Virus-Like Particles As Vaccines For Paramyxovirus

Statement Of Government Support

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Field Of Invention

The present invention relates to the field of viral vaccines. In one embodiment, the present invention contemplates a paramyxoviral vaccine effective against diseases such as, but not limited to, Newcastle disease, measles, parainfluenza virus 3, and respiratory syncytial virus. In one embodiment, the present invention contemplates a vaccine comprising Newcastle disease virus (NDV)-like particles (VLP). In one embodiment, the present invention contemplates a method comprising transfecting avian cells with cDNAs encoding major NDV structural proteins. In another embodiment, a method wherein particles resembling infectious virions are released with nearly 100% efficiency. In one embodiment, the particles are non-infectious and provide a safe and effective NDV vaccine.

Background

Over the last decade, a number of concerns have arisen related to safety issues regarding paramyxovirus vaccines that have had an adverse effect on the public's trust. These concerns affect not only parents whose children are the primary recipient of childhood disease vaccines, but also ranchers devoted to raising animals susceptible to various types of paramyxoviruses.

Historically, Newcastle disease has been a devastating disease of poultry, and in many countries the disease remains one of the major problems affecting existing or developing poultry industries. Even in countries where Newcastle disease may be considered to be controlled, an economic burden is still associated with vaccination and/or maintaining strict biosecurity measures. The variable nature of Newcastle disease virus strains in terms of virulence for poultry and the different susceptibilities of the different species of birds mean that for control and trade purposes, Newcastle disease requires careful definition. Confirmatory diagnosis of Newcastle

Disease requires the isolation and characterization of the virus involved. Currently Newcastle disease control is limited to prevention of introduction and spread, good biosecurity practices and/or live attenuated virus vaccination. Newcastle disease viruses may infect humans, usually causing transient conjunctivitis, but human-to-human spread has never been reported. Alexander D.J., "Newcastle disease and other avian paramyxoviruses" *Rev Sci Tech.* 19(2):443-62 (2000).

Historically, the live attenuated measles virus (MV) vaccine and the combination multivalent measles, mumps, and rubella (MMR) vaccine have had a positive impact on the health of children worldwide by preventing infectious disease. The induction of an effective antiviral immune response using these live attenuated virus vaccines, however, are known to result in a significant rate of adverse events (i.e., for example, autism). Kennedy et al., "Measles virus infection and vaccination: potential role in chronic illness and associated adverse events" *Crit Rev Immunol.* 24(2):129-56 (2004).

Healthy, and at risk, children are susceptible to the morbidity and mortality associated with viral-induced respiratory diseases, including respiratory syncytial virus (RSV) and influenza. Currently, the World Health Organization is attempting to develop and distribute effective vaccines to prevent/reduce key viral respiratory diseases. The progress, however, is slow and the risk/benefit ratio is high. A vaccination program for viral respiratory infections should include the prevention of lower respiratory tract infections and prevention of infection-associated morbidities, hospitalization and mortality. Presently, there are two influenza vaccines; i) a trivalent inactivated vaccine, and ii) a live, cold-adapted, attenuated vaccine. Compliancy, however, is relatively low (i.e., 10 – 30%). Because it is believed that the low compliancy is related to the known high risk of contaminated vaccines, those in the art recommend that research should continue into safe and effective vaccines for all childhood viral illnesses. Greenberg et al., "Immunization against viral respiratory disease: A review" *Pediatr Infect Dis J.* 23(11 Suppl):S254-61 (2004).

What is needed in the art is a low risk, highly effective paramyxovirus vaccine that is compatible with population-wide distribution marketing goals of low cost and high production rates.

Summary

The present invention relates to the field of viral vaccines. In one embodiment, the present invention contemplates a paramyxoviral vaccine effective against diseases such as, but not limited to, Newcastle disease, measles, parainfluenza virus 3, and respiratory syncytial virus. In one embodiment, the present invention contemplates a vaccine comprising Newcastle disease virus-like particles (VLP). In one embodiment, the present invention contemplates a method comprising transfecting avian cells with cDNAs encoding major NDV structural proteins. In another embodiment, a method wherein particles resembling infectious virions are released with nearly 100% efficiency. In one embodiment, the particles are non-infectious and provide a safe and effective NDV vaccine.

In one embodiment, the present invention contemplates a method, comprising; a) providing, i) an expression vector comprising DNA sequences encoding a Newcastle disease matrix protein; ii) a cell capable of being transfected by said vector; b) transfecting said cell with said vector under conditions such that Newcastle disease virus-like particles are generated. In one embodiment, the method further comprises the step c) harvesting said virus-like particles so as to create a cell-free preparation of particles. In one embodiment, the method further comprises the step d) administering a vaccine comprising said preparation of particles to a chicken. In one embodiment, the cell is part of a cell culture and said harvesting comprises obtaining said particles from the supernatant of said culture. In one embodiment, the cell culture comprises sub-confluent avian cells. In one embodiment, the vector further comprises DNA sequences encoding additional Newcastle disease viral proteins selected from the group consisting of a nucleocapsid protein, a fusion protein, and a hemagglutinin-neuraminidase protein. In one embodiment, the particles are free of Newcastle disease viral DNA.

In one embodiment, the present invention contemplates a transfected cell comprising an expression vector comprising DNA sequences encoding a Newcastle disease matrix protein capable of generating Newcastle disease virus-like particles.

In one embodiment, the present invention contemplates a cell-free preparation of virus like particles harvested from a transfected cell comprising an expression vector comprising DNA sequences encoding a Newcastle disease matrix protein capable of generating Newcastle disease virus-like particles.

In one embodiment, the present invention contemplates a method, comprising; a) providing, i) a vaccine comprising Newcastle disease virus-like particles, said particles comprising a Newcastle disease viral matrix protein; ii) a host susceptible to Newcastle disease; b) immunizing said host with said vaccine under conditions such that antibodies directed to said virus-like particle are produced. In one embodiment, the host is selected from the group consisting of avian, murine, and human. In one embodiment, the particles further comprise one or more additional Newcastle disease viral proteins selected from the group consisting of a fusion protein, a nucleocapsid protein and a hemagglutinin-neuraminidase protein.

In one embodiment, the present invention contemplates a vaccine comprising Newcastle disease virus-like particles, said particles comprising a Newcastle disease viral matrix protein. In one embodiment, the particles are free of Newcastle disease viral DNA. In one embodiment, the particles further comprise one or more additional viral proteins selected from the group consisting of a fusion protein, nucleocapsid protein and a hemagglutinin-neuraminidase protein.

In one embodiment, the present invention contemplates a vaccine comprising a Newcastle disease virus-like particle and a Newcastle disease matrix protein. In one embodiment, the vaccine further comprises at least two viral glycoproteins. In one embodiment, the glycoproteins are selected from the group consisting of a fusion protein and a hemagglutinin-neuraminidase protein. In one embodiment, the vaccine further comprises a nucleocapsid protein. In one embodiment, the matrix protein comprises a Late Domain. In one embodiment, the Late Domain comprises an FPIV sequence (SEQ ID NO:1). In one embodiment, the Late Domain comprises a PXXP sequence (SEQ ID NO:2). In one embodiment, the Late Domain comprises an YXXL sequence (SEQ ID NO:3). In one embodiment, the vaccine is non-infectious.

One embodiment of the present invention contemplates an avian vaccine comprising a Newcastle disease virus-like particle and a Newcastle disease matrix protein. In one embodiment, the vaccine further comprises at least two viral glycoproteins. In one embodiment, said glycoproteins are selected from the group comprising a fusion protein and a hemagglutinin-neuraminidase protein. In one embodiment, the vaccine further comprises a nucleocapsid protein. In one embodiment, said virus-like particle comprises a Paramyxovirus virus-like particle. In one embodiment, said Paramyxovirus virus-like particle comprises a Newcastle

disease virus-like particle. In one embodiment, said matrix protein comprises a Late Domain. In one embodiment, said Late Domain comprises an FPIV sequence (SEQ ID NO:1). In one embodiment, said Late Domain comprises a PXXP sequence (SEQ ID NO:2). In one embodiment, said Late Domain comprises an YXXL sequence (SEQ ID NO:3). In one embodiment, said virus-like particle is non-infectious.

In one embodiment, the present invention contemplates a method, comprising; a) providing, i) an expression vector comprising cDNA sequences encoding a Newcastle disease virus matrix protein and at least two viral glycoproteins; ii) a cell capable of being transfected by said vector; b) transfecting said cell by said vector under conditions that generate a Newcastle disease virus-like particle, wherein said particle comprises said matrix protein. In one embodiment, the cell comprises sub-confluent avian cells. In one embodiment, the expression vector comprises pCAGGS. In one embodiment, the glycoproteins are selected from the group consisting of a fusion protein and a hemagglutinin-neuraminidase protein. In one embodiment, the expression vector further comprises a cDNA sequence encoding a nucleocapsid protein. In one embodiment, the method further comprises releasing said virus-like particle at an efficiency of at least 85%. In one embodiment, the virus-like particle further comprises said at least two viral glycoproteins.

One embodiment of the present invention contemplates a method, comprising; a) providing, i) an expression vector comprising cDNA sequences encoding a Newcastle disease virus matrix protein and at least two viral glycoproteins; ii) a cell capable of being transfected by said vector; and b) transfecting said cell by said vector under conditions that generate an avian vaccine comprising a virus-like particle. In one embodiment, said cell comprises sub-confluent avian cells. In one embodiment, said cell comprises human cells. In one embodiment, said expression vector comprises pCAGGS. In one embodiment, said glycoproteins are selected from the group comprising a fusion protein and a hemagglutinin-neuraminidase protein. In one embodiment, the vector further comprises a cDNA sequence encoding a nucleocapsid protein. In one embodiment, the method further comprises releasing said virus-like particle at an efficiency of at least 85%. In one embodiment, said virus-like particle comprises said matrix protein and said at least two viral glycoproteins.

In one embodiment, the present invention contemplates a method, comprising; a) providing, i) a vaccine comprising a Newcastle disease virus-like particle and a Newcastle disease virus matrix protein and at least two viral glycoproteins; ii) a host capable of immunization by said virus-like particle; b) immunizing said host by said virus-like particle under conditions such that antibodies directed to said virus-like particle are produced. In one embodiment, the host is selected from the group consisting of avian, murine, and human. In one embodiment, the glycoproteins are selected from the group consisting of a fusion protein, and a hemagglutinin-neuraminidase protein. In one embodiment, the vaccine further comprises a nucleocapsid protein.

One embodiment of the present invention contemplates a method, comprising; a) providing, i) an avian vaccine comprising a Newcastle disease virus virus-like particle, a Newcastle disease virus matrix protein and at least two viral glycoproteins; ii) a host capable of immunization by said virus-like particle; b) immunizing said host by said vaccine under conditions such that antibodies directed to said virus-like particle are produced. In one embodiment, said host is selected from the group comprising avian, murine, and human. In one embodiment, said virus-like particle comprises a Newcastle disease virus-like particle. In one embodiment, said glycoproteins are selected from the group comprising a fusion protein, and a hemagglutinin-neuraminidase protein. In one embodiment, the vaccine further comprises a nucleocapsid protein.

In one embodiment, the present invention contemplates an VLP vaccine expression system comprising a first cDNA encoding a first viral protein gene from a first Newcastle disease virus strain; a second cDNA encoding a second viral protein gene from a second Newcastle disease virus strain; and a third cDNA encoding a third viral protein gene from a third strain. In one embodiment, the first viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the first strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one embodiment, the second viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the second strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one embodiment, the third viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the third strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one

embodiment, the present invention contemplates a method for detecting a viral protein gene incorporated into a VLP vaccine comprising contacting the viral protein gene with strain specific antibodies or incorporated sequence tags.

Definitions

The terms used within the present invention are generally used according to those definitions accepted by one having ordinary skill in the art, with the following exceptions:

The term "virus-like particle" as used herein, refers to a non-infective viral subunit either with, or without, viral proteins. For example, a virus-like particle may completely lack the DNA or RNA genome. Further, a virus-like particle comprising viral capsid proteins may undergo spontaneous self-assembly. Preparations of virus-like particles are contemplated in one embodiment, where the preparation is purified free of infectious virions (or at least substantially free, such that the preparation has insufficient numbers to be infectious).

The term "matrix protein", "membrane protein", or "M protein" as used herein, means any protein localized between the envelope and the nucleocapsid core and facilitates the organization and maintenance of the virion structure and budding processes.

The term "fusion protein" or "F protein" as used herein, means any protein that projects from the envelope surface and mediates host cell entry by inducing fusion between the viral envelope and the cell membrane. However, it is not intended that the present invention be limited to functional F proteins. For example, an F protein may be encoded by a mutant F gene such as, but not limited to, F-K115Q. F-K115Q is believed to eliminate the normal cleavage and subsequent activation of the fusion protein. F-K115Q mimics naturally occurring F-protein mutations in avirulent NDV strains, and in cell culture, eliminates any potential side effects of cell-cell fusion on the release of VLPs.

The term "nucleocapsid protein" or "NP protein" as used herein, means any protein that associates with genomic RNA (i.e., for example, one molecule per hexamer) and protects the RNA from nuclease digestion.

The term "haemagglutinin-neuraminidase protein", "HN protein", or G protein as used herein, means any protein that spans the viral envelope and projects from the surface as spikes to

facilitate cell attachment and entry (i.e., for example, by binding to sialic acid on a cell surface). These proteins possess both haemagglutination and neuraminidase activity.

The term "glycoprotein" as used herein, refers to any protein conjugated to a nonprotein group that comprises a carbohydrate.

The term "paramyxovirus" as used herein, refers to any virus of the Paramyxoviridae family of the Mononegavirales order; that are negative-sense single-stranded RNA viruses responsible for a number of human and animal diseases (i.e., for example, Newcastle disease). Paramyxoviruses include, but are not limited to, for example, Sendai virus, Newcastle disease virus, Mumps virus, Measles virus, Respiratory syncytial (RS) virus, rinderpest virus, distemper virus, simian parainfluenza virus (SV5), type I, II, and III human parainfluenza viruses, etc. Sendai viruses may be wild-type strains, mutant strains, laboratory-passaged strains, artificially constructed strains, or so on. Incomplete viruses such as the DI particle (J. Virol., 1994, 68, 8413-8417), synthesized oligonucleotides, and so on, may also be utilized as material for producing the vaccine of the present invention.

The term "Late Domain" as used herein, refers to any region in a viral protein that is involved in the budding of virus particles from a cell's plasma membrane. Late Domains comprise highly conserved motifs known to mediate protein-protein interactions between cellular proteins. For example, at least three classes of motifs comprise PTAP (SEQ ID NO:4), PPXY (SEQ ID NO:5), or YXXL (SEQ ID NO:3)(i.e., for example, a YANL sequence).

The term "vector" as used herein, refers to any nucleotide sequence comprising exogenous operative genes capable of expression within a cell. For example, a vector may comprise a nucleic acid encoding a viral matrix protein and at least two glycoproteins that are expressed within a human, avian, or insect cell culture system. For example, a baculovirus vector may be used to transfect various *Lepidoptera* species.

The term "transfect" or "transfecting" as used herein, refers to any mechanism by which a vector may be incorporated into a host cell. A successful transfection results in the capability of the host cell to express any operative genes carried by the vector. Transfections may be stable or transient. One example of a transient transfection comprises vector expression within a cell, wherein the vector is not integrated within the host cell genome. Alternatively, a stable

transfection comprises vector expression within a cell, wherein the vector is integrated within the host cell genome.

The term "host" as used herein, refers to any organism capable of becoming infected by a virus and immunized by a virus-like particle. A host may be an avian host (i.e., for example, a chicken) or a mammalian host (i.e., for example, human, mouse, dog, rat, cow, sheep, etc.).

The term "sequence tag" as used herein, refers to any atom or molecule that can be used to provide a detectable (preferably quantifiable) signal, and that can be attached to a nucleic acid or protein. "Sequence tags" may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. A "sequence tag" may be a charged moiety (positive or negative charge) or alternatively, may be charge neutral. "Sequence tags" can include or consist of a nucleic acid or protein sequence, so long as the sequence comprising the "sequence tag" is detectable.

The term "adjuvant" as used herein, refers to any compound which enhances or stimulates the immune response when administered with an antigen(s).

Brief Description Of The Figures

The following figures are presented only as an illustration of specific embodiments of the present invention and are not intended to be limiting.

Figure 1 presents exemplary data showing co-expression of NP, F, HN, and M proteins resulted in VLP formation and release. Radioactively labeled proteins in both the transfected (Panel A) and infected (Panel B) extracts were immunoprecipitated with a cocktail of antibodies specific for all viral proteins and precipitated labeled proteins are shown on the left side of each panel. VLP particles in cell supernatants were purified as described in Example 4. After flotation into sucrose gradients, each gradient fraction was immunoprecipitated with antibody cocktail (right side of each panel). The density of each fraction (g/cc) is shown at the bottom.

Panel A: avian cells, co-transfected with pCAGGS(-NP), (-M), (F- K115Q), and (-HN), were radioactively labeled with ^{35}S-methionine and ^{35}S-cysteine for 4 hours (P) and then chased in non-radioactive medium for 8 hours (C).

Panel B: avian cells, infected with NDV, strain AV, with a Multiplicity Of Infection (MOI) of 5 pfu for 5 hours, were pulse-labeled for 30 minutes and chased in non-radioactive medium for 8 hours.

Panel C shows the quantitation of efficiency of virion and VLP release as determined by the amount of M protein in the pulse and chase cell extracts. The results of 3 separate experiments were averaged and the standard deviation is shown.

Figure 2 presents exemplary data showing that M protein is sufficient for VLP release. Avian cells were transfected with pCAGGS-NP, -M, -F-K115Q, and -HN individually.

Panel A shows radioactively labeled proteins in the extracts at time of pulse (left) and chase (right). Particles in the supernatants of avian cells expressing NP, M, F, and HN individually, were concentrated and floated into sucrose gradients as described above in Figure 1.

Panel B shows the distribution in the gradients of radioactively labeled proteins derived from each supernatant.

Panel C shows the quantification of the amounts of each protein in VLPs. The results of three separate experiments were averaged and the standard deviation is shown.

Figure 3 presents exemplary data showing effects of NP, F, or HN protein co-expression with M protein on VLP release. Avian cells, transfected with all possible combinations of two NDV structural protein genes (i.e., pair wise combinations including, but not limited to, F+NP, F+M, F+HN, HN+NP, HN+M and NP+M, wherein F is F-K115Q). Labeling in a pulse-chase protocol is as described in Figure 1. Particles present in the supernatants were concentrated and then floated into sucrose gradients as described in Example 4.

Panel A shows labeled proteins in cell extracts at time of pulse (top) and chase (bottom).

Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail. Densities (g/cc) of the fractions are shown at the bottom. Gradients from transfections that did not contain M protein are not shown since there were no radioactively labeled proteins in those gradients.

Panel C shows the quantification of each protein in VLPs released from transfected avian cells. Results are the average of three experiments and the standard deviation is shown.

Figure 4 presents exemplary data showing effects of expressing all combinations of three viral proteins on VLP release. Avian cells, transfected with all possible combinations of three NDV structural protein genes, were labeled in a pulse-chase protocol and particles in the supernatant were concentrated and floated into a sucrose gradient as in Figure 1. The proteins in the cell extracts were immunoprecipitated with the antibody cocktail.

Panel A show labeled proteins in cell extracts at time of pulse (top) and chase (bottom).

Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail for some of the viral protein combinations. Densities (g/cc) of the fractions are shown at the bottom.

Panel C shows quantification of the amounts of each protein in VLPs.

Panel D shows the efficiency of VLP release based on the percent of pulse labeled M protein remaining in the chase extracts.

Panel E show the relative amounts of M protein in the pulse extracts.

Figure 5 presents exemplary data showing that dominant-negative mutants of CHMP3 and Vps4-E228Q, blocked release of M protein-containing particles.

Panel A, left, shows pulse labeled extracts of human 293T cells that were simultaneously transfected with pCAGGS-M (1.0 μg) and either pDsRed2-Nl vector (0.1, 0.5 and 1.0 μg) or pDsRed2-Nl-CHMP3-RFP (0.1, 0.5 and 1.0 μg). Panel A, right, shows the VLPs released from these cells after an 8 hour chase.

Panel B, left, shows extracts of pulse labeled cells that were simultaneously transfected with pCAGGS-M and either pBJ5 vector or pBJ5-Vps4A-E228Q-Flag. Panel B, right, shows the VLPs released from these cells after an 8 hour chase. Transfected 293T cells in both A and B were labeled in a pulse-chase protocol as described in Figure 1. Particles from supernatants were concentrated by centrifugation onto a sucrose pad as described in Example 4.

Panels C and D show percent VLPs released from cells transfected with pCAGGS-M and pDsRed2-Nl-CHMP3 or pBJ5-Vps4A-E228Q relative to those released from cells transfected with pCAGGS-M and vector only.

Panels E and F show the quantitation of protein expression (pulse label) in the cell extracts. Identical results were obtained in two separate experiments.

Figure 6 presents a schematic of one embodiment of the viral protein structure of a representative Paramyxovirus.

Figure 7 presents a schematic of one embodiment of an infectious cycle caused by a representative Paramyxovirus.

Figure 8 presents an amino acid sequence (SEQ ID NO:6) (Panel A) and a nucleotide sequence (SEQ ID NO:7) (Panel B) encoding a first Newcastle disease virus nucleocapsid protein (AB124608).

Figure 9 presents an amino acid sequence (SEQ ID NO:8) (Panel A) and a nucleotide sequence (SEQ ID NO:9) (Panel B) encoding a first Newcastle disease virus hemagglutinin-neuraminidase protein (AY288990).

Figure 10 presents a partial amino acid sequence (SEQ ID NO:10) (Panel A) and a partial nucleotide sequence (SEQ ID NO:11) (Panel B) encoding a first Newcastle disease virus fusion protein (Y18728).

Figure 11 presents an amino acid sequence (SEQ ID NO:12) (Panel A) and a nucleotide sequence (SEQ ID NO:13) (Panel B) encoding a first Newcastle disease virus matrix protein (AY728363).

Figures 12A/B present of a nucleotide sequence (SEQ ID NO:14)for a baculovirus expression vector (DQ003705).

Figure 13 presents two exemplary plasmids comprising a pCAGGS expression vector. Panel A: pCAGGS/MCS; Panel B: pJW4303 (US Pat. No. 5,916,879, herein incorporated by reference). It should be noted that the pCAGGS expression vector comprises a hybrid cytomegalovirus (CMV) beta actin promoter sequence.

Figure 14 presents exemplary autoradiograph data showing viral protein accumulation resulting from a pulse-chase experiment that compares virus release from avian and COS-7 cells. Panel A: F protein. Panel B: NP protein.

Figure 15 presents exemplary data showing the quantification pulse-chase autoradiography shown in Figure 14. Panel A: F protein. Panel B: NP protein. Diamonds: Avian cells. Squares: COS-7 cells.

Figure 16 presents exemplary autoradiograph data from purification of VLPs in sucrose gradients released from avian cells (Panel A) and from COS-7 cells (Panel B). Lanes 1 – 9 provide banding patterns in sucrose densities of 1.12 – 1.26, respectively. HN = heamagglutinin-neuraminidase protein. F_0 = fusion protein; NP = nucleocapsid protein; M = matrix protein.

Figure 17 presents an exemplary autoradiograph showing residual viral proteins in cell extract lysates following a pulse-chase experiment. Panel A: Avian cells. Panel B; COS-7 cells.

Figure 18 presents exemplary data demonstrating the improved efficiency of M protein VLP release from avian (Panel A) versus COS-7 primate cells (Panel B) when transfected only by an M protein cDNA. Radioactively labeled M protein (M arrow) is shown in each sucrose gradient density fraction (i.e., Lanes 1 – 9; 1.12 – 1.26) is shown.

Figure 19 presents exemplary densitometry data comparing a quantification of VLP particle release from avian (Panel A) and COS-7 primate cells (Panel B) after transfection by either NP, M, F-K115Q, and HN protein cDNAs individually, or transfected using a combination of NP, M, F-K115Q, and HN protein cDNAs, in combination (ALL).

Figure 20 presents an amino acid sequence (SEQ ID NO:15) (Panel A) and a nucleotide sequence (SEQ ID NO:16) (Panel B) encoding a second Newcastle disease virus hemagglutinin-neuraminidase mRNA (M22110).

Figure 21 presents an amino acid sequence (SEQ ID NO:17) (Panel A) and a nucleotide sequence (SEQ ID NO:18) (Panel B) encoding a third Newcastle disease virus hemagglutinin-neuraminidase protein (U37193).

Figure 22 presents an amino acid sequence (SEQ ID NO:19) (Panel A) and a nucleotide sequence (SEQ ID NO:20) (Panel B) encoding a second Newcastle disease virus fusion protein (M21881).

Figure 23 presents an amino acid sequence (SEQ ID NO:21) for a third Newcastle disease virus B1 fusion protein (AAG36978).

Figure 24 presents an amino acid sequence (SEQ ID NO:22) (Panel A) and a nucleotide sequence (SEQ ID NO:23) (Panel B) encoding a second Newcastle disease virus nucleocapsid protein. (AF060483).

Figure 25 presents an amino acid sequence (SEQ ID NO:24) (Panel A) and a nucleotide sequence (SEQ ID NO:25) (Panel B) encoding a second Newcastle disease virus matrix protein (M16622).

