JOURNAL OF VIROLOGY, May 2010, p. 4513–4523 Vol. 84, No. 9
0022-538X/10/$12.00 doi:10.1128/JVI.01931-09

Assembly and Biological and Immunological Properties of Newcastle Disease Virus-Like Particles[▽]

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Received 11 September 2009/Accepted 11 February 2010

Virus-like particles (VLPs) released from avian cells expressing the Newcastle disease virus (NDV) strain AV proteins NP, M, HN (hemagglutinin-neuraminidase), and F were characterized. The VLP-associated HN and F glycoproteins directed the attachment of VLPs to cell surfaces and fusion of VLP membranes with red blood cell membranes, indicating that they were assembled into VLPs in an authentic conformation. These particles were quantitatively prepared and used as an immunogen, without adjuvant, in BALB/c mice. The resulting immune responses, detected by enzyme-linked immunosorbent assay (ELISA), virus neutralization, and intracellular cytokine staining, were comparable to the responses to equivalent amounts of inactivated NDV vaccine virus. HN and F proteins from another strain of NDV, strain B1, could be incorporated into these VLPs. Foreign peptides were incorporated into these VLPs when fused to the NP or HN protein. The ectodomain of a foreign glycoprotein, the Nipah virus G protein, fused to the NDV HN protein cytoplasmic and transmembrane domains was incorporated into ND VLPs. Thus, ND VLPs are a potential NDV vaccine candidate. They may also serve as a platform to construct vaccines for other pathogens.

Vaccination is the most effective means of preventing virus infection and controlling the spread of a virus through a population. Most licensed viral vaccines are live, attenuated viruses or inactivated virus. Live, attenuated viruses offer long-lasting and protective immunity and are considered the most effective vaccines. However, these types of vaccines may cause serious disease in immunocompromised individuals, a significant concern due to the increase in this population in recent years (reviewed in references 11, 33, and 34). They can also cause disease in normal individuals, albeit at low frequency, due to reversion to virulent forms (41). It is also possible that recombination events between the vaccine virus and endemic avirulent viruses can produce a virulent virus (44). Recombinant live virus vaccines may also have unknown, novel properties and require significant amounts of testing to ensure that these new viruses pose no unforeseen hazards. An additional problem with these forms of live virus vaccines is the immunogenicity of the vector virus, a complication if a human virus is used as a vector (2).

Inactivated vaccines are safer but produce poorer and shorter-lived immune responses than live virus, in part due to alteration of the immunogenicity of the viral proteins during inactivation (reviewed in references 11 and 33). Inactivated virus vaccines are also thought to be less effective in stimulating cellular immune responses (11). Additionally, vaccination with some inactivated virus vaccines, notably those developed for respiratory syncytial virus (RSV) and measles virus, did not protect but actually exacerbated disease upon subsequent exposure to the live virus (reviewed in references 11 and 33). Some viruses are also difficult to produce in quantity because of their virulence in eggs (47) or the difficulty in growing them in tissue culture.

Other types of vaccines are subunit vaccines or DNA vaccines. Subunit vaccines are usually less effective and often require an adjuvant, which adds additional safety concerns (reviewed in reference 11). DNA vaccines, while having a great deal of potential, have not yet been licensed for use in humans (reviewed in reference 7). In human trials, immune responses are often reported to be weak without additional immunization (21).

Virus-like particles (VLPs) are increasingly being considered as potential viral vaccines (reviewed in references 15 and 34) because of their safety and efficacy. Indeed, two VLP vaccines are licensed for use in humans, the papillomavirus vaccine and the hepatitis B virus vaccine, and a number of other VLP vaccines are in testing (15). VLPs are large particles, the size of viruses, composed of repeating structures on their surfaces and in their cores, structures that mimic those of infectious viruses (15, 34). It has been noted that just these properties account, in part, for the very potent immunogenicity of viruses (15). VLPs are formed by the assembly of the structural proteins and lipids into particles but without the incorporation of the viral genome. Thus, VLPs are incapable of the multiple rounds of infection typical of an infectious virus, yet they retain the superb antigenicity of virus particles.

Paramyxoviruses are enveloped, negative-stranded RNA vi-

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▽ Published ahead of print on 24 February 2010.

ruses (4, 16, 19). Many members of this virus family are serious human or animal pathogens, and vaccines do not exist for many of them (4, 8, 9, 12, 16). It has been reported that VLPs can be produced upon the expression of structural proteins of several different paramyxoviruses (3, 5, 39, 42, 45, 46). For example, cells expressing the four major structural proteins, the viral NP, M, HN (hemagglutinin-neuraminidase), and F proteins, of the Newcastle disease virus (NDV) very efficiently release particles that resemble virus particles (37). We therefore explored the possibility that these Newcastle disease virus-like particles (ND VLPs) could be developed as vaccines. We report that VLPs contain biologically active glycoproteins, indicating that they have retained their authentic conformation during VLP assembly. These VLPs could be quantitatively prepared, and they stimulated both humoral and cellular immune responses in mice. We also explored the possibility that these VLPs could be used as a platform for the assembly of sequences from other viruses. We report that the HN and F proteins from a different strain of NDV can be efficiently incorporated into these VLPs. We found that short foreign peptide sequences can be assembled into ND VLPs when fused to two different NDV proteins. We have recently reported that the ectodomain of the RSV G protein can be incorporated into these VLPs and that immunization of mice with the G protein-containing VLPs stimulated protective anti-RSV immune responses (32). Here, we show that the ectodomain of another glycoprotein, the G protein of Nipah virus, (NiV) can be assembled into ND VLPs, demonstrating the versatility of these VLPs as a platform for the assembly of foreign sequences into particles.

MATERIALS AND METHODS

Cells, virus, and plasmids. ELL-0 avian fibroblasts obtained from the American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with penicillin-streptomycin and 10% fetal calf serum. NDV NP, M, F, and HN protein genes derived from the AV strain of NDV were inserted into the pCAGGS expression vector as previously described (37). NDV strains AV and B1 were grown and gradient purified as previously described (29). Strain AV, a select agent, was prepared and the titer determined by plaque assay in biosafety level 3 conditions by authorized personnel.

