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RESEARCH PAPER

Production of Influenza Virus-like Particles from Stably Transfected *Trichoplusia ni* BT1 TN-5B1-4 Cells

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Abstract We investigated the production of influenza A/Korea/01-2-9/2009 (H1N1) virus-like particles (VLPs) containing four structural proteins, matrix protein 1 (M1), matrix protein 2 (M2), neuraminidase (NA) and hemagglutinin (HA), using stably transfected *Trichoplusia ni* BT1 TN-5B1-4 (TN-5B1-4) cells. Recombinant M1, M2, NA and HA were expressed as bands with molecular weights of 28, 17, 60, and 70 kDa, respectively, in stably transfected TN-5B1-4 (TN-5B1-4/M1-M2-NA-HA) cells. VLPs were purified from the culture medium of the TN-5B1-4/M1-M2-NA-HA cells by pelleting on a sucrose cushion, followed by ultracentrifugation in a sucrose density gradient. The four structural proteins were released together from the TN-5B1-4/M1-M2-NA-HA cells, and were co-purified from the same fractions of the sucrose density gradient, indicating that recombinant M1, M2, NA, and HA self-assembled into VLPs in the TN-5B1-4/M1-M2-NA-HA cells. Recombinant baculoviruses (rBac/NA and rBac/HA) expressing recombinant NA and HA were generated and used to co-infect stably transfected TN-5B1-4 (TN-5B1-4/M1-M2) cells expressing recombinant M1 and M2, resulting in the production of high-molecular-weight VLPs and the release of self-assembled VLPs with diameters of 80 ~ 120 nm. The production of VLPs was greatly enhanced in the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA, compared with the TN-5B1-4/M1-M2-NA-HA cells. Our results suggest that a stably transfected-insect cell expression system might be useful for producing VLPs for the development of recombinant vaccines against influenza.

Keywords: *Trichoplusia ni* BT1 TN-5B1-4 cells, stable transfection, influenza, virus-like particles

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1. Introduction

Influenza is a serious respiratory disease caused by the influenza virus, which contains a segmented negative-sense RNA and belongs to the family *Orthomyxoviridae* [1]. The influenza virus is easily spreads as an aerosol and causes seasonal epidemics, resulting in about 3 ~ 5 million cases of severe illness and 250,000 ~ 500,000 deaths annually, which can rise to millions in a pandemic. Vaccination is the most effective method of preventing influenza infection. A method based on embryonated chicken eggs is commonly used to produce influenza vaccines. However, this method requires about 4 ~ 6 months for vaccine production, which could be fatal in a pandemic [2,3]. Supplying sufficient embryonated chicken eggs might also be difficult in the case of an emergency, or may be inappropriate if the influenza strains to be vaccinated against are lethal to birds [4]. Local or systemic allergic responses to residual egg proteins in the vaccines can also occur in some individuals. Therefore, new egg-independent strategies are required for the production of influenza vaccines. Several egg-independent methods of vaccine production, including cell-culture-derived whole

virus or subvirion vaccines, recombinant-protein-based vaccines, virus-like-particle vaccines, and DNA vaccines, have been developed [2,5]. Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of native viruses [6]. VLPs lack the viral genome, potentially yielding safer and cheaper vaccines. Influenza VLPs have been produced in several expression systems, including mammalian cells and baculovirus-infected insect cells [7,8]. Influenza VLPs induce high neutralizing antibody titers and strong protective immunity [9,10]. Intranasal immunization with influenza VLPs that include hemagglutinin (HA) induced mucosal immunoglobulin G and cellular immune responses, which were reactivated rapidly by viral challenge. Immune sera administered intranasally confered 100% protection from a lethal challenge with influenza virus [9]. Influenza VLPs containing matrix protein 1 (M1), matrix protein 2 (M2), neuraminidase (NA) and HA display functional characteristics of influenza virus, including its hemagglutination and neuraminidase activities, and elicit serum antibodies specific for influenza virus, which inhibit the replication of the influenza virus after challenge [10]. Influenza VLPs also induce broad-spectrum cross-protective antibody-based immunity against different subtypes of influenza viruses [11,12].

The recombinant baculovirus-infected insect cell expression system expressed high expression levels of recombinant proteins, allowing the large-scale manufacture of heterologous recombinant proteins [13]. Several VLPs have been produced *via* recombinant baculovirus-infected insect cell expression systems, and have displayed high immunogenicity, inducing both neutralizing antibodies and cellular immune responses [14]. Stably transfected insect cells also have a number of potential advantages over mammalian cells in the production of heterologous recombinant proteins [15]. Although influenza VLPs have been produced in insect cells using baculovirus-based expression systems, the production of influenza VLPs in stably transfected insect cells has not yet been investigated.

Trichoplusia ni BT1 TN-5B1-4 (also known as High Five; TN-5B1-4) cells, originating from the ovarian cells of the cabbage looper, are one of insect cells most wildly used for the production of recombinant proteins after the stable integration of exogenous vector constructs [16,17]. TN-5B1-4 cells have been reported to produce high amounts of protein, up to 20-fold higher than *Spodoptera frugiperda* 9 (Sf9) cells [18]. TN-5B1-4 cells produced influenza VLPs in a baculovirus expression system more efficiently than Sf9 cells [19], and are also suggested to be an excellent insect cell line for the production of influenza VLPs. To the best of our knowledge, the production of influenza VLPs in stably transfected TN-5B1-4 cells has not been reported. Here, we investigated the production of influenza VLPs composed of four structural proteins, M1, M2, NA, and HA, from influenza A/Korea/01-2-9/2009 (H1N1) in stably transfected TN-5B1-4 cells.

