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Expression of an RSV-gag virus-like particle in insect cell lines and silkworm larvae

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A B S T R A C T

Rous sarcoma virus group antigen protein-based virus-like particles (VLPs) are well known for their structural integrity and ease of handling. VLPs play an important role in drug delivery systems because they can be manipulated with ease. In this study, a new method was established for expressing *Rous sarcoma* virus group antigen protein based VLPs in silkworm larvae and establishing stably expressing insect cell lines. These VLPs have been isolated by ultracentrifugation using a sucrose step gradient of 10–60% (v/v), and their spherical structure has been confirmed using transmission electron microscopy (TEM). The spherical morphology is similar in both the silkworm larvae and in stably expressing cell lines. Silkworm larvae are better suited for producing *Rous sarcoma* virus group antigen protein-based VLPs on a large scale; yields from silkworm larvae were approximately 8.2-fold higher than yields from stable cell lines. These VLPs provide a new method for large-scale application in vaccine development and drug delivery systems.

1. Introduction

The *Rous sarcoma* virus (RSV) species, which belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae,* and genus *Alpharetrovirus* of single-stranded RNA viruses, was one of the first viruses found to cause cancer in chickens. Considerable research has been undertaken to elucidate the structure of the group antigen protein (gag), which is important for the assembly and packaging of the RNA into the virus and subsequent pinching from the host cell membrane. This protein is composed of mature matrix (MA), capsid (CA) and nucleocapsid (NC) proteins required to package the RNA genome and later cleaved off by viral proteases (Ma and Vogt, 2002). RSV-gag codes for a protein 701 amino acids in length that dimerizes on the cellular membrane to form VLPs in the range of 30–120 kDa. Research on RSV-gag has become more relevant due to the conserved nature of this protein, which is found in other viruses belonging to the retroviridae family.

Retrovirus-based vaccines have the potential to become the next generation of drug delivery systems (DDS). Retroviruses are known for their ability to enter the host cell utilizing the viron, which has evolved the ability to cross the host plasma membrane barrier efficiently (Waelti and Gluck, 1998). To date, viral-based gene therapy has been the focus of research and improvement but is not yet a well-established field; the use of only the virion or the viral envelope as a container for the transport of therapeutics to a cell or tissues has not been exploited efficiently (Kondo et al., 2008). VLPs are capsid proteins minus the genetic material of the virus; in short, they are hollow viral protein cages. Capsid proteins from almost any virus, minus the genetic material, have the innate ability to fold and self-assemble as VLPs when utilized in an expression system. This property has been of great interest from the standpoint of vaccine development (Roy and Noad, 2008). Neutralizing a threat from an epidemic virus like influenza is very important. This virus has the ability to mask its surface proteins, such as hemagglutinin (HA), very quickly and resurface thus causing a very high mortality rate (Fanning and Taubenberger, 1999). Presenting these active components of the virus for the development of antibodies or vaccines for treatment and detection is of prime importance. Concerns regarding the safety of recombinant viruses have prompted

Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; DDS, drug delivery system; ER, endoplasmic reticulum; QD525, Quantum dot 525 nm; RSV-gag, *Rous sarcoma* virus-group antigen; TEM, transmission electron microscope; VLPs, virus like particles.

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increasing interest in viral systems that carry no genetic material; VLPs are prime candidates for DDSs because they carry no genetic material.

Insect cells are good hosts and equally better expression systems to express different proteins for commercial and research applications that utilize baculovirus (Dojima et al., 2009). Until now, many researchers have used baculovirus expression systems to express virus like particles (VLPs) for use as vaccine delivery systems (Keller et al., 2008; Noad and Roy, 2003; Wills et al., 1989). Recently, silkworms have evolved as an efficient expression system able to express proteins at high levels using the *Bombyx mori* nucleopolyhedrovirus (BmNPV) species belonging to the *Baculoviridae* (Kato et al., 2010) family of double-stranded DNA viruses. It takes almost one month to express and purify milligram amounts of any protein using the BmNPV bacmid-based expression system in silkworm larvae (Dojima et al., 2010). Injecting two or more bacmids with two or more subunit proteins allows for co-expression and subsequent formation of complete functional proteins that can then be purified. Silkworm larvae are completely domesticated insects and they have been used for many centuries without affecting humans.

The current study focuses on the need to establish a system composed of stable insect cell line and silkworm-based expression systems to express a complete RSV-gag of approximately 75 kDa theoretical molecular weight, as well as RSV-gag lacking the PR region of approximately 61 kDa theoretical molecular weight. The short RSV-gag-577 codes for 577 amino acids, and its ability to form VLPs is well documented (Joshi and Vogt, 2000). Silkworms are able to express VLPs, and serve as a more potent system for the expression of VLPs in the future as compared to other bacterial systems (Yu et al., 2001). This is also important for large-scale application of VLPs; combining the robustness of an insect cell expression system with VLPs can make for an efficient vaccine/DDS.