HN and F protein cDNAs from the B1 strain of NDV were cloned by standard reverse transcription (RT)-PCR protocols using infected-cell RNA as template as previously described (27). The resulting DNAs were sequenced in their entirety to verify that they contained the correct sequence. The addition of hemagglutinin (HA) and FLAG sequence tags to the strain AV HN and F protein sequences, respectively, was accomplished by standard PCR protocols, and the resulting fusion genes were verified by sequencing. That the HN protein contained the HA sequence and the F protein the FLAG sequence was verified by Western analysis and by immunoprecipitation of the expressed proteins using anti-HA antibody or anti-FLAG antibodies.

The Nipah virus G protein cDNA was obtained from Paul Rota. It was moved from pTM1 into an XhoI- and Msc1-cut pCAGGS vector using the BamI (blunted with T4 DNA polymerase) and XhoI sites that flanked the G sequences in pTM1. Two different NDV HN-NiV G protein chimera genes were constructed by ligation of PCR-derived DNAs derived from pCAGGS-HN and pCAGGS-G. The HN sequences encoded amino acids 1 through 47, and the NiV G sequences encoded amino acids 71 through 601 (HN/NiVG#1) or 74 through 601 (HN/NiVG#2). The primers used to generate DNA encoding the HN cytoplasmic (CT) and transmembrane (TM) domains were GGTTATTGTGCT GTCGACTCATTTTGGC (forward primer) and CATACTATATGCCAGGG CGGCCGCAGAGATGGCTAAG (reverse primer). The product was digested with XhoI and NotI (a site introduced without changing the amino acid sequence). The forward primers used to generate DNA encoding the G protein ectodomain were GGATCTATCGTGATCATAGCGGCCGCTCTAGCGTAC AGCCAAAATTACAAAGATC (forward primer for chimera 1) or GTGATC ATAGTGATGAATGCGGCCGCCCTCGCATATTCCACAAGATCAACAG ACAATC (forward primer for chimera 2). Both primers introduced a NotI site without changing the amino acid sequence. The reverse primer was GCCAGA AGTCAGATGGCCAAGG. The PCR products were digested with NotI and Msc I. The two DNA fragments were ligated into an XhoI-MscI-digested pCAGGS vector. The resulting plasmids containing the chimeric protein genes were sequenced in their entirety to verify the gene junctions (illustrated in Fig. 9) and to ensure that no additional changes were introduced during the PCRs.

Antibodies. Polyclonal rabbit anti-NDV antibody was raised against UV-inactivated, purified NDV as previously described (24). The polyclonal antiserum specific to F protein was a mix of antibodies raised against peptides with sequences of the HR1 domain and the HR2 domain as previously described (6, 24). The polyclonal antiserum specific for the HN protein was raised against a peptide with HN protein sequences as previously described (28). The secondary antibodies utilized were goat anti-rabbit antibodies (Sigma). Anti-G protein polyclonal monospecific mouse antibody was obtained from Paul Rota. Anti-NDV M protein antibody is a monoclonal antibody obtained from Mark Peeples (10).

Transfections. Transfections were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. For small-scale transfections, a mixture of plasmid DNA (0.5 μg/35-mm plate) and Lipofectamine (5 μl/35-mm plate) in Opti-MEM medium (Gibco) was incubated at room temperature for 45 min and added to avian cells grown in 35-mm plates and previously washed with Opti-MEM. Cells were incubated for 5 h at 37°C, Opti-MEM was removed, and 2 ml of supplemented DMEM was added.

For quantitative preparations of VLPs, large-scale transfections of avian cells growing in T-150 flasks (1.8×10^7 cells) were utilized. For each T-150 flask, plasmid DNA (8 μg of each plasmid) in 1.6 ml of Opti-MEM and Lipofectamine (80 μl) in 3.2 ml of Opti-MEM were each incubated for 15 min at room temperature, mixed, and further incubated for 45 min at room temperature. Opti-MEM (11.2 ml) was mixed with the DNA-Lipofectamine complexes and added to cells in a T-150 flask that had been twice washed with Opti-MEM. Cells and DNA-Lipofectamine complexes were incubated for 5 h at 37°C, the complexes removed, and 15 ml of complete medium added.

Surface expression and biological activities of NDV glycoproteins. Quantification of the surface expression of HN and F proteins expressed in tissue culture cells was accomplished by biotinylation of surfaces of avian cells transfected with cDNA encoding the HN protein or the F protein as previously described (25). The fusion activity of F-FLAG protein expressed in tissue culture cells was measured in a content-mixing assay by quantifying the β-galactosidase activity activated in fused cells as previously described (25). The attachment activity of HN-HA protein expressed in tissue culture cells was measured by quantifying the binding of avian red blood cells (RBCs) as previously described (26, 28). Neuraminidase was measured as previously described (23, 30), using *N*-acetyl neuramin lactose as the substrate.

Metabolic labeling of cells and VLPs. At 36 h posttransfection, cells (in 35-mm plates) were washed with DMEM without methionine or cysteine. The medium was replaced with 0.7 ml DMEM without methionine or cysteine and supplemented with 100 μCi of a [^{35}S]methionine and [^{35}S]cysteine mixture (NEG-722 EASYTAG express protein labeling mix; Perkin-Elmer Life Sciences, Inc.). After 4 h of labeling, the labeling medium was replaced with complete medium and incubation was continued for an additional 8 to 12 h. VLPs in the cell supernatant were purified as previously described (37).