Figure 26 presents one embodiment of an amino acid sequence (SEQ ID NO:26) (Panel A) and a nucleotide sequence (SEQ ID NO:27) (Panel B) encoding a third Newcastle disease virus matrix protein (U25828).

Figures 27A – 27D present a nucleotide sequence (SEQ ID NO:28) of a Newcastle disease virus B1 complete genome (AF309418).

Figure 28 illustrates one method of constructing baculovirus recombinant DNA.

Figure 29 illustrates one ligation-independent cloning technique to produce a baculovirus recombinant DNA containing His-tag and S-tag sequence tags.

Figure 30 depicts a circular map of a wild-type AcNPV C6 genome containing 154 putative open reading frames. Genes marked with solid arrows are known and reported in protein sequence databases. hr = AcNPV repetitive homologous region positions.

Figure 31 illustrates seven (7) embodiments of a baculovirus transfer plasmid (pBAC).

Figure 32 presents one embodiment of an amino acid sequence (SEQ ID NO:29) (Panel A) and a nucleotide sequence (SEQ ID NO:30) (Panel B) encoding a first measles virus hemagglutinin protein (AY249267).

Figure 33 presents one embodiment of an amino acid sequence (SEQ ID NO:31) (Panel A) and a nucleotide sequence (SEQ ID NO:32) (Panel B) encoding a second measles virus hemagglutinin protein (AY249269).

Figure 34 presents one embodiment of an amino acid sequence (SEQ ID NO:33) (Panel A) and a nucleotide sequence (SEQ ID NO:34) (Panel B) encoding a third measles virus hemagglutinin protein (DQ011611).

Figure 35 presents one embodiment of an amino acid sequence (SEQ ID NO:35) (Panel A) and a nucleotide sequence (SEQ ID NO:36) (Panel B) encoding a first measles virus fusion protein (AJ133108).

Figure 36 presents one embodiment of an amino acid sequence (SEQ ID NO:37) (Panel A) and a nucleotide sequence (SEQ ID NO:38) (Panel B) encoding a second measles virus fusion protein (X05597).

Figure 37 presents one embodiment of an amino acid sequence (SEQ ID NO:39) (Panel A) and a nucleotide sequence (SEQ ID NO:40) (Panel B) encoding a third measles virus fusion protein (Y17840).

Figure 38 presents one embodiment of an amino acid sequence (SEQ ID NO:41) (Panel A) and a nucleotide sequence (SEQ ID NO:42) (Panel B) encoding a first measles virus nucleocapsid protein (M89921).

Figure 39 presents one embodiment of an amino acid sequence (SEQ ID NO:43) (Panel A) and a nucleotide sequence (SEQ ID NO:44) (Panel B) encoding a second measles virus nucleocapsid protein (AF171232).

Figure 40 presents one embodiment of an amino acid sequence (SEQ ID NO:45) (Panel A) and a nucleotide sequence (SEQ ID NO:46) (Panel B) encoding a third measles virus nucleocapsid protein (X01999).

Figure 41 presents one embodiment of an amino acid sequence (SEQ ID NO:47) (Panel A) and a nucleotide sequence (SEQ ID NO:48) (Panel B) encoding a first measles virus matrix protein (D12682).

Figure 42 presents one embodiment of an amino acid sequence (SEQ ID NO:49) (Panel A) and a nucleotide sequence (SEQ ID NO:50) (Panel B) encoding a second measles virus matrix protein (D12683).

Figure 43 presents one embodiment of an amino acid sequence (SEQ ID NO:51) (Panel A) and a nucleotide sequence (SEQ ID NO:52) (Panel B) encoding a third measles virus matrix protein (AY124779).

Figure 44 presents one embodiment of an amino acid sequence (SEQ ID NO:53) (Panel A) and a nucleotide sequence (SEQ ID NO:54) (Panel B) encoding a first respiratory syncytial virus G protein (i.e., for example, a glycoprotein G protein)(U92104).

Figure 45 presents one embodiment of an amino acid sequence (SEQ ID NO:55) (Panel A) and a nucleotide sequence (SEQ ID NO:56) (Panel B) encoding a second respiratory syncytial virus G protein (AY333361).

Figure 46 presents one embodiment of an amino acid sequence (SEQ ID NO:57) (Panel A) and a nucleotide sequence (SEQ ID NO:58) (Panel B) encoding a third respiratory syncytial virus G protein (AB117522).

Figure 47 presents one embodiment of an amino acid sequence (SEQ ID NO:59) (Panel A) and a nucleotide sequence (SEQ ID NO:60) (Panel B) encoding a first respiratory syncytial virus fusion protein (AY198177).

Figure 48 presents one embodiment of an amino acid sequence (SEQ ID NO:61) (Panel A) and a nucleotide sequence (SEQ ID NO:62) (Panel B) encoding a second respiratory syncytial virus fusion protein (Z26524).

Figure 49 presents one embodiment of an amino acid sequence (SEQ ID NO:63) (Panel A) and a nucleotide sequence (SEQ ID NO:64) (Panel B) encoding a third respiratory syncytial virus fusion protein (D00850).

Figure 50 presents one embodiment of an amino acid sequence (SEQ ID NO:65) (Panel A) and a nucleotide sequence (SEQ ID NO:66) (Panel B) encoding a first respiratory syncytial virus matrix protein (U02470).

Figure 51 presents one embodiment of an amino acid sequence (SEQ ID NO:67) (Panel A) and a nucleotide sequence (SEQ ID NO:68) (Panel B) encoding a second respiratory syncytial virus matrix protein (AY198177).

Figure 52 presents one embodiment of an amino acid sequence (SEQ ID NO:69) (Panel A) and a nucleotide sequence (SEQ ID NO:70) (Panel B) encoding a first respiratory syncytial virus nucleocapsid protein (U07233).

Figure 53 presents one embodiment of an amino acid sequence (SEQ ID NO:71) (Panel A) and a nucleotide sequence (SEQ ID NO:72) (Panel B) encoding a second respiratory syncytial virus nucleocapsid protein (X00001).

Figure 54 presents one embodiment of an amino acid sequence (SEQ ID NO:73) (Panel A) and a nucleotide sequence (SEQ ID NO:74) (Panel B) encoding a third respiratory syncytial virus nucleocapsid protein (S40504).

Figure 55 presents one embodiment of an amino acid sequence (SEQ ID NO:75) (Panel A) and a nucleotide sequence (SEQ ID NO:76) (Panel B) encoding a first parainfluenza virus 3 nucleocapsid protein (D10025).

Figure 56 presents one embodiment of an amino acid sequence (SEQ ID NO:77) (Panel A) and a nucleotide sequence (SEQ ID NO:78) (Panel B) encoding a first parainfluenza virus 3 fusion protein (D00016).

Figure 57 presents one embodiment of an amino acid sequence (SEQ ID NO:79) (Panel A) and a nucleotide sequence (SEQ ID NO:80) (Panel B) encoding a second parainfluenza virus 3 fusion protein (AF394241).

Figure 58 presents one embodiment of an amino acid sequence (SEQ ID NO:81) (Panel A) and a nucleotide sequence (SEQ ID NO:82) (Panel B) encoding a first parainfluenza virus 3 matrix protein (D00130).

Figure 59 presents one embodiment of an amino acid sequence (SEQ ID NO:83) (Panel A) and a nucleotide sequence (SEQ ID NO:84) (Panel B) encoding a first parainfluenza virus 3 hemagglutinin-neuraminidase protein (AB189960).

Figure 60 presents one embodiment of an amino acid sequence (SEQ ID NO:85) (Panel A) and a nucleotide sequence (SEQ ID NO:86) (Panel B) encoding a second parainfluenza virus 3 hemagglutinin-neuraminidase protein (AB189961).

Figure 61 presents one embodiment of an amino acid sequence (SEQ ID NO:87) (Panel A) and a nucleotide sequence (SEQ ID NO:88) (Panel B) encoding a third parainfluenza virus 3 hemagglutinin-neuraminidase protein (L25350).

Figure 62 presents exemplary data showing that M proteins may be encased in membranous particles. Avian cells were transfected with pCAGGS-M and radioactively labeled VLPs were isolated and purified. Extract (upper panel) and VLPs (middle panel) were treated with different concentrations (0.25, 0.5, 1, 5, 10, and 20 μg /ml; lanes 2 to 7 respectively) of Proteinase K for 30 minutes on ice. In parallel, VLPs were incubated in 1% Triton X-100 prior to Proteinase K treatment (bottom panel). After incubation with protease, reactions were stopped by adding 0.1 mM PMSF. M proteins were then immunoprecipitated.

Figure 63 presents exemplary data showing that M protein is required for VLP release. Avian cells were transfected with all possible combinations of cDNAs in pCAGGS vector encoding NP, F, and HN proteins in the absence of M cDNA (F-K115Q+HN, F-K115Q+NP, HN+NP, NP+F-K115Q+HN). Particles in cell supernatants were then purified. Panels show proteins present in each gradient fraction. Radioactively labeled infected cell extract was used as marker. Densities of fractions are shown at the bottom (g/cc).

Figure 64 presents exemplary data showing co-localization of M protein with F and HN proteins. The cell surface localization of NDV F and HN proteins and the cellular localization of M proteins were analyzed by immunofluorescence microscopy. Avian cells were either transfected individually (A) or with F-K115Q+M or HN+M (B), with NP+M+F-K115Q, NP+M+HN or M+F-K115Q+HN (C) and all 4 cDNAs (D). Nuclei were stained with DAPI (blue) 40 h post-transfection. Intact transfected cells were stained with rabbit anti-F protein antibodies or anti-HN protein antibodies as indicated in the panels. Cells were permeabilized with 0.05% Triton X-100 prior to incubation with anti-M protein antibody. Secondary antibodies were anti-rabbit Alexa 488 conjugate (green) and anti-mouse Alexa 568 conjugate (red). Images were merged using Adobe Photoshop.

Figure 65 presents exemplary data showing co-immunoprecipitation of viral proteins in VLPs. Radioactively labeled VLPs generated from cells expressing NP+M+F-K115Q+HN (A), M+F-K115Q+HN (B), NP+M+F-K115Q (C) and NP+M+HN (D) were lysed in TNE buffer with 1% Triton X -100. Lysed VLPs were then incubated with excess amounts of cocktail of anti-F protein antibodies (anti-HR1, anti-HR2, anti-Ftail, anti-F2-96 and monoclonal anti-F

(G5)), anti-HN protein antibodies (mix of monoclonal antibodies), anti-M protein monoclonal antibody or cocktail of NDV-specific antibodies for overnight at 4°C. No antibody as well as pre-immune sera were used as negative controls. Immune complexes were precipitated with prewashed Pansorbin A for at least 2 h at 4°C with constant mixing. Samples were washed three times in cold TNE with 0.5% Triton X-100. All steps of co-immunoprecipitation were accomplished at 4°C. Proteins were resolved by SDS-PAGE gel electrophoresis. Results show one of three independent experiments, all with identical results.

Figure 66 presents exemplary data showing protein-protein interactions in VLPs. Inset: Various embodiments of viral protein-protein interactions detected by co-immunoprecipitation of proteins in VLPs. Also shown are illustrative potential interactions that may result in assembly of VLPs formed by co-expression of all combinations of NP, F, and HN proteins with M protein.

Figure 67 presents exemplary data showing VLPs released from 293T cells. 293T cells transfected with pCAGGS M (Panel A) or with mixture of pCAGGS-NP, -M, -F-K155Q, and –HN (Panel B), were radioactively labeled with [^{35}S] methionine and [^{35}S] cysteine for 4 hours (P) and then chased in non-radioactive medium for 8 hours (C). Proteins present in cell lysates were immunoprecipitated with a cocktail of antibodies specific for all viral proteins and the precipitated labeled proteins are shown on the left side of each panel. Particles in cell supernatants were then purified. After flotation into sucrose gradients (right side of each panel), each gradient fraction was immunoprecipitated with the antibody cocktail. The density of each fraction (g/cc) is shown at the bottom.

Figure 68 presents exemplary data showing the effect of wild type and dominant-negative mutant protein of the VPS pathway M protein VLP release. Panel A shows cell extracts of 293T cells (top) and corresponding released particles (bottom) from cells co-transfected with pCAGGS-M and either pDsRed2-N1 vector (lane 1), pBJ5-WT-CHMP3 (lane 2) or pDsRed2-N1-CHMP3-RFP (lane 3). Panel C shows cell extracts of 293T cells (top) and corresponding released particles (bottom) from cells co-transfected with pCAGGS-M and either pBJ5 vector (lane 1), pBJ5-WT-Vps4A (lane 2) or pBJ5-Vps4A-E228Q (lane 3). Panel E shows extracts of 293T cells (top) and corresponding VLPs (bottom) from cells co-transfected with pCAGGS-M and either pDsRed2-N1 vector (lane 1), pBJ5-AIP1-HA (lane 2) or pDsRed2-N1-AIP1-HA-CHMP3-RFP (lane 3). Extracts are from pulse labeled cells. VLPs are released from pulse labeled cells during an 8-hour nonradioactive chase. Particles were then purified. Proteins were

immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels B, D and F show quantification of particles released relative to those released from wild type VPS protein controls. Identical results were obtained in two separate experiments.

Figure 69 presents exemplary data showing the effect of dominant negative mutants of CHMP3, Vps4A and AIP1 on the release of complete VLPs. Panel A shows extracts of 293T cells (lanes 1-3) and corresponding released VLPs (lanes 4-6) from cells co-transfected with NDV cDNAs, encoding NP, M, HN, and F proteins, and either pDsRed2-N1 vector (lanes 1 and 4), pBJ5-WT-CHMP3 (lanes 2 and 5) or pDsRed2-N1-CHMP3-RFP (lanes 3 and 6). Panel C shows extracts of 293T cells (lanes 1-3) and corresponding released VLPS (lanes 4-6) from cells co-transfected with the mixture of four NDV cDNAs and either pBJ5 vector (lanes 1 and 4), pBJ5-WT-Vps4A (lanes 2 and 5) or pBJ5-Vps4A-E228Q (lanes 3 and 6). Panel E shows extracts of 293T cells (lanes 1-3) and corresponding VLPs (lanes 4-6) from cells co-transfected with the mixture of NDV cDNAs and either pDsRed2-N1 vector (lanes 1 and 4), pBJ5-AIP1-HA (lanes 2 and 5) or pDsRed2-N1-AIP1-HA-RFP (lanes 3 and 6). Extracts are from pulse labeled cells. VLPs are released from pulse labeled cells during an 8-hour nonradioactive chase. Particles were then purified. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels B, D, and F show quantification of VLPs released relative to vector and to wild type Vps protein controls. Identical results were obtained in two separate experiments.

Figure 70 presents exemplary data demonstrating the functionality of the L domain in NDV M protein. Panel A shows wild type M protein, mutant M proteins with alanine substitutions at amino acid positions 216 and 219 (M-$A_{216}A_{219}$) or 232 and 235 (M-$A_{232}A_{235}$), and YPDL or PTAP substitutions at positions 232 - 235. Panel B shows extract (top) and VLPs released (bottom) from 293T cells expressing wild type or mutant M proteins. Panel D shows extract (left) and VLPs released (right) from 293T cells expressing NP, F and HN proteins with either wild type or mutant M proteins. Particles were then purified. Proteins were immunoprecipitated using NDV protein-specific antibodies and resolved by SDS-PAGE. Panels C and E shows quantification of VLPs released relative to wild type M protein. Identical results were obtained in two separate experiments.

Figure 71 presents exemplary data showing the incorporation of AIP1 in VLPs. 293T cells were transfected with pCAGGS M and either empty vector, or vector with HA-tagged AIP1. Panel A shows radioactively labeled M protein precipitated from cell extracts (anti-M IP) and VLPs using M protein-specific monoclonal antibody. HA-AIP1 (N-terminally tagged) and AIP1-HA (C-terminally tagged) were detected in extracts and VLPs by immunoblotting using HA antibody conjugated with peroxidase (anti-HA-IB). Panel B shows precipitated radiolabeled M protein and AIP1-HA from cell extracts (top) and VLPs (bottom).

Figure 72 presents exemplary data comparing the protein content of purified NDV virus and VLPs without prior immunoprecipitation.

Figure 73 presents exemplary electron micrographs showing virus (B1) (upper panel), M protein-only VLPs (middle panel) and NP, M, F, and HN VLPs (lower panel).

Figure 74 presents exemplary data showing a silver stain of virus (B1) when grown in eggs as compared to VLPs prepared from a large scale tissue culture.

Detailed Description Of The Invention

The present invention relates to the field of viral vaccines. In one embodiment, the present invention contemplates a paramyxoviral vaccine effective against diseases such as, but not limited to, Newcastle disease, measles, parainfluenza virus 3, and respiratory syncytial virus. In one embodiment, the present invention contemplates a vaccine comprising Newcastle disease virus-like particles (VLP). In one embodiment, the present invention contemplates a method comprising transfecting avian cells with cDNAs encoding major NDV structural proteins. In another embodiment, a method wherein particles resembling infectious virions are released with nearly 100% efficiency. In one embodiment, the particles are non-infectious and provide a safe and effective NDV vaccine.

Paramyxoviruses have a negative, single-stranded RNA genome which is usually linear. Paramyxovirus morphology comprises a relatively spherical shape having diameters ranging between approximately 150 – 350 nanometers (nm). Generally, the genomes are packaged with nucleoprotein into ribonucleoprotein cores. Polymerase proteins may also be associated with these ribonucleoprotein cores which play a role in early infection replication and transcription

processes. The matrix protein is a prominent feature of paramyxoviruses and lines the inner face of the viral membrane. Transmembrane proteins (i.e., for example, heamaglutinin, fusion or neuraminidase proteins) all form homo-oligomeric complexes (i.e., known in the art as spike proteins) and assist with virus assembly localized at the host cell plasma membrane. Garoff et al., "Virus Maturation By Budding" *Microbiol Mol Biol Rev* 62:1171-1190 (1998).

I. Viral Structure And Assembly

Paramyxoviruses are enveloped and known to assemble their virion components at the plasma membrane of infected cells and subsequently release progeny particles by the process of budding. Newcastle disease virus (NDV), measles, parainfluenza virus 3, and respiratory syncytial virus all belong to *Paramyxoviridae,* characterized as an enveloped virus with a genomic negative-stranded RNA (i.e., for example, approximately 16KB) that is packaged with nucleoprotein into a ribonucleoprotein (RNP) core.

The paramyxovirus RNP core also contains the polymerase complex, which is composed of a Phosphoprotein and Large Polymerase. The RNP core is encased in a membrane which contains two transmembrane glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins, as well as the matrix (M) protein, which is associated with the inner surface of the lipid-containing viral envelope. Lamb *et al.*, "Paramyxoviridae: The Viruses and Their Replication" pp. 1305-1340. In: Fields Virology, Third Edition, Vol. 1., Eds: D. M. K. &. P. M. Howley, LippincottWilliams & Wilkins, Philadelphia (2001).

The matrix protein of many enveloped RNA viruses are believed to play a role in virus assembly and budding. Freed, E. O., "The HIV-TSGI0l interface: recent advances in a budding field" *Trends Microbiol.* 11:56-9 (2003); Jasenosky et al., "Filovirus budding" *Virus Res.* 106:1B1-8 (2004); Jayakar et al., "Rhabdovirus assembly and budding" *Virus Res.* 106:117-32 (2004); Peeples M. E., "Paramyxovirus M proteins: pulling it all together and taking it on the road" pp. 427-456. In: The Paramyxoviruses, Ed: D. W. Kingsbury, Plenum, New York, N.Y (1991); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); Schmitt et al., "Escaping from the cell: assembly and budding of negative-strand RNA viruses" *Cuff Top Microbiol Immunol* 283:145-96 (2004); and Takimoto et al., "Molecular mechanism of paramyxovirus budding" *Virus Res.* 106:133-45 (2004). However, expression of the retroviral gag precursor protein, in the absence of other viral components, also

results in the assembly and release of gag virus-like particles (VLPs) from the plasma membrane. Delchambre et al., "The GAG precursor of simian immunodeficiency virus assembles into virus-like particles" *EMBO J* 8:2653-60 (1989); Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Gheysen et al., "Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells" *Cell* 59:103-12 (1989); and Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004). It has been unclear, therefore, which NDV proteins are sufficient and necessary to direct viral particle formation and release.

A. M Proteins

In one embodiment, the present invention contemplates a method comprising an M protein from a paramyxovirus, without any additional glycoproteins, wherein VLPs are created.

M proteins isolated from:

i) Ebola virus (Jasenosky et al., "Filovirus budding" *Virus Res.* 106:1B1-8 (2004); Jasenosky et al., "Ebola virus VP40-induced particle formation and association with the lipid bilayer" *J. Virol.* 75:5205-14 (2001); and Timmins et al., "Vesicular release of Ebola virus matrix protein VP40" *Virology* 283: 1-6 (2001));

ii) vesicular stomatitis virus (Jayakar et al., "Rhabdovirus assembly and budding" *Virus Res.* 106:117-32 (2004); Li et al., "Viral liposomes released from insect cells infected with recombinant baculovirus expressing the matrix protein of vesicular stomatitis virus" *J. Virol.* 67:4415-20 (1993); and Sakaguchi et al., "Double-layered membrane vesicles released from mammalian cells infected with Sendai virus expressing the matrix protein of vesicular stomatitis virus" *Virology* 263:230-43 (1999))

and, iii) influenza virus (Gomez-Puertas et al., "Influenza virus matrix protein is the major driving force in virus budding" *J. Virol.*74:11538-47 (2000)), when expressed alone, assemble into and are released as VLPs.

Conversely, M protein-deficient rabies virus is known to be severely impaired in virion formation. Mebatsion et al., "Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G" *J. Virol.* 73:242-50 (1999).

Studies in several paramyxovirus systems have also suggested a role for the M protein in virus assembly and budding. Measles virus (MV) and Sendai virus (SV) modified by reverse genetics to lack the M protein genes were impaired in budding. Cathomen et al., "A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain" *EMBO J* 17:3899-3908 (1998); and Inoue et al., "A new Sendai virus vector deficient in the matrix gene does not form virus particles and shows extensive cell-to-cell spreading" *J. Virol.* 77:6419-29 (2003), respectively, Moreover, MV containing mutant M protein derived from subacute sclerosing panencephalitis (SSPE) virus was also defective in budding. Patterson et al., "Evidence that the hypermutated M protein of a subacute sclerosing panencephalitis measles virus actively contributes to the chronic progressive CNS disease" *Virology* 291:215-25 (2001).

Recent studies of paramyxovirus assembly have also focused on identifying the viral protein requirements for assembly and budding of VLPs and have demonstrated a role for the M protein. The human parainfluenza virus type 1 (hPIV1) M protein and the SV M protein expressed alone induced budding of VLPs from the plasma membrane. Coronel et al., "Human parainfluenza virus type 1 matrix and nucleoprotein genes transiently expressed in 12 mammalian cells induce the release of virus-like particles containing 13 nucleocapsid-like structures" *J. Virol.* 73:7035-8 (1999); Sakaguchi et al., "Double-layered membrane vesicles released from mammalian cells infected with Sendai virus expressing the matrix protein of vesicular stomatitis virus" *Virology* 263:230-43 (1999); Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004); and Takimoto et al., "Role of matrix and fusion proteins in budding of Sendai virus" *J. Virol.* 75: 11384- 91 (2001). Expression of M protein was also required for Simian Virus 5 (SV5) VLP formation. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002).

However, in contrast to PIY1 and SV, the SV5 M protein was not sufficient for VLP release. Rather, simultaneous expression of SV5 M protein, together with NP and either of the glycoproteins was required. Although existing reports agree upon a role for M protein as a budding organizer in paramyxovirus particle release, there are differences in the protein requirements for assembly and budding of virions. The budding capacities of retrovirus gag protein, Ebola virus M protein, and influenza Ml protein are attributed, in part, to Late Domains

(*infra*). Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Freed, E. O., "Viral late domains" *J. Virol.* 76:4679-87 (2002); Jasenosky et al., "Filovirus budding" *Virus Res.* 106:1B1-8 (2004); Jayakar et al., "Rhabdovirus assembly and budding" *Virus Res.* 106:117-32 (2004); Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); Nayak et al., "Assembly and budding of influenza virus" *Virus Res* 106:147-65 (2004); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); Schmitt et al., "Escaping from the cell: assembly and budding of negative-strand RNA viruses" *Cuff Top Microbiol Immunol* 283:145-96 (2004); Strack et al., "AIP 1/ALIX is a binding partner for HIV - 1 p6 and EIA V p9 functioning in virus budding" *Cell* 114:689-99 (2003); and von Schwedler et al., "The protein network of HIV budding" *Cell* 4:701-13 (2003).

