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Proteomic Characterization of Influenza H5N1 virus-like particles and their protective immunogenicity

Jae-Min Song², Chi-Won Choi¹, Sang-Oh Kwon¹, Richard. W. Compans², Sang-Moo Kang², and Seung Il Kim¹

¹Division of Life Science, Korea Basic Science Institute, Daejeon, 305-333, South Korea

²Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA30322, USA

Abstract

Recombinant virus-like particles (VLPs) have been shown to induce protective immunity. Despite of their potential significance as promising vaccine candidates, the protein composition of VLPs produced in insect cells has not been well characterized. Here we report a proteomic analysis of influenza VLPs containing hemagglutinin (HA) and matrix M1 proteins from a human isolate of avian influenza H5N1 virus (H5 VLPs) produced in insect cells using the recombinant baculovirus expression system. Comprehensive proteomic analysis of purified H5 VLPs identified viral proteins and 37 additional host-derived proteins, many of which are known to be present in other enveloped viruses. Proteins involved in different cellular structures and functions were found to be present in H5 VLPs including those from the cytoskeleton, translation, chaperone, and metabolism. Immunization with purified H5 VLPs induced protective immunity, which was comparable to the inactivated whole virus containing all viral components. Unpurified H5 VLPs containing excess amounts of non-influenza soluble proteins also conferred 100% protection against lethal challenge although lower immune responses were induced. These results provide important implications consistent with the idea that VLP production in insect cells may involve similar cellular machinery as other RNA enveloped viruses during synthesis, assembly, trafficking, and budding processes.

Keywords

Influenza H5N1 virus-like particles; vaccine; 1-DE-LC-MS/MS; proteome

Introduction

Influenza is a major infectious disease of humans and animals causing significant morbidity and mortality worldwide. Based on World Health Organization reports (<http://www.who.int/mediacentre/factsheets/fs211>), it is estimated that seasonal influenza epidemics affect 5 to 15% of the global population annually and are responsible for more than 3–5 million hospitalizations and about 250,000 to 500,000 deaths per year. Influenza virus circulating in various reservoirs including humans, birds, and pigs shows continuous mutation, which provides a source for the emergence of novel strains. For example, the

Correspondence: Seung Il Kim, PhD, Division of Life Science, Korea Basic Science Institute, 52, Yeo-eun-Dong, Yusong-Ku, Daejeon, 305-333, South Korea, Phone: 82-42-865-3451 ksi@kbsi.re.kr Fax: 82-42-865-3419. Sang-Moo Kang, PhD, Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA30322, USA, Phone: 1-404-712-1735 skang2@emory.edu Fax: 1-404-727-8250.

SM Kang and SI KIM are co-corresponding authors

novel swine-origin 2009 H1N1 influenza virus was declared to be the first pandemic in the 21st century. Since the emergence of a highly pathogenic H5N1 influenza virus in Hong Kong in 1997, recurrent outbreaks of avian influenza viruses (H5N1) pose a greater threat because human cases were shown to have high mortality up to 60%, which is 100 to 1000 fold higher compared to seasonal influenza viruses (http://www.who.int/csr/disease/avian_influenza).

Preventive vaccination is the most efficient way of preventing seasonal or pandemic outbreaks of influenza. The current licensed influenza vaccines are mainly manufactured in the fertilized chicken eggs as a production system. This method of vaccine manufacturing capacity is restricted by the availability of eggs, which may be insufficient to meet the urgent requirements for vaccine during pandemic outbreaks of influenza ^{1, 2}. Pandemic potential H5N1 virus inactivated vaccines were shown to induce antibodies that protect ferrets or nonhuman primates, and to be safe and tolerable in human trials ³⁻⁶. In addition, the efficacy of live attenuated H5N1 influenza virus vaccines has been proved in mice, ferrets, and nonhuman primate models ⁷⁻⁹. Currently licensed vaccines induce antibodies primarily to the viral HA and display lower protective rates in the elderly and may be poorly immunogenic in young children ^{1, 10, 11}. Therefore, the development of effective vaccines using alternative technologies is highly needed to meet the demand for pandemic influenza preparedness and surge capacity following a newly identified pandemic influenza outbreak.

As for advanced vaccines for a wide range of viruses that cause disease in humans, noninfectious virus-like particles (VLPs) that self-assemble by spontaneous interactions of viral structural proteins have been suggested as a promising vaccine candidate platform ¹²⁻¹⁴. Importantly, a VLP-based human papillomavirus (HPV) vaccine produced in a yeast or an insect cell system was approved for human vaccination ^{15, 16}. Influenza VLPs expressed by recombinant baculovirus (rBV) systems that are capable of conferring protective immunity against homologous or heterologous strains of pandemic potential or pandemic influenza virus, have been previously demonstrated ¹⁷⁻²².