Large-scale VLP purification. At various times posttransfection, cell supernatants from 2×10^8 to 7×10^8 cells were collected and cell debris was removed by centrifugation at 5,000 rpm (Sorvall GSA SLA-1500 rotor). VLPs in the supernatant were pelleted by centrifugation in a type 19 rotor (Beckman) at 18,000 rpm for 12 h. The resulting pellet was resuspended in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA), dounce homogenized, and layered on top of a discontinuous sucrose gradient composed of 2 ml 65% sucrose and 4 ml 20% sucrose. The gradients were centrifuged in an SW 28 rotor (Beckman) at 24,000 rpm for 6 h. The fluffy layer at the 20 to 65% sucrose interface, containing the VLPs, was collected, mixed with two volumes of 80% sucrose, and placed on top of a 1-ml layer of 80% sucrose in an SW41 Beckman centrifuge tube, and then layers of 3.5 ml of 50% sucrose and 2 ml of 10% sucrose were laid on top. The gradients were centrifuged for 18 h at 38,000 rpm. The VLPs, which float to the interface of the 50% and 10% sucrose layers, were collected and concentrated by centrifugation in an SW50.1 rotor for 16 h at 38,000 rpm. All sucrose solutions were measured as weight/volume and dissolved in TNE buffer, and all centrifugations were done at 4°C.

NDV UV inactivation. Purified virus (strain B1) was diluted in 2 ml of PBS in a 60-mm tissue culture dish and placed on a rotating platform 10 cm from a germicidal lamp (G15T8; Sylvania) for 20 min, a time previously determined to

inactivate 100% of the virus as measured by plaque assay. The efficacy of UV inactivation was confirmed in a plaque assay. Virus was inactivated prior to inoculation into animals in order to comply with USDA regulations.

Polyacrylamide gel electrophoresis, silver staining, and Western analysis. To prepare cell extracts, cells were washed in cold phosphate-buffered saline (PBS) and lysed in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM $NaCl_2$, and 5 mM EDTA) containing 1% Triton X-100, 0.5% sodium deoxycholate, and 2.5 mg/ml of *N*-ethylmaleimide (NEM). VLPs or purified virus were also lysed in TNE buffer.

Proteins in extracts, virus, or VLPs were resolved on 8% polyacrylamide gels (SDS-PAGE) as previously described (14). Silver staining of proteins in the polyacrylamide gels was accomplished as recommended by the manufacturer (Pierce). For quantification of individual proteins in the polyacrylamide gels, different concentrations of bovine serum albumin (BSA) were electrophoresed on the same gel. A standard curve based on the densitometry of the BSA (Fluor-S Multi Imager; Bio-Rad) was used to determine the concentrations of each of the proteins in the purified VLPs or virus. For Western analysis, proteins in the polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes and detected as previously described (14).

Virus neutralization. Mouse sera were complement inactivated and then diluted in DMEM without serum. Purified NDV strain AV was diluted to approximately 75 to 150 PFU in 100 μl. Dilutions of mouse sera in 100 μl were added to the virus and incubated for 1 h at 37°C. The mixture was then added to prewashed, confluent monolayers of avian cells growing in 12-well tissue culture dishes, and the cells were incubated at 37°C for 1 h. The antibody-virus mixture was removed, and 2 ml of agarose overlay were added to each well as previously described. Plates were incubated for 3 days.

Hemagglutination. Red blood cells (RBCs) (guinea pig; Bio-link, Inc.) were washed three times in PBS and resuspended in PBS at a concentration of 5.3×10^7 cells/ml. Twofold serial dilutions of VLPs or virus in PBS were prepared, and 10 μl of each dispensed into microtiter plate wells. To each well, 75 μl of the RBCs was added, and the plate was incubated for 1 h at 4°C and then photographed.

VLP membrane fusion. Hemifusion of VLPs to cell membranes was measured by dequenching of the fluorescent dye R18 (octadecyl rhodamine B; Molecular Probes) loaded into VLP membranes as has been previously described (18). Purified ND VLPs were treated with acetylated trypsin (5 μg/ml) for 10 min on ice to cleave the F protein, followed by the addition of soybean trypsin inhibitor (10 μg/ml). The VLPs were then incubated with R18 at room temperature in the dark for 1 h. R18-loaded VLPs were purified away from excess R18 by sedimentation through 20% sucrose to a 20 to 65% interface. ND VLPs were then incubated with chilled guinea pig erythrocytes (Bio-Link, Inc.) in 24-well plates for 1 h on ice at 4°C in the dark. Upon incubation of plates at 37°C, R18 fluorescence (excitation at 560 nm and emission at 590 nm) from wells of VLP-erythrocytes was read at 7-min intervals with a SpectraMax Gemini XS (Molecular Devices) and SoftMax Pro software (Molecular Devices). Experimental values were normalized for loading of R18 and by the amounts of input particles as determined by amounts of M protein. Similar results were obtained using the HN protein content for normalization.

VLP neuraminidase. The neuraminidase activities of HN proteins in purified NDV (B1 strain) and VLPs were determined in particle suspensions as previously described (23, 30), using *N*-acetyl neuramin lactose as the substrate.

Animal immunization protocols. Mice, 4-week-old BALB/c males from Taconic laboratories, were housed (groups of 5) under pathogen-free conditions in microisolator cages at the University of Massachusetts Medical Center animal quarters. Mice (groups of 5) were immunized and boosted by intraperitoneal (IP) inoculation of different concentrations of VLPs or UV-inactivated NDV protein (10, 20, or 40 μg total particle protein/mouse) resuspended in 0.5 ml of PBS containing 30% sucrose. Boosts (10 μg total protein/mouse) were accomplished at 27 days after initial immunization. Blood was collected from tail veins by standard protocols.

Determination of antibody titers by ELISA. The antigens used as targets in enzyme-linked immunosorbent assays (ELISAs) were purified NDV (1 μg protein/well). All antigens were placed in carbonate buffer, pH 9.6, added to microtiter plates (Costar), and incubated overnight at 4°C.

