 2009; Roldao et al., 2011). A variety of enhancer protein fusing expression systems reported to improve protein expression are now available. These systems contain maltose binding protein (MBP) (Pengelley et al., 2006), glutathione S transferase (GST) (Romier et al., 2006), small ubiquitin-like modifier (SUMO) (Liu et al., 2008), KDEL retention signal (Gomez-Casado et al., 2011) and baculovirus polyhedrin fragments (Bae et al., 2013; Marumoto et al., 1987; Roh et al., 2010).

Among these, we previously reported a hyper-enhanced BEVS using *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) polyhedrin fragment-fused target proteins (Bae et al., 2013). This improved BEVS showed that target proteins enhanced green fluorescent protein and classical swine fever virus were dramatically increased by fusion expression with polyhedrin amino acids 19 to 110, and its activity was confirmed using a fluorescence spectrophotometer and experimental guinea pigs. However, there is a need to improve the shorter polyhedrin fragment in this system because the 19 to 110 region of polyhedrin is capable of forming a supramolecular self-assembly into a nuclear occlusion-like particle (Jarvis et al., 1991) and is relatively large in size as fusion tag.

In this study, we generated a number of recombinant AcMNPVs to express fusion forms of enhanced green fluorescence protein (EGFP), firefly luciferase, gD of porcine Aujeszky's disease virus and ORF2 of porcine circovirus with several domains between amino acids 19 to 110 of polyhedrin. The results showed that all of the tested partial polyhedrin fusions diffused the target proteins in the cytosol. Specifically, the fusion with amino acids 32 to 59 showed the highest expression of EGFP compared to the fusion with amino acids 19 to 110 of polyhedrin. However, fusion expression with ORF2 of porcine circovirus could not increase the expression efficiency. Our results suggest that the 32 to 59 amino acid

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region of polyhedrin is the most suitable fusion partner for the production of target protein in BEVS.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* continuous cell line IPLBSF-21 (Sf21 cells) was maintained at 27 °C in SF900 II serum-free medium (Gibco, USA). The AcMNPV-C6 strain was used in this study. Routine cell culture maintenance and virus production procedures were performed according to the published procedures (King and Possee, 1992).

Construction of recombinant transfer vector

All DNA manipulations were performed following standard procedures. Polymerase chain reaction (PCR) was performed with the Takara PCR Thermal Cycler Dice® mini (Takara, Japan). Primers used in this study (Table 1) were synthesized at Bioneer (Bioneer, Korea). The constructs were cloned into pBacPAK9 (Clontech, USA), downstream of the polyhedrin promoter. The previously constructed pB9-EGFP (Bae et al., 2013) and pB9-19-110-EGFP (Bae et al., 2013) were used as PCR template DNA in this study. Polyhedrin 32 to 110 and 86 to 110 coding region with EGFP were amplified from pB9-19-110-EGFP using the PCR primers Polh 32-F, Polh 86-F and EGFP-R and cloned into the *Eco*RI and *Pst*I sites of pBacPAK9, resulting in pB9-32-110-EGFP and pB9-86-110-EGFP for rAc32-110-EGFP and rAc86-110-EGFP. Specific deletions within polyhedrin fragments 19-59, 19-35 and 60-85 were generated from pB9-19-110-EGFP by PCR and splicing by overlap extension (SOE), as previously described (Warrens et al., 1997). Briefly, two nested primers were designed to be complementary and carried two segments corresponding to flanking sequences upstream and downstream of the region to be mutated. The sequences of the nested primers used in the PCR reactions utilized the PCR primer sets Polh 59-R (P2) and Polh 59-F (P3); Polh 35-R (P2) and Polh 35-F (P3); Polh 59/86-R (P2) and Polh 59/86-F (P3); and Polh 85-R (P2) and Polh 85-F (P3). Each of the primers was used in reactions with upstream or downstream outside primers Polh1 19-110-F (P1), Polh 60-F (P1) or EGFP-R (P4). The products were then mixed, denatured and used as the template in a third PCR amplification using only the outside primers (P1, P4). The final PCR products were cloned into the intermediate plasmid, and then, each partial polyhedrin with the EGFP fragment was cloned into the *Eco*RI and *Pst*I sites of pBacPAK9, resulting in pB9-19-59-EGFP, pB9-19-35-EGFP, pB9-60-85-EGFP and pB9-32-59 + 86-110-EGFP for rAc19-59-EGFP, rAc19-35-EGFP, rAc60-85-EGFP and rAc32-59 + 86-110-EGFP, respectively. Polyhedrin 32 to 59 coding region with EGFP was amplified from pB9-19-59-EGFP using the PCR primers Polh 32-F and EGFP-R and cloned into pBacPAK9, resulting in pB9-32-59-EGFP for rAc32-59-EGFP.