2. Materials and Methods

2.1. Cell line, plasmids, and enzymes

TN-5B1-4 cells were grown at 27ºC in a T-25 culture flask (Nunc, Roskilde, Denmark) in Express Five SFM (serum-free medium; Gibco BRL, Grand Island, NY, USA). The TN-5B1-4 cells were a gift from Dr. Granados of the Boyce Thompson Institute for Plant Research (Ithaca, NY, USA). *Spodoptera frugiperda* 9 (Sf9) cells were grown at 27ºC in a T-25 culture flask in Grace insect cell culture medium (Gibco BRL) with 10% (v/v) fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA). pIZT-V5-His (3.3 kb; Invitrogen, Carlsbad, CA, USA) and pCoHygro (4.5 kb; Invitrogen) were used as the basic plasmids to construct pIZTx2 and pICHTx2, respectively, which contain dual *Orgyia pseudotsugata* immediate-early 2 (*OpIE2*) promoter and terminator. pIZT/V5-His contains the *OpIE2* promoter, a V5 epitope tag, a polyhistidine region, an OpIE2 terminator and a zeocin-resistance gene under the control of the *OpIE1* and *EM7* promoters. pCoHygro contains the bacterial hygromycin B phosphotransferase (*hyg*) gene under the control of the constitutive *Drosophila* Copia 5' long terminal repeat (LTR) promoter. pIZTx2 was developed to generate stably transfected TN-5B1-4 cells expressing recombinant M1 and M2. pICHTx2 was developed to express recombinant NA and HA from stably transfected TN-5B1-4 cells expressing M1 and M2. pFastBac1 (4.8 kb; Invitrogen) was used to establish recombinant baculoviruses. pFastBac1 contains transposition elements Tn7R and Tn7L, a polyhedron promoter, SV40 terminator, and a gentamycin-resistance gene. The *Escherichia coli* DH5a strain was used as the primary host in which to construct and propagate the plasmids. The *E. coli* DH10Bac strain containing a baculovirus shuttle vector (bacmid) and a helper plasmid was used to generate recombinant bacmids, after the transposition of the pFastBac1 expression constructs. *Eschericha coli* DH5α transformed with pIZTx2, pICHTx2, or pFastBac1 was routinely grown and maintained in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.3) containing antibiotics, with agitation at 37ºC. DNA restriction enzymes from Promega (Madison, WI, USA) or Takara (Shiga, Japan) were used according to the manufacturer's instructions.

2.2. Constructions of expression plasmids

The RNA genome of influenza virus A/Korea/01-2-9/2009

(H1N1) was provided by the Center for Genome Science, National Institute of Health (Korea). Full-length *M1*, *M2*, *NA*, and *HA* DNA fragments were amplified with reverse transcription–polymerase chain reaction (RT-PCR) using the One-Step RT-PCR System (Invitrogen) and oligonucleotide primers (*M1* sense 5'-ACTAGTATGGCTAGTC TTCTAACCGAGGTCGAAACGTACGTTCTTTC-3', *M1* antisense 5'-GAATTCTCACTTGAATCGCTGCATCTGC ACTCCCATTCG-3'; *M2* sense 5'-GCGGCCGCCATGGC TAGTCTTCTAACCGAGGTCGAAACG-3', *M2* antisense 5'-TCTAGATTACTCTAGCTCTATGTTGACAAAATGA CC-3'; *NA* sense 5'-ACTAGTATGGCTAATCCAAACCA AAAGATAATAACC-3', *NA* antisense 5'-GAATTCTTAC TTGTCAATGGTAAATGGCAACTCAGC-3'; and *HA* sense 5'-GCGGCCGCGCTATGGCTAAGGCAATACTAGTAG TTCTGC-3', *HA* antisense 5'-CCGCGGTTAAATACATAT CCTACACTGTAGAGACCC-3'). PCR was performed in a thermal cycler (PE Biosystems, Foster City, CA, USA) using LA Taq Polymerase (Takara) in a 50 μL volume. The amplified *M1*, *M2*, *NA*, and *HA* fragments were inserted into a T/A cloning vector, pGEM-T (Promega), to yield pGEM-T/M1, pGEM-T/M2, pGEM-T/HA, and pGEM-T/NA.