After binding of the target antigen, wells were blocked in 50 μl PBS containing 1% BSA at room temperature for 1 to 2 h, washed three times in PBS, and drained. Serial dilutions of mouse sera were added to the microtiter wells in 50 μl of PBS-BSA and incubated for 1 h at room temperature. After the mouse sera were removed and the wells washed three times, a biotinylated anti-mouse antibody (Sigma) in 50 μl of PBS-BSA was added and the microtiter plates were incubated for 1 h at room temperature. The microtiter plates were then washed three times in PBS, and horseradish peroxidase (HRP)-conjugated neutravidin (1:4,000 dilution) (Pierce) was added in 50 μl of PBS-BSA. The microtiter plates were incubated for 1 h at room temperature and washed four times in PBS. TMB (3,3′,5,5′-tetramethylbenzidene) substrate (Sigma) in 50 μl was added to each well and incubated for 15 to 20 min. The reaction was stopped with 50 μl 1N H_2S0_4, and the optical densities (ODs) were read in a plate reader (Molecular Devices).

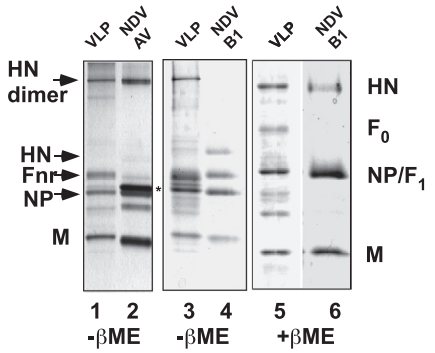

FIG. 1. Proteins in ND VLPs. Proteins in purified ND VLPs (VLP) and egg-grown, purified NDV (virus) were separated on polyacrylamide gels and visualized by silver staining. Lanes 1, 3, and 5 show proteins in VLPs from three different preparations. Proteins in purified virus are shown in lanes 2 (strain AV), 4 (strain B1), and 6 (strain B1). Lanes 1 to 4 show proteins electrophoresed in the absence of reducing agent (−βME). Lanes 5 and 6 show proteins electrophoresed in the presence of reducing agent (+βME). HN, hemagglutinin-neuraminidase protein, either dimer or monomer; F_0, uncleaved fusion protein; NP, nucleocapsid protein; M, membrane protein; Fnr, nonreduced fusion protein (mix of uncleaved F_0 and disulfide-linked F_1-F_2). The asterisk alongside lane 2 indicates BSA that copurifies with some preparations of virus.

Antibody titers were defined as the dilution of antiserum that resulted in OD values 5 times greater than the background.

Intracellular cytokine staining. At 23 days after the second immunization, mice were sacrificed, spleens recovered, and splenocytes harvested from each spleen using standard protocols (for example, see reference 31). The recovered splenocytes were cocultured separately for 7 days with uninfected, UV-irradiated P815 cells or UV-irradiated P815 cells that had been infected with NDV strain B1 for 9 h and then washed three times to remove virus. After 7 days, spleen cells were mixed with infected P815 cells, uninfected P815 cells, or anti-CD3. Cells were washed, resuspended in medium with GolgiPlug (BD Biosciences Pharmingen), incubated for 5 h at 37°C, washed, incubated with anti-CD8 or anti-CD4 for 30 min at 4°C, washed, and then incubated with Cytoperm/Cytofix (BD Biosciences Pharmingen) for 30 min at 4°C. After washing, the cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% BSA and 0.02% sodium azide) and incubated with antibody specific for gamma interferon (IFN-γ) for 30 min at 4°C. After washing, the percentages of $CD4^+$, IFN-γ^+ cells, of $CD8^+$, IFN-γ^+ cells, and of $CD3^+$ cells were determined by flow cytometry.

RESULTS

Characterization of ND VLPs. For the quantitative preparation of VLPs required for their use as immunogens, avian cells were transfected with cDNAs encoding the NDV HN, F, NP, and M proteins as previously described (37). A mutation was introduced into the cleavage site of the F protein (F-K115Q) in order to inhibit cytopathic effects due to syncytium formation in the transfected cells (20). VLPs released from 2×10^8 to 6×10^8 transfected avian cells were purified, and the VLP-associated proteins were compared to virion proteins of two different NDV strains, AV and B1 (Fig. 1). The cDNAs used to produce VLPs were derived from the AV strain of NDV, a virulent strain (reviewed in reference 29). Thus, the

TABLE 1. Protein concentrations and ratios in VLPs and virus[a]

Protein	VLP prepn						All VLPs (avg ratio ± SD)	NDV strain		
	1		2		3			B1[b]		AV (ratio[d])
	μg/ml	Ratio	μg/ml	Ratio	μg/ml	Ratio		μg/ml	Ratio	
HN	177	2.46	109	1.73	92	1.53	1.89 ± 0.37	50	0.66	1.06 ± 0.2
F	120	1.66	85	1.35	53	0.88	1.29 ± 0.32	80[c]	1.07	0.43 ± 0.2[c]
NP	140	1.92	98	1.55	92	1.50	1.65 ± 0.18	100[c]	1.33	0.53 ± 0.1[c]
M	72	1.00	63	1.00	60	1.00	1.0	75	1.00	1.00
Total Protein	509		355		297			305		

[a] Determined by densitometer scans of silver-stained polyacrylamide gels. Values assume that all proteins bind silver stain equally.
[b] Values are from 3 dozen infected eggs.
[c] Determined using nonreducing gels.
[d] Values are from a single stock prepared in 20 dozen eggs. Standard deviations show variations in 3 to 5 determinations of protein ratios.

VLPs were compared to virion proteins in this strain of NDV (lanes 1 and 2). Strain B1 is an NDV vaccine strain (reviewed in reference 29). The VLP proteins were also compared with this strain of NDV (lanes 3 to 6), which was purified in parallel. Proteins were electrophoresed in the absence (lanes 1 to 4) and presence (lanes 5 and 6) of a reducing agent (2-mercaptoethanol [βME]). While the F protein in VLPs is the uncleaved F_0 protein, due to a mutation of the F protein cleavage site, the F protein in the egg-grown virus, either AV or B1, is cleaved into F_1, which, in reducing gels, migrates with NP (lane 6), and F_2, which migrates off the gel (reviewed in reference 29). To resolve the virion-associated F protein, proteins were electrophoresed in the absence of reducing agent since the cleaved F protein is a disulfide-linked heterodimer (F_1-F_2) in nonreducing gels (Fnr) and can be separated from NP (lanes 2 and 4).