The protein composition of a vaccine often serves as an important guideline in characterizing the vaccine as well as mechanistic insight into how the vaccine is synthesized. VLPs are commonly considered to be composed of viral structural proteins that are required for maintaining the virus architecture. This conventional view is now being changed due to enhanced proteomics techniques and the availability of annotated genomic sequences for several mammalian species. Recent proteomic studies demonstrated that enveloped viruses in particular have the capability of incorporating numerous host proteins, both into the interior and the lipid envelope of virus particle ^{23, 24}. However, detailed proteomic information on influenza VLPs is not well known despite extensive preclinical vaccine studies. This present study reports the proteomic analysis as well as protective immunity of influenza VLPs containing HA derived from a human isolate of avian influenza H5N1 virus (H5 VLPs). Proteomic analysis of H5 VLPs identified numerous cellular and vector derived proteins as well as the influenza H5N1 viral proteins HA and M1.

Materials and Methods

Cells and Viruses

Spodoptera frugiperda SF9 cells which were used for production of recombinant baculoviruses (rBVs) and VLPs were purchased from the American Type Culture Collection (ATCC, CRL-1711) and maintained in SF900-II SFM medium at 27 °C incubator. A reverse genetic engineered reassortant influenza H5N1 virus which has hemagglutinin derived from A/Indonesia/5/2005 (H5N1) and other 7 genes derived A/PR/8/34 (H1N1) virus was generated as described ^{25, 26}. This reassortant H5N1 virus was propagated in the allantoic

cavity and used as an ELISA antigen and challenge experiments as described previously^{27, 28}.

Preparation of influenza H5 VLP

Influenza H5 VLPs containing HA and M1 proteins were produced using the rBV expression system as previously described^{19, 28}. Briefly, to generate the rBVs expressing the influenza H5 HA protein, a full length HA cDNA was derived from influenza H5N1 virus (A/Indonesia/05/2005), cloned into pFastBac, and then transferred into Bacmid recombinant BV DNA (rAcNPV) by transformation with DH10Bac cells. This H5 HA protein contains a deletion of polybasic amino acids in the cleavage site. The rBV expressing influenza H5 HA protein was generated by bacmid transfection with sf9 insect cells and harvested from culture supernatant 2 days post transfection. To generate influenza H5 VLP, SF9 insect cells were co-infected with rBVs expressing HA and M1 proteins at a multiplication of infection of 3 and 1 respectively. Approximately 36 hours after infection of SF9 cells with rBVs, culture media containing released VLPs were collected and clarified by low speed centrifugation ($2,000 \times g$, 30min, 4 °C). Culture supernatants were concentrated and filtrated by Quixstand bench-top system (GE Healthcare) using a hollow fiber cartridge of 300 kDa molecular weight cut-off. Further purification was performed by 30% and 60% sucrose layer gradient ultracentrifugation ($28,000 \times g$, for 60 min). The protein concentration of H5 VLPs was quantified by a protein assay kit (Bio-rad, Irvine, CA) and biological activity was determined by a hemagglutination assay as previously described¹⁹. Briefly, the highest dilution factor of H5 VLP samples or inactivated H5N1 virus that prevents aggregated precipitation of 1% horse erythrocytes was determined to present hemagglutination activity units (HAU) as an indicator of vaccine activity²⁹.

SDS-PAGE and in-gel digestion

The protein components of purified VLPs were separated by SDS-PAGE. The protein samples (10 µg) were separated by 12% SDS-PAGE using mini-PROTEAN (BIO-RAD) and the gels were stained with Coomassie Brilliant Blue R-250. The separated proteins of VLPs were sliced into 10 fractions according to molecular weight. Each sliced gel fragment was used for the in-gel digestion according to previous methods³⁰. Reduction and alkylation of cysteines were performed by incubating sample proteins in 10 mM DTT/100 mM ammonium bicarbonate and then 55 mM iodoacetamide/100 mM ammonium bicarbonate. After washing and buffer exchange of alkylated proteins in the gel with 50mM ammonium bicarbonate, proteins were digested with 10 µl trypsin (0.1 mg/ml, Promega) at 37°C for 16 hrs. The tryptic peptides were recovered using two extraction steps using 50 mM ammonium bicarbonate and then 50% (v/v) acetonitrile containing 5% (v/v) trifluoroacetic acid (TFA). The digested peptides were resolved in 15 µl of sample solution containing 0.02% formic acid and 0.5% acetic acid.

Mass spectrometry (MS)/MS analysis using an LCQ Deca XP

The peptide samples were concentrated on a MGU30-C18 trapping column (LC Packings). Peptides were eluted from the column and directed onto a 10 cm×5 µm i.d. C18 reverse phase column (PROXEON, Denmark) at a flow rate of 100 nl/min. Peptides were eluted by a gradient of 0~65% acetonitrile for 80 min. All MS (mass spectrometry) and MS/MS spectra in the LCQ-Deca XP ESI ion trap mass spectrometer (Thermo Finnigan) were acquired in a data-dependent mode. Each full MS (m/z range of 400 to 2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum with dynamic exclusion enabled.