To construct pIZTx2, the *OpIE2* promoter and terminator fragments were amplified with PCR from pIZT-V5-His (*OpIE2* promoter sense 5'-GATATCTCATGATGAT AAACAATGTATGGTGC-3', *OpIE2* promoter antisense 5'-GCGGCCGCAAGCTTTAAATTCGAACAGATGC-3'; *OpIE2* terminator sense 5'-GAATTCGTTTATCTGACTA AATCTTAGTTTG-3', *OpIE2* terminator antisense 5'-GATATCGAGCATCGATCCCACGCGCTTG-3'). pIZTx2 was constructed by inserting the *Eco*RI- and *Eco*RV-digested *OpIE2* terminator fragment and the *Eco*RV- and *Not*I-digested *OpIE2* promoter fragment between the *Eco*RI and *Not*I sites of pIZT-V5-His (Fig. 1A). The *Spe*I- and *Eco*RI-digested *M1* fragment from pGEM-T/M1 was cloned into the *Spe*I and *Eco*RI sites of pIZTx2 to generate pIZTx2/M1. pIZTx2/M1-M2 was constructed by inserting the *Not*I- and *Xba*I-digested *M2* fragment from pGEM-T/M2 between the *Not*I and *Xba*I sites of pIZTx2/M1 (Fig. 1B). The *Spe*I- and *Eco*RI-digested *NA* fragment from pGEM-T/NA was also cloned into the *Spe*I and *Eco*RI sites of pIZTx2 to generate pIZTx2/NA. pIZTx2/NA-HA was constructed by inserting the *Not*I- and *Sac*II-digested *HA* fragment from pGEM-T/HA between the *Not*I and *Sac*II sites of pIZTx2/NA (Fig. 1C). The *NA–HA* DNA fragment (*pOpIE2-NA-OpIE2 pA-pOpIE2-HA-OpIE2 pA*) including the *OpIE2* promoter and terminator, was amplified with PCR from the pIZTx2/NA-HA plasmid (sense 5'-GCATGCTCATCAATGTATCTTATCATGTCTGGATCA-

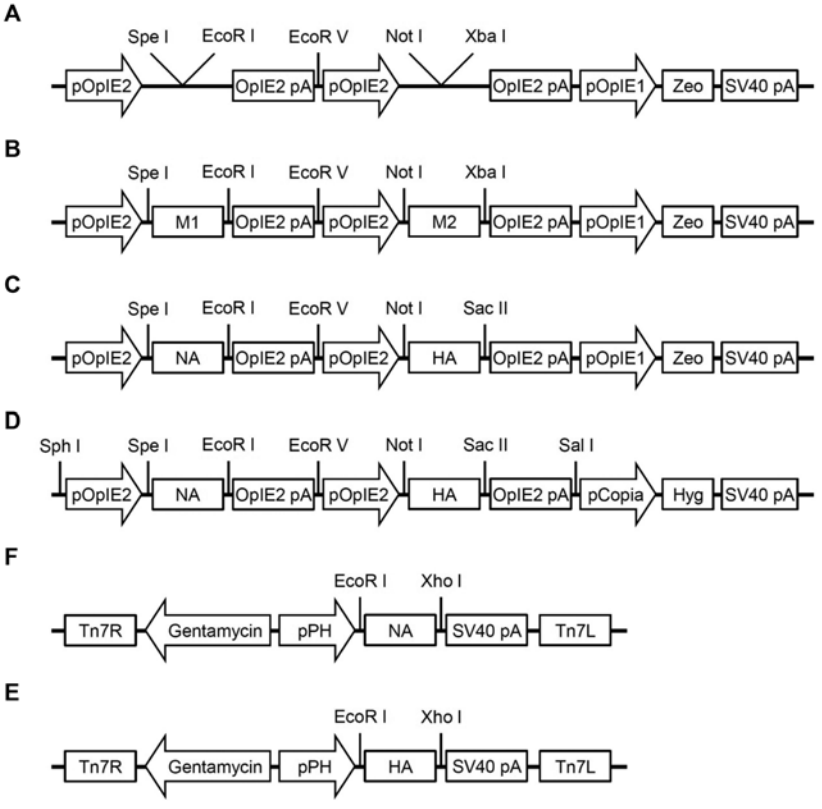

Fig. 1. Schematic representation of the expression plasmids. (A) pIZT/x2. (B) pIZTx2/M1-M2. (C) pIZTx2/NA-HA. (D) pICHTx2/NA-HA. (E) pFastBac1/NA. (F) pFastBac1/HA.

3', antisense 5'-GTCGACCTGTGGATAACCGTATTACC GCCTTTGAGTGAG-3'). pICHTx2 was constructed by the inserting the *Sph*I- and *Sal*I-digested *pOpIE2-NA-OpIE2 pA-pOpIE2-HA-OpIE2 pA* fragment between the *Sph*1 and *Sal*I sites of pCoHygro (Fig. 1D).

To construct the baculovirus expression vectors pFastBac1/HA and pFastBac1/NA, the full-length *HA* and *NA* fragments were amplified with PCR from pGEM-T/HA and pGEM-T/NA, respectively (*HA* sense 5'-GAATTCATGGC TAAGGCAATACTAGTAGTTCTGC-3', *HA* antisense 5'-CTCGAGTTAAATACATATCCTACACTGTAGAGACC C-3'; *NA* sense 5'-GAATTCATGGCTAATCCAAACCAA AAGATAATAACC-3', *NA* antisense 5'-CTCGAGTTACT TGTCAATGGTAAATGGCAACTCAGC-3'). The *HA* and *NA* fragments were cloned into pGEM-T to yield pGEM-T/HA and pGEM-T/NA, respectively. The *Eco*RI- and *Xho*I-digested fragments from pGEM-T/HA and pGEM-T/NA were inserted between the *Eco*RI and *Xho*I sites of pFastBac1 to generate pFastBac1/HA and pFastBac1/NA, respectively (Figs. 1E and 1F). The proper orientation and reading frames of the inserted genes in all the recombinant plasmids were confirmed with both restriction enzyme mapping and DNA sequencing.