Figure 1 shows that the VLPs (lanes 1, 3, and 5) contained primarily the four major NDV structural proteins, NP, M, HN, and F, and, like the virus, there was minimal cellular protein content in the particles. That these proteins are NDV proteins is shown by their comigration with proteins in purified virions. In addition, it has been previously shown that these proteins are specifically immunoprecipitated with anti-NDV antibodies (37). Figure 1, lanes 1 and 3, also shows that the VLP-associated HN protein is a disulfide-linked dimer, typical of the HN protein in NDV strain AV (lane 2) (28). The HN protein in the B1 strain of NDV is not disulfide linked and migrates as a monomer in nonreducing gels (lane 4). The ratios of proteins in VLPs are, in general, similar to the ratios in virus, although the F protein content, relative to that of M protein, is increased in VLPs compared to the ratio in the AV strain but similar to that in the B1 strain.

Table 1 shows a quantitative analysis of individual protein content in three different VLP preparations and in purified virus. Different preparations of VLPs contained similar ratios of viral proteins, and the ratios were similar to those of the B1 virus, purified in parallel, with the exception of the relative amounts of HN protein. B1 virus contained slightly less HN protein relative to the amount of M protein. The ratio of proteins in NDV AV strain virions is also shown in Table 1 and confirms that the VLPs contained an increase in F protein relative to the amount of M protein.

Glycoproteins incorporated into ND VLPs are functional. To determine if VLP-associated glycoproteins were assembled into particles in a native conformation, the functional activities of HN and F proteins were measured. HN protein attachment activity was measured by cell binding and by hemagglutination. Figure 2 shows the binding of purified, radioactively labeled ND VLPs to avian cell monolayers. Nearly 100% of the input ND VLPs (lane 1) bound to cells (lane 4). This binding was inhibited with anti-NDV antibody (lane 3), a result consistent with specific binding mediated by the HN protein. Figure 3 shows that ND VLPs are capable of hemagglutinating red

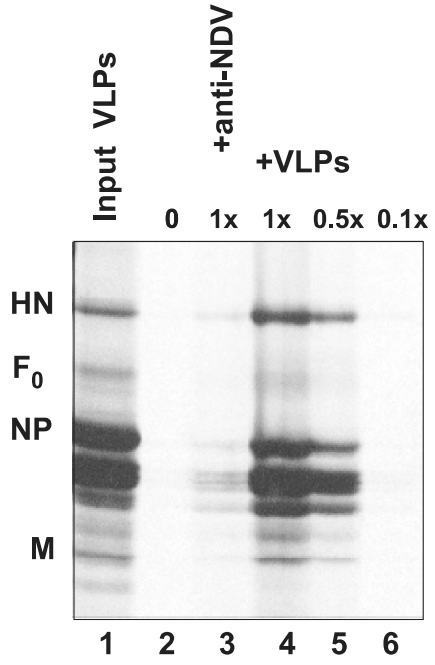

FIG. 2. Attachment activity of ND VLPs. Different amounts (1×, 0.5×, and 0.1×) of radioactively labeled ND VLPs were added to avian cell monolayers on ice and incubated for 30 min. Unbound particles were removed, and the cells washed with PBS and lysed. Viral proteins in the resulting cell extracts were electrophoresed on polyacrylamide gels and visualized by autoradiography. Lane 1, input VLPs (1×); lane 2, negative control (particles purified from supernatants of vector-transfected cells); lane 4, 1× VLPs added to cells; lane 5, 0.5× VLPs added to cells; lane 6, 0.1× VLPs added to cells. Lane 3 shows the binding of 1× VLPs to cells in the presence of anti-NDV antiserum. The relative ratios of proteins in VLPs shown in Fig. 2 appear different than those shown in Fig. 1 due to the variations in numbers of methionine and cysteine residues in the NDV proteins.

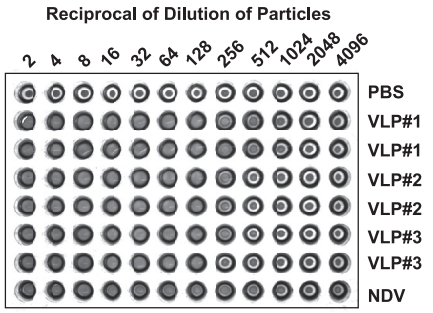

FIG. 3. Hemagglutination activities of ND VLPs. The hemagglutination titer was determined as described in Materials and Methods. Twofold dilutions of the three preparations of ND VLPs (VLP#1, VLP#2, and VLP#3) described in Table 1 or of purified NDV (strain B1) were added, in duplicate, to wells of a microtiter plate. RBCs were added to each well. Shown is a digitally acquired photograph of the plate.

blood cells, a classic property of virus particles that is the result of specific binding of the HN protein to sialic acid receptors on red blood cell surfaces (16, 19). The HA titers of equivalent amounts of VLPs and virus were similar. VLP preparations 2 and 3 had protein concentrations similar to that of B1 virus (Table 1), and the HA titers were similar (both with titers of 256). VLP preparation 1 had a higher protein concentration than the virus preparation and a higher HA titer (titer of 512).

The VLP-associated HN protein also retained neuraminidase activity typical of virus particles. The neuraminidase activity associated with VLPs was compared to that in a dilution of NDV virions that contained equivalent amounts of HN protein as determined by silver staining. The VLP-associated neuraminidase activity was 111% (±31% [standard deviation]) that of virus-associated activity (average of six determinations).

The fusion activity of VLP-associated F protein was assessed using a well-established fusion assay which measures the dequenching of the fluorescent lipid R18 incorporated into effector membranes upon binding to target membranes (17). The effectiveness of this assay for measuring fusion directed by NDV has been previously documented (18). Fusion of R18-labeled VLP membranes with cell membranes was indicated by an increase in fluorescence signal over time at 37°C (Fig. 4). That this fusion was specific to the F protein was shown by the absence of fusion of particles containing only the NDV NP, M, and HN proteins. Thus, both HN and F proteins assembled into ND VLPs retained the function of virion-associated proteins.