B. Late Domains

Late Domains are short peptide motifs that mediate interactions with a member of the class E proteins, which are involved in the vacuolar protein sorting (VPS) pathway. The Late Domain promotes budding by interacting with components of the cellular machinery responsible for sorting cargo into multivesicular bodies (MVB). The formation of MVB vesicles and the budding of a virus are topologically similar processes. Available evidence suggests that enveloped RNA viruses bud by co-opting the cellular machinery that is normally used to create MVB inside the cell. Carter, C. A., "Tsg101: HIV-1 's ticket to ride" *Trends Microbiol.* 10:203-205 (2002); Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Freed, E. O., "The HIV-TSGI01 interface: recent advances in a budding field" *Trends Microbiol.* 11:56-9 (2003); Freed, E. O., "Viral late domains" *J. Virol.* 76:4679-87 (2002); Garrus et al., "Tsg 1-01 and the vacuolar protein sorting pathway are essential for HIV-1 budding" *Cell* 107:55-65 (2001); Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); Pornillos et al., "*HN* Gag mimics the TsgI01-recruiting activity of the human Hrs protein" *J Cell Biol* 162:425-34 (2003); Strack et al., "AIP 1/ALIX is a binding partner for HIV -1 p6 and EIA V p9 functioning in virus budding" *Cell* 114:689-99 (2003); von Schwedler et al., "The protein network of HIV budding" *Cell* 4:701-13 (2003). Martindale, D., "Budding viral hijackers co-opt the endocytic machinery to make a getaway" *J Biol.* 3:2 (2003); and Simons et al., "The budding mechanisms of enveloped animal viruses" *J. Gen. Virol.* 50:1-21 (1980).

In one embodiment, the present invention contemplates that dominant negative mutant protein component of the VPS pathway may also inhibit particle release. In one embodiment, an YXXL (SEQ ID NO:3) sequence in the NDV M protein has properties of a Late Domain. Although it is not necessary to understand the mechanism of an invention, it is believed that the YXXL mutation abolishes particle release while substitution of late domains such as YPDL and/or PTAP fully restore particle release.

C. Budding

Within the paramyxovirus family, it is known that the VPS pathway is involved in the SV5 budding. It was shown that a dominant-negative mutation VPS4(E228Q) (an ATPase required for recycling protein complexes involved in the VPS pathway) inhibited budding of SV5 virions as well as VLPs. Schmitt et al., "Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus" *J. Virol.* 79:2988-97 (2005). Since it is known that VPS4(E228Q) also inhibits the VPS pathway, one may believe that the VPS pathway is involved in SV5 budding. In addition, a putative Late Domain in SV5 M was identified. However, SV5 M protein is not sufficient for VLP formation and release, complicating the interpretation of this result. Thus, the general rules for assembly and release of paramyxoviruses are not yet clear. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002). Open questions include: i) the further definition of paramyxovirus late domains in viral structural proteins, ii) the role or contribution of each viral protein in virus assembly, and iii) the cellular factors involved in the assembly and budding process.

Various embodiments of the present invention answer these questions. In one embodiment, the present invention contemplates a method for producing NDV VLPs from cells transfected with nucleic acids encoding viral structural proteins. In another embodiment, the present invention contemplates transfecting with nucleic acid encoding an NDV M protein that is both necessary and sufficient for release of lipid-containing particles (i.e., for example VLPs). In another embodiment, the present invention contemplates that the most efficient incorporation (i.e., for example, almost 100%) of other viral proteins into VLPs requires the expression of M protein with at least two other NDV proteins. For example, it is known that dominant-negative mutations of CHMP3 and Vps4 proteins (both components of the host VPS system) inhibited

release of VLPs. Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); Strack et al., "AIP 1/ALIX is a binding partner for HIV -1 p6 and EIA V p9 functioning in virus budding" *Cell* 114:689-99 (2003); and von Schwedler et al., "The protein network of HIV budding" *Cell* 4:701-13 (2003). It is further contemplated that AIP1 is also incorporated into VLPs thereby playing a role in NDV particle budding.

D. Dominant Negative Mutations

The dominant negative Vps4 protein may block release of SV5 virions or VLPs composed of NP, HN, F, and M proteins, implicating the VPS system in paramyxovirus release. Schmitt et al., "Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus" *J. Virol.* 79:2988-2997 (2005). Confirming these results, a dominant negative version of Vps4, Vps4 A-E228Q, blocked NDV VLP release. Martin-Serrano et al., "Role of ESCRT-I in retroviral budding" *J Virol* 77:4794-4804 (2003); Strack et al., "AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003); and von Schwedler et al., "The protein network of HIV budding" *Cell* 114:701-713 (2003)).

Although it is not necessary to understand the mechanism of an invention, it is believed that the results demonstrated herein show that these dominant negative proteins blocked release of particles containing only M protein. For example, a dominant negative version of CHMP3, a subunit of the ESCRT III complex (1), and a dominant negative mutant of AIP1, a protein that binds both ESCT I and III proteins, inhibited NDV VLP release as well as release of particles containing only M protein. This inhibition was not due to over expression of the protein since transfection of the wild type versions of these proteins had little effect on M particle release. These results show that an intact VPS pathway facilitates NDV VLP budding. Furthermore, these results indicate that the VPS pathway is involved in M particle release.

Many studies have demonstrated that L domains in the matrix proteins of viruses mediate their interaction with specific molecules of the VPS pathway. Bieniasz, P. D., "Late budding domains and host proteins in enveloped virus release" *Virology* 344:55-63 (2006); Freed, E. O., "Viral late domains" *J. Virol.* 76:4679-4687 (2002); and Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol* 20:395-425 (2004). Three L domain motifs, PTAP, YPXL, and PPXY (Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-579 (2002)), have been identified in retroviruses (Puffer et al., "Equine infectious anemia virus

utilizes a YXXL motif within the late assembly domain of the Gag p9 protein" *J Virol* 71:6541-6546 (1997)), rhabdoviruses and filoviruses (Irie et al., "Budding of PPxY-containing rhabdoviruses is not dependent on host proteins TGS101 and VPS4A" *J Virol* 78:2657-2665 (2004)). An YRKL sequence has been identified as a late domain in orthomyxoviruses (Hui et al., "YRKL sequence of influenza virus M1 functions as the L domain motif and interacts with VPS28 and Cdc42" *J Virol* 80:2291-2308 (2006)).

Binding of the PTAP sequence to TSG101 (tumor susceptibility gene 101) protein, a component of ESCRT I, has been reported. Huang et al., "p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease" *J Virol* 69:6810-6818 (1995). Further, the YPXL sequence has been shown to interact with AP2 (adaptor protein 2) and AIP1. Chen et al., "Functions of early (AP-2) and late (AIP1/ALIX) endocytic proteins in equine infectious anemia virus budding" *J Biol Chem* (2005); and Strack et al., "AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003), respectively. The YRKL sequence in the influenza virus M1 protein binds to VSP28, an ESCRT 1 protein that binds tsg101, as well as Cdc42, a member of the Rho family of GTP-binding proteins. The PPXY motif binds to Nedd4-like (neural precursor cell expressed, developmentally down regulated gene 4) ubiquitin ligases. Vana et al., "Role of Nedd4 and ubiquitination of Rous sarcoma virus Gag in budding of virus-like particles from cells" *J Virol* 78:13943-13953 (2004); and Xiang et al., "Fine mapping and characterization of the Rous sarcoma virus Pr76gag late assembly domain" *J Virol* 70:5695-5700 (1996)).

Paramyxovirus M proteins do not have a PTAP, an YPXL, an YRKL, or a PPXY motif. The sequence FPIV, however, in the SV5 M protein may be a late domain in paramyxoviruses. Mutation of FPIV inhibited release of particles and addition of this sequence in a retrovirus gag construct stimulated the release of particles. However, since the SV5 M protein is not sufficient for SV5 particle release, FPIV is not believed to function independently as a late domain in the context of this paramyxovirus M protein. Schmitt et al., "Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus" *J. Virol.* 79:2988-2997 (2005).

Thus, it is not clear how SV5 uses the VPS pathway or how the FPIV sequence might function as a late domain. Sequence analysis of the NDV M protein shows the presence of this FPIV motif. In addition, NDV M protein contains a PKSP and a YANL sequence, not found in

the SV5 M protein. In one embodiment, the present invention contemplates a YANL motif comprising properties of an L domain. In one embodiment, a YANL mutation reduces M protein particle release. Although it is not necessary to understand the mechanism of an invention, it is believed that substitution of a YANL mutation with other known late domains (i.e., for example, PTAP or YPDL) particle release may become fully restored.

It is further believed that inhibition of particle release by mutation of the YANL sequence is not likely due only to effects on protein folding. The data provided herein suggests that the NDV M protein may access the VPS pathway using either type of late domain, an YPDL or a PTAP domain and that the FPIV sequence in the NDV M protein may not function as a late domain independent of the YANL sequence since the YANL mutant protein M-A_{232}-A_{235} has a wild type FPIV sequence.

YPDL late domains have been shown to interact with the VPS protein AIP1. In one embodiment, the present invention contemplates that AIP1 protein is found in released particles containing only M protein.

The M protein of Sendai virus has also been shown to be sufficient for release of particles (Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004); and Takimoto et al., "Role of matrix and fusion proteins in budding of Sendai virus" *J. Virol.* 75:11384-11391 (2001)). The Sendai virus M protein has an YLDL sequence, which could serve as a late domain for SV M protein. As noted above, the SV5 M protein is not sufficient for release of neither particles nor does it has an YXXL motif. Schmitt et al., "Requirements for budding of paramyxovirus simian virus 5 virus-like particles" *J Virol* 76:3952-3964 (2002). However, the SV5 NP protein has a number of YXXL motifs including a YPLL sequence. Alternatively, an SV5 late domain may be present on the SV5 NP rather than the M protein. Indeed, it has been reported that SV5 VLP release is significantly enhanced by the expression of the SV5 NP protein with M protein as well as a glycoprotein. Schmitt et al., "Requirements for budding of paramyxovirus simian virus 5 virus-like particles" *J Virol* 76:3952-3964 (2002). Consequently, it is clear that differential requirements for the release of particles in different paramyxovirus systems exist and may be due in part to different distributions of the late domains on structural proteins. Nevertheless, the present invention contemplates that the host cell VPS

pathway facilitates M protein budding and that the YANL motif in the NDV M protein has the properties of a late domain.

II. Virus-Like Particle (VLP) Formation And Release

In one embodiment, the present invention contemplates transfecting a host cell with nucleic acid encoding only a paramyxovirus M protein so that the transfected cells express the matrix protein and create paramyxoviral VLPs. In another embodiment, the present invention contemplates co-expression of two or more paramyxovirus glycoproteins including, but not limited to, NP, F-K115Q, and/or HN proteins (together with M protein) under conditions such that paramyxovirus VLP formation and release occurs.

The present invention contemplates conditions for the efficient generation of VLPs of a virulent paramyxoviral strain. In one embodiment, the paramyxoviral strain comprises the group including, but not limited to, Newcastle disease, measles, parainfluenza virus 3, or respiratory syncytial virus. In another embodiment, the VLPs comprise the same major antigens as infectious virus. In another embodiment, the VLPs comprise major antigens having the same ratios as infectious virus. In one embodiment, the major antigens are selected from the group comprising nucleocapsid protein, membrane/matrix protein, hemagglutinin-neuraminidase protein, and fusion protein.

The production of VLPs in accordance with embodiments of the present invention is much simpler and likely more cost effective than currently available live or attenuated virus vaccines. VLPs can be harvested from cell supernatants and purified by the same protocols used to purify virus. VLPs can be engineered to increase the spectrum of immune responses. The VLPs can also be engineered so that the immune response can be distinguished from that induced by an infection.

A. VLP Release Characteristics

In one embodiment, VLPs are released from cells co-expressing the major structural proteins of paramyxoviruses. In one embodiment, NDV VLP particles are released from a chicken fibroblast cell line co-expressing NP, M, F and HN proteins that can be purified and characterized. In one embodiment, an uncleaved version of F protein eliminated any potential effects of cell-to-cell fusion on virus release. In one embodiment, avian cells are used because

birds are the natural host of NDV. For example, as detailed in the Examples below, cells (i.e., for example, avian or human) were co-transfected with plasmids encoding NDV viral proteins using concentrations of DNA previously determined to result in expression levels and ratios of proteins comparable to infected cells. Cells were then pulse-labeled with ^{35}S- methionine and ^{35}S-cysteine and then chased for 8 hours (a time also resulting in maximal particle release). VLPs in the cell supernatants were isolated and fractionated by sucrose density ultracentrifugation.

In one embodiment, the efficiency of paramyxoviral VLP release from cells expressing at least four viral proteins (85%) was comparable to the efficiency of infectious particle release from paramyxovirus -infected cells (92%). Although it is not necessary to understand the mechanism of an invention, it is believed that this result suggests that four paramyxovirus proteins (i.e., for example, M protein, NP protein, F, protein, or HN protein) may provide an efficient formation of particles. It is further believed that the viral Large Polymerase or Phosphoprotein proteins have little quantitative effect on virus release.

Although it is not necessary to understand the mechanism of an invention, it is believed that paramyxoviral VLPs, which can be isolated on sucrose gradients, have a relatively homogeneous density that is slightly less than the average density of an authentic virus. Although it is not necessary to understand the mechanism of an invention, it is believed that this result is likely due to the absence of the viral genomic RNA in the particles. It is further believed, therefore, that the VLPs are non-infectious.

Although it is not necessary to understand the mechanism of an invention, it is believed that paramyxoviral VLPs are likely folded into conformations virtually identical to an authentic virus and are packaged into particles in a manner identical to paramyxoviral particles. As a result, these particles should be as antigenic as authentic virus. VLPs do not, however, contain the viral genome, since the cells (i.e., for example, avian or human), which are forming and releasing these particles, are not infected with virus. Therefore, VLPs cannot be infectious and cannot cause disease.

B. M Protein Function

In one embodiment, a paramyxovirus M protein is both sufficient and necessary for VLP particle release. In one embodiment, the paramyxovirus is selected from the group including, but

not limited to, Newcastle disease virus, measles virus, parainfluenza virus 3, and syncytial respiratory virus. That is to say, expression of the M protein alone resulted in very efficient release of M protein containing paramyxovirus VLP particles. For example, the efficiency of M protein release is comparable to that observed when at least four proteins were co-expressed. Although it is not necessary to understand the mechanism of an invention, it is believed that this result suggests that it is the M protein that directs the budding of paramyxovirus VLPs. Furthermore, VLPs are released when only M protein is present. Consequently, significant VLP particle release will not occur the absence of M protein even if viral protein expression (or co-expression of a combination of viral proteins) is present. For example, cells expressing HN protein, alone, released only trace amounts of a very light density HN protein-containing material into cell supernatants, and it is unlikely that this material reflects a significant component of virus assembly. In one embodiment, the present invention contemplates that no NDV protein, other than M protein, can function independently in the release of lipid containing particles that reflect virus assembly.

Although it is not necessary to understand the mechanism of an invention, it is believed that VLP particles released from cells expressing only M protein have very heterogeneous densities because this budding occurs indiscriminately from different cell membranes or from different plasma membrane domains and, consequently, contain different lipid-to-protein ratios due to variable M protein oligomerization. For example, particles formed from monomer M protein may have a higher lipid to protein ratio than particles formed from M protein in an oligomeric state. It is known that M proteins of other negative stranded RNA viruses can form oligomeric structures. Garoff et al., "Virus maturation by budding" *Microbiol Mol Biol Rev* 62:1171-90 (1998); and Panch et al., "In vivo oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding" *Proc Natl Acad Sci USA* 100:15936-41 (2003).

C. Glycoprotein Function

Formation of infectious paramyxovirus virions is believed to involve the incorporation of both the HN and F glycoproteins. In one embodiment, the present invention contemplates a composition comprising glycoprotein incorporation into a paramyxovirus VLP when M protein is co-expressed with at least two glycoproteins. Single glycoprotein co-expression (i.e., for example HN+M or F+M) resulted in only trace amounts of either HN or F glycoprotein

incorporated into VLP particles. Further, when HN and F glycoproteins were co-expressed with M protein, the glycoprotein incorporation levels were comparable to that observed with co-expression of at least four proteins.

Although it is not necessary to understand the mechanism of an invention, it is believed that these results indicate that the M protein binds more efficiently with a complex of HN and F glycoproteins. This possibility is also supported by observations that co-expression of these two glycoproteins with M protein resulted in paramyxovirus VLPs having a more homogenous and decreased density. M protein VLP particles generally have a very heterogeneous density. Co-expression of M protein with either glycoprotein, alone, did not change the general density of M protein containing particles. It is believed that these results indicate that interactions of M protein with an HN-F protein complex affected the protein to lipid ratio of the VLPs or affected the membrane from which the particles were released.

It should be noted that not just any combination of M protein and viral glycoproteins produce paramyxovirus VLPs in good yield as contemplated herein. For example, co-expression of a single glycoprotein and an M protein results in a 40-60% VLP release suppression when compared to VLP release observed after: i) co-expression with all four proteins; ii) expression of an M protein with at least two glycoproteins; and iii) expression of M protein alone. Empirical studies revealed that this release suppression is relieved by co-expression of M protein with NP and another glycoprotein.

Although it is not necessary to understand the mechanism of an invention, it is believed that VLP release suppression by a single glycoprotein + M protein is consistent with observations that NP + M protein VLP release is: i) 70% lower when compared to release from cells expressing at least four proteins; and ii) 80% lower when compared to release from cells expressing only M protein. Although it is not necessary to understand the mechanism of an invention, it is believed that the large amount of NP in the cytoplasm may pull M protein away from the plasma membrane, thereby preventing its association with this membrane and, therefore, budding of particles. Consequently, one hypothesis suggests that co-expression with another glycoprotein may redirect both NP and M protein to a cellular membrane thereby relieving VLP release suppression.

D. Vacuolar Protein Sorting (VPS) System And Multivesicular Buds (MVBs)

Although it is not necessary to understand the mechanism of an invention, it is believed that paramyxovirus M protein-dependent VLP release uses the host vacuolar protein sorting (VPS) system. The VPS system has been reported to mediate budding of other enveloped viruses. Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); and Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002).

Budding of retroviruses, filoviruses, and influenza viruses are thought to depend upon the host cell VPS pathway. The VPS pathway also serves to form MVBs. Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Jasenosky et al., "Filovirus budding" *Virus Res.* 106:1B1-8 (2004); Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); Freed, E. O., "Viral late domains" *J. Virol.* 76:4679-87 (2002); and Schmitt et al., "Escaping from the cell: assembly and budding of negative-strand RNA viruses" *Cuff Top Microbiol Immunol* 283:145-96 (2004). MVBs are formed by invagination of endosomal membranes into the endosomallumen thereby creating a vesicle inside a vesicle. Martindale, D., "Budding viral hijackers co-opt the endocytic machinery to make a getaway" *J Biol.* 3:2 (2003). The topology of MVB formation is similar to that of virus budding from plasma membrane.

It has been proposed that viral proteins usurp this host cell machinery to direct virus budding. Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Martindale, D., "Budding viral hijackers co-opt the endocytic machinery to make a getaway" *J Biol.* 3:2 (2003); and Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004). Currently, research suggests that the formation of MVBs involves three protein complexes, first characterized in yeast, and are collectively known as the Endosomal Sorting Complexes Required for Transport (i.e., for example, ESCRT I, II, and III). Babst et al., "ESCRT -III: an endosome-associated heterooligomeric protein complex 4 required for MVB sorting" *Dev Cell* 3:271-282 (2002); Jiang et al., "Multivesicular bodies: a mechanism to package lytic and storage functions in one organelle?" *Trends Cell Biol.* 12:362-7 (2002); Katzmann et al., "Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I" *Cell* 106:145-55 (2001); and Katzmann et al.,

"Vps27 recruits ESCRT machinery to endosomes during MVB sorting" *J Cell Biol.* 162:413-23 (2003). In addition, Vps4 protein (i.e., for example, an ATPase) is required for the dissociation of the full ESCRT complex. Raiborg et al., "Protein sorting into multivesicular endosomes" *Cuff Opin Cell Biol* 15:446-55 (2003).

E. VLP Release Inhibition

Studies with a number of virus types, most prominently retroviruses, have shown that cellular proteins involved in the formation of MVBs are recruited by retrovirus gag proteins and other matrix-like proteins by interactions of viral Late Domains with a component of the VPS pathway. Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); 44. It has been found that dominant negative mutants of Vps4, CHMP3, and CHMP2 can block retrovirus release. Strack et al., "PIP1/ALIX is a binding partner for HIV-1p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003).

Although it is not necessary to understand the mechanism of an invention, it is believed that a dominant-negative mutation of Vps4 or Vps4 A-E228Q is capable of blocking M protein paramyxovirus VLP release. It is further believed that a dominant-negative mutation of CHMP3 (i.e., for example, a subunit of the ESCRT III complex) inhibits M protein paramyxovirus VLP release. These observations indicate not only that the VPS pathway is involved in paramyxoviral budding (i.e., for example, VLP release) but that it is the M protein that directly interacts with the VPS pathway.

It has recently been reported that SV5 VLP and virion release are also inhibited by expression of the dominant negative form of VSP4 implicating the VPS pathway in SV5 assembly and release. Schmitt et al., "Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus" *J. Virol.* 79:2988-97 (2005). Furthermore, the sequence FPIV (SEQ ID NO:1) in the SV5 M protein is believed to be a Late Domain. The SV5 M protein, however, is known not to be sufficient for particle release. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002). Consequently, it is not clear how SV5 uses the VPS pathway or how this sequence might function as a Late Domain.

In one embodiment, a sequence analysis of an NDV M protein also shows the presence of an FPIV motif (SEQ ID NO:1). In one embodiment, an NDV M protein further comprises a PXXP motif (SEQ ID NO:2) and an YXXL motif (SEQ ID NO:3), sequences not found in the SV5 M protein. Other motifs identified in the art might also be candidate Late Domains for other paramyxovirus M proteins; i.e., domains that could function in budding independent of other viral proteins. Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); and Freed, E. O., "Viral late domains" *J. Virol.* 76:4679-87 (2002).

F. Host-Specific VLP Expression

Virus-like particle expression from human 293T cells have been reported in three other paramyxovirus systems (Sendai virus (SV), PIV1, and SV5) at efficiencies ranging between 18% to 70%. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002); Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004); and Takimoto et al., "Role of matrix and fusion proteins in budding of Sendai virus" *J. Virol.* 75: 11384- 91 (2001).

In one embodiment, the present invention contemplates a method comprising improving the efficiency of paramyxovirus VLP release by using cells from the natural host of the virus. In one embodiment, a paramyxovirus is selected from the group including, but not limited to, Newcastle disease virus, measles virus, parainfluenza virus 3, or respiratory syncytial virus. In one embodiment, a M protein paramyxovirus VLP is released from avian cells with an efficiency of 90%. In another embodiment, M protein paramyxovirus VLP is released from human 293T cells with an efficiency of 50%. Furthermore, the efficiency of release of both M protein VLPs, as well as complete VLPs, from COS cells was significantly lower than release from avian cells; a difference that is not due to a lower expression level of viral proteins in COS cells versus avian cells. Although it is not necessary to understand the mechanism of an invention, it is believed that differences between the efficiencies of paramyxovirus VLP formation may be due to a host cell-specific dependency.

It is known that the protein requirements for VLP formation in other paramyxovirus systems also vary. For example, paramyxovirus systems comprising M proteins of SV, hPIV1 and SV5 are considered involved in directing virus assembly and budding, but there are

differences in the role of M protein in actual particle formation. Coronel et al., "Human parainfluenza virus type 1 matrix and nucleoprotein genes transiently expressed in 12 mammalian cells induce the release of virus-like particles containing 13 nucleocapsid-like structures" *J. Virol.* 73:7035-8 (1999); Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002); Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004); and Takimoto et al., "Role of matrix and fusion proteins in budding of Sendai virus" *J. Virol.* 75: 11384- 91 (2001). Similar to NDV M protein, the SV and hPIVl M proteins were sufficient for particle release, the SV5 M protein, however, was not sufficient. SV5 M protein co-expression with NP and at least one glycoprotein was required for efficient formation and release of SV5 VLPs. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002).

In one embodiment, the present invention contemplates that only M protein, and no other paramyxovirus protein, can solely direct VLP particle release. Previous studies do indicate that SV F protein may exhibit an autonomous exocytosis activity demonstrated by the release of vesicles containing the only the F protein. Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004); and Takimoto et al., "Role of matrix and fusion proteins in budding of Sendai virus" *J. Virol.* 75: 11384- 91 (2001).

In contrast, cells contemplated by the present invention expressing the NDV F protein, alone, did not release F protein-containing material, and cells expressing HN protein, alone, released only trace amounts of very light density material HN protein containing material into the cell supernatants. These observations are similar to other reports showing that expression of SV5 F or HN glycoproteins, alone, did not result in VLP particle release. Schmitt et al., "Requirements for budding of paramyxovirus simian virus virus-like particles" *J Virol* 76:3952-64 (2002). Although it is not necessary to understand the mechanism of an invention, it is believed that despite observations that SV F and other enveloped negative strand virus glycoproteins have been shown to exhibit budding activity, no Late Domains have been identified in any viral glycoproteins. Schmitt et al., "Escaping from the cell: assembly and budding of negative-strand RNA viruses" *Cuff Top Microbiol Immunol* 283:145-96 (2004).

Embodiments of the present invention comprising co-expression of M protein and NP is also in contrast with those reported in the SV system. For example, simultaneous expression of SV M and NP is known to result in the release of VLPs containing both viral proteins. Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004).

G. Protein-Protein Interactions

The present invention contemplates using NDV as a prototype paramyxovirus in order to clarify the role of each paramyxovirus protein in particle assembly and release. Using this model, certain embodiments integrate a definition of the viral protein requirements for assembly and release of VLPs with a characterization of the protein-protein interactions in VLPs formed with different combinations of viral proteins.

Further, in some embodiments the present invention contemplates a co-localization of M protein with the viral glycoproteins in plasma membranes. Although it is not necessary to understand the mechanism of an invention, it is believed that the data presented herein show that particle assembly involves a network of specific protein-protein interactions and likely correct targeting of proteins to specific cellular domains.