2.3. Stable transfection of TN-5B1-4 cells and generation of recombinant baculoviruses

Exponentially growing TN-5B1-4 cells were transfected with pIZTx2/M1-M2 using the lipofectin method as described elsewhere [20]. Stably transfected polyclonal cell populations of TN-5B1-4 cells were isolated after 4 weeks of selection with 200 μg/mL zeocin. To generate stably transfected TN-5B1-4 cells expressing recombinant M1, M2, NA, and HA, exponentially growing TN-5B1-4/M1-M2 cells were transfected with pICHTx2/NA-HA using the lipofectin method. Stably transfected polyclonal cell populations of TN-5B1-4/M1-M2 cells were isolated after 4 weeks of selection with 100 μg/mL hygromycin B in Express Five SFM. The stably transfected TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-NA-HA cells were grown at 27ºC in T-25 culture flasks in 4 mL of Express Five SFM containing 50 μg/mL zeocin and/or 50 μg/mL hygromycin B. TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-NA-HA cells were cultured for 5 days in multiple T-25 culture flasks to analyze the expression of recombinant M1, M2, NA, and HA. The TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-NA-HA cells were centrifuged at 800 × *g* for 5 min to recover the cells. The harvested cells were lysed with RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation at 16,000 × g for 20 min, protein extracts were collected and analyzed with a western blot analysis.

Recombinant bacmids were produced with site-specific homologous recombination following the transformation of competent *E. coli* DH10Bac cells (Invitrogen) with pFastBac1/HA and pFastBac1/NA. The recombinant bacmid DNA was extracted from the recombinant *E. coli* DH10Bac cells and used to transfect Sf9 cells seeded in six-well plates using CellFectin® II Reagent (Invitrogen). The resulting recombinant baculoviruses were collected from the culture medium on day 3 after transfection, as recommended by the manufacturer. After three-repeated infections of the recombinant baculoviruses, the recombinant baculoviruses were recovered from the cells and plaque-purified. Plaque isolates expressing recombinant HA and NA were amplified by infecting Sf9 cells seeded in T-75 culture flasks. Three days after infection, the culture medium was harvested by centrifugation at 800 × *g* for 5 min and stored at 4ºC until use. The titers of the recombinant baculovirus stocks were determined with the BacPAK™ Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA, USA).

2.4. Purification of VLPs from stably transfected TN-5B1-4/M1-M2-NA-HA cells and recombinant-baculovirus-infected TN-5B1-4 cells

To produce VLPs, TN-5B1-4/M1-M2-NA-HA cells were cultured for 5 days in multiple T-75 culture flasks. The recombinant baculoviruses, at a multiplicity of infection (MOI) of 3, were used to infect TN-5B1-4/M1-M2 cells in multiple T-75 culture flasks, which were then cultured for 3 days. The culture media of the TN-5B1-4/M1-M2-NA-HA cells and recombinant-baculoviruses-infected TN-5B1-4/M1-M2 cells were harvested by centrifugation at 800 × *g* for 5 min, filtrated with a 0.45 μm syringe filter (PALL Life Science, Port Washington, NY, USA), and then layered onto a 30% (w/v) sucrose–TNE [10 mM Tris-HCl (pH 4), 100 mM NaCl, 1 mM EDTA] cushion. After ultracentrifugation at 100,000 × *g* for 6 h at 4ºC in an Optima L-100K ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA), the resulting pellet was resuspended in phosphate-buffered saline (PBS; pH 7.2), loaded onto a 30 ~ 60% (w/v) discontinuous sucrose density gradient, and sedimented by ultracentrifugation at 100,000 × *g* for 3 h at 4ºC. The fractions were collected and analyzed with western blotting.

The sucrose density gradient fractions that included the VLPs were pooled and used for gel filtration chromatography with Superose 6 resin (GE Healthcare, Uppsala, Sweden) in PBS (pH 7.2) in a column 15 mm in diameter with a bed volume of 20 mL. The column was equilibrated with PBS and calibrated with dyes, including Dextran Blue 2,000 and cytochrome c, with apparent molecular weights of 2,000 and 12 kDa, respectively (Amersham Biosciences, Piscataway, NJ, USA). An aliquot of the pooled sucrose gradient fractions was loaded onto the column at a flow

rate of 0.2 mL/min. The fractions (2 mL) were collected and analyzed with western blotting.

2.5. Electron microscopy

An aliquot of the pooled sucrose gradient fractions was adsorbed on carbon-coated nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA), and stained with 2% uranyl acetate for 1 min. Excess stain was wicked away with a piece of paper and the sample air dried for 1 ~ 3 min. The stained grids were viewed with a transmission electron microscope (LIBRA 120; Carl Zeiss, Germany) at a magnification of × 100,000.

2.6. Hemagglutination assay

The hemagglutination assay was performed as described in elsewhere [19]. Briefly, a series of 2-fold dilutions of VLPs (50 μL, pelleted eluates of sucrose cushion ultracentrifugation and fractions from sucrose gradient ultracentrifugation) in PBS (pH 7.2) were mixed with 25 μL of 1% (v/v) chicken red blood cells (Innovative Research, Novi, MI, USA) and incubated for 40 min at room temperature. The hemagglutination titer was defined as the highest dilution yielding complete hemagglutination.