Bioinformatic analysis for protein identification

For protein identification, the complete genome sequences of *Autographa californica* nucleopolyhedrovirus (NC_001623) from NCBI (<http://www.ncbi.nih.gov>) and the ESTs sequences of SF9 insect cell from SPODOBASE (<http://bioweb.ensam.inra.fr/Spodobase/>) were used as the database for MS/MS analysis. The sequences of M1 (AF115287_1) and HA proteins (derived from A/Indonesia/5/2005 H5N1 virus) were added into the database. MS/MS spectra were analyzed by MASCOT software ver. 2.2 (Matrix science, www.Matrixscience.com). For validation of identified proteins, FDR (false discovery ratio) of fewer than 10% was used by Mascot decoy database³¹. The mass tolerance of parent ion or fragment ion was 1.5 Da. Carbamidomethylation of cysteine and oxidation of methionine were considered in MS/MS analysis as variable modification of tryptic peptides.

Vaccines and virus challenge

BALB/c mice (8 weeks old, female) (Harlan Laboratories, Indianapolis, IN) were housed in the animal facility of Emory University, and used for immunization and lethal challenge studies. Groups of mice (n=10) were intramuscularly immunized once with 50 μ l PBS solution containing 30 HAU of either purified VLP vaccine, partially or unpurified VLPs or inactivated whole influenza A/H5N1 virus. For infection, isoflurane-anesthetized mice were inoculated intranasally with 5MLD₅₀ of reassortant influenza H5N1 virus (A/Indonesia/5/2005) and body weight changes were monitored for two weeks. All animal experiments were carried out following an approved IACUC protocol (Institutional Animal Care and Use Committee of Emory University).

Serum antibody responses

Blood samples were collected at 4 weeks after a single dose immunization. Influenza virus specific antibody responses were determined by ELISA using inactivated influenza H5N1 virus as a coating antigen as previously described²⁸. Briefly, 96 well microtiter plates were coated with inactivated influenza H5N1 virus (2 μ g/ml) and washed three times with PBS containing 0.05% Tween 20 (PBST). After blocking with 3% BSA for 2 h at 37 °C, serial dilutions of serum samples were added and incubated for 1 h at 37 °C. Then HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a antibodies were used as a secondary antibody to determine total IgG and isotype antibodies specific to virus. O-phenylenediamine (OPD) was used as a substrate and the absorbance was read at 450 nm.

Statistical analysis

To determine the statistical significance, a two-tailed paired Student's *t* test using Graphpad PRISM software was performed when two groups of animals were compared. A *p* value less than 0.05 was considered to be significant.

Results

Production and purification of influenza H5 VLP

Influenza H5 VLPs containing A/Indonesia/05/2005 H5 HA and M1 proteins (H5 VLPs) were produced and harvested from culture supernatants of sf9 insect cells co-infected with two rBVs expressing HA and M1 protein separately. Host cells and cell debris were removed from culture supernatants by centrifugation and this clarified preparation of H5 VLPs showed a significant HA titer of 776 per mg total VLP protein prior to purification processes (unpurified H5 VLPs) (Table 1). As shown in Table 1, the growth media contained a high level of non-influenza viral proteins. To remove soluble proteins and to concentrate VLPs, we performed ultra-filtration using a hollow fiber filter cartridge with molecular weight cut-off value of 3,000,000 Da. Based on the quantification of total VLP

proteins, we found that more than 80% of non-viral proteins and 90% of the volume of culture supernatants were removed during the partial purification processes of re-circulating filtration and concentration (Table 1). There was no HA activity in the eluted solution from the hollow fiber cartridge, indicating that the HA activity is associated with VLPs. To obtain highly purified influenza H5 VLPs, the concentrated VLP solution was loaded on discontinuous sucrose density gradients and a visible white band was collected from 30% and 60% sucrose layers after ultra-centrifugation. Finally approximately 99.5 % (3283 of 3300 mg) of proteins present in the initial unpurified H5 VLPs were removed during the purification processes (Table 1). In contrast, most of the HA activity was preserved in the re-collected fluid and the unit HA activity (per mg total VLP protein) increased about 167 fold during the purification processes (Table 1). Therefore, rBV expressed HA proteins in insect cells are likely to be targeted onto the SF9 cell membrane and incorporated into VLPs in a functionally active form. These results suggest that VLPs are easily purified to high quality by removing over 99% of non-particulate proteins present in the culture supernatants.

SF9 host cell derived proteins are associated with H5 VLPs

Seasonal and pandemic influenza VLPs produced in insect cells were shown to confer protection, which suggests that influenza VLPs are a promising vaccine candidate. However, the protein components of VLPs produced in insect cells remain unknown. To identify protein components of purified H5 VLPs by MS/MS analysis, the proteins of VLPs separated on the 12% SDS-polyacrylamide gel were divided into 10 fractions and were subjected to in-gel protease digestion to generate polypeptides (Fig. 1A). Most tryptic peptides were detected at a high frequency (three times out of three analyses), and those detected more than two out of three times analysis were included for identification of proteins. Based on the MS/MS analysis using an LCQ Deca XP, over 37 proteins originated from SF9 host cells and were found to be associated with H5 VLPs (Table 2). The EST database of SF9 cell contains 5822 sequences. Because the EST database of SF9 cells used for protein identification was not annotated, sequence homology analysis of each EST sequence was performed using the Uniprot insert database.