In one embodiment, the present invention contemplates, VLP protein interactions form with all combinations of three and four proteins (i.e., for example, when defined by co-immunoprecipitation). In another embodiment, cell surface HN and F proteins are co-localized with M protein when expressed in different combinations with M and NP proteins. In another embodiment, co-expression of two viral proteins with M protein also significantly increased the co-localization of M protein with either HN or F proteins in the plasma membrane indicating increased interactions with M protein.

To define these protein-protein interactions, VLPs formed with different combinations of three and four proteins were solubilized with nonionic detergent and proteins precipitated with cocktails of monospecific antibodies for M, HN, or F proteins. First, each antibody cocktail precipitated all proteins from VLPs formed with M, HN, F and NP, although the efficiency of precipitation for each protein varied with the antibody specificity. Although it is not necessary to understand the mechanism of an invention, it is believed that these results are consistent with a network of interactions between all four proteins such that precipitation of one resulted in the precipitation of the other three proteins but with efficiencies that varied determined by how

directly a protein was linked to the precipitated protein.

Protein-protein interactions were more clearly defined by immunoprecipitation of proteins from VLPs formed with all combinations of three proteins. These results show a specific interaction between HN and M proteins, between NP and M protein, and between F protein and NP. (See, Figure 66). A direct interaction between F protein and M protein was not directly observed but there is likely a weak interaction between F and HN proteins, since anti-F protein antibodies precipitated HN protein from VLPs containing M, HN, and F proteins. The apparent inability for F and M proteins to interact suggest that incorporation of F protein into these VLPs may be mediated by interactions with an HN protein. Alternatively, an interaction between HN protein and NP may also facilitate incorporation processes.

Thus, when all four proteins are co-expressed, NP and HN protein are incorporated into VLPs by a direct interaction with M protein. (See, Figure 66). Although it is not necessary to understand the mechanism of an invention, it is believed that F protein is likely incorporated indirectly due to interactions with NP and HN protein. It is further believed that an ordered complex of the four proteins is supported by a co-localization of M protein with F protein and M protein with HN protein in the plasma membrane when all four proteins are co-expressed.

However, when only F is expressed with M protein, F protein was likely not significantly incorporated into VLPs because a direct interaction between these two proteins was not observed. (See, Figure 66). Supporting this conclusion is the observation that there was no co-localization of F and M proteins in the plasma membrane in these cells.

In spite of direct associations of M with NP, there was little NP protein incorporation into VLPs when NP and M proteins were co-expressed in the pair-wise combination. Previous reports that show that the M protein of Sendai virus is recruited in the cytoplasm by the viral nucleocapsid. Stricker et al., "The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding" *J Gen Virol* 75 (Pt 5):1031-1042 (1994). Perhaps NP causes the retargeting of M protein to this compartment. Indeed, co-expression of M protein with NP resulted in a 2.5 fold suppression of M protein containing VLP release, a result also consistent with retention of M protein in cells by NP protein.

Although co-immunoprecipitations of VLP proteins formed with M, HN, and F protein

indicated a direct interaction of HN protein with M protein, there were only low levels of incorporation of HN protein into VLPs when HN and M proteins were co-expressed in a pair-wise combination. Furthermore, there was little co-localization of the two proteins in the plasma membrane. Perhaps, in the absence of other proteins, HN and M proteins show minimal co-localization in the same regions of the cell, thereby preventing their association. Alternatively, it is also possible that the conformation of the HN protein transmembrane or cytoplasmic tail may be different in the absence of expression of F protein or NP protein inhibiting association of HN protein with M protein. The 50% reduction of M protein VLPs upon co- expression of HN protein with M protein cannot be presently explained but similar results have been previously reported in Sendai virus system. Sugahara et al., "Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein" *Virology* 325:1-10 (2004).

It should be realized that immunoprecipitation is not necessary to produce purified VLPs. In one embodiment, the present invention contemplates a VLP preparation comprising pure viral proteins. Protein compositions were compared between purified NDV whole virus and VLPs that have not undergone immunoprecipitation. The data shows that the VLP preparation does not contain any proteins that are not present in the whole virus preparation. See, Figure 72. Consequently, the VLPs are as pure as the whole virus.

Although it is not necessary to understand the mechanism of an invention, it is believed that VLPs formed with NP, M and F proteins are likely due to interactions between M and NP and interactions between F and NP. (See, Figure 66). For example, F protein may relocate NP to the plasma membrane drawing M to specific domains containing F protein. Indeed, data presented herein show that addition of NP increases the co-localization of M protein with F protein in the plasma membrane. It is further believed that VLPs formed with NP, M and HN proteins likely form due to interactions of both HN protein and NP with M protein. Data presented herein, show that expression of NP with HN and M proteins increase the co-localization of M and HN proteins in the plasma membrane. One possible hypothesis suggests that NP-M protein interactions alter the conformation of M thereby facilitating its interaction with HN protein. Indeed, surface HN protein in the presence of NP appears more punctuate along the cell edges.

This network of interactions proposed above could account for the conclusions that the

cytoplasmic domains (CT) of the HN and F proteins have redundant functions. Schmitt et al., "Requirements for budding of paramyxovirus simian virus 5 virus-like particles" *J Virol* 76:3952-3964 (2002). For example, the CT domain of the F protein may target NP-M complexes to the plasma membrane by interactions with NP protein while the HN protein CT domain targets these complexes by virtue of direct interactions with M protein.

The interaction of M protein and NP suggested by the data herein is supported by studies using Sendai virus. Stricker et al., "The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding" *J Gen Virol* 75 (Pt 5):1031-1042 (1994). Further, a possible interaction of HN protein with other viral protein is consistent with numerous studies suggesting an interaction of M protein with viral glycoproteins in paramyxovirus-infected cells or in cells transfected with paramyxovirus cDNAs. Ali et al., 'Assembly of Sendai virus: M protein interacts with F and HN proteins and with the cytoplasmic tail and transmembrane domain of F protein" *Virology* 276:289-303 (2000); Ghildyal et al., "Interaction between the respiratory syncytial virus G glycoprotein cytoplasmic domain and the matrix protein" *J Gen Virol* 86:1879-1884 (2005); Henderson et al., "Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins" *Virology* 300:244-254 (2002); Sanderson et al., "Sendai virus assembly: M protein binds to viral glycoproteins in transit through the secretory pathway" *J Virol* 67:651-663 (1993); and Yoshida et al., "Membrane (M) protein of HVJ (Sendai virus) - Its role in virus assembly" *Virology* 71:143-161 (1976). Indeed, it has been reported that the respiratory syncytial virus G protein specifically interacts with M protein. However, there are no previous reports of a direct interaction between F protein and NP. It is possible that interactions between viral proteins vary within paramyxoviruses and the requirements for formation of VLPs may depend upon the distribution of late domains on the viral proteins. The results presented herein are consistent with the proposal that the NDV M protein buds and releases indiscriminately from different cellular membranes in the absence of other viral proteins. Although it is not necessary to understand the mechanism of an invention, it is believed that when both glycoproteins and M proteins are present in the plasma membrane, the M protein-plasma membrane association has an improved stability. It is further believed that NP association with F and M protein may also further stabilize and organize the network of interactions within the assembling particle.

This protein-protein interacting network hypothesis has support from observations comparing electron micrographs of whole virus (B1) with VLPs formed only with M protein, and VLPs formed with NP, M, F, and HN proteins. See, Figure 73. When all four viral proteins are present, the VLP size and shape is very similar to the whole virus. However, an M protein-only VLP size and shape is more hetergeneous when compare to the whole virus but is still remarkably similar.

In one embodiment, the present invention contemplates a VLP production system for NDV. In one embodiment, the M protein facilitates NDV VLP budding such that NDV VLP budding is virtually non-existent in the absence of M protein. In other embodiments, specific protein-protein interactions occur in VLPs involved in the ordered assembly of particles. In one embodiment, an interaction between M and HN or F and NP directs the targeting of M and NP into assembly sites within the plasma membrane.

III. Paramyxoviral Diseases

The present invention is not limited to NDV, measles, parainfluenza virus 3, and respiratory syncytial paramyxovirus diseases. Many other paramyxoviruses diseases are also within the scope of this invention. For example, both human diseases (See Table 1) and animal diseases (See Table 2) are contemplated.

Table 1: Paramyxovirus-Mediated Human Diseases Susceptible To VLP Vaccination

Virus Type	Disease Type	Current Vaccination
Parainfluenza (1, 2, 3, and 4)	Acute Respiratory Infection	None
Mumps	Childhood Disease	Live Attenuated Virus
Measles	Childhood Disease	Live Attenuated Virus
Respiratory Syncytial	Serious Respiratory Infection	None
Nipah	Emerging Infection Acute Neurological Disease	None
Hendra	Emerging Infection Acute Neurological Disease	None
Metapneumovirus	Acute Respiratory Infection	None

Table 2: Paramyxovirus-Mediated Animal Diseases Susceptible To VLP Vaccination

Virus Type	Animal Species
Canine Distemper	Dogs
Rhinderpest	Cattle
Pneumoviruses	Birds

A. Newcastle Disease

Newcastle disease virus (NDV) is an avian pathogen. There are different strains of this virus that have been isolated in many regions of the world. Some strains are avirulent and are used as live attenuated vaccines. Others are virulent and cause severe systemic disease in birds with a high mortality rate. Because of the threat to the poultry industry, the United States government has classified virulent NDV strains as select agents under the Patriots Act.

Most chickens in the United States are vaccinated with an avirulent NDV strain. The current vaccine, however, is not ideal. The vaccine, a live attenuated virus, infects chickens and causes a mild respiratory disease. As a result, vaccinated birds have a lower body weight and lower egg production than unvaccinated birds. For this reason, many other countries do not vaccinate against NDV. Thus, there are periodic outbreaks of the disease in these countries forcing massive bird slaughter to contain the disease. Flocks of vaccinated chickens can also be susceptible to some NDV virulent strains. Consequently, there have been Newcastle disease virus outbreaks in the United States. For example, there was an NDV outbreak in California in 2001-2002.

What is needed is a NDV vaccine that does not have negative productivity consequences and can induce a broader range of protection than currently used vaccines.

In birds, clinical evidence of NDV includes, but is not limited to, the respiratory, neurological and gastrointestinal systems. Clinical signs suggestive of Newcastle disease, are observed mainly in young birds. Common symptoms include, but are not limited to, inability to walk or fly, walking in circles, paralysis, twisted necks, depression, and high frequency of sudden death. In mammals, symptoms of Newcastle disease may include, but are not limited to, acute conjuctivitis.

A significant problem of the currently utilized NDV vaccines is a failure to protect against all NDV strains. Currently, inactivated NDV vaccines (i.e., attenuated) are sometimes used to vaccinate flocks of birds. While eliminating the detrimental effects of a live virus vaccination, these vaccines still have the disadvantage that they do not stimulate a broad spectrum of immune responses. Further, incomplete attenuation results in a percentage of vaccinated birds contracting Newcastle disease. These vaccines are also more expensive than

embodiments contemplated by the present invention due to the increased manipulation required for inactivation and the monitoring of the effectiveness of inactivation.

Another problem with currently used vaccines, either live virus or inactivated virus, is that it is difficult to distinguish between birds that have been vaccinated and those that have been infected with a wild virus. The present invention contemplates antigens incorporated into a VLP preparation comprising a sequence tag. In one embodiment, the sequence tag may be detected in vivo, thereby identifying a vaccinated animal.

B. Measles

Measles is believed to be a childhood infection characterized by fever, cough, coryza (i.e., for example, an upper respiratory tract infection or inflammation), and often conjunctivitis followed by a maculopapular rash. It has been observed that the severity of the disease varies with the strain of the virus as well as the health status of the infected children. In most children, recovery is complete. However, there is a low incidence of neurological complications of varying severity. Furthermore, malnourishment or another underlying disease can significantly increase the severity of the disease. In addition, the infection is immunosuppressive resulting in increased susceptibility of the child to other life threatening infections, particularly in a third world setting.

The currently used vaccine is a live, attenuated virus that is effective in generating a protective immune response. However, the age of immunization is problematic. Vaccination too early results in a poor antibody response due to maternal antibody. Increasing the dose to overcome this effect results in immunosuppression and increased susceptibility to other potentially life threatening infections. Vaccination at a later age places the infant at a risk of acquiring the disease prior to immunization but after the maternal antibody level declines. Thus there is a need for a vaccine that will generate an effective immune response in the face of material antibody and, more importantly, a vaccine that will not be immunosuppressive at any dosage. In one embodiment, the present invention contemplates that VLPs are a candidate for such a vaccine.

Certain embodiments of the present invention provide virus-like particles (VLPs) as a safe, broad-spectrum, and effective vaccine to protect mammals from a measles virus. Additionally, these embodiments provide systems and protocols for the large-scale, economical production of a measles VLP vaccine (i.e., for example, to be useful as a vaccine, VLP production must be easy and economical).

The present invention contemplates conditions for the generation of VLPs of a measles virus strain. In another embodiment, the VLPs comprise the same major antigens as infectious virus (but, of course, lack the complete viral genome). In another embodiment, the VLPs comprise major antigens having the same ratios as infectious virus. In one embodiment, the major antigens are selected from the group comprising nucleocapsid protein, membrane/matrix protein, hemagglutinin protein, and fusion protein.

Other embodiments of the present invention provide antigens derived from many different measles strains that may be incorporated into a single VLP preparation. A significant problem of the currently utilized measles vaccines is a failure to protect against all measles strains.

Measles is thought to be a highly contagious viral illness having primary symptoms including, but not limited to, fever, cough, conjunctivitis (i.e., redness and irritation in membranes of the eyes), and spreading rash. The viral infection may be spread by contact with droplets from the nose, mouth, or throat of an infected person. The incubation period is 8 to 12 days before symptoms generally appear.

Immunity to the disease occurs after vaccination or active infection. Currently, vaccination is limited to attenuated live virus that has a significant risk of causing measles in the vaccinated subject. Further some believe that the Measles-Mumps-Rubella vaccine can cause autism. Before widespread immunization, measles was so common during childhood that the majority of the population had been infected by age 20. Measles cases dropped over the last several decades to virtually none in the U.S. and Canada because of widespread immunization, but rates are currently on the rise. Public fear, therefore, results in lower vaccination rates that can cause outbreaks of measles, mumps, and rubella -- which can be serious. One advantage of one embodiment of the present invention is that a VLP non-replicating measles vaccine carries no risk of infection. The VLP vaccine is thus expected to generate a much higher compliance rate and subsequently the measles occurrence should drop dramatically.

In one embodiment, measles symptoms include, but are not limited to, sore throat, runny nose, cough, muscle pain, fever, bloodshot eyes, tiny white spots inside the mouth (called Koplik's spots), photophobia (light sensitivity), a rash appearing around the fifth day of the disease and lasting 4 – 7 days that usually starts on the head and spreads to other areas,

progressing downward (the rash may be a maculopapular rash appearing as both macules (flat, discolored areas) and papules (solid, red, elevated areas) that later merge together (confluent)), further the rash may itch.

There is no specific treatment of measles, though some children may require supplementation with Vitamin A. Symptoms may be relieved with bed rest, acetaminophen, and humidified air. The probable outcome is excellent in uncomplicated cases. However, pneumonia or encephalitis are possible complications.

C. Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is believed to be the single most common cause of hospitalization for respiratory infection of infants and young children worldwide. Re-infection also commonly occurs. RSV attack rates for all infant populations is estimated between 100% and 83% and an estimated 50% of these experience two or more infections during the first two years of life (reviewed in Collins, et al, Respiratory Syncytial Virus, in Fields Virology, Ed. Knipe, D. and Howley, P. Lippincott Williams and Wilkins, 2001). RSV is also increasingly recognized as a serious pathogen for the elderly.

Currently, there is no vaccine available for this pathogen. Early trials with a formalin inactivated virus preparation had the disastrous effect of enhancing the severity of disease upon exposure to the live virus. In addition, protein subunit vaccines had a similar effect in experimental animals. It is speculated that proteins in an abnormal conformation, either induced by formalin treatment or by expression and purification of individual proteins, resulted in a loss of epitopes that stimulated a protective immune response. Animal studies suggested that immunopathology was due to immune cells (reviewed in Collins, et al, Respiratory Syncytial Virus, in Fields Virology, Ed. Knipe, D. and Howley, P. Lippincott Williams and Wilkins, 2001. VLPs formed with RSV proteins will likely incorporate viral proteins in their native conformation. These immunogens have the potential to stimulate a protective immune response and to avoid the adverse effects of unfolded proteins.

Certain embodiments of the present invention provide virus-like particles (VLPs) as a safe, broad-spectrum, and effective vaccine to protect mammals from Respiratory Syncytial Virus (RSV). Additionally, these embodiments provide systems and protocols for the large-

scale, economical production of RSV VLP vaccines (i.e., for example, to be useful as a vaccine, VLP production must be easy and economical).

The present invention contemplates conditions for the efficient generation of VLPs of a virulent RSV strain. In another embodiment, the VLPs comprise the same major antigens as infectious virus. In another embodiment, the VLPs comprise major antigens having the same ratios as infectious virus. In one embodiment, the major antigens are selected from the group comprising nucleocapsid protein, membrane/matrix protein, G or attachment protein, and fusion protein.

Other embodiments of the present invention provide antigens derived from many different RSV strains that may be incorporated into a single VLP preparation. A significant problem of the currently utilized RSV vaccines is a failure to protect against all RSV strains.

Respiratory syncytial virus (RSV) is believed to be a very common virus that causes mild cold-like symptoms in adults and older healthy children. RSV may cause serious respiratory infections in young babies, especially those born prematurely, who have heart or lung disease, or who are immunocompromised.

RSV is believed to be the most common respiratory pathogen in infants and young children. Specifically, RSV is believe to infect nearly all infants by the age of two years. Seasonal outbreaks of acute respiratory illness occur each year, on a schedule that is somewhat predictable in each region. The season typically begins in the fall and runs into the spring.

RSV may be spread easily by physical contact including, but not limited to, touching, kissing, and shaking hands with an infected subject. Although it is not necessary to understand the mechanism of an invention, it is believed that RSV transmission is usually by contact with contaminated secretions, which may involve tiny droplets or objects that droplets have touched. RSV can live for half an hour or more on the skin surface. It is also believed that RSV can also live up to five hours on countertops and for several hours on used tissues, consequently, RSV often spreads very rapidly in crowded households and day care centers.

In one embodiment, the present invention contemplates a VLP RSV vaccine that prevents the development of infant and young adult diseases such as, but not limited to, pneumonia, bronchiolitis (inflammation of the small airways of the lungs), and tracheobronchitis (croup). In

one embodiment, the present invention contemplates a VLP RSV vaccine that prevents the development of a mild respiratory illness in healthy adults and older children.

The lack of a safe and effective RSV vaccine poses a significant public safety and health risk. For example, it is believed that each year up to 125,000 infants are hospitalized due to severe RSV disease; and about 1-2% of these infants die. Further, infants that are: i) born prematurely; ii) suffering chronic lung disease; iii) immunocompromised; or iv) afflicted with certain forms of heart disease are at increased risk for severe RSV disease. Even adults who are exposed to tobacco smoke, attend daycare, live in crowded conditions, or have school-age siblings are also at higher risk of contracting RSV.

In one embodiment, the present invention contemplates RSV symptoms including, but not limited to, nasal congestion, nasal flaring, cough, rapid breathing (tachypnea), breathing difficulty or labored breathing, shortness of breath, cyanosis (bluish discoloration of skin caused by lack of oxygen), wheezing, fever, or croupy cough (often described as a "seal bark" cough). It should be recognized that symptoms are variable and differ with age. For example,, infants less than one year old are most severely affected and often have the most trouble breathing. Conversely, older children usually have only mild, cold-like symptoms. In general, symptoms usually appear 4-6 days after exposure.

Because there is no known treatment for an active RSV infection, those in the art have considered preventative drugs. For example, Synagis® (palivizumab) has been approved for prevention of RSV disease in children younger than 24 months of age who are at high risk for serious RSV disease. Synagis® however, must be prescribed and given as a monthly shot to provide complete protection.

D. Parainfluenza 3 (PIV 3)

PIV3 is believed to be a common cause of respiratory disease (rhinitis, pharyngitis, laryngitis, bronchiolitis, and pneumonia). This virus is the second most common cause of respiratory infection in hospitalized pediatric patients. No vaccines are available for PIV 3. A number of different approaches to vaccination have been considered but none has resulted in a licensed vaccine. (reviewed in Chanock, et al, Parainfluenza Viruses, in Fields Virology, Ed. Knipe, D. and Howley, P. Lippincott Williams and Wilkins, 2001).

Physiologically, PIV 3 usually infects the upper and lower respiratory systems. Currently, five

serotypes of Parainfluenza virus are known (1, 2, 3, 4a, and 4b), all of which are associated with causing disease. Children are believed highly susceptible to Parainfluenza and may be responsible for approximately 40 percent to 50 percent of all cases of croup, and 10 percent to 15 percent of bronchiolitis and bronchitis and some pneumonias. In the general population, the incidence of parainfluenza is unknown but suspected to be very high. Illness causing only a runny nose and cold-like symptoms may pass as a simple cold rather than parainfluenza. Risk factors include young age. By school age most children have been exposed to parainfluenza virus. Most adults have antibodies against parainfluenza although they can get repeat infections.

Laryngotracheobronchitis (i.e., for example, croup) is believed to be a common clinical manifestation of parainfluenza virus infection. Parainfluenza viruses are found uncommonly associated with other respiratory tract infections in children such as tracheobronchitis, bronchiolitis, and bronchopneumonia. Occasionally, a mild non-specific illness is seen after parainfluenza virus infection. Parainfluenza viruses produce disease throughout the year, but peak prevalence rates occur during wintertime outbreaks of respiratory tract infections, especially croup, in children throughout the temperate zones of the northern and southern hemispheres. Parainfluenza virus infections are primarily childhood diseases, the highest age-specific attack rates for croup occur in children below the age of 3 years. Serotype 3 infections occur earliest and most frequently, so that 50% of children in the US are infected during the first year of life and almost all by 6 years, as determined by seroepidemiological studies.

Parainfluenza viruses generally enters a host through the inhalation of infected droplet nuclei. Virus multiplication occurs throughout the tracheobronchial tree, inducing the production of mucus. The vocal cords of the larynx become grossly swollen, causing obstruction to the inflow of air, which is manifested by inspiratory stridor. In adults, the virus is usually limited to causing inflammation in the upper parts of the respiratory tract. In infants and young children, the bronchi, bronchioles and lungs are occasionally involved, which may reflect on the small size of the airways and the relative immunological immaturity. Viraemia is neither an essential nor a common phase of infection.

Typically, children may exhibit a croupy cough, inspiratory stridor, hoarse voice or cry and respiratory difficulty on inspiration, and are usually afebrile. About 80% of patients exhibit a cough and runny nose 1 to 3 days before the onset of the cough. Respiratory rhonchi are heard frequently throughout the lung fields. Radiological examination is usually normal. Occasionally

the epiglottitis is grossly swollen and reddened. Severe airway obstruction may ensue, necessitating an emergency tracheotomy.

IV. VLP Vaccines

Paramyxovirus VLP vaccines are novel in the art. While virosome vaccines are known, these vaccines require disrupting a purified virus, extracting the genome, and reassembling particles with the viral proteins and lipids to form lipid particles containing viral proteins. This approach is very costly. Also, since the starting material is live virus, there is a danger of contaminating the vaccine with live virus. In addition, the resulting vaccine is likely not a broad-spectrum vaccine. Furthermore, the immune response to this vaccine cannot be distinguished from a virus infection.

Paramyxovirus VLPs are believed to be a highly effective type of subunit vaccine that mimics the overall virus structure without containing genetic material that results in host cell infection. For example, a virus-like particle may completely lack the DNA or RNA genome while maintaining the authentic conformation of viral capsid proteins. Consequently, the VLP is non-infectious. Further, a virus-like particle comprising viral capsid proteins may undergo spontaneous self-assembly similar to authentic viruses. It is known, however, that polyomavirus VLP preparations are among the least developed in the art. Noad et al., "Virus-like particles as immunogens" *Trends Microbiol* 11:438-444 (2003).

In one embodiment, the present invention contemplates a vaccine comprising a paramyxovirus VLP. In one embodiment, the paramyxovirus is selected from the group including, but not limited to, Newcastle disease, measles, parainfluenza virus 3, or respiratory syncytial virus. In one embodiment, the VLP comprises an M protein. In another embodiment, the VLP further comprises at least two glycoproteins. In one embodiment, the glycoproteins are selected from the group consisting of F protein and HN protein.

A. Newcastle Disease Virus

Certain embodiments of the present invention provide virus-like particles (VLPs) as a safe, broad-spectrum, and effective vaccine to protect poultry from Newcastle disease virus. Additionally, these embodiments provide systems and protocols for the large-scale, economical

production of VLPs (i.e., for example, to be useful as a vaccine, VLP production must be easy and economical).

A silver stain comparision of whole virus (B1) grown in eggs are compared to VLPs grown in large scale tissue culture demonstrates that VLPs may be produced in microgram quantities (i.e., sufficient for immunogenicity testing in mice). See, Figure 74. VLPs have been rapidly purified from large amounts of media to faciliate large scale VLP production techniques. See, Table 3.