2.7. Western blotting analysis

Protein samples (protein extracts of TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-HA-NA, pelleted eluates of sucrose cushion ultracentrifugation, and fractions from sucrose gradient ultracentrifugation and gel filtration chromatography) were separated by electrophoresis on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (PALL Life Science). The membranes were pre-incubated with blocking solution [3% (w/v) non-fat dried milk in PBS-T (PBS with 0.05% (v/v) Tween-20)] for 1 h at room temperature, and then incubated overnight at 4°C with mouse anti-H1N1 M1 monoclonal antibody (1:5,000 dilution in blocking solution; AbD Serotec, Kidlington, UK), rabbit anti-H1N1 M2 polyclonal antibody (1:5,000 dilution in blocking solution; GeneTex, Irvine, CA, USA), mouse anti-H1N1 NA monoclonal antibody (1:5,000 dilution in blocking solution; Sino Biological Inc., Beijing, China), or rabbit anti-H1N1 HA monoclonal antibody (1:2,000 dilution in blocking solution; Sino Biological Inc.). The membranes were washed three times with PBS-T and incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:5,000 dilution in blocking solution; Santa Cruz Biotech., Santa Cruz, CA, USA). After the membranes were washed with PBS-T, the protein bands were detected with enhanced chemiluminescence using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.).

3. Results and Discussion

3.1. Establishment of stably transfected TN-5B1-4 cells expressing recombinant M1, M2, NA, and HA

To establish stably transfected TN-5B1-4 cells expressing the four structural proteins of the influenza virus, two insect expression vectors, pIZTx2/M1-M2 (Fig. 1B) and pICHTx2/NA-HA (Fig. 1D), were designed. The vector pIZTx2/M1-M2 contains two gene expression cassettes for the expression of recombinant M1 and M2, placed under the control of the *OpIE2* promoter. The other vector, pICHTx2/NA-HA, contains two gene expression cassettes for the expression of recombinant NA and HA, also placed under the control of the *OpIE2* promoter. pIZTx2/M1-M2 and pICHTx2/NA-HA also contain zeocin- and hygromycin-B-resistance gene expression cassettes, respectively. Stably transfected TN-5B1-4 cells were obtained by the transfection of cells with pIZTx2/M1-M2, followed by selection with zeocin. The expression of recombinant M1 and M2 was examined in the stably transfected TN-5B1-4 cells. The intracellular proteins were prepared and the presence of recombinant M1 and M2 was determined with a western blot analysis (Fig. 2). Recombinant M1 and M2 were expressed as single bands with molecular masses of approximately 28 and 17 kDa, respectively. The stably transfected TN-5B1-4 cells were designated as TN-5B1-4/M1-M2 and used as the founder cells to establish stably transfected TN-5B1-4 cells expressing the four structural proteins M1, M2, NA, and HA. TN-5B1-4/M1-M2 cells were transfected with the pICHTx2/NA-HA vector, and the stably transfected cells were selected in culture medium containing both zeocin and hygromycin B. The stably transfected TN-5B1-4/M1-M2 cells obtained were designated

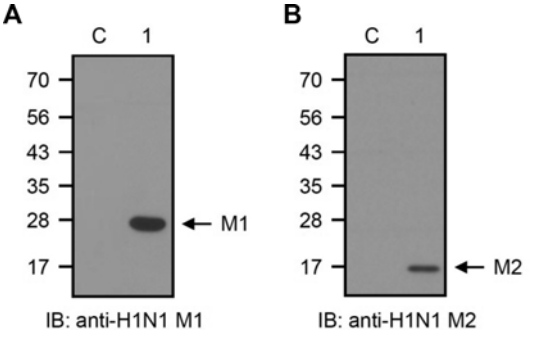

Fig. 2. Expression of recombinant M1 and M2 in TN-5B1-4/M1-M2 cells. Intracellular protein extracts were collected from TN-5B1-4/M1-M2 cells. The expression of recombinant M1 and M2 was confirmed with SDS polyacrylamide gel electrophoresis followed by a western blot analysis using a mouse anti-H1N1 M1 monoclonal antibody (A) and a rabbit anti-H1N1 M2 polyclonal antibody (B). Lane C, intracellular protein extract from untransfected TN-5B1-4 cells; lane 1, intracellular protein extract from TN-5B1-4/M1-M2 cells.