Antibody responses to ND VLPs. To determine if ND VLPs could stimulate immune responses in experimental animals, different concentrations of purified VLPs (10, 20, and 40 μg of total VLP protein) were injected into the intraperitoneal cavities of BALB/c mice. No adjuvant was used. In order to compare immune responses stimulated by VLPs with those stimulated by virus, the same concentrations of UV-inactivated NDV were utilized as an immunogen in a second set of mice. All mice received a boost of 10 μg of either VLPs or virus at 27 days after the initial immunization. NDV strain B1 was used as the virus control for these experiments since this NDV strain is used as a vaccine.

The NDV-specific antibody titers in serum from each mouse

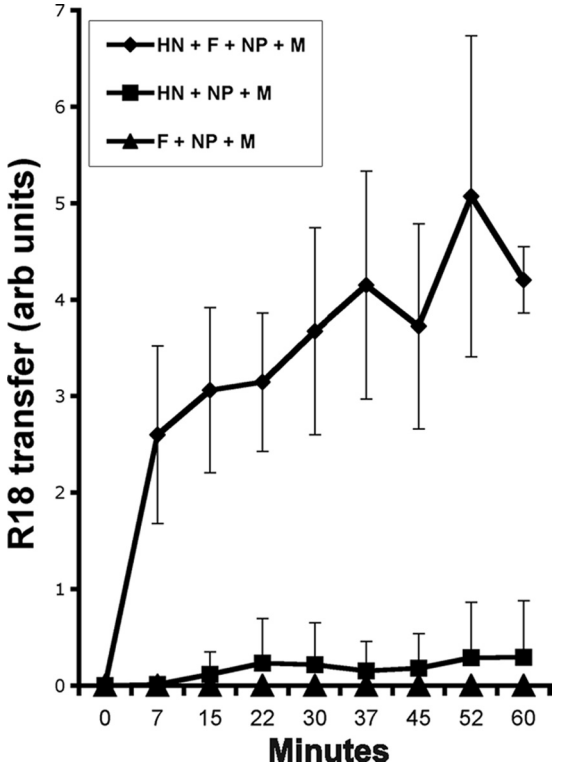

FIG. 4. Membrane fusion activities of ND VLPs. Purified VLPs formed with NP, HN, F, and M proteins, VLPs formed with NP, HN, and M proteins (37), and VLPs formed with F, NP, and M proteins (37) were loaded with R18 as described in Materials and Methods. The VLPs were added to RBCs on ice and warmed to 37°C. The figure shows the increase in fluorescence with time after transfer to 37°C. The data were normalized for the R18 loading in each VLP as determined by fluorescence after chemical dequenching induced by the addition of Triton X-100. Shown are the averages of the results of three experiments and error bars indicating standard deviations.

over time after immunization were determined by ELISA as described in Materials and Methods (Fig. 5). The results show that VLPs were at least as efficient in stimulating total anti-NDV antibodies as virus. The titers of antibodies to VLP proteins increased slightly faster than the titers resulting from virus immunization. The maximal titers detected from both VLP- and virus-immunized mice were similar. While the results shown in Fig. 5 were obtained using as the target antigen the proteins in purified NDV strain AV, the antibody titers after virus immunization were not increased when proteins from purified NDV strain B1 were used as the target antigen (not shown).

To determine if VLPs stimulated neutralizing antibodies, the ability of sera from VLP-immunized mice to neutralize virus in an *in vitro* plaque reduction assay was measured. The results in Table 2 show that sera from mice immunized with either VLPs or virus contained neutralizing antibody. The sera derived from VLP-immunized mice were at least as effective in neutralization as sera from NDV-vaccinated mice.

T cell responses to VLP immunization. To determine the effectiveness of VLPs in stimulating T cell responses in vaccinated animals, splenocytes from mice immunized with two different concentrations of VLPs were harvested at 49 days

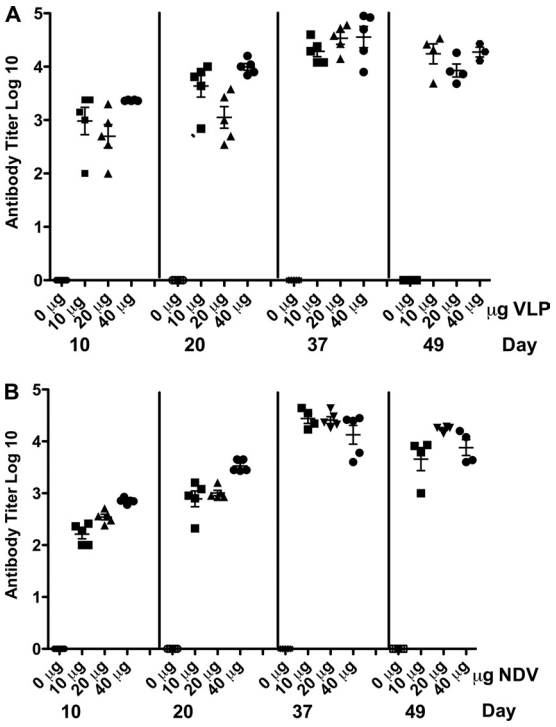

FIG. 5. ELISA titers of NDV-specific antibodies after immunization with ND VLPs or NDV. Groups of five mice were immunized with the total protein concentrations of VLPs or virus indicated at the bottom of each panel. All mice received a boost of 10 μg of total VLP or virus protein at day 26. Sera were collected at 10, 20, 37, and 49 days postimmunization. The figure shows the titers of antibodies with the use of total NDV proteins of purified NDV strain AV as the target antigen. Titer was defined as the reciprocal dilution of antibody that resulted in an OD that was 5-fold greater than that of the background. Top, titers obtained after ND VLP immunization; bottom, titers obtained after immunization with UV-inactivated NDV. Horizontal bars, mean; vertical bars, standard deviation.

postimmunization and restimulated *in vitro* with irradiated, NDV-infected cells. After stimulation *in vitro*, the percentages of $CD8^+$ and $CD4^+$ T cells that were secreting IFN-γ were determined by flow cytometry. Figure 6, top panel, shows that comparable amounts of $CD8^+$, IFN-γ^+ cells were detected in spleens from mice immunized with VLPs and from mice immunized with UV-inactivated virus. Similarly the percentages of $CD4^+$, IFN-γ^+ T cells from both sets of mice were comparable (Fig. 6, bottom panel). Thus, VLPs were as effective as virus in stimulating murine T cell responses.