A variety of proteins important for cell biology was identified. As representatively shown in Fig. 1A, these host proteins include heat shock protein 90 known to be involved in protein folding and cytoskeleton proteins such as alpha/beta tubulins and a cytoplasmic actin. Also, H5 VLPs were found to be associated with host proteins involved in vesicular trafficking (ADP-ribosylation factor, vesicle-associated membrane protein, vacuolar protein sorting 28, myosin II essential light chain), ribosomal proteins, putative ubiquitin/ribosomal protein S27Ae fusion protein, and cell signaling related proteins (heterotrimeric guanine nucleotide binding protein gamma subunit-like protein, Rho1) (Table 2). In addition, many proteins with enzyme activities were found. A range of additional proteins include those related to reduction-oxidation controls, metabolism, and signaling (Table 2). Therefore, these results suggest that many host proteins with various biological functions are associated with and/or incorporated into VLPs during their formation in host cells.

Identification of baculovirus vector derived proteins

The information on the genome database containing 156 known genes of *Autographa californica* nucleopolyhedrovirus made it possible to identify baculovirus-derived proteins in H5 VLPs (Table 3). As expected, many vector-derived proteins were also found to be in H5 VLPs. The proteomic analysis shows the presence of structural proteins that are known to be involved in virus or VLP formation (Table 3 and Fig. 1B). These structural proteins include occlusion derived and polyhedron associated proteins (AcOrf-102, -114), capsid or capsid associated proteins, and envelope proteins (Table 3). Also, proteins with regulatory and/or enzyme activity functions were found. However, an essential 78-kDa phosphoprotein and a

6.9-kDa major core protein with a small arginine-rich polypeptide were not found, which are known to be important for the formation of nucleoprotein complex of baculovirions^{32, 33}. Nonetheless, these results indicate that there are some baculovirus-derived proteins in the preparation of purified H5 VLPs.

Verification of H5 and M1 proteins incorporated into H5 VLPs

Proteome analysis results showed that H5 and M1 proteins were located in the fraction numbers 8 and 5 at the respectively expected location. The sequence coverage of H5 and M1 proteins were 46% and 70%, respectively, indicating their high accuracy. However, our analysis results showed that the thick protein band in fraction number 8 contained a baculovirus envelope glycoprotein as well as influenza H5 HA proteins. M1 protein was found to be a major protein in fraction number 5 although this fraction also included several proteins such as glutathione S-transferase sigma and apoptosis inhibitor proteins (Fig. 1).

Immunogenicity of differentially purified H5 VLPs in mice

To investigate the effect of non-influenza components on inducing protective immunity, BALB/c mice were immunized intramuscularly with purified H5 VLPs, or partially purified or unpurified H5VLPs. Different levels of purity of H5 VLP preparations used for immunization were shown in Table 1. Also, a control group of mice that was immunized with inactivated whole H5N1 virus produced from fertilized egg substrates was included as a comparative control (Fig. 2). The doses of H5 VLPs and inactivated H5N1 virus vaccines were normalized based on the amount of HA, which is equivalent to 30 HAU in PBS. Mouse sera were collected at 4 weeks post single immunization and the levels of virus specific antibodies were determined by ELISA. All groups of VLP-vaccinated mice induced robust antibody responses specific to H5N1 influenza virus. Nonetheless, we observed differences among groups immunized with H5 VLPs containing different levels of non-influenza components. There were decreases in IgG antibody responses in groups of mice immunized with unpurified VLPs ($p=0.011$) or partially purified VLPs ($p=0.045$) compared to the group of purified VLPs that showed the highest level of antibodies specific to H5N1 virus (Fig. 2A). Importantly, immunization with H5 VLPs induced virus-specific antibodies at similar or higher levels compared to those by inactivated whole H5N1 virus. Interestingly, it was noted that partially purified VLPs also elicited levels of antibody responses which were not statistically different compared to purified inactivated virus vaccine.

Cytokines secreted by subsets of antigen-specific helper T cells regulate the production of different IgG isotypes. Thus the distribution of IgG antibody isotypes elicited by vaccination is indicative of the type of T cell immune response. Immunization with H5 VLPs induced the IgG2a isotype dominantly regardless of different levels of non-influenza components (Fig. 2B). In contrast, influenza VLPs produced in mammalian cells were previously demonstrated to elicit IgG1 isotype antibody predominantly³⁴. Interestingly, a similar pattern of IgG2a dominant antibody responses was elicited by both whole inactivated virus vaccines prepared from Madin-Darby canine kidney (MDCK) cells in a previous study³⁴ or propagated in egg substrates in this study. Therefore, these results suggest that different patterns of immune responses observed may be reflected by different vectors and/or cell types producing VLPs.