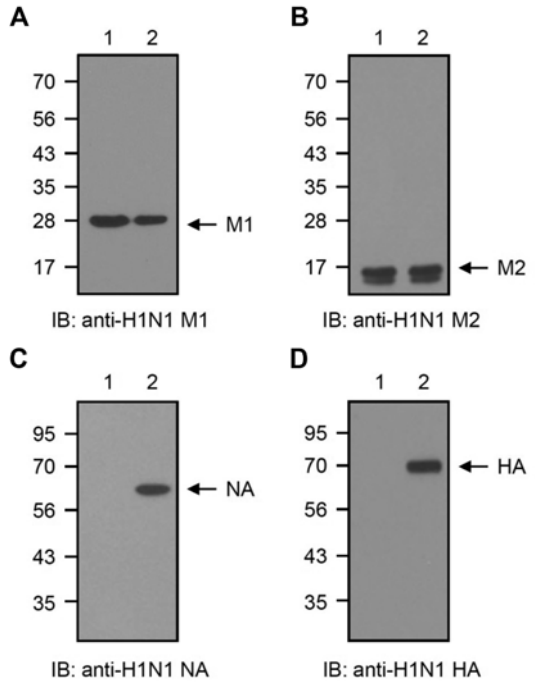

Fig. 3. Expression of recombinant M1, M2, NA, and HA in TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-NA-HA cells. Intracellular protein extracts were collected from TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2-NA-HA cells. The expression of recombinant M1, M2, NA, and HA was determined with a western blot analysis using a mouse anti-H1N1 M1 monoclonal antibody (A), rabbit anti-H1N1 M2 polyclonal antibody (B), mouse anti-H1N1 NA monoclonal antibody (C), or rabbit anti-H1N1 HA monoclonal antibody (D). Lanes 1 and 2, intracellular protein extracts from TN-5B1-4/M1-M2 and TN-5B1-4/M1-M2/NA-HA cells, respectively.

as TN-5B1-4/M1-M2-NA-HA cells. The expression of recombinant M1, M2, NA and HA in the TN-5B1-4/M1-M2-NA-HA cells was examined with western blot analysis (Fig. 3). The expression of the four recombinant envelope proteins, M1, M2, NA, and HA, was detected, and each appeared as a single band with a molecular mass of approximately 28, 17, 60 and 70 kDa, respectively.

3.2. Production of VLPs from stably transfected TN-5B-4/M1-M2-NA-HA cells

To confirm the production of VLPs from TN-5B1-4/M1-M2-NA-HA cells, the cells were cultured for 5 days in T-75 culture flasks. The culture medium was collected and pelleted by ultracentrifugation on a 30% sucrose cushion. The presence of recombinant M1, M2, NA, and HA was then confirmed with a western blot analysis (Fig. 4A). The pelleted eluates included recombinant M1, M2, NA, and HA. When 100 mL of culture medium was ultracentrifuged on a 30% sucrose cushion, about 70 μg of protein was obtained (Table 1). The hemagglutination activity of the pelleted eluates was approximately 100 HAU/mg. To further confirm the production of VLPs, the pelleted eluates were fractionated by ultracentrifugation on a 30% ~ 60% discontinuous sucrose density gradient. The fractions were analyzed with western blotting to detect the four recombinant proteins (Fig. 4B). Recombinant M1 and NA were detected in fractions 4 and 5, corresponding to sucrose densities of 41.3 ~ 45.0%. Recombinant HA was detected in fractions 4, 5, 6, and 7 corresponding to sucrose densities of 41.3 ~ 52.5%, and recombinant M2 was detected in fractions 4, 5, and 6 corresponding to sucrose densities of 41.3 ~ 48.8%. These results demonstrate that all four influenza proteins were released together from the TN-5B1-4/M1-M2-NA-HA cells and were co-purified from the same fractions of the sucrose density gradient, implying that the four influenza proteins self-assembled into VLPs in the TN-5B1-4/M1-M2-NA-HA cells. The influenza VLPs produced in mammalian expression systems contain two

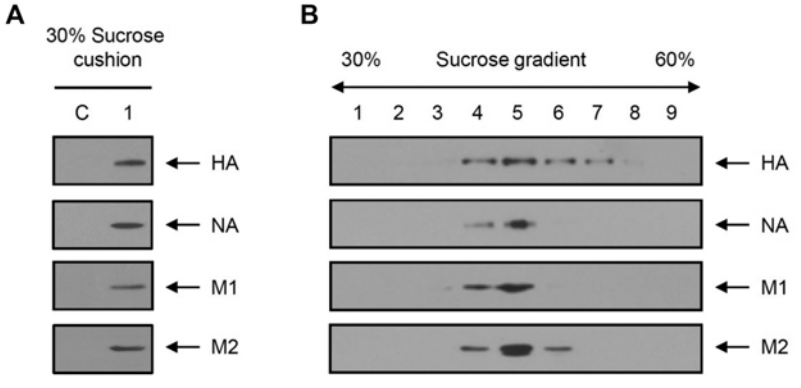

Fig. 4. Purification of VLPs from TN-5B1-4/M1-M2-NA-HA cells by ultracentrifugation. (A) The culture media from TN-5B1-4 and TN-5B1-4/M1-M2-NA-HA cells were collected and pelleted by ultracentrifugation on a 30% sucrose cushion. The presence of recombinant M1, M2, HA, and NA in the pelleted eluates was determined with western blot analyses. Lane C, pelleted eluate of TN-5B1-4 cells; lane 1, pelleted eluate of TN-5B1-4/M1-M2-NA-HA cells. (B) The pelleted eluate was fractionated by 30 ~ 60% sucrose density gradient ultracentrifugation. The presence of recombinant M1, M2, HA, and NA in the fractions was confirmed with a western blot analysis.