Table 3: Large Scale VLP Preparations

Particle		ng/μl	total volume	Total protein(μg)
B1 virus	HN	23.05	1ml	23.05
	F	11.09		11.09
	NP	100.32		100.09
	M	75.08		75.08
				209.54 total
VLP prep1	HN	177.35	1.1 ml	195.08
	F	349.56		384.52
	NP	140.19		154.21
	M	72.02		79.22
				813.04 total
VLP prep2	HN	109.70	0.5 ml	54.85
	F	85.42		42.71
	NP	98.24		49.71
	M	63.50		31.75
				178.43 total
VLP prep 3	HN	92.55	0.2 ml	18.4
	F	53.54		10.70
	NP	92.13		18.26
	M	60.89		12.18
				59.54 total

Preparation 1 was contaiminated with albumin, which co-migrates with F protein. Therefore, the amounts of F in Preparation 1 appear enhanced when compared to NP. This albumin contamination was successfully eliminated in Preparations 2 & 3

Although it is not necessary to understand the mechanism of an invention, it is believed that virus (B1) grown in eggs (as is standard in the art) are deficient in the HN and F glycoproteins (typical of avirulent (AV) virus particles), unlike the presently disclosed VLP production methods in which virus (AV) VLP comprise HN and F glycoproteins. In one embodiment, the present invention contemplates an improved vaccine comprising an NVD VLP comprising HN and F glycoproteins.

NDV subunit protein expression has been reported in the art. For example, electron microscopic examination of negatively stained extracellular fluids (ECF) from *Spodoptera frugiperda* cell cultures infected with a recombinant baculovirus expressing the Newcastle disease virus (NDV) haemagglutinin-neuraminidase (HN) revealed NDV-like envelopes which resembled the envelopes of authentic NDV. Immunogold staining with anti-NDV HN monoclonal antibodies demonstrated HN antigen in spikes on the NDV-like envelopes. The ECF from the recombinant-infected cultures also contained baculovirus particles which resembled standard baculovirus particles except that some showed polar protrusions of the envelope. Unlike the embodiments contemplated in the present invention, it was concluded that NDV HN, in the absence of the matrix protein (i.e., M protein), might be able to initiate and control the production of viral envelopes which are morphologically identical to those of authentic NDV. Nagy et al., "Synthesis of Newcastle disease virus (NDV)-like envelopes in insect cells infected with a recombinant baculovirus expressing the haemagglutinin-neuraminidase of NDV" *J Gen Virol.* 72:753-756 (1991).

In one embodiment, the present invention contemplates a method comprising a commercially usable NDV VLP vaccine. In one embodiment, producing a NDV VLP vaccine is economical and efficient. In another embodiment, immunization with an NDV VLP vaccine stimulates production of a broad spectrum of protective antibodies. In one embodiment, an avian cell line continuously expresses at least four NDV glycoproteins

In one embodiment, the present invention contemplates a method producing NDV VLP vaccines in a transient expression system. In one embodiment, the system comprises avian cells

transfected with nucleic acid (e.g., in plasmids, expression vectors, etc) encoding at least one NDV viral glycoprotein. In one embodiment, the system comprises an avian cell line with select viral genes as part of the avian cell chromosome, wherein the incorporated viral gene continually releases NDV VLP particles useful for vaccines. In one embodiment, the viral gene comprises a viral glycoprotein. In one embodiment, the viral glycoprotein is selected from the group comprising NP protein, M protein, F-K115Q protein, or HN protein.

In one embodiment, the present invention contemplates a method of generating VLPs comprising antigens for many different NDV strains of NDV. Although it is not necessary to understand the mechanism of an invention, it is believed that an integrated NDV vaccine confers a broader protection range than that generated by current vaccines. In one embodiment, the present invention contemplates an VLP vaccine expression system comprising a first cDNA encoding a first viral protein gene from a first strain; a second cDNA encoding a second viral protein gene from a second strain; and a third cDNA encoding a third viral protein gene from a third strain. In one embodiment, the first viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the first strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one embodiment, the second viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the second strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one embodiment, the third viral protein gene is selected from the group comprising HN protein, F protein, NP protein or M protein. In one embodiment, the third strain is selected from the group comprising strain Hertz, strain AV, or strain B1. In one embodiment, the present invention contemplates a method for detecting a viral protein gene incorporated into a VLP vaccine comprising contacting the viral protein gene with strain specific antibodies or incorporated sequence tags.

In one embodiment, the present invention contemplates a method comprising a baculovirus expression system producing NDV VLP vaccines. Although it is not necessary to understand the mechanism of an invention, it is believed that baculovirus expression systems are capable the highest levels of expression of a protein of all expression systems available. In one embodiment, a baculovirus expression system produces milligrams of VLP vaccine. In one embodiment, a baculovirus expression vector encodes an NDV VLP vaccine. In one embodiment, an insect cell is transfected with a baculovirus expression system encoding an NDV

VLP vaccine. In one embodiment, a baculovirus vector comprises at least four NDV structural proteins. For a VLP to be a realistic vaccine candidate, it needs to be produced in a safe expression system that is amenable to large-scale production. An insect-ceil-based protein production system has many advantages for VLP production. The first is that large amounts of recombinant proteins can be produced in high-density cell culture conditions in eukaryotic cells, resulting in high recovery of correctly folded antigen. Second, as the insect cells used for vaccine production can be cultured without mammalian-cell-derived supplements, the risk of culturing opportunistic pathogens is minimized. Third, the baculovirus used for recombinant protein expression has a narrow host range that includes only a few species of *Lepidoptera*, and therefore represents no threat to vaccinated individuals. Fourth, baculovirus is easily inactivated by simple chemical treatment, and is localized mainly in the nucleus and culture media of insect cell preparations, whereas most VLPs are purified from cytoplasmic extracts. Finally, the baculovirus system can be scaled- up for large-scale vaccine production.

B. Measles

In one embodiment, the present invention contemplates a measles vaccine comprising a measles virus like particle, wherein said particle comprises a measles matrix protein. In one embodiment, the vaccine further comprises at least two measles glycoproteins.

The use of VLP vaccines have been proposed for the measles paramyxovirus virus, but only retrovirus HIV VLP production was demonstrated in yeast cells. Morikawa Y., "Virus-like micrograins and process of producing the same" *United States Patent Application Publ. No. 20040009193* (2004). This proposed technique is limited to VLP expression in eukaryotic bacterial cells and does not suggest either baculovirus or mammalian cell culture techniques. Further, there is no showing that these eukaryotic VLP vaccines are, in fact, safe and effective. More importantly, Morikawa's VLP measles vaccines relies upon type IV budding as described by Garoff et al., supra. Some embodiments described herein clearly demonstrate that the ribonucleic acid core is not required for paramyxovirus budding; as Garoff et al. teaches.

Another approach suggested as useful for the development of a paramyxovirus measles vaccine involves gene therapy techniques by administering a DNA vaccine. Robinson et al., "Compositions and methods for generating an immune response" *United States Patent Application Publ. No. 20040105871* (2004). This technique has been demonstrated by the stable

transfection of a host genome with an expression cassette comprising an HIV DNA VLP vaccine. *See also,* Mazzara et al., "Self assembled, defective, nonself-propagating viral particles" *United States Patent No. 5,804,196* (1998)(herein incorporated by reference)

. An alternative gene therapy approach suggests incorporating live attenuated measles virus into an expression vector to produce a vaccine, either in vivo or in vitro. VLPs, however, are not contemplated for measles virus vaccines. Herold J., "SARS-coronavirus virus-like particles and methods of use" *United States Patent Application Publ. No. 20050002953* (2005).

C. Respiratory Syncytial Virus

In one embodiment, the present invention contemplates a respiratory syncytial virus vaccine comprising a respiratory syncytial virus like particle, wherein said particle comprises a respiratory syncytial virus matrix protein. In one embodiment, the vaccine further comprises at least two respiratory syncytial virus glycoproteins.

VLPs have been disclosed for the production and use of HIV-related vaccines. In passing, it is suggested that many other virus (i.e., respiratory syncytial virus and measles virus) might also be compatible with the disclosed technology. No detail, however, is presented to support these speculations. Barnett et al., Expression of HIV polypeptides and production of virus-like particles" *United States Patent No. 6,602,705* (2003).

It has also been suggested that it might be possible to produce respiratory syncytial virus VLP vaccines in a manner identical to Bluetongue VLPs comprising the VP3, VP7, VP2, and VP5 genes. Ermak et al., "Oral immunization with multiple particulate antigen delivery system" *United States Patent No. 5,690,938* (1997)(herein incorporated by reference). Aside from this brief mention, Ermak does not provide any technical information regarding paramyxoviruses, and is limited to the Orbivirus genus (Reoviridae family).

In vivo mouse cytotoxic lymphocyte responses (i.e., an immunization response) are hypothesized to occur following exposure to recombinant HIV-1-IIIB gp160 envelope glycoprotein complexed to microspheres and administered as a vaccine. Rock, K. L., "Compositions and methods for inducing cytotoxic T lymphocyte responses by immunization with protein antigens" *United States Patent No. 6,328,972* (2001). Rock suggests that VLPs having antigens to either respiratory syncytial virus or measles virus might also stimulate these cytotoxic lymphocytes to generate an immune response. There is, however, no discussion, of

any technical details or expectations of success regarding this approach. In fact, Rock does not show any data relevant to VLP vaccines for any antigen.

D. Parainfluenza 3 Virus

In one embodiment, the present invention contemplates a parainfluenza 3 virus vaccine comprising a parainfluenza 3 virus like particle, wherein said particle comprises a parainfluenza 3 virus matrix protein. In one embodiment, the vaccine further comprises at least two parainfluenza 3 glycoproteins.

E. Enhancement Of VLP Vaccines

Vaccine or treatment compositions of the invention may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories, and in some cases, oral formulations or formulations suitable for distribution as aerosols. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25-70%.

In the case of the oral formulations, the manipulation of T-cell subsets employing adjuvants, antigen packaging, or the addition of individual cytokines to various formulation can result in improved oral vaccines with optimized immune responses.

1. Adjuvants

The present invention further contemplates immunization with or without adjuvant. In one embodiment, the present invention contemplates a co-administration of a paramxyovirus VLP vaccine and an adjuvant, wherein the resultant immune response is enhanced. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant--or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. Another preferred use of adjuvant is the use of Gerbu adjuvant (GMDP; C.C. Biotech Corp.). The invention also contemplates the use of RIBI fowl adjuvant (MPL; RIBI

Immunochemical Research, Inc.). Other adjuvants include, but are not limited to, potassium alum, aluminum phosphate, aluminum hydroxide, QS21 (Cambridge Biotech), Titer Max adjuvant (CytRx), or Quil A adjuvant.

2. Cytokines

In one embodiment, the present invention contemplates a co-administration of a paramxyovirus VLP vaccine and a cytokine, wherein the resultant immune response is enhanced. Although it is not necessary to understand the mechanism of an invention, it is believed that cytokines may modulate proliferation, growth, and differentiation of hematopoietic stem cells that ultimately produce vaccine related antibodies. In one embodiment, a cytokine may be selected from the group comprising interleukin-12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-18 (IL-18), alpha, beta, or gamma-interferon (α,β,γ-IFN) or chemokines. Especially preferred cytokines include IL-12 and GM-CSF. The cytokines can be used in various combinations to fine-tune the response of an animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to control or eliminate a paramyxovirus infection.

V. VLP Vaccine Expression Systems

In one embodiment, the present invention contemplates methods to produce VLP vaccines economically and at high production rates. In one embodiment, the present invention contemplates a method comprising transfecting a cell culture with a nucleic acid expression vector comprising a paramyxovirus VLP vaccine cassette. In one embodiment, the cell culture comprises avian cells (i.e., for example, ELL-0 cells). In one embodiment, the cell culture comprises a viruses (i.e., for example, baculovirus).

A. Avian Continuous Cell Culture Expression Systems

In one embodiment, the present invention contemplates a method comprising expressing paramyxoviral proteins using an avian cell culture (i.e., for example, ELL-0 cell culture). In one embodiment, the cell culture continuously expresses the proteins. In one embodiment, the paramyxoviral proteins are selected from the group including, but not limited to, Newcastle disease viral protein, measles virus proteins, parainfluenza virus 3, or respiratory syncytial virus

proteins. In one embodiment, the paramyxoviral proteins are selected from the group including, but not limited to, matrix (M) proteins, nucleocapsid (NP) proteins, fusion (F) proteins, or heamagglutinin-neuraminidase (NM) proteins (and combinations thereof).

To generate avian cell lines expressing paramyxoviral proteins, it is useful to integrate the viral genes into an avian cell chromosome. The use of retrovirus vectors is a useful approach to accomplish this integration. Avian cells can be infected with a retrovirus containing a paramyxovirus gene and, as part of the retrovirus replication cycle, the retrovirus genome with the paramyxovirus gene will integrate into the cell chromosome. Four avian cell lines will be made: i) avian cells expressing M, NP, F, and HN proteins; ii) avian cells expressing M, NP, and F; iii) avian cells expressing M, NP, and HN proteins; and iv) avian cells expressing M, HN, and F proteins.

The retrovirus vector may be constructed such that the vector is unable to direct the formation of new, progeny retroviruses in the avian cells (i.e., non-replicability). The general approach for such studies is as follows. The paramyxovirus genes are cloned into a vector with the retrovirus ends (LTRs) and the packaging signal. This vector is, however, replication incompetent due to the lack of essential genes for that process (i.e., for example, gag or pol).

The vector DNA is transfected into a packaging cell line (i.e., for example, GP-293), a cell line expressing the retroviral structural proteins; gag, pol, and env. Also transfected with the vector is another DNA encoding the vesicular stomatitis virus (VSV) G protein (i.e., for example, pVSV-G). These cells then replicate retrovirus vectors and package the vector RNAs in an envelope with the env protein as well as the VSV-G protein (called a pseudotype). These cells release particles, which are then purified and used to infect avian cells. The presence of the VSV-G protein allows these particles to initiate infection in the avian cells and expands the host range of the retrovirus.

Following transfection, the vector RNA is converted to DNA, which is then integrated into the avian cell chromosome. Because the avian cells are not expressing gag or pol, the retrovirus infection does not proceed and no progeny virus are released. The transfected avian cells thus continuously express the integrated paramyxoviral genes, but not retrovirus genes.

This protocol will be repeated to sequentially integrate each of the four paramyxovirus proteins. Cell lines will be characterized for expression of the paramyxovirus genes and the release of VLPs from these cell lines will be verified.

Vectors and packaging cell lines (pantropic retrovirus expression system) to accomplish these steps are available from Clontech (BD Biosciences Clontech). In addition, there is available a vector (Q vector) which is engineered so that transcription of the target gene is driven by an internal promoter once the expression cassette is integrated into the avian cell genome. The Q vectors reduce the likelihood that cellular sequences located adjacent to the vector integration site will interfere with the expression of the paramyxovirus genes or that these sequences are abnormally expressed due to proximity with the retroviral LTR.

B. Baculovirus Expression Systems

In one embodiment, the present invention may be practiced using the BacVector® system (Novagen). This system uses the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) containing inserted genes to express proteins in an insect cell line (i.e., for example, Sf9). See Figure 30. The present invention is not limited to one method of integrating target genes in to the AcNPV genome. Numerous different transfer plasmids may be used. For example, by co-transfecting cells with AcNPV DNA and the transfer plasmid, viruses can be isolated to have the genes inserted into the virus genome by homologous recombination (i.e., for example, using BacVector® Triple Cut Virus DNA, Novagen). See Figure 28. In one embodiment, target genes (i.e., for example, NDV, measles, parainfluenza virus 3, or respiratory syncytial viral particle proteins) maybe cloned into a pBAC transfer plasmid to produce recombinant baculovirus vectors. In one embodiment, the recombination may comprise a ligation-independent cloning (LIC) technique. See Figure 29. For example, a LIC transfer plasmid pBAC/pBACgus-2cp may encode an upstream His-Tag and S-Tag peptide having an enterokinase (ek) cleavage site. The recombination is facilitated by primer sequences comprising: sense strand, 5' to ATG: GACGACGACAAG (SEQ ID NO:89); antisense strand, 5' to TTA: GAGGAGAAGCCCGG (SEQ ID NO:90).

Upon transfection, the BacVector® DNA will not produce virus unless there is a recombination event between the virus DNA and the transfer plasmid; i.e., a recombination that repairs the circular viral DNA required for replication. In one embodiment, the transfer plasmid

comprises pBAC4x-1 (Novagen). See Figure 31. Although it is not necessary to understand the mechanism of an invention, it is believed that pBAC4x-1 is constructed such that up to four (4) genes can be inserted into a single plasmid and, therefore, a single AcNPV. It is also believed that each gene is expressed using either the polh or the p10 promoters; promoters that can result in very high levels of protein expression from 24-72 hours post-infection. The pBAC4x-1 transfer vector was designed for expression of multi-subunit protein complexes and is capable of expressing the NDV M, NP, HN, and F genes either singly or in any combination.

Subsequent to co-transformation using a transfer plasmid and virus DNA , the infected cells (i.e., for example, Sf9) form plaques and express virus particles. These plaques are then isolated, wherein the expressed virus particles are purified and characterized for inserted protein gene expression. In one embodiment, the present invention contemplates an infected cell expressing virus particles comprising NDV, measles, parainfluenza virus 3, or respiratory syncytial protein genes, wherein the cell was transformed with baculovirus transfer plasmid. In one embodiment, the expression is characterized for optimal conditions, and times of expression, to support large-scale VLP preparation.

AcNPV-infected cells are known to produce extremely high quantities of the major very late gene products; polyhedrin (polh) and p10; 40–50% of the total cellular protein consists of these two gene products by the end of the infection cycle. Very late in infection (i.e., occurring after the budding and release phase), in both insects and in tissue culture, a large majority of the cell's transcriptional activity is dedicated to the polh and p10 promoters, which makes them ideal for use to drive the high-level expression of introduced target genes that replace these viral genes. Yields of up to 100 mg target protein per 10^9 cells can be obtained.

The convenience of baculoviral expression systems has improved by developing viruses having Bsu36 I restriction sites positioned within an essential gene (i.e., for example, ORF 1629) downstream of the AcNPV polyhedrin gene and in the upstream ORF 603. such that digestion releases a fragment containing a sequence necessary for virus growth. Kitts et al., *BioTechniques* 14:810-817 (1993). When insect cells are co-transfected with an appropriate recombinant transfer plasmid and Bsu36 I-cut virus DNA, the necessary ORF 1629 sequence is supplied by the transfer plasmid through homologous recombination. The vast majority of the progeny viruses derived from these co-transfections contain the repaired virus with the target gene, thus

minimizing the need to screen and multiply plaque purify recombinants. Alternatively, other baculoviral expression systems utilize other essential genes. For example, the progenitor BacVector-1000® and BacVector-2000® viruses from which the high efficiency BacVector-1000 and –2000 Triple Cut Virus DNAs® are prepared for cotransfections have the lacZ gene (β-galactosidase) in lieu of AcNPV polyhedrin gene. These lacZ-negative recombinants can be distinguished easily from any residual parental viruses, which are visualized as blue plaques when stained with X-Gal.

LacZ recombinants form clear plaques on staining with X-Gal, since the target gene replaces lacZ when the transfer plasmid recombines with the viral genome. A third Bsu36 I site within the lacZ gene further reduces the likelihood of reforming the parental virus. In practice and under optimal conditions, the commercially available baculovirus transfection technology produces plaques that are approximately > 95% recombinant.

The recent elucidation of the complete sequence of the 133,894bp AcNPV genome has revealed a total of some 154 potential genes. See Figure 30. A large number of these genes are unnecessary for growth of the virus in tissue culture. These non-essential genes are known to compete with target genes for cellular resources and can be deleterious to the expression of some gene products. It is preferable to use a baculovirus expression system wherein competing non-essential genes have been deleted.

In one embodiment, the present invention contemplates using pBAC transfer plasmids designed for the expression of target proteins (i.e., for example, NDV, measles, parainfluenza virus 3, or respiratory syncytial viral proteins). Several potential pBAC transfer plasmids are shown in Figure 31. For example, two vector backbones (shown at the top) differ only by the presence of the reporter β-glucuronidase (gus) gene driven by the p6.9 promoter (i.e., for example, the pBACgus series). Because the gus gene and P6.9 are carried with the target gene into the baculovirus genome, recombinants produce β-glucuronidase and can be identified by staining with X-gluc. The corresponding transfer plasmids lacking the gus indicator gene are about 2kbp smaller in size and may produce higher cloning efficiencies with some large inserts.

Additionally, LIC vectors including, but not limited to, pBAC-2cp and pBACgus-2cp plasmids are ready for annealing with appropriately prepared inserts. See Figure 31. In practice, a target sequence is generated by PCR using primers extended with defined sequences. See

Figure 29. For example, vector compatible cohesive ends (13 and 14bp on the N- and C-terminal coding sequences, respectively) are produced by treatment with T4 DNA polymerase in the presence of dATP. The 3'–5' exonuclease activity of the enzyme digests one strand of the duplex until a dT residue is encountered in the complementary strand, whereupon the available dA is added by the polymerase activity. Aslanidis et al., *Nucleic Acids Res.* 18:6069-6074 (1990). The treated insert and pBAC LIC transfer plasmid are briefly annealed, and the mixture transformed into NovaBlue Competent Cells.

The prepared vectors allow fusion of target genes at the most desirable position relative to the enterokinase cleavage site following the His-Tag and S-Tag fusion sequences. Inserts may be placed such that vector-encoded sequences can be completely removed by enterokinase cleavage. See Figure 29. In addition, the configuration of restriction sites in the multiple cloning region allows direct subcloning of inserts from many pET bacterial vectors into pBAC-1 or -2 series plasmids. The His-Tag sequence may be incorporated into, for example, the pBAC-1 or -2 vectors and encodes a consecutive stretch of 6 histidines. Alternatively, a S-Tag sequence encodes a 15 AA domain of ribonuclease A, which has a strong affinity for the 104 AA S-protein. Richards et al., In: The Enzymes, Vol. IV (Boyer, P.D., Ed.), pp. 647-806, Academic Press, New York (1971). This highly specific protein-protein interaction forms the basis for sensitive detection of fusion proteins with S-protein-reporter molecule conjugates. Chemiluminescent detection of S-Tag fusion proteins may be observed using an S-protein HRP conjugate and SuperSignal™ CL-HRP substrate. (S-Tag Rapid Assay Kit, Novagen).

The pBAC4x vectors are designed for coexpression of up to 4 genes in the same cell. These vectors are extremely useful for expression of multisubunit proteins, multiple copies of a gene, multiprotein complexes, and for studies of protein-protein interactions. Weyer et al., *J. Gen. Virol.* 72:2967-2974 (1991); Belyaev et al., *Nucleic Acids Res.* 21:1219-1223 (1993); and Belyaev et al., *Gene* 156:229-233 (1995).

It is known that baculoviral expression technology may be developed into an eukaryotic virus display system. Boublik et al., *Bio/Technology* 13:1079-1084 (1995). By appropriately engineering the AcNPV major surface glycoprotein (i.e., for example, gp64) functional proteins, including glycoproteins, can be expressed on the virus surface. A pBACsurf-1 transfer plasmid may be designed for in-frame insertion of target genes between the gp64 signal sequence and the

mature protein coding sequence, under the control of the polh promoter. See Figure 31. With this system, it is possible to construct and screen virus libraries of complex proteins for desired functional characteristics.

In one embodiment, the present invention contemplates using baculovirus expression technology to infect an Sf9 insect cell culture to express NDV, measles, parainfluenza virus 3, or respiratory syncytial viral proteins. These cells may be adapted for serum or serum-free monolayer, suspension, or fermentation culture, and ready for direct infection, transfection and plaque assay.

Extracts of wild-type AcNPV infected and uninfected Sf9 cells are useful for blocking non-specific binding of antibodies and other reagents to virus and insect cell proteins. The extracts are also useful for running as negative controls on Western blots, ELISA, binding assays, or enzymatic assays in which target proteins are analyzed in cell lysates.

In one embodiment, the present invention contemplates a VLP vaccine comprising proteins from different paramyxovirus strains. In one embodiment, the paramyxovirus strain is selected from the group including, but not limited to, Newcastle disease virus, measles virus, parainfluenza virus 3, or respiratory syncytial virus. In one embodiment, the NDV strain is virulent. In another embodiment, the virulent NDV strain may be selected from the group ~~comprising strain AV and strain Hertz. In one embodiment, the NDV strain is avirulent. In~~ another embodiment, the avirulent strain comprises strain B1.

In one embodiment, the present invention contemplates a composition comprising a cDNA clones encoding at least one paramyxovirus structural protein. In one embodiment, the structural protein comprises an HN glycoprotein. In one embodiment, the paramyxovirus is selected from the group including, but not limited to, Newcastle disease virus, measles virus, parainfluenza virus 3, or respiratory syncytial virus. In one embodiment, the clone is derived from a virulent NDV strain. In another embodiment, the virulent NDV strain may be selected from the group comprising strain AV and strain Hertz. In another embodiment, the clone is derived from an avirulent NDV strain. In one embodiment, the avirulent NDV strain comprises strain B1.

VI. VLP Vaccine Sequence Tags

In another embodiment, the present invention contemplates a paramyxovirus VLP vaccine such as, but not limited to, a Newcastle disease virus VLP vaccine, a measles virus VLP vaccine, a parainfluenza virus 3 VLP vaccine, or a respiratory syncytial virus VLP vaccine, wherein said vaccine comprises a sequence tag. In one embodiment, the vaccine is administered to a host. In one embodiment, the sequence tag is detected.