Protective efficacy of differentially purified H5 VLPs in mice

We have determined the HI antibody titers in different groups and found that their HI titers were very low in a range of 0 to 20. Considering low hemagglutination inhibition (HI) titers induced in vaccinated mice, it thus is important to determine protective efficacy of vaccinated mice with H5 VLPs containing different levels of non-influenza components. At 5 weeks after a single dose of immunization, mice were challenged with a lethal dose

(5MLD₅₀) of homologous H5N1 virus. After infection with a given dose, naïve mice showed a severe loss of body weight and all died. In contrast, both groups of mice that received a single dose of highly purified or partially purified H5 VLPs showed no loss in body weight and were 100% protected as observed in the inactivated whole virus group (Fig. 3). Meanwhile, the group of mice immunized with unpurified H5 VLPs displayed a moderate loss in body weight up to 8% transiently (Fig. 3A) and then recovered to normal weight although 100% mice in this group survived (Fig. 3B). These results suggest that both highly and partially purified H5 VLPs provide comparable protective immunity as the inactivated whole viral vaccine, and that even a single dose of unpurified H5 VLPs can provide 100% survival protection in a mouse model.

Discussion

Influenza VLP vaccines produced by the rBV expression system in insect cells are known to provide protective immunity against influenza virus and thus have been suggested to be a promising vaccine candidate. Despite the fact that VLPs can be clinically relevant vaccines, there is significant gap in our understanding of detailed information on the VLP formation and composition. The present study has described the proteomic analysis as well as immunogenicity of influenza H5 VLPs.

In a previous study, 36 host-encoded proteins were detected in influenza virus particles propagated in MDCK cells³⁵. Coronavirus infectious bronchitis virus particles were shown to associate with 60 host proteins of embryonated chicken eggs³⁶. Our proteomic analysis of H5 VLPs identified 37 cellular proteins assigned to functional descriptions. It is important to note that of these 37 insect cell proteins identified in this study, 22 have been reported to be present in other types of enveloped virus particles (Table 2), despite using different cell types, diverse viruses, and different analytical methods. These viruses and VLPs seem to share a fundamental feature, suggesting that these host proteins may be involved in the processes of virus and VLP production. This common feature is that all these viruses and VLPs are produced by budding, resulting in the acquisition of an envelope derived from host cells. A possible explanation is that some host proteins that are found to be commonly incorporated may play a role in particular stages during the formation of enveloped viruses and VLPs. It is also likely that VLPs are produced and released into culture supernatants by following a common or similar cellular pathway as other enveloped viruses.

Most of the 37 insect cell derived proteins could be classified into functional groups including cytoskeletal proteins, heat shock proteins, ribosomal proteins and metabolic enzymes. Among them, cytoskeletal proteins such as tubulin and actin are involved in the transport of viral components in the cell³⁷ and also are known to be required for viral gene expression particularly for RNA viruses which encode few proteins^{38, 39}. The reported viruses include human immunodeficiency (HIV-1)⁴⁰, vaccinia virus⁴¹, human cytomegalovirus (HCMV) particles⁴², Epstein-Barr virus⁴³, Moloney murine leukemia virus⁴⁴, bronchitis virus particles³⁶, and influenza virus³⁵. Viral proteins of VLPs are produced in the cytosol of host cells. Incorporation of viral proteins into VLPs may be dependent on cellular transportation machinery which consists of cytoskeletal and vesicular trafficking proteins as shown in Table 2. For example, cytoskeletal proteins were found in virions of HIV-1⁴⁰, and α/β tubulins in several enveloped viruses including influenza virus and VLPs^{34, 35}. Therefore, regardless of cell types, cytoskeletal proteins may play important roles for the formation of VLPs similar to the cases of enveloped virus production.

Heat-shock proteins (HSPs) have been known to be multifunctional proteins with a role in the folding and unfolding of proteins, vesicular transport processes, and preventing protein aggregation in the cytosol. HSPs were shown to interact with various viral proteins during

virus production⁴⁵, and may be involved in the assembly of adenovirus⁴⁶, enterovirus⁴⁷, and vaccinia virus⁴⁸. HSP 90 was found in influenza H5 VLPs produced in insect cells, which is consistent with the results for influenza VLPs and HIV-1 produced in mammalian cells^{34, 40}.

Interestingly, H5 VLPs were also found to contain fatty acid-binding protein 3 (FABP3) which was reported to be present in HIV-1 virions⁴⁰. FABP is a family of carrier proteins for long-chain fatty acid and accomplishes lipid organization. Influenza HA protein has a fatty acid binding moiety⁴⁹ although its potential role in H5 VLP formation is not clear. We also identified less well known host proteins including ribosomal proteins, signal transduction related proteins (G-proteins, Rho1), and glyceraldehyde 3-phosphate dehydrogenase which are described for HIV-1 virions produced in monocyte-derived macrophages⁴⁰. In addition, H5 VLPs were found to contain proteins previously undetected in other virions, which are related to ubiquitination, oxidation-reduction, translation regulator, and calcium modulation (Calmodulin). It is not clear what roles these proteins might have during the production of VLPs in insect cells.