Table 1. Comparison of influenza VLP production and hemagglutination activity in TN-5B1-4/M1-M2-NA-HA cells and TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA

	Culture volume (mL)	Precipitation on 30% sucrose cushion	
		Protein content (mg)	Hemagglutination activity (HAU/mg)
TN-5B1-4/M1-M2-NA-HA	100	0.07	100
TN-5B1-4/M1-M2 co-infected with rBac/NA and rBac/HA	100	1.23	3,200

HA polypeptides, HA1 and HA2, with molecular masses of 40 and 27 kDa, respectively [10]. The recombinant HA derived from TN-5B1-4/M1-M2-NA-HA cells was in the uncleaved HA0 precursor form, with a molecular weight of approximately 70 kDa. No significant amounts of processed HA1 and HA2 polypeptides were detected. It has been reported that HA is not progressed to HA1 and HA2 polypeptides in influenza VLPs produced in baculovirus-infected insect cells [10]. However, the antibodies induced by these VLPs in mice efficiently recognized the HA1 and HA2 polypeptides derived from wild-type influenza viruses. Furthermore, the conformation of the HA0 precursor on the outer surfaces of the VLPs did not abrogate the self-assembly or hemagglutination functions of the VLPs [10].

3.3. Manufacture of recombinant baculoviruses expressing recombinant NA and HA

To develop an alternative strategy for producing influenza VLPs using stably transfected TN-5B1-4 cells, recombinant baculoviruses expressing influenza NA and HA were generated using the pFastBac1 bacmid transfer method, as described in the Materials and Methods. To confirm the expression of recombinant NA and HA in the recombinant-baculovirus-infected TN-5B1-4 cells, the TN-5B1-4 cells were infected with recombinant baculovirus and cultured for 3 days. The intracellular proteins were prepared and the presence of recombinant NA and HA was determined with a western blot analysis (Fig. 5). Recombinant NA and HA were expressed as single bands with molecular masses of 60 and 70 kDa, respectively. These molecular masses are consistent with those of the recombinant NA and HA expressed from the stably transfected TN-5B1-4 cells. The recombinant baculoviruses expressing recombinant NA and HA were designated as rBac/NA and rBac/HA, respectively.

3.4. Production of VLPs from recombinant-baculovirus-infected TN-5B-4/M1-M2 Cells

To estimate the production of VLPs from the recombinant-baculovirus-infected TN-5B1-4/M1-M2 cells, the cells were co-infected with rBac/NA and rBac/HA at an MOI of 3. After incubation for 3 days, the culture medium was collected and pelleted by ultracentrifugation on a 30% sucrose cushion. The presence of recombinant M1, M2, NA, and HA was confirmed with a western blot analysis (Fig. 6A). The pelleted eluates from the culture medium of the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA contained the recombinant M1, M2, NA, and HA proteins. The sucrose cushion pellet from the TN-5B1-4/M1-M2 cells infected with the control baculovirus (rBac, recombinant baculovirus derived from the pFastBac1 vector) contained only recombinant M1 and M2. When 100 mL of the culture medium was ultracentrifuged on a 30% sucrose cushion, about 1.23 mg of total protein was obtained (Table 1). The hemagglutination activity of the pelleted eluates was approximately 3,200 HAU/mg. Based on the protein content and hemagglutination activity, the production of VLPs was strongly increased in the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA compared with that in TN-5B1-4/M1-M2-NA-HA cells. To further investigate the production of VLPs, the pelleted eluate containing recombinant M1, M2, NA, and HA was fractionated by ultracentrifugation on a 30 ~ 60% discontinuous sucrose density gradient. The fractions were analyzed with western blotting (Fig. 6B). Recombinant M1, M2, and NA were detected in fractions 4 and 5 corresponding to sucrose densities of 41.3 ~ 45.0%. Recombinant HA was detected in fractions 4, 5, 6, and 7 corresponding to sucrose densities of 41.3 ~ 52.5%. The

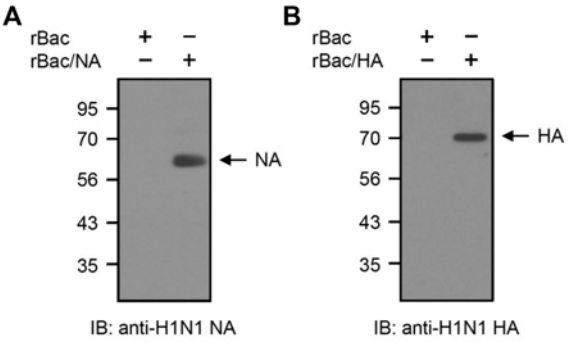

Fig. 5. Expression of recombinant NA and HA from recombinant-baculovirus-infected TN-5B1-4 cells. Intracellular protein extracts were collected from recombinant-baculovirus-infected TN-5B1-4 cells. The expression of recombinant NA, and HA was detected with a western blot analysis using a mouse anti-H1N1 NA monoclonal antibody (A) and a rabbit anti-H1N1 HA monoclonal antibody (B). rBac, recombinant baculovirus derived from pFastBac1; rBac/NA and rBac/HA, recombinant baculoviruses derived from pFastBac1/NA and pFastBac1/HA, respectively.