In one embodiment, the present invention contemplates a vector comprising at least one cDNA encoding a paramyxoviral protein, wherein said cDNA comprises a sequence tag. In one embodiment, the cDNA is transfected into a host cell. In one embodiment, the cDNA is incorporated into a host genome. In another embodiment, the cDNA resides in the host cytoplasm. In one embodiment, the sequence tag is detected.

A. Antibody Tags

The present invention contemplates some embodiments comprising a paramyxoviral glycoprotein expressed with a terminal sequence tag. In one embodiment, the tag comprises FLAG, HA and MYC tags.

In response to the rapidly growing field of proteomics, the use of recombinant proteins has increased greatly in recent years. Recombinant hybrids contain a polypeptide fusion partner, termed affinity tag (i.e., for example, a sequence tag), to facilitate the purification of the target polypeptides. The advantages of using fusion proteins to facilitate purification and detection of recombinant proteins are well-recognized. The present invention is compatible with various affinity sequence tags including, but not limited to, Arg-tag, calmodulin-binding peptide, cellulose-binding domain, DsbA, c-myc-tag, glutathione S-transferase, FLAG-tag, HAT-tag, His-tag, maltose-binding protein, NusA, S-tag, SBP-tag, Strep-tag, and thioredoxin. Terpe K., "Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems" *Appl Microbiol Biotechnol.* 60:523-33 (2003).

FLAG, HA, and MYC are short amino acid sequences for which there are commercially available antibodies (i.e., for example, ELISA kits). In one embodiment, a F protein comprises a terminal FLAG tag. In one embodiment, the terminal comprises the C-terminal. In another embodiment, the terminal comprises the N-terminal. Although it is not necessary to understand the mechanism of an invention, it is believed that F or HN viral proteins comprising a terminal

sequence tag (i.e., for example, FLAG or HA) are completely functional. It is further believed that when an F protein (or any other viral protein) comprising a terminal tag is incorporated into a VLP, immunized animals will make antibodies not only to the F protein, but also to the terminal tag (i.e., for example, a FLAG amino acid sequence).

Antibodies specific for sequence tags have affinities for specific protein sequences, known as an epitopes. An epitope has the property that it selectively interacts with molecules and/or materials containing acceptor groups. The present invention is compatible with many epitope sequences reported in the literature including, but not limited to, HisX6 (HHHHHH) (SEQ ID NO:91) (ClonTech), C-myc (-EQKLISEEDL) (SEQ ID NO:92) (Roche-BM), FLAG (DYKDDDDK) (SEQ ID NO:93) (Stratagene), SteptTag (WSHPQFEK) (SEQ ID NO:94) (Sigma-Genosys), and HA Tag (YPYDVPDYA) (SEQ ID NO:95) (Roche-BM).

The FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (SEQ ID NO:93) has been used as an epitope tag in a variety of cell types. For example, the modification of the cytomegalovirus (CMV) promoter containing vector, pCMV5, created two transient expression vectors designed for secretion and intracellular expression of FLAG-fusion proteins in mammalian cells. As a functional test, the bacterial alkaline phosphatase gene was cloned into both vectors, and anti-FLAG monoclonal antibodies were used for detection of FLAG epitope-tagged bacterial alkaline phosphatase in mammalian cells. In addition, secreted bacterial alkaline phosphatase was purified from the extracellular medium by anti-FLAG affinity chromatography. Chubet et al., "Vectors for expression and secretion of FLAG epitope-tagged proteins in mammalian cells" *Biotechniques* 20:136-41 (1996).

The net negatively charged HA-tag sequence (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) (SEQ ID NO:95) from the hemagglutinin influenza virus has proven useful in tagging proteins related to a wide variety of proteomic applications. In one embodiment, embodiment the present invention contemplates an improved HA epitope tag. Although it is not necessary to understand the mechanism of an invention, it is believed that the ability to metabolically label proteins with ^{35}S-methionine facilitates the analysis of protein synthesis and turnover. However, efficient labeling of proteins in vivo is often limited by a low number of available methionine residues, or by deleterious side-effects associated with protein overexpression. To overcome these limitations, a methionine-rich variant of the widely used HA tag, called HAM, maybe useful with ectopically expressed proteins. In one embodiment, the present invention contemplates the

development of a series of vectors, and corresponding antisera, for the expression and detection of HAM-tagged VLP viral proteins. These HAM tags improve the sensitivity of ^{35}S-methionine labeling and permit the analysis of Myc oncoprotein turnover even when HAM-tagged Myc is expressed at levels comparable to that of the endogenous protein. Because of the improved sensitivity provided by the HAM tag, the vectors described herein should be useful for the detection of radiolabeled VLP proteins. Herbst et al., "HAM: a new epitope-tag for in vivo protein labeling" *Mol Biol Rep*. 27:203-8 (2000).

Alternatively, antibodies may be generated to recognize specific sequences within a protein or oligonucleotide. Such antibodies may be polyclonal or monoclonal. For example, specific sequences to a carcinoembryonic antigen may be detectable by antibodies. Barnett et al., "Antibody preparations specifically binding to unique determinants of CEA antigens or fragments thereof and use of the antibody preparations in immunoassays" *US Pat No. 6,013,772* (2000)(herein incorporated by reference). Similarly, antibodies may be raised to specific nucleotide sequences. Tchen et al., "Probe containing a modified nucleic acid recognizable by specific antibodies and use of this probe to detect and characterize a homologous DNA sequence" *US Pat. No. 5,098,825* (1992)(herein incorporated by reference).

Numerous immunoassays may be used according to the present invention. The readout systems capable of being employed in these assays are numerous and non-limiting examples of such systems include fluorescent and colorimetric enzyme systems, radioisotopic labeling and detection and chemiluminescent systems. For example, an antibody preparation having a sequence-specific affinity for a sequence-tagged NDV viral protein (preferably a VLP particle protein) is attached to a solid phase (i.e., for example, a microtiter plate or latex beads). This antibody-VLP protein complex is then washed to remove unbound VLP particle proteins. After washing, color or fluorescence is developed by adding a chromogenic or fluorogenic substrate to activate the VLP protein sequence tag. The amount of color or fluorescence developed is proportional to the amount of VLP protein in the sample.

B. Chemical Tags

Sequence tags (i.e., nucleotide and/or protein sequences) also include molecules which will be recognized by the enzymes of the transcription and/or translation process without steric or electrostatic interference. Detection of sequence tags may occur through release of a label.

Such labels may include, but are not limited to one or more of any of dyes, radiolabels, binding moieties such as biotin, mass tags, such as metal ions or chemical groups, charge tags, such as polyamines or charged dyes, haptens such as digoxgenin, luminogenic, phosphorescent or fluorogenic moieties, and fluorescent dyes, either alone or in combination with moieties that can suppress or shift emission spectra, such as by fluorescence resonance energy transfer (FRET) or collisional fluorescence energy transfer. Aizenstein et al., "Methods and compositions for detecting target sequences" *US Pat No. 6,913,881* (2005)(herein incorporated by reference).

When TdT or polyA polymerase is employed, an oligonucleotide may contain a 5′ end label. The invention is not limited by the nature of the 5′ end label; a wide variety of suitable 5′ end labels are known to the art and include biotin, fluorescein, tetrachlorofluorescein, hexachlorofluorescein, Cy3 amidite, Cy5 amidite and digoxigenin. A radioisotope label (e.g., a 32P or 35S-labelled nucleotide) may be placed at either the 5′ or 3′ end of the oligonucleotide or alternatively, distributed throughout the oligonucleotide (i.e., a uniformly labeled oligonucleotide). A biotinylated oligonucleotide may be detected by probing with a streptavidin molecule that is coupled to an indicator (e.g., alkaline phosphatase or a fluorophore) or a hapten such as dioxigenin and may be detected using a specific antibody coupled to a similar indicator. The reactive group may also be a specific configuration or sequence of nucleotides that can bind or otherwise interact with a secondary agent, such as another nucleic acid, and enzyme, or an antibody.

To be useful, sequence tags must possess certain physical and physio-chemical properties. First, a sequence tag must be suitable for incorporation into either a growing peptide chain or oligonucleotide. This may be determined by the presence of chemical groups which will participate in peptide or phosphodiester bond formation. Second, sequence tags should be attachable to a tRNA molecule or a nucleic acid polymerase complex. Third, sequence tags should have one or more physical properties that facilitate detection and possibly isolation of nascent proteins or oligonucleotides. Useful physical properties include a characteristic electromagnetic spectral property such as emission or absorbance, magnetism, electron spin resonance, electrical capacitance, dielectric constant or electrical conductivity.

Useful sequence tags comprise native amino acids coupled with a detectable label, detectable non-native amino acids, detectable amino acid analogs and detectable amino acid

derivatives. Labels and other detectable moieties may be ferromagnetic, paramagnetic, diamagnetic, luminescent, electrochemiluminescent, fluorescent, phosphorescent, chromatic or have a distinctive mass. Fluorescent moieties which are useful as sequence tags include dansyl fluorophores, coumarins and coumarin derivatives, fluorescent acridinium moieties and benzopyrene based fluorophores. Preferably, the fluorescent marker has a high quantum yield of fluorescence at a wavelength different from native amino acids and more preferably has high quantum yield of fluorescence can be excited in both the UV and visible portion of the spectrum. Upon excitation at a preselected wavelength, the marker is detectable at low concentrations either visually or using conventional fluorescence detection methods. Electrochemiluminescent markers such as ruthenium chelates and its derivatives or nitroxide amino acids and their derivatives are preferred when extreme sensitivity is desired. DiCesare et al., *BioTechniques* 15:152-59 (1993). These sequence tags are detectable at the femtomolar ranges and below.

In addition to fluorescence, properties based on the interaction and response of a sequence tag to electromagnetic fields, radiation, light absorption (i.e., for example, UV, visible and infrared), resonance Raman spectroscopy, electron spin resonance activity, nuclear magnetic resonances, and mass spectrometry. Electromagnetic spectroscopic properties of a sequence tag are preferably not possessed by a naturally occurring compound and, therefore, are readily distinguishable. For example, the amino acid tryptophan absorbs near 290 nm, and has fluorescent emission near 340 nm. Thus, tryptophan analogs with absorption and/or fluorescence properties that are sufficiently different from tryptophan can be used to facilitate their detection in proteins.

For example, many different modified amino acids which can be used as sequence tags are commercially available (Sigma Chemical; St. Louis, Mo.; Molecular Probes; Eugene, Oreg.). One such sequence tag is N-ε -dansyllysine and may created by the misaminoacylation of a dansyl fluorophore to a tRNA molecule. Another such sequence tag is a fluorescent amino acid analog based on the highly fluorescent molecule coumarin. This fluorophore has a much higher fluorescence quantum yield than dansyl chloride and can facilitate detection of much lower levels. Rothschild et al., "Methods for the detection, analysis and isolation of nascent proteins" *US Pat. No. 6,875,592* (2005)(herein incorporated by reference).

Sequence tags for a protein can be chemically synthesized from a native amino acid and a molecule with marker properties which cannot normally function as an amino acid. For example a highly fluorescent molecule can be chemically linked to a native amino acid group. The chemical modification can occur on the amino acid side-chain, leaving the carboxyl and amino functionalities free to participate in a polypeptide bond formation. For example, a highly fluorescent dansyl chloride can be linked to the nucleophilic side chains of a variety of amino acids including lysine, arginine, tyrosine, cysteine, histidine, etc., mainly as a sulfonamide for amino groups or sulfate bonds to yield fluorescent derivatives. Such derivatization leaves the ability to form peptide bond intact, allowing the normal incorporation of dansyllysine into a protein.

In one embodiment, the present invention contemplates a fluorophore comprising a dipyrromethene boron difluoride (BODIPY) derivative. The core structure of all BODIPY fluorophores is 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. See U.S. Pat. Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; 5,433,896; 5,451,663 (all hereby incorporated by reference). All BODIPY fluorophores have a high extinction coefficient, high fluorescence quantum yield, spectra that are insensitive to solvent polarity and pH, narrow emission bandwidth resulting in a higher peak intensity compared to other dyes such as fluorescein, absence of ionic charge and enhanced photostability compared to fluorescein. The addition of substituents to the basic BODIPY structure which cause additional conjugation can be used to shift the wavelength of excitation or emission to convenient wavelengths compatible with the means of detection.

A variety of BODIPY molecules are commercially available in an amine reactive form which can be used to derivatize aminoacylated tRNAs. One example of a compound from this family which exhibits superior properties for incorporation of a detectable sequence tag into nascent proteins is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY-FL). When the sulfonated N-hydroxysuccinimide (NHS) derivative of BODIPY-FL is used to place a sequence tag on an E. coli initiator $tRNA^{fmet}$, the labeled protein can be easily detected on polyacrylamide gels after electrophoresis using a standard UV-transilluminator and photographic or CCD imaging system. This can be accomplished by using purified $tRNA^{fmet}$ which is first aminoacylated with methionine and then the α-amino group of methionine is specifically modified using NHS-BODIPY. Varshney et al., "Direct analysis of aminoacylation levels of tRNA in vitro" *J. Biol. Chem.* 266:24712-24718 (1991).

C. Unique Sequence Tags

Serial Analysis of Gene Expression (SAGE) is a technique that allows a rapid, detailed analysis of thousands of transcripts. SAGE is based on two principles. First, a short nucleotide sequence tag (i.e., for example, 9 to 10 base pairs (bps)) contains sufficient information to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. For example, a sequence as short as 9 bp can distinguish 262,144 transcripts given a random nucleotide distribution at the tag site, whereas current estimates suggest that even the human genome encodes only about 80,000 transcripts. Second, concatenation of short sequence tags allows the efficient analysis of transcripts in a serial manner by the sequencing of multiple tags within a single clone. As with serial communication by computers, wherein information is transmitted as a continuous string of data, serial analysis of the sequence tags requires a means to establish the register and boundaries of each tag.

Double-stranded cDNA may then be synthesized from mRNA by means of a biotinylated oligo(dT) primer. The cDNA is then cleaved with a restriction endonuclease (anchoring enzyme) that can be expected to cleave most transcripts at least once. Typically, restriction endonucleases with 4-bp recognition sites are used for this purpose because they cleave every 256 bp on average, whereas most transcripts are considerably larger. The most 3' portion of the cleaved cDNA is then isolated by binding to streptavidin beads. This process provides a unique site on each transcript that corresponds to the restriction site located closest to the polyadenylated [poly(A)] tail. The cDNA is then divided in half and ligated via the anchoring restriction site to one of two linkers containing a type IIS (tagging enzyme). Type IIS restriction endonucleases cleaves at a defined distance up to 20 bp away from their asymmetric recognition sites. The linkers are designed so that cleavage of the ligation products with the tagging enzyme results in release of the linker with a short piece of the cDNA.

For example, a combination of anchoring enzyme and tagging enzyme that would yield a 9-bp tag can be cured. After blunt ends are created, the two pools of released tags are ligated to each other. Ligated tags then serve as templates for polymerase chain reaction (PCR) amplification with primers specific to each linker. This step serves several purposes in addition to allowing amplification of the tag sequences. First, it provides for orientation and punctuation of the tag sequence in a very compact manner. The resulting amplification products contain two

tags (one ditag) linked tail to tail, flanked by sites for the anchoring enzyme. In the final sequencing template, this results in 4 bp of punctuation per ditag. Second and most importantly, the analysis of ditags, formed before any amplification steps, provides a means to completely eliminate potential distortions introduced by PCR. Because the probability of any two tags being coupled in the same ditag is small, even for abundant transcripts, repeated ditags potentially produced by biased PCR can be excluded from analysis without substantially altering the final results. Cleavage of the PCR product with the anchoring enzyme allows for the isolation of ditags that can then be concentrated by ligation, cloned, and sequenced.

In addition to providing quantitative information on the abundance of known transcripts, SAGE can be used to identify NDV expressed genes. SAGE can provide both quantitative and qualitative data about gene expression. The combination of different anchoring enzymes with various recognition sites and type IIS enzymes with cleavage sites 5 to 20 bp from their recognition elements lends great flexibility to this strategy.

D. Direct Detection Technology

When a sufficient amount of a nucleic acid to be detected is available, there are advantages to detecting that sequence directly, instead of making more copies of that target, (e.g., as in PCR and LCR). Most notably, a method that does not amplify the signal exponentially is more amenable to quantitative analysis. Even if the signal is enhanced by attaching multiple dyes to a single oligonucleotide, the correlation between the final signal intensity and amount of target is direct. Such a system has an additional advantage that the products of the reaction will not themselves promote further reaction, so contamination of lab surfaces by the products is not as much of a concern. Traditional methods of direct detection including Northern and Southern blotting and RNase protection assays usually require the use of radioactivity and are not amenable to automation. Recently devised techniques have sought to eliminate the use of radioactivity and/or improve the sensitivity in automatable formats. Two examples are the "Cycling Probe Reaction" (CPR), and "Branched DNA" (bDNA).

The cycling probe reaction (CPR), uses a long chimeric oligonucleotide in which a central portion is made of RNA while the two termini are made of DNA. Duck et al., *BioTech.*, 9:142 (1990). Hybridization of the probe to a target DNA and exposure to a thermostable RNase H causes the RNA portion to be digested. This destabilizes the remaining DNA portions of the

duplex, releasing the remainder of the probe from the target DNA and allowing another probe molecule to repeat the process. The signal, in the form of cleaved probe molecules, accumulates at a linear rate. While the repeating process increases the signal, the RNA portion of the oligonucleotide is vulnerable to RNases that may be carried through sample preparation.

Branched DNA (bDNA) involves oligonucleotides with branched structures that allow each individual oligonucleotide to carry 35 to 40 labels (e.g., alkaline phosphatase enzymes). Urdea et al., *Gene* 61:253-264 (1987). While this enhances the signal from a hybridization event, signal from non-specific binding is similarly increased.

VII. *In Vivo* Vaccination

In one embodiment, the present invention contemplates a paramyxovirus VLP vaccine comprising at least one viral glycoprotein wherein the vaccine is antigenic. In one embodiment, the vaccine stimulates an immune response to diseases including, but not limited to, Newcastle disease, measles, parainfluenza virus 3, or respiratory syncytial virus infection. In one embodiment, the present invention contemplates a method comprising administering a purified antigenic paramyxovirus VLP vaccine to a host (i.e., for example, a mouse or chicken) under conditions that generate an immune response. In one embodiment, the immune response is characterized by measuring the serum glycoprotein antibody levels. In one embodiment, the viral glycoprotein comprises an NDV glycoprotein. In one embodiment, the viral glycoprotein comprises a measles virus glycoprotein. In one embodiment, the viral glycoprotein comprises a respiratory syncytial virus glycoprotein.

In one embodiment, the present invention contemplates a method comprising administering a purified antigenic NVD, measles, parainfluenza virus 3, or respiratory syncytial virus VLP vaccine to a chicken to create a vaccinated chicken. In one embodiment, the method further comprises administering a live virus challenge to the vaccinated chicken. In one embodiment, the method further comprises determining the NDV infection rate to the vaccinated chicken.

Experimental

The following examples are only illustrative of specific embodiments of the present invention and are not intended as limiting.

Example 1: Cell Cultures

This example describes the cell cultures used in the Examples below to construct specific embodiments of the present invention.

A spontaneously transformed fibroblast cell line derived from the East Lansing strain (ELL-O) of chicken embryos (UMNSAH/DF-l) was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal calf serum (FCS).

Human renal epithelial cells expressing the SV 40 T antigen (293T) were also propagated in DMEM supplemented with 10% FCS, penicillin-streptomycin, vitamins, non-essential amino acids, and glutamine. NDV, strain A V, was propagated in embryonated chicken eggs by standard protocols.

Example 2: Plasmids

This example describes the types of plasmids used in the Examples below to construct various embodiments of the present invention.

NDV cDNA sequences encoding NP (i.e., for example, SEQ ID NO:23), M (i.e., for example, SEQ ID NO:27), HN (i.e., for example, SEQ ID NO:18), and uncleaved F (i.e., for example, SEQ ID NO:20 or, alternatively, an F-K115Q) proteins were subcloned into the expression vector pCAGGS to generate pCAGGS-NP, pCAGGS-M, pCAGGS-HN and pCAGGS-F-KI15Q, respectively. Miyazaki et al., "Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5" *Gene* 79:269-77 (1989); and Niwa et al., "Efficient selection for high-expression transfectants with a NDVel eukaryotic vector" *Gene* 108:193-9 (1991).

F protein cDNA contains a point mutation in the cleavage site sequence, F-KI15Q, which eliminates the furin recognition site. Li et al., "Effect of cleavage mutants on syncytium formation directed by the wild-type fusion protein of Newcastle disease virus" *J. Virol.* 72:3789-95 (1998).

pBJ5 expression vector containing the gene encoding a Flag-tagged Vps4A with E228Q mutation and pDsRed2-Nl vector (Clontech) containing the gene encoding the CHMP3-RFP fusion protein were previously described. Strack et al., "PIP1/ALIX is a binding partner for HIV-1p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003).

Example 3: Transfection, Infection, And Metabolic Labeling

This examples describes the basic techniques used to develop and express various embodiments of the present invention.

Transfections of sub-confluent ELL-O cells and/or 293T cells were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The following amounts of plasmid DNA were used per 35mm dish: 1.0 μg pCAGGS-NP, 1.0 μg pCAGGS-M, 0.75 μg pCAGGS-F-KI15Q, and 1.0 μg pCAGGS-HN, either alone or in mixtures. These amounts were previously determined to yield levels of expression similar to cells infected with NDV at a multiplicity of infection of 5.

A total of 3.75 μg of plasmid DNA per 35 mm plate was used in all transfection experiments. When only one, two, or three cDNAs were used, the total amount of transfected DNA was kept constant by adding vector pCAGGS DNA. For each transfection, a mixture of DNA and 5 μl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) was incubated at room temperature for 45 minutes, and added to cells previously washed with OptiMEM. The cells were incubated for 5 hours, the Lipofectamine-DNA complexes were removed, and 2 ml of supplemented DMEM was added.

After 36 hours, the medium was replaced with 0.7 ml DMEM without cysteine and methionine and supplemented with 100 μCi of ^{35}S-methionine and ^{35}S-cysteine mixture (NEG-772 EASYTAG™ Express Protein Labeling Mix, ^{35}S, Perkin Elmer Life Sciences Inc.). After 4 hours of pulse label, one set of transfected plates was lysed, while in another set the medium was

replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemicals Corporation). After 8 hours of chase, the medium was collected and the cells were lysed in 0.5 ml lysis buffer containing Triton-DOC (1 % Triton, 1 % sodium deoxycholate) and 25 mg N-ethylmaleimide (NEM). Cells were harvested with a cell scraper and homogenized by passing through a 26 gauge needle 10 to 15 times.

Sub-confluent 293T cells were simultaneously transfected with pCAGGS-M and different concentrations of either pBJ5-Vps4-E228Q-Flag or pDsRed2-NI-CHMP3. Corresponding empty vectors were used as control. Cells were incubated for 36 hours and the same pulse-chase protocol was performed as described above.

ELL-O cells were infected at an MOI of 5 pfu for 5 hours, labeled with ^{35}S-methionine and ^{35}S-cysteine mixture for 30 min, and chased in nonradioactive medium for 8 hours as described above. Cell supernatant was harvested and virions purified as described below. Cells were lysed and homogenized as described above.

Example 4: VLP Purification And Isolation

Virus and VLP, as well as virions, were purified from cell supernatants in protocols previously reported. Levinson et al., "Radiation studies of avian tumor viruses and Newcastle disease virus" *Virology* 28:533-542 (1966). The cell supernatants were centrifuged at 5000 rpm for 5 min at 4°C, overlaid on top of a block gradient consisting of 3.5 ml 20% and 0.5 ml 65% sucrose solutions in TNE buffer (25 mM Tris- HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and re-centrifuged at 40,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The sucrose gradient interface (containing concentrated particles) was collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, and overlaid on top of 1.0 ml 80% sucrose cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.5 ml of 10% sucrose) were layered on top of the sample. The gradient was centrifuged at 38,000 rpm for 20 h at 4°C. The gradient was collected from the bottom into one 1 ml fraction and eight 0.5 ml fractions using a polystaltic pump. Densities of each fraction were determined using a refractometer. VLPs derived from expression of all combinations of proteins were prepared in a single experiment, thus enabling direct comparison of results.

The experiments were repeated three times. Immunoprecipitation and polyacrylamide gel electrophoresis. Immunoprecipitation was accomplished by combining one volume of cell

lysate or sucrose gradient fraction with two volumes of TNE buffer. Samples were incubated with specific antibodies for 16 hours at 4°C. Antisera used to precipitate NP, F and HN were rabbit polyclonal antibody raised against UV inactivated NDV by standard protocols; anti-HR1 and anti-HR2 McGinnes et al., "Newcastle disease virus HN protein alters the conformation of the F protein at cell surfaces" *J. Virol.* 76:12622-33 (2002); anti-F2-96 and anti-A. McGinnes et al., "Role of carbohydrate processing and calnexin binding in the folding and activity of the HN protein of Newcastle disease virus" *Virus Res* 53:175-85 (1998).

Anti-F2-96 was raised against a glutathione S-transferase (GST) fusion protein that contained the F protein sequences from amino acid 96 to 117. Antiserum used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein. Faeberg et al., "Strain variation and nuclear association of 20 NDV Matrix protein" *J Virol.* 62:586-593 (1988). Immune complexes (ICs) were adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% 9 Tween-20 and 0.4% sodium dodecyl sulfate (SDS). ICs were resuspended in SDS- polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCI [pH 6.8], 2% SDS, 10% glycerol, 0.4 % Bromphenol blue) with 1 M J3 mercaptoethanol (BME) and boiled.