The H5 VLPs described in this study were derived by rBVs expressing influenza HA and M1 proteins. Despite the clinically relevant vaccine candidates based on VLPs, vector-derived components in VLPs have not been described previously. In addition to HA and M1, our proteomic analysis identified 20 baculovirus-derived proteins (Table 3), which include capsid and envelope structural proteins, and some regulatory proteins. Baculovirus proteins are not required for influenza VLP formation since influenza VLPs can be produced in mammalian cells³⁴. It is likely that the process of budding does lend itself to the entrapment of proteins that are present at the budding site as the H5 VLPs are being assembled. It was noted that some proteins essential for baculovirus formation including 78-kDa phosphoprotein and a 6.9-kDa major core protein with arginine-rich polypeptides were not found in the H5 VLP preparation, indicating that baculovirus co-purification with VLPs is minimal.

It is interesting to note that some host and viral proteins appear more than once at similar sizes in the same fraction or different fractions. For example, proteins involved in vesicular trafficking/actin cytoskeleton, translation, enzymes, and protein folding were observed with various isoforms in the same fraction (Table 2). Also, structural and capsid viral proteins were detected more than once in the same or different molecular fractions (Table 3). It is speculated that multiple isoform proteins of similar or different sizes are involved in host cellular functions and viral protein production, assembly, trafficking, budding and release. This phenomenon was similarly reported in previous studies on viral particle and infected host cell proteomics^{34, 40, 50}.

It is important to determine the effects of non-influenza components on protective vaccine efficacy since many vaccines contain heterogeneous components in addition to the vaccine antigenic targets. Unpurified H5 VLPs contain over 160 fold more non-VLP proteins compared to the purified VLPs used for proteomic analysis. Most of non-influenza proteins in unpurified or partially purified proteins originated from the culture media (Table 1). The presence of excess media proteins in VLP vaccines seems to moderately interfere with immune responses and slightly lower the protective efficacy as shown by weight loss. It is possible that an excess of non-influenza proteins may decrease the effective interactions of HA antigen with immune cells. In support of this possibility, partially purified H5 VLPs (35 fold more non-influenza proteins compared to purified VLPs) induced similar protective efficacy despite lower levels of antibody responses. Also, we observed that vaccination with 3 – 4 fold higher doses of unpurified H5 VLPs induced complete protection without any loss in body weight (data not shown).

For the first time, proteomic analysis of rBV-derived H5 VLPs identified significant numbers of proteins derived from insect cells and baculovirus. Despite the presence of host and vector derived proteins, the H5 VLP vaccine conferred higher or non-inferior protective efficacy compared to the whole inactivated viral vaccine. Split vaccines are a more common format for seasonal vaccination in the US and are known to be less immunogenic compared to a whole viral vaccine^{51, 52}. Our preliminary studies suggest that rBV-derived influenza VLP vaccines are superior to licensed split vaccine in inducing protective immunity (data not shown). In addition, rBV-derived VLPs were demonstrated to activate innate immune cells such as dendritic cells^{53–55}. Importantly, this study shows that rBV-derived VLPs elicited IgG2a dominant isotype antibodies indicative of T helper type 1 immune responses in contrast to the mammalian produced VLPs³⁴. IgG2a antibody is known to interact efficiently with complement and Fc receptors by virtue of its Fc domain properties contributing to effective viral clearance^{56–59}.

Probably due to single immunizations with low doses of H5 VLP vaccines (30 HAU vaccines are equivalent to approximately 0.25 ug total protein of purified VLPs), low HI titers observed in vaccinated mice are consistent with previous studies^{17, 28}. Also, in a previous study with different doses of H5 VLPs, 4 fold differences in dosage did not result in significant differences in lung viral titers although vaccinated groups showed lower lung viral titers compared to the mock control²⁸. It was also demonstrated that protection against H5N1 virus was observed in the absence of serum HI activities in vaccinated mice¹⁷ and that HI antibody levels were very low after a single dose of the H5N1 live attenuated virus vaccine⁹. Importantly, it is worth noting that a single dose of rBV-derived H5 VLPs conferred complete protection as observed in the present study whereas two doses were previously used with mammalian cell-produced influenza VLPs³⁴. Therefore, in summary, proteomic information on influenza VLPs will be very helpful in elucidating the mechanisms by which VLPs are formed and released, as well as new insights on how VLPs induce protective immunity.

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Abbreviations

1-DE-LC-MS/MS	one-dimensional electrophoresis-liquid chromatography-tandem mass spectrometry
VLP	virus-like particle
HA	hemagglutinin
HAU	hemagglutination activity units
MDCK	Madin-Darby canine kidney
HSP	heat-shock protein
FABP3	fatty acid-binding protein 3

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Figure 1.