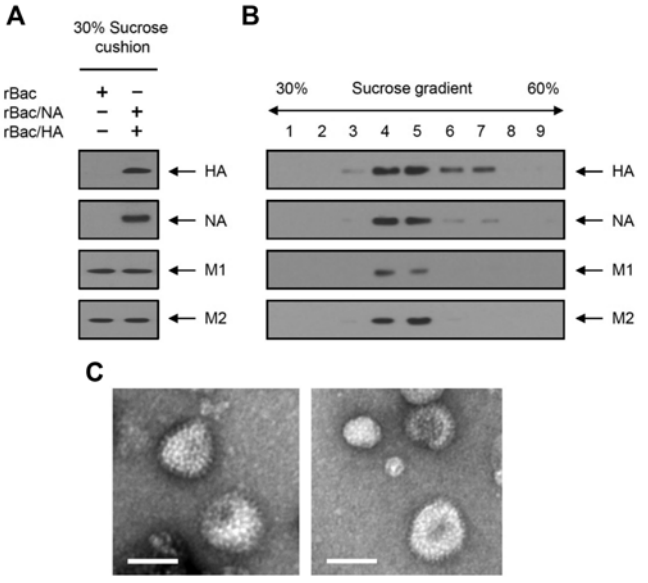

Fig. 6. Purification of VLPs from rBac/NA- and rBac/HA-infected TN-5B1-4/M1-M2 cells by ultracentrifugation and morphological analysis of the VLPs with electron microscopy. (A) The culture media of the rBac/NA- and rBac/HA-infected TN-5B1-4/M1-M2 cells were collected and pelleted by ultracentrifugation on a 30% sucrose cushion. The presence of recombinant M1, M2, HA, and NA in the pelleted eluates was detected with a western blot analysis. (B) The pelleted eluate was fractionated with 30 ~ 60% sucrose density gradient ultracentrifugation. The presence of recombinant M1, M2, HA, and NA in the fractions was detected with a western blot analysis. (C) Electron microscopic analysis of a negatively stained mixture of fractions 4 and 5. Bar represents 100 nm.

distributions of the recombinant influenza proteins in the sucrose gradient fractions were similar to those of the proteins isolated from TN-5B1-4/M1-M2-NA-HA cells. Most of the recombinant influenza proteins were detected in fractions 4 and 5. The morphology of the VLPs in fractions 4 and 5 of the sucrose density gradient was examined with electron microscopy (Fig. 6C). The negatively-stained samples revealed the presence of VLPs with diameters of approximately 80 ~ 120 nm, which is consistent with the morphology and size of the influenza VLPs produced with other mammalian and baculovirus–insect expression systems [19,21].

To confirm that the influenza M1, M2, NA, and HA proteins had self-assembled into high-molecular-weight VLPs, fractions 4 and 5 from the culture medium of the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA were pooled and loaded onto a Superose 6 column. The column was calibrated with Dextran Blue 2,000 (MW 2,000 kDa) and cytochrome c (MW 12 kDa). Recombinant M1, M2, NA, and HA were detected in fractions 10 and 11 (Fig. 7). Fraction 11 corresponds to the Dextran Blue 2,000 fraction. This indicates that the influenza M1, M2, NA, and

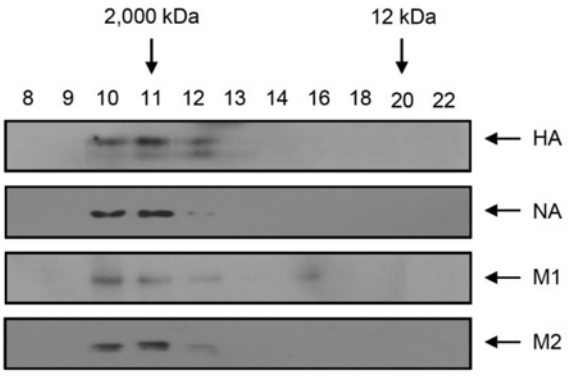

Fig. 7. Characterization of VLPs using Superose 6 chromatography. Sucrose density gradient fractions 4 and 5 were pooled and loaded onto a Superose 6 column calibrated with Dextran Blue 2,000 (MW 2,000 kDa) and cytochrome c (MW 12 kDa). The presence of recombinant M1, M2, HA, and NA in the fractions was determined with a western blot analysis. The positions at which Dextran Blue 2,000 and cytochrome c eluted are indicated with arrows.

HA proteins produced in the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA had self-assembled into high-molecular-weight VLPs.

4. Conclusion

Stably transfected TN-5B1-4 (TN-5B1-4/M1-M2-NA-HA) cells were established to express four structural proteins of the influenza virus A/Korea/01-2-9/2009 (H1N1). The four structural proteins self-assembled into VLPs and were released to the culture medium. We also developed an alternative strategy to produce VLPs using stably transfected TN-5B1-4 cells and a recombinant baculovirus expression system. When stably transfected TN-5B1-4 (TN-5B1-4/M1-M2) cells expressing recombinant M1 and M2 were co-infected with recombinant baculoviruses (rBac/NA and rBac/HA) expressing recombinant NA and HA, high-molecular-weight VLPs with diameters of 80 ~ 120 nm self-assembled and were released into the culture medium. The production of VLPs was strongly enhanced in the TN-5B1-4/M1-M2 cells co-infected with rBac/NA and rBac/HA compared with that in the TN-5B1-4/M1-M2-NA-HA cells. Taken together, our results suggest that a stably transfected insect cell expression system combined with a recombinant baculovirus expression system can efficiently produce VLPs for the development of recombinant vaccines against influenza virus.

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