Proteins were separated in 8% polyacrylamide-SDS gel and detected by autoradiography. Quantification of resulting autoradiographs was accomplished using a Fluor-STM MultiImager (BioRad).

Example 5: High Efficiency VLP Release

Co-expression of NP, M, F and HN proteins resulted in the release of VLPs with a density of 1.19 to 1.16 g/cc (Figure 1, panel A). Virus particles purified in parallel from NDV, strain AV, infected cells had a density of 1.21 to 1.19 g/cc (Figure 1, panel B). Although it is not necessary to understand the mechanism of an invention, it is believed that the slightly lighter density of VLPs compared to authentic virus is likely due to the absence of the virion RNA within the VLPs. The efficiencies of VLP and virus release were calculated as the percentage of M protein remaining in the cell extracts after the chase relative to the amount of protein in the pulse. The loss of M protein from cells in the chase portion of the experiment is due to release

from cells as VLPs or virions. By this criterion, the efficiency of VLP release was 85%, while the efficiency of NDV release was 92% (Figure 1, panel C). The results show that release of VLPs is almost as efficient as release of virions.

These results demonstrate that NDV VLPs are efficiently assembled and released from avian cells expressing the four major structural proteins. In one embodiment, M protein is sufficient for VLP release. Minimum protein requirements for VLP formation, were determined by individually assessing the capability of each protein to direct particle release. Cells expressing each of the viral proteins individually were radioactively labeled in a pulse-chase protocol and VLPs were isolated as described above.

Example 6: M Protein Dependent VLP Release

VLPs are released only from cells expressing the M protein. Figure 2, Panel B. Almost no M protein is detectable in cell extracts after the 8 hour chase. Figure 2A, right panel. Although it is not necessary to understand the mechanism of an invention, it is believed that this indicates that much of the pulse-labeled protein was released from cells. It is further believed that by comparing the levels of M protein in the pulse labeled extract and the chase extract, the efficiency of release was calculated to be 90%.

In contrast, most of the pulse labeled NP, F and HN proteins remained in extracts after the chase (Figure 2A). Significant amounts of VLPs were also not detected in the corresponding cell supernatant (Figure 2, panel B) although there was a trace of very light density material released from HN protein expressing cells. Figure 2, panel C, shows the quantification of VLPs produced from cells expressing each protein individually. Interestingly, the amount of M protein-containing particles from cells expressing M protein alone was greater than when all four structural proteins were expressed. However, the M protein-only VLPs had a very heterogeneous density, with values ranging from 1.23 to 1.12 g/cc (Figure 2, panel B). These results reveal that M protein is sufficient for the release of particles.

Example 7: M Protein Dependent VLP Release: Pair Wise Combinations

As shown in Example 6, M protein is required for VLP release. To determine the contribution of NP, F or HN proteins to M protein-driven VLP formation, VLPs from cells expressing all possible combinations of two proteins were isolated and characterized as described above. Cells expressing any combination of proteins without M protein did not release VLPs (Figure 3; panel C). Furthermore, in the absence of M protein, NP, F and HN proteins expressed in pair wise combinations were retained in cell extracts after the 8 hour chase (Figure 3A). This finding suggests that M protein is required for particle release. Pair wise expression of NP, F, or HN proteins with M protein resulted in the release of VLPs containing both proteins (Figure 3, panel B). Intriguingly, however, there was only trace amounts of NP, F or HN proteins and M protein was the predominant protein in the VLPs (Figure 3, panel B).

The distribution of NP, F, or HN proteins in the gradients was identical to that of M protein (Figure 3, panel B). In addition, the VLP densities were very heterogeneous and were much like that of M protein-only VLPs. Surprisingly also, the amount of M protein containing VLPs was significantly decreased (by about 2 to 2.5 fold) upon co-expression of M protein with either NP, F, or HN proteins (Figure 3, panel C). These results suggest that NP, F, or HN proteins can individually suppress M protein-driven VLP release.

Example 8: M Protein Dependent VLP Protein Incorporation

Efficient incorporation of other viral proteins into VLPs requires the expression of M protein and at least two of the other proteins. To examine the effects of expression of three viral proteins on particle release, cells were transfected with all possible combinations of three cDNAs. Again, VLPs were only released from cells expressing M protein. Expression of NP, F, and HN proteins without the M protein did not result in the release of any particles (Figure 4, panel C). This finding further strengthens our conclusion that the M protein is required for release of VLPs.

In contrast to the expression of a single glycoprotein with the M protein, co-expression of both F and HN glycoproteins with M protein resulted in significantly increased incorporation of both glycoproteins into VLPs (Figure 4, panels B and C). The F and HN proteins were detected in the same gradient fractions as M protein. Furthermore, the densities of the VLPs were more

homogenous compared to those generated from cells expressing M protein alone (compare Figure 4, panel B and Figure 2, panel B) or M protein with a single glycoprotein. These results indicate that expression of both F and HN proteins with M protein is necessary for efficient incorporation of either glycoprotein into particles.

Expression of M protein with NP and either F or HN protein resulted in increased incorporation of NP as well as the glycoprotein into VLPs (Figure 4, panels B and C). The distribution of NP protein-containing particles in the gradient was similar to that of VLPs released from cells expressing all four structural proteins (compare Figure 1, panel A and Figure 4, panel B). Importantly, the densities of these particles were more homogenous compared to particles released from cells expressing M alone, and were analogous to the density of the authentic virus or complete VLPs (compare Figure 4, panel B, and Figure 1, panel B). Overall, these results indicate that M protein is necessary and sufficient for particle release and that expression of M protein with at least two other proteins is required for efficient incorporation of other proteins into VLPs.

Example 9: VLP Release Inhibition

Host cell VPS pathway is involved in VLP formation and release. Previous studies have implicated the VPS pathway in budding of other enveloped RNA viruses. Demirov et al., "Retrovirus budding" *Virus Res* 106:87 - 102 (2004); Pornillos et al., "Mechanisms of enveloped RNA virus budding" *Trends Cell Biol.* 12:569-79 (2002); and Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol.* 20:395-425 (2004). This pathway might be involved in M protein-driven VLP release because CHMP3 is a subunit of the ESCRT III complex. von Schwedler et al., "The protein network of HIV budding" *Cell* 4:701-13 (2003).

Fusion of CHMP3 with RFP transforms it into a dominant-negative protein which inhibits HIV -1 gag VLP release. Strack et al., "PIP1/ALIX is a binding partner for HIV-1p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003). Simultaneous expression of the M protein with CHMP3-RFP resulted in 98.5% inhibition of VLP release (Figure 5, panels A and C). Expression of another dominant-negative component of the VPS pathway, Vps4A-E228Q with M protein, yielded the same result, with 96.2% inhibition (Figure 5, panels B and D). Expression of both dominant-negative CHMP3 and Vps4A did not suppress the expression

of M protein (Figure 5, panels E and F). Thus an intact host cell VPS pathway is essential for M protein VLP release.

Example 10: Cell Type Dependent Effects on Virus and VLP release

This example provides exemplary data showing that VLP release is dependent upon the host cell type. Host cell type affects basic VLP release mechanisms as well as overall VLP release efficiencies.

Basic Release Mechanisms

VLP release from avian cells (ELL-0) was compared with VLP release from primate cells (COS-7 cells). To compare virus particle release from these cells, equal numbers of avian cells and COS-7 cells were infected with NDV at an MOI = 5. The cells were radioactively labeled in a pulse and then subjected to a nonradioactive chase. Virions were harvested from the cell supernatant at various times during the chase and the proteins in the virus particles resolved by polyacrylamide gel electrophoresis.

An autoradiograph of the NP and F proteins in virus particles at different times of chase are shown in Figure 14A and Figure 14B, respectively (top gel: avian; bottom gel: COS-7). A quantification of the levels of each protein is shown in Figure 15A and Figure 15B, respectively. Clearly, the amounts of virus released from avian cells were higher than amounts released from COS-7 cells and the rate of release from avian cells was faster than the rate of release from COS-7 cells. This difference between avian and primate cells was not due to differences in the levels of protein expression in the two cell types. The levels of total viral proteins made during the pulse label were higher in COS-7 cells than avian cells (not shown), a result that suggests that virus entry, replication and translation were at least as efficient in COS-7 as in avian cells.

These data show that the rate of virus particle release is faster in avian cells than primate cells and the amounts of virus released from avian cells are significantly higher than amounts released from primate cells.

Release Efficiencies

To determine if avian cells were also more efficient in the release of VLPs, equal numbers of avian cells and COS-7 cells were transfected with cDNAs encoding the NP, M, HN,

and F-K115Q proteins of NDV. Cells were radioactively labeled for four (4) hours (i.e., pulsed) and then subjected to a non-radioactive incubation for eight (8) hours (i.e., chased). VLPs were subsequently isolated from the cell supernatant. VLPs in the supernatants were purified by flotation into sucrose gradients.

Sucrose gradients were generated that contain VLPs released from avian cells and COS-7 cells, respectively. See Figure 16A and 16B, respectively. Clearly, the data show that more VLPs were released from avian cells than from COS-7 cells.

Cell lysate extracts from avian cell and COS-7 cells were prepared after the pulse-label and after the nonradioactive chase. See Figure 17A and 17B, respectively. Importantly, the HN, F, and M proteins were no longer present in avian cell extracts after the nonradioactive chase. This observation is consistent with a more efficient release from avian cells and/or incorporation into VLPs. Conversely, significant levels of these viral proteins remained in the COS-7 cell extracts. This observation is consistent with viral protein retention in COS-7 cells and a lower release of the viral proteins into particles. Clearly, the data demonstrate that VLP release is more efficient from avian cells than from COS-7 cells.

Example 11: Comparison Of Specific Viral Protein-Induced VLP Release

This example demonstrates that VLPs are also more efficiently released from avian cells when transfected with NDV containing only an M protein.

VLP particle release was determined from cells transfected with only M protein cDNA as described above. A sucrose density gradient purification of M protein VLPs were generated from both avian and COS-7 primate cells. See Figure 18A and Figure 18 B, respectively. Clearly, the amounts of VLP M proteins released from avian cells were significantly higher, and therefore more efficient, than VLP M proteins released from primate cells.

Further, equal numbers of cells were transfected with either NP protein cDNA, M protein cDNA, F-K115Q protein cDNA, or HN protein cDNA alone. Alternatively, the experiment used cells transfected with a vector having all four (4) viral protein cDNAs in combination. VLPs were then prepared as described above. A sucrose gradient purification was generated for each transfection and particle release was determined by densitometry. When the

various viral protein cDNAs were transfected individually, only M protein resulted in any VLP viral protein release (i.e., only M protein). When a cell was transfected with all four proteins, VLP viral protein release contained all four proteins. In both cases, released VLPs contained greater amounts of viral proteins in avian cells versus COS-7 cells. See Figure 19A and Figure 19B, respectively. Clearly, release efficiency of both M protein VLPs and complete VLPs is better from avian cells than COS-7 cells.

Example 12: Generation of Antibodies To VLP Viral Vaccines

I. Monoclonal Antibodies

Balb/c mice are immunized with multiple I.P. inoculations of a KLH conjugated NDV viral peptide. Splenocytes from immunized animals are then fused with the mouse myeloma AG8 using standard protocols. Wunderlich et al., *J. Immunol. Methods* 147:1-11 (1992). Supernatants from resultant hybridomas are then screened for immunoreactivity to an ovalbumin-coupled NDV viral peptide using standard ELISA protocols known in the art. Hybridomas positive for the expression of immunoreactive MAbs are cloned at least twice by limiting dilution and MAb isotype analysis performed. Purified MAb IgG will be prepared from ascites fluid using protein-A affinity chromatography. After fusion, screening will show a plurality of positive parental signals, from which monoclonal antibody producing clones may be prepared.

Immunoprecipitation/Scintillation Assay for Hybridoma Screening

To develop and screen for monoclonal antibodies which recognize the VLP viral protein in solution rather, than when attached to a solid phase, an assay will be developed in which immunoprecipitation of an ^{35}S-methionine-labeled in vitro-translated VLP viral protein is measured. A standard amount of in vitro translated VLP viral protein is allowed to form antibody/antigen complexes in a solution which can be optimized for ionic strength, pH, and detergent composition. After the immune complexes are precipitated with Protein G (Omnisorb cells) and washed extensively, bound radioactivity is counted in a liquid scintillation counter; background is subtracted and the efficiency of precipitation calculated. This Immunoprecipitation/Scintillation assay (IPSA) allows for both the rapid identification and characterization of antibodies, and will be used to test a variety of monoclonal VLP viral protein

antibodies. The assay is applicable, in general, to monoclonal hybridoma supernatants as well as polyclonal sera to identify antibodies which can be used for immunoprecipitations.

Briefly, approximately $1.5x10^5$ DPMs of ^{35}S-methionine-labeled in vitro-translated VLP viral proteins are added to 10 µl of a 10X immunoprecipitation buffer (150 mM NaCl, 10% NP-40, 5% deoxycholic acid, 1% SDS, 500 mM Tris pH 8). To this, 90 µl of monoclonal cell supernatant from the monoclonal fusion of interest is added and allowed to react for 2 hrs at 4°C. After 2 hrs, 40 µl of a 10% solution of Omnisorb cells (Calbiochem) equilibrated in 1X immunoprecipitation buffer (RIPA buffer; 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH8) is added and allowed to react for an additional 2 hrs at 4°C with rocking. The cells are pelleted by centrifugation for 5 min at 4500 g and 4°C, and washed 3X with 800 µl cold 1X immunoprecipitation buffer. Pellets were quantitatively transferred to scintillation vials and counted in a Beckman LS6000 scintillation counter in the Auto DPM mode. The percentage of VLP viral protein immunoprecipitated may then be calculated.

Characterization of VLP viral protein MAbs

To further characterize a best cell line, a competition immunoprecipitation/scintillation assay (Competition IPSA) may be performed. In this variation, a clone producing monoclonal antibodies to a VLP viral protein was added to an approximate 200 fold molar excess of unlabelled competitor peptide at the same time as labeled in vitro translated VLP viral protein. As expected, peptides to the suspected epitope regions will be compared with peptides that are not suspected of representing the epitope regions. A high percentage of competition in assays containing the suspected epitope regions will verify the VLP viral protein monoclonal antibody binding specificity.

II. Antisera

Antisera used to precipitate viral proteins were a cocktail of anti-NDV antibodies. Antiserum used to precipitate NP was rabbit polyclonal antibody raised against UV inactivated NDV by standard protocols. Antisera used to precipitate F protein were raised against glutathione S-transferase (GST) fusion proteins that contained amino acid sequences 130 to 173 (anti-HR1) (McGinnes et al., "Newcastle disease virus HN protein alters the conformation of the F protein at cell surfaces" *J. Virol.* 76:12622-12633 (2002).), 470 to 500 (anti-HR2) (Dolganiuc et al., "Role of the cytoplasmic domain of the Newcastle disease virus fusion protein in

association with lipid rafts" *J Virol* 77:12968-12979 (2003)), or 96 to 117 (anti-F_2-96). Antiserum used to precipitate HN protein was raised against HN protein sequences from amino acid 96 to 117 (anti-A) (McGinnes et al., "Role of carbohydrate processing and calnexin binding in the folding and activity of the HN protein of Newcastle disease virus" *Virus Res* 53:175-185 (1998)). Antiserum used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein (Faeberg et al., "Strain variation and nuclear association of NDV Matrix protein" *J. Virol.* 62:586-593 (1988)). Antibody used to precipitate HA-tagged proteins was a mouse monoclonal HA antibody conjugated to agarose beads (Sigma). Secondary antibody used for immunoblotting was a peroxidase conjugated mouse monoclonal anti-HA antibody (Sigma).

Example 13: Construction Of Recombinant Baculovirus Vectors

This example describes a general methodology from the construction of recombinant baculovirus vectors.

A general scheme for constructing baculovirus recombinants is shown in Figure 28. As a first step, the target gene (i.e., for example, an NDV particle protein), shown as a PCR-derived DNA, is cloned downstream of a copy of an AcNPV promoter in a suitable plasmid transfer vector (i.e., for example, pBAC4x-1). The transfer vector has upstream and downstream segments of baculovirus DNA flanking the promoter and target gene.

A selected clone of the derived recombinant transfer vector is grown in a bacterial cell culture (i.e., for example, *E. coli*), avian cell culture (i.e., for example, ELL-O), or a human cell culture (i.e., for example, 293T) and the resulting recombinant plasmid DNA is characterized and purified.

In the second step, the purified recombinant transfer plasmid is co-transfected with linearized virus DNA into insect cells (i.e., for example, Sf9) to construct the recombinant baculovirus. The flanking regions of the transfer vector participate in homologous recombination with the virus DNA sequences during virus replication and introduce the target gene into the baculovirus genome at a specific locus (usually polyhedrin or p10, depending on the transfer plasmid).

Following transfection and plaque purification to remove parental virus, a high titer virus stock is prepared from the appropriate recombinant. Once a high titer virus stock is obtained, it is employed to determine the optimal times for target protein expression (depending on the promoter and the properties of the gene product). After these parameters are established, a large scale culture is prepared and used for protein production.

Example 14: Production Of Measles VLP Vaccine

This example presents a protocol that will result in the production of VLP vaccines specific for the measles virus.

Vectors: MV cDNA sequences encoding NP (i.e., for example, SEQ ID NO:42), M (i.e., for examp SEQ ID NO:48), HA (i.e., for example, SEQ ID NO:30), and uncleaved F (i.e., for example, SEQ I NO:36) proteins will be subcloned into the expression vector pCAGGS to generate pCAGGS-NP, pCAGGS-M, pCAGGS-HA and pCAGGS-F-K111G, respectively. The cDNA encoding the MV F protein will be mutated to eliminate the furin recognition site at amino acid 108-112. The mutation introduce a glycine in place of lysine at amino acid 111, the position analogous to the K115Q mutat in the NDV F protein. Elimination of cleavage of the F protein will inhibit the ability of the F prote fuse. Absence of cell-cell fusion in the culture will likely increase the yield of VLPs.

Cell lines: Measles virus is released efficiently from human and primate cell lines but not murine ce lines (Vincent, et al Virology 265: 185). Thus Hela cells (human cervical carcinoma cells), 293 cel (human embryonic kidney cells), VERO cells (African green monkey kidney cells) and COS-7 (prin cells will be used.

Transfection, infection and metabolic labeling: Transfections of sub confluent cells will be accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The followir amounts of plasmid DNA will be used per 35mm dish: 1.0 μg pCAGGS-NP, 1.0 μg pCAGGS-M, 0. μg pCAGGS-F-K111G, and 1.0 μg pCAGGS-HA. A total of 3.75 μg of plasmid DNA per 35mm pl will be used in all transfection experiments. When only one, two, or three cDNAs are used, the total amount of transfected DNA will be kept constant by adding vector pCAGGS DNA. For each

transfection, a mixture of DNA and 5 µl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) be incubated at room temperature for 45 minutes, and added to cells previously washed with Opti The cells will be incubated for 5 hours, the Lipofectamine-DNA complexes removed, and 2 ml of supplemented DMEM added. After 36 hours, the medium will be replaced with 0.7 ml DMEM wi cysteine and methionine and supplemented with 100 µCi of ^{35}S-methionine and ^{35}S-cysteine mixtu (NEG-772 EASYTAG™ Express Protein Labeling Mix, ^{35}S, Perkin Elmer Life Sciences Inc.). Af hours of pulse label, one set of transfected plates will be lysed, while in another set the medium wi replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemi Corporation). After 8 hours of chase, the cell supernatant will be collected. In addition, the cells w sonicated to release cell-associated VLPs. The resulting cell supernatants will be combined. The will be lysed in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH7.4) contair Triton-DOC (1% Triton, 1% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). Cells be harvested with a cell scraper and homogenized by passing through a 26-gauge needle 10 to 15 ti

To determine if the VPS pathway is involved in VLP budding, sub confluent 293T cells wil simultaneously transfected with pCAGGS-M and different concentrations of either pBJ5-Vps4-E22 Flag or pDsRed2-N1-CHMP3. Corresponding empty vectors will be used as control. Cells will be incubated for 36 hours and the same pulse-chase protocol was performed as described above.

To generate virus particles for controls, primate or human cells will be infected at an MOI o pfu for 30 hours and labeled with ^{35}S-methionine and ^{35}S-cysteine mixture for 4 hours, and chased i nonradioactive medium for 8 hours as described above. Cell supernatant will be harvested and viric purified as described below. Cells will be lysed and homogenized as described above.

Virus and VLP purification: VLPs as well as virions will be purified from cell supernatants in proto previously developed for virus purification. The cell supernatants will be clarified by centrifugation 5000 rpm for 5 min at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 6 sucrose solutions in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and centrifuged at 40,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The interface (containing concentrated particles) will be collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, an overlaid on top of 1.0 ml 80% sucrose cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.5 of 10% sucrose) will be layered on top of the sample. The gradient will be centrifuged at 38,000 rpn 20 h at 4°C. The gradient will be collected from the bottom into one 1ml fraction and eight 0.5 ml

fractions using a polystaltic pump. Densities of each fraction will be determined using a refractom VLPs derived from expression of all combinations of proteins will be prepared in a single experim thus enabling direct comparison of results.

Immunoprecipitation and polyacrylamide gel electrophoresis: Immunoprecipitation will be accomplished by combining one volume of cell lysate or sucrose gradient fraction with two volumes of TNE buffer. Samples were incubated with MV specific polyclonal antibodies for 16 hours at 4°C. Antiserum used to precipitate NP, F and HA will be rabbit polyclonal antibody raised against UV inactivated MV by standard protocols. Immune complexes (ICs) will be adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). ICs will be resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.4 % Bromphenol blue) with 1 M β-mercaptoethanol (BME) and boiled. Proteins will be separated on 8% polyacrylamide-SDS gel and subjected to autoradiography. Quantification of resulting autoradiographs will be accomplished using a Fluor-S™ MultiImager (BioRad).

Example 15: Production Of Respiratory Syncytial Virus VLP Vaccine

This example presents a protocol that will result in the production of VLP vaccines specific for the respiratory syncytial virus (RSV).

Vectors: RSV cDNA sequences encoding NP (i.e., for example, SEQ ID NO:70), M (i.e., for exam SEQ ID NO:66 or, alternatively, M2-1), G (i.e., for example, SEQ ID NO:54), and an uncleaved F (for example, SEQ ID NO:60) protein will be subcloned into the expression vector pCAGGS to gene pCAGGS-NP, pCAGGS-M2-1, pCAGGS-G and pCAGGS-F-R108N/R109N, respectively. The cDI encoding the RSV F protein will be mutated to eliminate one of the two furin recognition sites at am acids 106-109 and 131-136, as previously reported (Gonzalez-Reyes, et al, PNAS 98: 9859). Elimination of cleavage will inhibit the ability of the F protein to fuse. The absence of cell-cell fusic will likely increase the release of VLPs. A double mutation, R108N/R109N, eliminates one cleavage

and inhibits the fusion activity of the protein (Gonzalez-Reyes, et al, PNAS 98: 9859). Additiona proteins not found in other paramyxoviruses are NS1, NS2, M2-2, and SH, but all have been show be nonessential for virus assembly (reviewed in Collins, et al, Respiratory Syncytial Virus, in Fiel Virology, Ed. Knipe, D. and Howley, P. Lippincott Williams and Wilkins, 2001). G protein is als nonessential for assembly but likely contributes to a protective immune response to the virus.

Cell lines: RSV grows efficiently in a variety of cell lines from human and animal sources. Howe HEp-2 cells (a Hela cell variant) are the most efficient in production of virus (reviewed in Collins, Respiratory Syncytial Virus, in Fields Virology, Ed. Knipe, D. and Howley, P. Lippincott William Wilkins, 2001), thus these cells will be used. A549 cells (type II alveolar epithelial lung carcinom cells), also reported to be permissive for RSV, will be used as well.

Transfection, infection and metabolic labeling: Transfections of sub confluent cells will be accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The followi amounts of plasmid DNA will be used per 35mm dish: 1.0 μg pCAGGS-NP, 1.0 μg pCAGGS-M2- 0.75 μg pCAGGS-F-R108N/R109N, and 1.0 μg pCAGGS-G. A total of 3.75 μg of plasmid DNA p 35mm plate will be used in all transfection experiments. When only one, two, or three cDNAs are u the total amount of transfected DNA will be kept constant by adding vector pCAGGS DNA. For ea transfection, a mixture of DNA and 5 μl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) w be incubated at room temperature for 45 minutes, and added to cells previously washed with OptiM The cells will be incubated for 5 hours, the Lipofectamine-DNA complexes removed, and 2 ml of supplemented DMEM added. After 36 hours, the medium will be replaced with 0.7 ml DMEM with cysteine and methionine and supplemented with 100 μCi of ^{35}S-methionine and ^{35}S-cysteine mixture (NEG-772 EASYTAG™ Express Protein Labeling Mix, ^{35}S, Perkin Elmer Life Sciences Inc.). Afte hours of pulse label, one set of transfected plates will be lysed, while in another set the medium will replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemic Corporation). After 8 hours of chase, the medium will be collected. In addition, the cells will sonic to release cell associated VLPs. The resulting cell supernatants will be combined. The cells will be lysed in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) containing Trit DOC (1% Triton, 1% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). Cells will be harvested with a cell scraper and homogenized by passing through a 26-gauge needle 10 to 15 times.