Representative proteins identified in H5 VLPs. Total proteins of purified H5 VLPs were resolved by 12% SDS-PAGE and stained with Coomassie blue. Molecular masses of protein markers are shown on the left. Each fraction of gel as marked with a number on the right was subjected to proteomic analysis to identify its components as described in the Materials and Methods. Total 10 sliced fractions are shown in these figures. Representative proteins identified in each band were presented. (A) Representative SF9 insect cell-derived proteins identified in each fraction. (B) Representative baculovirus vector-derived proteins identified in each fraction.

Figure 2.

Antibody responses of H5 VLPs and inactivated whole virus. (A) Influenza virus (reassortant H5N1 virus) specific IgG antibody responses. BALB/c mice (n=10 per group) were intramuscularly immunized with a single dose (30HAU) of H5 VLP vaccines in different stages of purification (Table 1) or inactivated whole virus (reassortant H5N1 virus). At 4 weeks after single vaccination, influenza H5N1 virus specific IgG antibodies in sera (100 X dilution) are presented in optical density values at 450nm (OD₄₅₀). Purified VLP; after sucrose gradient purification, partially purified VLP; after ultra-filtration, Unpurified VLP; after low speed centrifugation. Inactivated virus; inactivated whole reassortant H5N1 virus. (B) Influenza H5N1 virus-specific isotype antibodies elicited by H5 VLPs or inactivated whole virus.

Figure 3.

Protective efficacy of H5 VLPs and inactivated whole virus. Groups of BALB/c mice that were immunized with a single dose of VLPs or inactivated virus were challenged with a lethal dose (5 LD₅₀) of reassortant influenza H5N1 virus at week 5 post vaccination. Body weight (A) and survival rates (B) were monitored daily for 2 weeks, and average values are shown (n=10).

Table 1
Analysis of total protein and biological HA activity of influenza H5 VLP upon purification processes

purification process	total protein		HA activity		fold increases of HA activities ²
	total protein amounts (mg)	concentrations (mg/ml)	fold increase of protein purities ¹	total HA activities (HAU)	unit HA activities (HAU/mg)
cell culture media	3000±110	3.0±0.1	N.A. ⁶	N.A	N.A.
unpurified VLP ³	3300±931	3.3±0.9	1	2,560,000	776
partially purified VLP ⁴	663±189	3.9±1.1	4.9	2,328,000	3,510
purified VLP ⁵	16.8±3.6	3.4±0.7	196	2,176,000	130,000

¹ Fold increases of protein purities were expressed by reverse ratios of total protein amounts based on starting unpurified VLPs during each purification step.

² Fold increases of unit HA activity were estimated by unit HA activities of each steps divided by unit HA activity of starting unpurified VLPs.

³ Unpurified VLP means culture supernatants of rBV infected insect cells after low speed centrifugation to remove cell debris.

⁴ Partially purified VLPs were obtained after ultra-filtration of starting unpurified VLP samples.

⁵ Purified VLPs were obtained by sucrose gradient ultra-centrifugation of partially purified VLPs.

⁶ Not applicable

Table 2

SF9 insect cell proteins in influenza VLP

protein types	function	description of identified proteins	Mw ¹	fraction No ²	accession No.	references ³
cytoskeletal proteins	vesicular trafficking	ADP-ribosylation factor	10,823	3	SF9L01296_F3	HIV
		ADP-ribosylation factor	24,158	3	SF9L03944_F3	HIV
		vesicle-associated membrane protein	16,410	3	SF9L06864_F1	HIV
		vacuolar protein sorting 28	23,141	4	SF9L00950_F2	HIV
		myosin II essential light chain	22,716	3	SF9L04607_F2	
actin cytoskeleton		alpha tubulin	29,234	7	SF9L06055_F1	HCMV, EBV, VV, MoMLV, Influenza
		alpha-tubulin	22,220	7	SF9L00324_F2	HCMV, EBV, VV, MoMLV, Influenza
		Arp2/3 complex subunit	22,315	3	SF9L02454_F2	HIV
		beta-tubulin	10,580	7	SF9L07745_F1	HCMV, EBV, VV, MoMLV, HIV, KSHV, Influenza
		beta-tubulin	16,641	7	SF9L01568_F1	HCMV, EBV, VV, MoMLV, HIV, KSHV, Influenza
		cytoplasmic actin	22,771	7	SF9L07198_F1	IBV
ribosomal proteins	translation	40S ribosomal protein S24	18,486	3	SF9L00421_F3	HIV
		60S acidic ribosomal protein P1	17,540	3	SF9L00321_F2	HIV
		60S acidic ribosomal protein P2	14,867	3	SF9L02648_F1	HIV
		ribosomal protein L40	12,769	1	SF9L05931_F1	HIV
heat-shock proteins	protein folding	putative ubiquitin/ribosomal protein S27Ae fusion protein	11,339	1	SF9L04046_F3	
		heat shock protein 90	19,074	9	SF9L03941_F2	HIV, EBV, IBV, Influenza
		heat shock protein 90	15,384	9	SF9L03405_F1	HIV, EBV, IBV, Influenza
G proteins	signal transducing	heat shock protein 90	19,324	9	SF9L03550_F1	HIV, EBV, IBV, Influenza
		heterotrimeric G-protein gamma subunit-like protein	19,498	1	SF9L00232_F2	HIV, IBV
		putative Rho1	25,839	4	SF9L00343_F1	HIV
enzymes	reduction oxidation control	glutaredoxin	18,549	2	SF9L01853_F2	MoMLV
		glutathione S-transferase 2	10,946	4	SF9L02598_F2	
		glutathione S-transferase sigma	25,239	5	SF9L06774_F2	