2. Materials and methods

2.1. Cell line, medium, silkworm larvae and culture

Tn-5B1-4 cells from *Trichoplusia ni* were purchased from Invitrogen (San Diego, CA, USA) and grown in 25 cm^2 tissue culture flasks (Falcon) and 100 ml flasks. Suspension cultures were set up in 100 ml flasks with a working volume of 15 ml in Express Five serum-free medium (SFM) (Invitrogen) supplemented with 1% (v/v) antibiotic–antimycotic (Invitrogen) (Deo and Park, 2006). A scale-up of the suspension culture was done under similar culture conditions using 500 ml flasks with culture volumes of 100 ml. Fifth instar larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were reared on an artificial diet (Silkmate S2, Nihon Yokohama, Japan) in a chamber (MLR-351H, Sanyo, Tokyo, Japan) at 27 °C and 65% humidity.

2.2. Construction of vectors

The pIB/V5-His-Dest Gateway vector was used for stable expression in insect cells (Invitrogen, Carlsbad, CA, USA). The *RSV*-gag cDNA was PCR amplified using pRep(A) (ATCC catalogue number 87702) as a template. The *RSV-gag*-577 insert was PCR amplified using the following primers: Forward primer, 5′-CACC ATGGAAGCCGTCATAAAGGTG-3′; reverse primer, 5′-TTA CGA GAC GGC AGG TGG CTC AGG-3′. The *RSV-gag*-701 insert was PCR amplified using the following primers: Forward primer, 5′-CACC ATGGAAGCCGTCATAAAGGTG-3′; reverse primer, 5′-CTA TAA ATT TGT CAA GCG GAG-3′. PCR products were inserted into the entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) to give pENTR/RSV-gag-701 and pENTR/RSV-gag-577, respectively. The sequences of all the PCR fragments inserted into pENTR/D-TOPO were confirmed by dideoxynucleotide chain termination sequencing (Sanger et al., 1977) using the Thermo Sequenase Cycle Sequencing kit (USB, Cleveland, Ohio, USA). pENTR/RSV-gag-577 was used to prepare the expression plasmid, pIB/V5-His-RSV-gag-577, via the LR reaction as per the protocol of stable cell line technology (Invitrogen).

For expression of RSV-gag in silkworm larvae, the BmNPV bacmid was used. *RSV-gag*-701 and *RSV-gag*-577 from pENTR/RSV-gag-701 and pENTR/RSV-gag-577, respectively, were transferred to pDEST8 via the LR reaction according to the manufacturers' protocol to make pDEST8/RSV-gag-701 and pDEST8/RSV-gag-577 respectively. These constructs were then used to transform DH10 BmNPV *Chi*$^-$-*CP*$^-$ competent cells to make recombinant bacmids (Deo et al., 2006). White colonies of recombinant bacmids carrying the *RSV-gag*-701 and *RSV-gag*-577 were isolated and the resulting bacmids were designated as BmNPV bacmid/RSV-gag-701 and BmNPV bacmid/RSV-gag-577, respectively. These were then inoculated into 3 ml LB medium containing the antibiotics used for screening. After culturing in LB medium with antibiotics for 36 h, the BmNPV bacmids were isolated and confirmed using standard M13 primers. Positive transformants were re-inoculated in 100 ml LB medium and a large amount was isolated for injection into fifth instar silkworm larvae.

2.3. Injecting silkworms

Each silkworm was injected with 40 μl of recombinant bacmid DNA solutions containing 10 μg of BmNPV bacmid DNA and 10% (v/v) DMREI-C reagent (Invitrogen) in PBS using 1-ml syringes. On the 7th day post-injection, the silkworm's hemolymph was harvested in falcon tubes containing 2 mM phenylthiourea to inhibit melanization. These samples were then aliquoted into 1 ml Eppendorf tubes and stored at −80 °C.

2.4. Serial screening of stable cell lines

Tn-5B1-4 cells were seeded into 6 well plates at 1×10^4 cells per well and allowed to grow overnight in the incubator. The cells were washed with serum-free medium and then overlaid with 1 ml containing vectors and Lipofectin mixed as per the kit protocol (Invitrogen). The cells were then incubated for 4 h in the incubator with mild shaking at intervals of 30 min. After 4 h, the medium was removed and replaced with fresh Express Five SFM. These cells were incubated and allowed to grow for 2 days. After 2 days, Express Five SFM containing 80 μg/ml Blasticidin (Invitrogen) was added, and the medium was replaced every 3rd day. The foci of resistant polyclonal stable cell lines were picked up carefully, maintaining homogeneity for each transformed cell line, and seeded separately into 96-well plates (Falcon, Lincoln Park, NJ, USA) (Deo and Park, 2006). After 1 week, cells exhibiting confluent growth were seeded into 24-well plates with a 2-cm^2 surface area (Falcon). After 2 weeks, cells exhibiting confluent growth were selected, and their supernatant was removed and exchanged with fresh medium. The supernatant was used for western blot analysis and positive cell lines were selected and further seeded into six-well plates with a 9.6 cm^2 surface area (Falcon).

2.5. Genomic DNA extraction and confirmation by PCR

Genomic DNA was extracted from the transformed cells using a FlexiGene DNA kit (Qiagen, Valencia, CA, USA). For analysis of the genomic *RSV-gag* cDNA, PCR was performed using the primers used previously for amplifying the cDNA under similar conditions. PCR products were resolved on 0.75% (w/v) agarose gels by electrophoresis.