To determine if the VPS pathway is involved in VLP budding, sub confluent HEp-2 cells w simultaneously transfected with pCAGGS-M2-1 and different concentrations of either pBJ5-Vps4-E228Q-Flag or pDsRed2-N1-CHMP3. Corresponding empty vectors will be used as control. Cells be incubated for 36 hours and the same pulse-chase protocol was performed as described above.

To generate virus particles for controls, cells will be infected at an MOI of 10 pfu for 30 ho and labeled with ^{35}S-methionine and ^{35}S-cysteine mixture for 4 hours, and chased in nonradioactive medium for 8 hours as described above. Cell supernatant will be harvested and virions purified as described below. Cells will be lysed and homogenized as described above.

Virus and VLP purification: VLPs as well as virions will be purified from cell supernatants in proto previously developed for virus purification. The cell supernatants will be clarified by centrifugation 5000 rpm for 5 min at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 6: sucrose solutions in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and centrifuged at 40,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The interface (containing concentrated particles) will be collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, ar overlaid on top of 1.0 ml 80% sucrose cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.: of 10% sucrose) will be layered on top of the sample. The gradient will be centrifuged at 38,000 rpn 20 h at 4°C. The gradient will be collected from the bottom into one 1ml fraction and eight 0.5 ml fractions using a polystaltic pump. Densities of each fraction will be determined using a refractomet VLPs derived from expression of all combinations of proteins will be prepared in a single experimen thus enabling direct comparison of results.

Immunoprecipitation and polyacrylamide gel electrophoresis: Immunoprecipitation will be accomplished by combining one volume of cell lysate or sucrose gradient fraction with two volumes of TNE buffer. Samples will be incubated with RSV specific polyclonal antibodies for 16 hours at 4°C. Antiserum to be used is commercially available from several sources. Immune complexes (ICs) will be adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). ICs will be resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.4 % Bromphenol blue) with 1 M β-mercaptoethanol

(BME) and boiled. Proteins will be separated on 8% polyacrylamide-SDS gel and subjected to autoradiography. Quantification of resulting autoradiographs will be accomplished using a Fluor-S™ MultiImager (BioRad).

Example 16: Production Of Parainfluenza 3 VLP Vaccine

This example presents a protocol that will result in the production of VLP vaccines specific for the parainfluenza 3 (PIV).

Vectors: PIV3 cDNA sequences encoding NP (i.e., for example, SEQ ID NO:76), M (i.e., for exam SEQ ID NO:80), HN (i.e., for example, SEQ ID NO:84), and an uncleaved F (i.e., for example, SE NO:78) protein will be subcloned into the expression vector pCAGGS to generate pCAGGS-NP, pCAGGS-M, pCAGGS-HN and pCAGGS-F, respectively. The cDNA encoding the PIV3 F protein be mutated to eliminate the furin recognition site at amino acid 109. The lysine at amino acid 108 be changed to glycine. Elimination of cleavage will inhibit the ability of the F protein to fuse. The absence of cell-cell fusion will likely increase the release of VLPs.

Cell lines: PIV 3 grows efficiently in a variety of cell lines from human and primate sources. Thus cells (human cervical carcinoma cells), 293 cells (human embryonic kidney cells), VERO cells (Afr green monkey kidney cells) and COS-7 (primate) cells will be used. (reviewed in Chanock, et al, Parainfluenza Viruses, in Fields Virology, Ed. Knipe, D. and Howley, P. Lippincott Williams and Wilkins, 2001. LLC-MK2 (rhesus kidney cells) and NCI-H292 (human lung carcinoma) cells will a be used as they have been successfully used to generate virus.

Transfection, infection and metabolic labeling: Transfections of sub confluent cells will be accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The followi amounts of plasmid DNA will be used per 35mm dish: 1.0 μg pCAGGS-NP, 1.0 μg pCAGGS-M, 0. μg pCAGGS-F-K108G, and 1.0 μg pCAGGS-HN. A total of 3.75 μg of plasmid DNA per 35mm pl will be used in all transfection experiments. When only one, two, or three cDNAs are used, the total amount of transfected DNA will be kept constant by adding vector pCAGGS DNA. For each transfection, a mixture of DNA and 5 μl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) wi

be incubated at room temperature for 45 minutes, and added to cells previously washed with OptiM
The cells will be incubated for 5 hours, the Lipofectamine-DNA complexes removed, and 2 ml of
supplemented DMEM added. After 36 hours, the medium will be replaced with 0.7 ml DMEM wi
cysteine and methionine and supplemented with 100 μCi of ^{35}S-methionine and ^{35}S-cysteine mixtu
(NEG-772 EASYTAG™ Express Protein Labeling Mix, ^{35}S, Perkin Elmer Life Sciences Inc.). Af
hours of pulse label, one set of transfected plates will be lysed, while in another set the medium wil
replaced with 1.0 ml of supplemented DMEM with 0.1 mM cold methionine (Nutritional Biochemi
Corporation). After 8 hours of chase, the cell supernatant will be collected. The cells will be lysed
0.5 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH7.4) containing Triton-DOC
Triton, 1% sodium deoxycholate) and 1.25 mg N-ethylmaleimide (NEM). Cells will be harvested v
cell scraper and homogenized by passing through a 26-gauge needle 10 to 15 times.

To determine if the VPS pathway is involved in VLP budding, sub confluent HEp-2 cells w
simultaneously transfected with pCAGGS-M and different concentrations of either pBJ5-Vps4-E22
Flag or pDsRed2-N1-CHMP3. Corresponding empty vectors will be used as control. Cells will be
incubated for 36 hours and the same pulse-chase protocol was performed as described above.

To generate virus particles for controls, cells will be infected at an MOI of 10 pfu for 30 hou
and labeled with ^{35}S-methionine and ^{35}S-cysteine mixture for 4 hours, and chased in nonradioactive
medium for 8 hours as described above. Cell supernatant will be harvested and virions purified as
described below. Cells will be lysed and homogenized as described above.

Virus and VLP purification: VLPs as well as virions will be purified from cell supernatants in proto
previously developed for virus purification. The cell supernatants will be clarified by centrifugation
5000 rpm for 5 min at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 6
sucrose solutions in TNE buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA), and
centrifuged at 40,000 rpm for 12 hours at 4°C using a SW50.1 rotor (Beckman). The interface
(containing concentrated particles) will be collected in 0.5 ml, mixed with 2.0 ml of 80% sucrose, ar
overlaid on top of 1.0 ml 80% sucrose cushion. Additional layers of sucrose (1.0 ml of 50 % and 0.
of 10% sucrose) will be layered on top of the sample. The gradient will be centrifuged at 38,000 rpr
20 h at 4°C. The gradient will be collected from the bottom into one 1ml fraction and eight 0.5 ml
fractions using a polystaltic pump. Densities of each fraction will be determined using a refractomet
VLPs derived from expression of all combinations of proteins will be prepared in a single experimer

thus enabling direct comparison of results.

Immunoprecipitation and polyacrylamide gel electrophoresis: Immunoprecipitation will be accomplished by combining one volume of cell lysate or sucrose gradient fraction with two volumes of TNE buffer. Samples will be incubated with PIV3 specific polyclonal antibodies for 16 hours at 4°C. Antiserum to be used is commercially available from several sources. Immune complexes (ICs) will be adsorbed to Protein A (Pansorbin Cells, CALBIOCHEM) for 2 hours at 4°C, pelleted, and then washed three times in immunoprecipitation (IP) wash buffer (phosphate buffer saline (PBS) containing 0.5% Tween-20 and 0.4% sodium dodecyl sulfate (SDS)). ICs will be resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.4 % Bromphenol blue) with 1 M β-mercaptoethanol (BME) and boiled. Proteins will be separated on 8% polyacrylamide-SDS gel and subjected to autoradiography. Quantification of resulting autoradiographs will be accomplished using a Fluor-S™ MultiImager (BioRad).

Example 17
Site-Specific Mutagenesis Of Late Domains

Mutations in the M protein PKSP and YANL sequences at amino acids 216 and 219 and amino acids 232 and 235 were introduced by PCR to yield M-$A_{216}A_{219}$ and M-$A_{232}A_{235}$, respectively. Specific sited-directed mutagenic primers were designed to substitute the proline residues at positions 216 and 219 and tyrosine and leucine residues at positions 232 and 235, respectively, with alanine. Additional mutant M genes were constructed by substituting PTAP or YPDL sequences for YANL at amino acid positions 232 to 235. The entire genes of each M protein mutant DNA were sequenced to verify that no additional mutation was introduced by the mutagenesis protocol. Mutations generated are illustrated in Figure 70.

Example 18

VLP Release From 293T Cells

This example evaluates the effects on particle release of available dominant negative mutant human VPS proteins and whether human renal epithelial cells (293T) could support the release of NDV VLPs.

VLP particles were released from 293T cells expressing M protein alone (top panel) or 293T cells co-expressing NP, M, F-K115Q and HN proteins (bottom panel). Figure 67, Panel A. Particles released from 293T cells expressing M protein alone were very heterogeneous with respect to density (Figure 67, panel A, top panel), very similar to particles released from avian cells expressing M protein alone (data not shown). In contrast, VLPs released from 293T cells expressing all 4 major structural proteins were more homogenous in density. These particles were slightly less dense (1.18 g/cc) than the authentic virus (1.2 g/cc; (Lamb et al., In: Paramyxoviridae: The Viruses and Their Replication, Third edition ed, vol. 1. LippincottWilliams & Wilkins, Philadelphia (2001))) due to absence of genomic RNA.

These combined results show that M protein VLPs and complete VLPs were released from 293T cells. However, the efficiency of release of particles from 293T cells, as measured by the percentage of pulse labeled M protein remaining in cells after a long nonradioactive chase, was lower than VLP release from avian cells (50% vs. 84%, respectively, data not shown).

Example 19

Dominant Negative VPS Protein Mutants Inhibit Particle Release

This example was designed to determine if inhibition of particle release was due only to over expression of dominant negative VPS proteins.

293T cells were transfected with vector control, wild type CHMP3, wild type Vps4A, wild type AIP1, dominant negative (dn) CHMP3, dn Vps4A, and dn AIP1.

The wild type forms of each VPS protein had little effect on particle release. M protein particle release was inhibited by dn-CHMP3 to about 90%. (Figure 68, Panels A and B). Vps4A-E228Q inhibited M protein VLP release by about 90% (Figure 68, Panels C and D), and AIP-1-RFP inhibited particle release by 90% (Figure 68, Panels E and F). The dominant

negative forms of CHMP3, Vps4A, and AIP1, but not the wild type forms, inhibited the release of VLPs containing all four viral proteins. Figure 69.

These combined results show that the inhibition of VLP release was not due to over expression of the VPS protein, but rather due to specific effect of the dn mutant proteins. These results support the conclusion that an intact VPS pathway facilitates M protein particle release.

Example 20
YANL Sequence Mutations Inhibit VLP Release

This example presents data showing that the L domain of an NDV M protein plays a role in particle budding. For example, the sequence of a NDV M protein has two possible L domain sequences, PKSP and YANL, which are similar to the classical L domains PTAP and YPXL, respectively (Freed, E. O., "Mechanisms of enveloped virus release" *Virus Res* 106:85-86 (2004)). The data below shows that by inducing mutations in these L domain sequences, VLP release maybe inhibited.

The proline residues in the PKSP sequence were substituted with alanine (M-$A_{216}A_{219}$); and the tyrosine and leucine in the YANL sequence were substituted with alanine (M-$A_{232}A_{235}$) (Figure 70, Panel A). These mutant M proteins were expressed either individually (Figure 70, Panel B, extracts) or in combination with NP, F-K115Q and HN proteins (Figure 70, Panel D, extracts). Particles were released from cells expressing the M-$A_{216}A_{219}$ mutant at levels comparable to cells expressing wild type M protein. Figure 5, Panels B-E.

In contrast, there was a significant reduction of particles released from cells expressing the M-$A_{232}A_{235}$ mutant (Figure 70, Panel B). Similarly, co-expression M-$A_{232}A_{235}$ mutant protein with NP, F-K115Q and HN proteins resulted in 80% reduction in particles released (Figure 70, Panel D, compare lanes 6 and 8 and Panel E). Amounts of VLPs released from cells co-expressing the M-$A_{216}A_{219}$ mutant protein with NP, F-K115Q and HN proteins were comparable to wild type levels (Figure 70, Panel D, lanes 6 and 7).

To determine if the inhibition of particle release by mutation of the YANL sequence was due to elimination of L domain activity or defects in conformation of the M protein, the YANL sequence was substituted separately with two known classical L domain sequences, YPDL and

PTAP (Morita et al., "Retrovirus budding" *Annu Rev Cell Dev Biol* 20:395-425 (2004); Strack et al., "AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003)).

Both the YPDL and PTAP sequences supported release of the NDV M protein particles. Figure 705, Panels B & C. The amounts of particles released from NDV M protein containing the substituted YPDL and PTAP motif were comparable to wild type levels. These results strongly indicate that the YANL sequence at position 232 to 235 in the NDV M protein functions as an L domain.

Retrovirus particles, which have a gag protein with an YPXL L domain, contain AIP1 (Strack et al., "AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding" *Cell* 114:689-699 (2003)) and may represent a polypeptide with an approximate size of 100kD in the SDS-PAGE gels containing NDV VLP proteins or virion proteins. AIP1 was incorporated into NDV particles and VLPs, thereby co-expressing M protein with an HA-tagged AIP1 at either the N-terminal (HA-AIP1) or the C-terminal (AIP1-HA), or with vector alone. M protein particles were released from both cells expressing M protein with vector and cells expressing M protein and either HA-tagged AIP1. Figure 71, Panel A. The expression of HA-AIP1 and AIP1-HA were at comparable levels (Figure 71, panel A, IB extract gel, lanes 2 and 3). However, only AIP1-HA incorporated into VLPs (Figure 71, panel A, IB VLP gel lane 3). AIP1-HA can also be precipitated from purified disrupted VLPs. Figure 71, Panel B, right.

These results demonstrated that AIP1 is incorporated into VLPs and suggest that AIP1 may be interacting directly or indirectly with the M protein in particles.

Example 21
Co-Immunoprecipitation

Purified VLPs were incubated in ice cold TNE buffer (25mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 2.5 mg/ml N-ethylmaleimide for 15 minutes. Excess primary antibody was added and VLPs were incubated at 4°C overnight. Pansorbin cells, blocked overnight in TNE buffer containing 1% Triton X-100 and 5 mg bovine serum albumin (BSA) and then prewashed in TNE containing 1% Triton X-100 and 1 mg/ml BSA, were added in excess as determined in preliminary experiments, and incubation was continued at 4°C with

constant mixing for at least 2 h. Immune complexes were collected by centrifugation (10,000 rpm for 30 seconds in a microcentrifuge) and washed three times in ice-cold TNE containing 0.5% Triton X-100. The pelleted complexes were resuspended in gel sample buffer.

Example 22
Protease Protection Assay

Protease digestion of M protein from avian cell extracts and VLPs was accomplished by adding 0.25, 0.5, 1, 5, 10, and 20 μg of proteinase K per ml of sample and incubating for 30 min on ice. In parallel, VLPs were also made 0.5 % with respect to Triton X-100 prior to incubation with proteinase K. After digestion, phenylmethylsulfonyl fluoride (PMSF) (0.1 M) was added. For subsequent immunoprecipitation, the reaction mixtures were made 1% with respect to Triton X-100 and 0.5% with respect to sodium deoxycholate.

Example 23
Immunofluorescence Microscopy

Avian cells, grown in 35 mm dish containing glass coverslips, were transfected with different combinations of NDV cDNAs as described above. After 40 hours, nuclei were stained with 5 μg/ml 4',6-Diamidino-2-phenylindole (DAPI) for 30 min at 37°C. Cells were washed twice with ice-cold immunofluoresecence (IF) buffer (PBS containing 1% bovine serum albumin, 0.02 % sodium azide, and 5 mM $CaCl_2$), fixed with 2% paraformaldehyde, blocked with IF buffer for 2 hours, and incubated for 1 hour at 4°C in IF buffer containing polyclonal antibodies against HN and F proteins.

Cells were washed twice with ice-cold IF buffer, permeabilized with 0.05% Triton X-100, blocked with IF buffer for at least 2 hours and incubated for 1 hour at 4°C in IF buffer containing purified ascites fluids containing anti-M protein monoclonal antibody (52-E5). Cells were then washed twice with ice-cold buffer followed by incubation for 1 hour at 4°C in IF buffer containing fluorescein conjugated goat anti-rabbit IgG (Alexa® 488; Molecular Probes) and rhodamine conjugated goat anti-mouse IgG (Alexa® 568; Molecular Probes) secondary antibodies. Cells were washed with ice-cold IF buffer, mounted onto slides using a mounting medium (Vectashield®, Vector Labs, Inc) for immunofluorescence microscopy. Fluorescence

images were acquired using a Nikon fluorescence microscope and Openlab® software and processed using Adobe Photoshop®.

Example 24
Membrane Associated M Protein

This example provides data confirming sucrose gradient data suggesting that M protein may be associated with membranes by incubation with a protease.

VLPs and cell extracts were either left untreated (Figure 62, lane 1) or treated with different concentrations of Proteinase K (lanes 2 to 7). As expected, the M protein in cell extracts was sensitive to low concentrations of protease (Figure 62 upper panel). The lower band below the M protein is a protease digestion product indicating that M protein has a protease resistant core. However, M proteins in VLPs were largely protected from protease digestion (Figure 62, middle panel). In contrast, disruption of the particle membrane with detergent resulted in digestion of the M protein (Figure 62, lower panel).

Taken together, these results demonstrated that the M protein VLPs are membrane-bound particles.

Example 25
M Protein Mediated VLP Release

This example extends the data relevant to M protein sufficiency for VLP release by studying the release of VLPs in the absence of an M protein gene.

Cells were transfected with all possible combinations of NP, F, and HN cDNAs in the absence of the M gene. Cells expressing any combination of proteins without M protein did not release VLPs. Figure 63. Furthermore, in the absence of M protein, NP, F and HN proteins (expressed in pair-wise combinations) were retained in cell extracts after the 8 hour chase (Figure 3; Panel A: lanes 2, 4 and 5, and Panel C).

These results strongly suggest that VLP release is mediated by the M protein.

Example 26
M Protein/Glycoprotein Co-Localization

This example explores further the role played by each protein in VLP assembly. Specifically, the plasma membrane localization of M, F and HN proteins was determined by immunofluorescence.

Transfected cells were incubated with anti-F protein or anti-HN protein antibodies prior to cell permeabilization to limit binding of antibodies to cell surface F or HN proteins. Cells were then permeabilized using 0.05% Triton X-100 and then incubated with M protein specific antibody.

Vector-transfected control cells and as well as cells expressing individually M, F-K115Q or HN proteins, demonstrated that the F-K115Q and HN proteins were diffusely distributed on the surface of the cells (Figure 64, Panel A). M protein exhibited diffuse cytoplasmic staining as well as punctate structures of various sizes (Figure 64, Panel A; anti-M image and merged image). Co-expression of either F or HN proteins with M protein, however, had little effect on the distribution of M protein, F protein, or HN protein. Further, little to no co-localization of F or HN glycoproteins with M protein was observed. Figure 64, Panel B. These findings correlate with the very low incorporation of F or HN proteins into M protein containing VLPs after pair-wise co-expression.

Co-expression of M protein with at least two other proteins slightly changed the distribution of M protein. For example, M protein co-expression with F and HN proteins increased the co-localization of M protein with either F or HN proteins. Figure 64, Panel C. This result is consistent with an increased incorporation of HN, F, or NP proteins when two proteins are co-expressed with M protein.

When all four proteins were co-expressed, the distribution of M protein was changed to more punctuate structures distributed mostly along the edges of the cells. Further, most of the F or HN protein signal co-localized with the M protein. Figure 64, Panel D. Although it is not necessary to understand the mechanism of an invention, it is believed that this result is consistent with a more ordered assembly of VLPs when all four proteins are co-expressed.

Altogether, these results suggest that co-localization of viral proteins is detected with expression of three proteins and is most dramatic when NP, M, F and HN proteins are co-

expressed. These results also suggest that there are specific protein-protein interactions involved in assembling particles.

Example 27
VLP Viral Protein Interactions

This example provides identification of several specific protein interactions involved in VLP assembly using co-immunoprecipitation techniques.

Radioactively labeled VLPs formed with different combinations of proteins were solubilized in 1% Triton X-100 and the proteins present were precipitated, separately, with cocktails of monospecific antibodies for M, HN or F proteins. Proteins were also precipitated with a mix of antibodies with specificities for all proteins in order to precipitate total VLP proteins (lane 6).

First, each antibody cocktail precipitated all proteins from VLPs formed with M, HN, F and NP, although the efficiency of precipitation for each protein varied with the antibody specificity (Figure 65 , Panel A). Although it is not necessary to understand the mechanism of an invention, it is believed that these results are consistent with a network of interactions between all four proteins such that precipitation of one resulted in the precipitation of the other three proteins.

The results also suggested that proteins indirectly linked to the precipitated protein were less efficiently precipitated than a protein directly linked to a precipitated protein. For example, anti-F protein antibody precipitated NP very efficiently but M protein very inefficiently (lane 3). This observation suggests that there may be a direct link between F protein and NP, but not F protein and M protein.

The protein interactions in VLPs were more clearly defined by precipitation of proteins from VLPs formed with all combinations of three proteins. In VLPs released from cells expressing M, F-K115Q and HN proteins, anti-F protein precipitated only F protein and traces of HN protein (Figure 65, Panel B, lane 3). This result indicates that the F protein does not directly complex with the M protein.

Anti-HN protein antibody co-precipitated M protein and HN protein (Figure 65, panel B, lane 4). Likewise, anti-M protein antibody co-precipitated HN protein and M protein (Figure 65,

panel B, lane 5). These results strongly suggest that the M protein interacts with HN protein but not with the F protein.

VLPs were also released containing NP, M and F-K115Q proteins. Anti-F protein antibody co-precipitated NP and F protein, but not M protein. (Figure 65, panel C, lane 3). Anti-M protein antibody co-precipitated NP and M protein, but not F protein (Figure 65, panel C, lane 4). These observations indicate that M protein directly interacts with NP and that the F protein interacts with NP and confirm that F and M protein do not interact.

Although it is not necessary to understand the mechanism of an invention, it is believed that anti-M protein antibody does not indirectly precipitate detectible amounts of F protein because an inefficient precipitation of NP protein may decrease the amounts of F protein precipitated to very low levels. Alternatively, NP-NP interactions required to precipitate F protein with anti-M protein antibody may be disrupted by VLP lysis. For example, when VLPs containing NP, M and HN were used, complexes formed with anti-HN protein antibody contained NP and M proteins as well as HN protein (Figure 65, panel D, lane 3). In addition, anti-M protein antibody precipitated NP and HN proteins (Figure 65, panel D, lane 4). These observations are consistent with the conclusion that the M protein interacts with both NP and HN proteins. It is further contemplated that, in one embodiment, HN protein and NP protein may interact.

Overall, results of co-immunoprecipitation of proteins in VLPs as well as results of cellular co-localization studies provide a rational basis for the incorporation of viral proteins into VLPs and suggest that specific protein interactions are involved in the assembly of an NDV virus-like particle.

Claims

We claim:

1. A method, comprising;

   a) providing,

      i) an expression vector comprising DNA sequences encoding a Newcastle disease matrix protein ;

      ii) a cell capable of being transfected by said vector;

   b) transfecting said cell with said vector under conditions such that Newcastle disease virus-like particles are generated.

2. The method of Claim 1, further comprising the step c) harvesting said virus-like particles so as to create a cell-free preparation of particles.

3. The method of Claim 2, further comprising the step d) administering a vaccine comprising said preparation of particles to a chicken.

4. The method of Claim 2, wherein said cell is part of a cell culture and said harvesting comprises obtaining said particles from the supernatant of said culture.

5. The method of Claim 4, wherein said cell culture comprises avian cells.

6. The method of Claim 1, wherein said vector further comprises DNA sequences encoding additional Newcastle disease viral proteins selected from the group consisting of a nucleocapsid protein, a fusion protein, and a hemagglutinin-neuraminidase protein.

7. The method of Claim 1, wherein said particles are free of Newcastle disease viral DNA.

8. The transfected cell of Claim 1.

9. The preparation of particles created according to Claim 2.

10. A method, comprising;

a) providing,

i) a vaccine comprising Newcastle disease virus-like particles, said particles comprising a Newcastle disease viral matrix protein;

ii) a host susceptible to Newcastle disease;

b) immunizing said host with said vaccine under conditions such that antibodies directed to said virus-like particle are produced.

11. The method of Claim 10, wherein said host is selected from the group consisting of avian, murine, and human.

12. The method of Claim 10, wherein said particles further comprise one or more additional Newcastle disease viral proteins selected from the group consisting of a fusion protein, a nucleocapsid protein and a hemagglutinin-neuraminidase protein.

13. A vaccine comprising Newcastle disease virus-like particles, said particles comprising a Newcastle disease viral matrix protein.

14. The vaccine of Claim 13, wherein said particles are free of Newcastle disease viral DNA.

15. The vaccine of Claim 13, wherein said particles further comprise one or more additional viral proteins selected from the group consisting of a fusion protein, nucleocapsid protein and a hemagglutinin-neuraminidase protein.

5