protein types	function	description of identified proteins	Mw ¹	fraction No ²	accession No.	references ³
		glutathione S-transferase sigma	25,805	4	SF9L00010_F1	
	metabolism	glyceraldehyde 3-phosphate dehydrogenase	21,389	6	SF9L06272_F1	EBV, MoMLV, KSHV, Influenza
	protein folding	peptidyl-prolyl cis-trans isomerase	21,007	3	SF9L01437_F2	
	signal transducing	protein tyrosine phosphatase prl	17,025	4	SF9L09318_F3	
	RNA metabolism	TruB pseudouridine (psi) synthase homolog 2	20,781	5	SF9L03132_F3	
	ATP hydrolysis	vacuolar ATPase subunit C	12,383	7	SF9L01769_F2	HIV, MoMLV
	ser/thr protein kinase	casein kinase II subunit alpha	20,520	6	SF9L02636_F2	
regulatory proteins	lipid organization	fatty acid-binding protein 3	13,451	2	SF9L09628_F2	HIV
	signal transducing	14-3-3 zeta	24,315	5	SF9L00839_F1	
	RNA metabolism	Rbp1-like RNA-binding protein PB	17,511	4	SF9L00686_F1	
	translation regulation	eukaryotic translation initiation factor 5A	17,148	4	SF9L01654_F3	
	calcium modulation	calmodulin [Culicoides sonorensis]	20,098	3	SF9L02874_F2	
viral proteins		AF115287_1 matrix protein ⁴	27,821	5	AAF75113.1	
		HA protein ⁵	59,879	8	ksi_HA0001	

¹The molecular weights were estimated by EST sequence.

²Numbers of gel sliced fractions as shown in Fig. 1

³References: Human immunodeficiency (HIV-1) ⁴⁰, vaccinia virus ⁴¹, human cytomegalovirus (HCMV) particles ⁴², Epstein-Barr virus (EBV) ⁴³, Moloney murine leukemia virus (MoMLV) ⁴⁴, Infectious bronchitis virus (IBV) ³⁶, Influenza virus ³⁵, Kaposi's sarcoma-associated herpesvirus (KSHV) ⁶⁰

^{4&5}Sequence of M1 and HA protein was added for the MS/MS analysis.

Baculovirus (*Autographa californica* nucleopolyhedrovirus) proteins in influenza VLP

Table 3

protein types	function	description of identified protein	Mw	fraction No	accession No.
Structural proteins	ODV-associated protein	AcOrf-102	13,334	3	NP_054132.1
		AcOrf-114 peptide	49,291	7	NP_054144.1
	polyhedron associated proteins	fibrous body protein	10,309	1	NP_054167.1
Capsid proteins	capsid protein	occlusion-derived virus envelope/capsid protein	33,527	6	NP_054175.1
		viral capsid associated protein	60,711	9	NP_054038.1
		viral capsid associated protein	79,876	9	NP_054134.1
	nucleocapsid protein	viral capsid protein	22,109	4	NP_054159.1
Envelope proteins		major viral capsid protein	38,950	6	NP_054119.1
	envelope protein	major budded virus envelope glycoprotein	58,564	8	NP_054158.1
		copia-like envelope protein	79,855	10	NP_054052.1
		occlusion-derived virus envelope protein	25,525	4	NP_054124.1
Regulatory proteins	baculoviral inhibition of apoptosis	apoptosis inhibitor	34,827	6	NP_054165.1
	DNA synthesis regulator	DNA synthesis regulator	52,634	7	NP_054169.1
	protein folding	AcOrf-51 peptide	37,531	6	NP_054080.1
	regulate phosphotyrosine levels in signal transduction	protein tyrosine phosphatase	19,288	3	NP_054030.1
	regulation of transporter protein	AcOrf-58 peptide	6,815	4	NP_054088.1
	ubiquitin pathway	viral ubiquitin	8,653	1	NP_054064.1
	integrase/recombinase	very late expression factor 1	44,362	7	NP_054107.1
	conversion of superoxide radicals to molecular oxygen	superoxide dismutase	16,182	3	NP_054060.1
	transfer of sugar moieties	ecdysteroid UDP-glucosyl transferase	57,031	8	NP_054044.1