Incorporation of different NDV HN and F proteins into ND VLPs. The VLPs described above were assembled with proteins from the AV strain of NDV. To determine if the glycoproteins of another NDV strain could be assembled into these VLPs, the HN or F protein cDNAs from the B1 strain of NDV were expressed in avian cells along with the cDNAs from the AV strain. Figure 7, left panel, shows the expression of the viral proteins in transfected cells. Figure 7, right panel, shows proteins in VLPs purified from the supernatants of the transfected cells. The B1 HN protein was incorporated into ND VLPs along with the AV NP, M, and F proteins (lane 8). While the incorporation of the B1 HN protein into VLPs was reduced compared to the incorporation of the AV HN protein (the ratio of HN to M protein was decreased by 70% compared to the AV HN:M ratio), this reduction correlated to the reduced expression of the B1 HN protein in cells, which was also reduced by 70% compared to AV HN protein expression (lanes 3 and 5). The B1 F protein was incorporated into ND VLPs with the AV NP, M, and HN proteins (lane 9) at levels comparable to the incorporation of the AV F protein (compare lanes 7 and 9). Furthermore, both the B1 HN and F proteins were incorporated into particles with the AV NP and M proteins (lane 10). Again, incorporation of the B1 HN protein was reduced but the B1 F protein incorporation was similar to AV F protein incorporation as determined by F:M protein ratios in each VLP preparation. Thus, there is not a stringent, strain-specific requirement for the assembly of glycoproteins into these particles and any reduction in incorporation likely relates to expression levels.

Incorporation of foreign peptide sequences into ND VLPs. The incorporation of foreign sequences into the VLPs would expand their use for presentation of antigens. To determine if foreign peptide sequences could be specifically incorporated into ND VLPs, three approaches were explored. First, short peptide sequences were fused to the carboxyl terminus of the HN or F protein. To test this approach, the HA sequence tag was fused to the carboxyl terminus of the HN protein, and the FLAG sequence tag was fused to the carboxyl terminus of the F protein. Table 3 shows the quantification of the surface expression and biological activities of these proteins compared to those of the wild-type proteins. Neither sequence tag inhibited the expression of the glycoproteins (Fig. 8, panel A, left). The surface expression of F-FLAG was very similar to that of the wild-type protein (Table 3). The HN-HA protein surface expression was reduced, on average, by 23%. The FLAG sequence at the carboxyl terminus of the F protein had no effect

TABLE 2. Virus neutralization

Immunogen (μg)	Serum dilution[a]	NDV titer[b] after incubation with serum
No serum		1.91×10^{10}
Preimmune sera	1:4	1.49×10^{10}
	1:8	1.65×10^{10}
	1:16	1.41×10^{10}
VLP, 20	1:4	$<5 \times 10^{7}$
	1:8	5.00×10^{7}
	1:16	3.00×10^{8}
VLP, 40	1:4	$<5 \times 10^{7}$
	1:8	$<5 \times 10^{7}$
	1:16	$<5 \times 10^{7}$
NDV, 20	1:4	1.50×10^{8}
	1:8	7.00×10^{7}
	1:16	1.65×10^{9}
NDV, 40	1:4	$<5 \times 10^{7}$
	1:8	1.10×10^{9}
	1:16	2.90×10^{9}
Rabbit anti-NDV antibody	1:8	$<5 \times 10^{7}$

[a] Sera from mice in each group (five mice) were pooled for this analysis.
[b] Each titer is the average of two separate determinations.

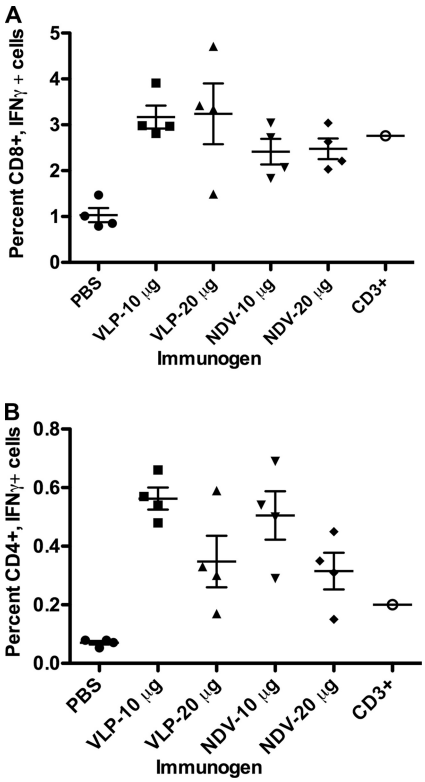

FIG. 6. Activation of T cells by ND VLPs. Activation of CD8 or CD4 T cells to secrete IFN-γ was measured by intracellular cytokine staining. Splenocytes from groups of four mice immunized with different concentrations of ND VLPs or UV-inactivated virus (shown at the bottom of each panel) were stimulated *in vitro* as described in Materials and Methods. The percentages of total cells that were $CD8^+$, IFN-γ^+ (A) or $CD4^+$, IFN-γ^+ (B) were determined by flow cytometry. The percentage of cells stimulated with anti-CD3 antibody is shown as a positive control. The mean and standard error of the mean, calculated using Prism Graph Pad software, are show for each set of mice.