The gD and ORF2 genes from the Aujeszky's disease virus (ADV) and porcine circovirus type 2 (PCV2) were generated by PCR with the primers ADV gD-F and ADV gD-R and PCV2-ORF2-F and PCV2-ORF2-R, respectively. The previously constructed pB9-PCV2ORF2 (Lee et al., 2012) containing ORF2 gene and pET-gD (Yun et al., 2011) containing gD gene were used as PCR template DNA in this study. The firefly, *Photinus pyralis*, luciferase gene was amplified from the pGL3-Control vector (Promega, USA) using primers Luc-F nd Luc-R. Plasmids pB9-ADV-gD, pB9-Luciferase and pB9-PCV2-ORF2 were constructed with synthesized DNAs and cloned into the *Eco*RI and *Pst*I sites of pBacPAK9 and the PCV2-ORF2 gene was inserted into the *Bgl*II and *Xho*I sites. The polyhedrin 19 to 110 and 32 to 59 coding regions were amplified using the PCR primer set Polh2 19-110-F/R and Polh2 32-59-F/R, respectively. Both PCR products were digested with the *Bgl*II-*Eco*RI and individually ligated it to *Bam*HI-*Eco*RI digested pBacPAK9 (*Bgl*II and *Bam*HI have compatible cohesive ends). And then, the gD, luciferase and PCV2-ORF2 genes were subcloned into two expression vector. Resulting in pB9-19-110-ADV-gD, pB9-19-110-PCV2-ORF2, pB9-19-110-Luc, pB9-32-59-ADV-gD, pB9-32-59-PCV2-ORF2 and pB9-32-59-Luc for rAc19-110-ADV-gD, rAc19-110-PCV2-ORF2, rAc19-110-Luc, rAc32-59-ADV-gD, rAc32-59-PCV2-ORF2 and rAc32-59-Luc, respectively.

Table 1
Primers used for the amplification in this study.

Name of primer[a]	Primer sequence[b]
EGFP-R (P4)	**CTGCAG**TTACTTGTACAGCTCGTCCATGCC
Polh1 19-110-F (P1)	**GAATTC***ATA*ATGAAGTACTACAAAAATTTAGGTG
Polh 32-F (P1)	**GAATTC***ATA*ATGAAGCGCAAGAAGCACTTC
Polh 86-F	**GAATTC***ATA*ATGGTCGTTGGATGGAA
Polh 59-R (P2)	GCTCACCATGGTAGGATCCTCAGCCACTAGG
Polh 59-F (P3)	GCTGCGGATCCTACCATGGTGAGCAAGGG
Polh 35-R (P2)	CTTGCTCACCATGGTCTTCTTGCGCTTAGCG
Polh 35-F (P3)	AAGCGCAAGAAGACCATGGTGAGCAAGG
Polh 59/86-R (P2)	CTTTCCATCCAACGACAGGATCCTCAGCCAC
Polh 59/86-F (P3)	GCTGAGGATCCTGTCGTTGGATGGAAAG
Polh 85-R (P2)	GCTCACCATGGTAAGCTTCATCGTGTCGG
Polh 85-F (P3)	ACGATGAAGCTTACCATGGTGAGCAAGGG
Polh 60-F (P1)	**GAATTC***ATA*TTCCTGGGACC
ADV gD-F	**GAATTC**ATGCTGCTCGCAGCGCTATTG
ADV gD-R	**CTGCAG**TTACGGACCGGGCTG
PCV2-ORF2-F	**AGATCT**ATGACGTATCCAAGGAG
PCV2-ORF2-R	**CTCGAG**TTAGGGGTTAAGTGGG
Luc-F	**GAATTC**ATGGAAGACGCCAAAAAC
Luc-R	**CTGCAG**TTACACGGCGATCTTTCC
Polh2 19-110-F	**AGATCT***ATA*ATGAAGTACTACAAAAATTTAGGTG
Polh2 19-110-R	**GAATTC**AACAATGGGGAAGCTGTCTTC
Polh2 32-59-F	**AGATCT***ATA*ATGAAGCGCAAGAAGCACTTC
Polh2 32-59-R	**GAATTC**AGGATCCTCAGCCAC

[a] For each truncation construct, forward primer (F) is in the forward orientation relative to the coding strand, and reverse primer (R) is in the reverse orientation relative to the coding strand.
[b] Restriction enzyme site shown in bold; Kozak consensus translation initiation sequence shown in italics.

Generation of recombinant baculoviruses and expression of recombinant proteins

Recombinant AcMNPVs were generated by co-transfection with each recombinant transfer plasmid and a defective viral genome, bAcGOZA DNA (Je et al., 2001). Transfection was performed using Cellfectin II™ (Invitrogen, USA) reagent according to the manufacturer's instructions, and the recombinant viruses were purified and propagated in Sf21 cells as previously described (O'Reilly et al., 1992). For expression studies, Sf9 cells were infected with recombinant baculoviruses at 5 MOI (multiplicity of infection) and then incubated for 3 days. The expressed recombinant proteins form the infected cell lysates were analyzed by SDS-PAGE and Western blot analysis using EGFP monoclonal antibody (abm, Canada) and luciferase monoclonal antibody (Cell Signaling, USA).

Fluorescence spectrometer

Wild-type and EGFP fusion recombinant AcMNPV-infected cells were harvested by centrifugation at 1000 $\times g$ for 10 min, and the cell pellet was resuspended in 1 ml of PBS after washing with the same buffer. The lysate was prepared by incubating the cells with 900 µl of lysis buffer for 30 min on ice followed by sonication. Immediately, 100 µl of 1 M sodium carbonate was added, and the resulting mixture was incubated at 37 °C for up to 1 h. Then, 2 ml of PBS was added. Measurements were performed at room temperature in quartz cuvettes with a minimum test volume of 3 ml. The fluorescence intensity of the resulting mixture samples was measured using a K2™ fluorescence spectrometer (ISS Inc., USA) with an excitation filter of 450 nm and an emission filter of 510 nm. A minimum of three trials was conducted.

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