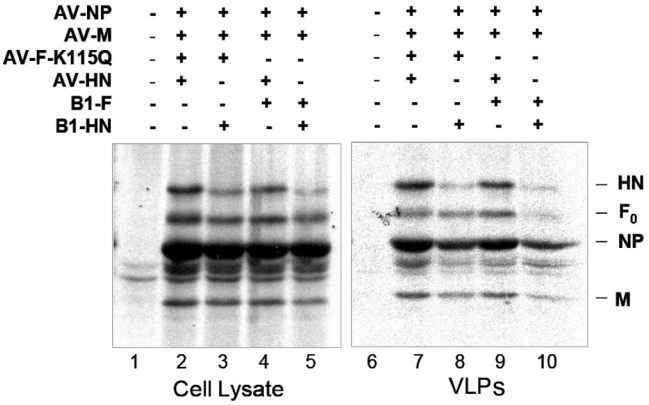

FIG. 7. Incorporation of HN and F proteins from NDV strain B1 into ND VLPs. Avian cells were transfected with the cDNAs encoding the NP and M proteins from strain AV, as well as various combinations of cDNAs encoding HN and F proteins from either strain AV or strain B1, as indicated at the top of the figure. Two sets of cells were pulse labeled with [^{35}S]methionine and then chased with nonradioactive medium as described in Materials and Methods. Radioactively labeled proteins in cell lysates of one set of cells prepared at the end of the pulse labeling are shown in the left panel. VLPs harvested at the end of the nonradioactive chase in the other set of cells were purified, and the proteins present in extracts and VLPs were precipitated with a cocktail of antibodies (anti-NDV antibody, anti-HN protein antibody, anti-F protein antibodies, and anti-M protein). Proteins in the precipitate were resolved on polyacrylamide gels and detected by autoradiography.

on the fusion activity of the F protein, while the HA tag did decrease the binding and neuraminidase activities of the HN protein even if these activities were normalized to surface expression. However, the HA tag did not depress the fusion promotion activity of the HN protein but, surprisingly, enhanced it. The mechanisms involved in this fusion enhancement are a subject of current investigation.

The incorporation of these tagged proteins into VLPs is shown in Fig. 8, panel A, right. The FLAG-tagged F protein was not incorporated into particles. This result suggests that a foreign sequence fused to the cytoplasmic tail of the F protein can block the assembly of F protein into particles. The inhibition of F protein assembly had no effect on assembly of the HN protein into VLPs. The HA-tagged HN protein was, however, incorporated into particles. There was a reduction in the amount of HN-HA detected in VLPs compared to the amount in HN protein. However, this reduction was likely due to a decreased expression of M protein in transfected cells in this experiment. Other experiments did not show this reduction. Indeed, the HN-HA:M protein ratio in VLPs shown in Fig. 8 was identical to the HN:M protein ratio, and the ratios were very similar in two other, separate experiments. Thus, the fusion of foreign sequences to the carboxyl terminus of HN protein is a potential approach for the introduction of additional epitopes into ND VLPs.

In a second approach to the incorporation of foreign epitopes into ND VLPs, a sequence tag was fused to the amino-terminal end of the NP (HA-NP) or, separately, to the carboxyl terminus (NP-HA). The HA sequence tag fused to either the amino terminus or the carboxyl terminus of NP had little effect on the incorporation of NP into the VLPs (Fig. 8, panel B). The sequence tag also had little effect on the incorporation of the glycoproteins.

In a third approach to the incorporation of foreign sequences into ND VLPs, the hypothesis that the entire ectodomain of a foreign glycoprotein could be incorporated into ND VLPs was tested using the ectodomain of the Nipah virus G protein. The approach was to fuse the ectodomain sequence to the transmembrane (TM) and cytoplasmic domain (CT) of an NDV glycoprotein. Since the Nipah virus G protein and the

TABLE 3. Activities of sequence-tagged glycoproteins[e]

Protein	Surface expression	Attachment[a]	Neuraminidase[a]	Fusion promotion/fusion
HN	100	100	100	100
HN-HA	77 ± 33[b]	37 ± 12[d]	48 ± 14[d]	164 ± 3[d]
F	100	NA	NA	100
F-FLAG	105 ± 22[c]	NA	NA	110 ± 2[d]

[a] NA, not applicable.
[b] Average and standard deviation of eight determinations.
[c] Average and standard deviation of six determinations.
[d] Average and standard deviation of four determinations.
[e] Expressed as percent wild type.

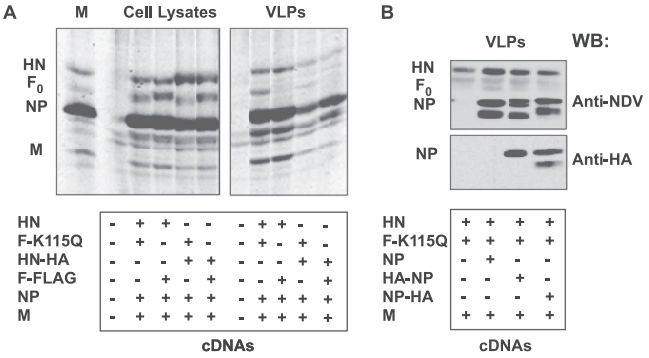

FIG. 8. Incorporation of peptides into ND VLPs. Avian cells were transfected with cDNAs encoding the NDV M protein, as well as various combinations of untagged and HA-tagged HN protein, untagged and FLAG-tagged F protein, and untagged and HA-tagged NP protein, as indicated below each panel. F protein cDNA containing a mutation in the cleavage site (20), as described in Results, was used in order to resolve the F protein separately from NP on polyacrylamide gels. (A) VLPs were radioactively labeled as described in Materials and Methods and the legend to Fig. 7. Proteins present in the pulse-labeled cell extracts (left panel) or in purified VLPs (right panel), harvested from supernatants of cells subjected to a radioactive pulse and a nonradioactive chase, were detected by autoradiography of polyacrylamide gels containing proteins precipitated using a polyclonal antibody cocktail. (B) Avian cells were transfected with the cDNAs indicated below the panel. VLPs were harvested at 24 h posttransfection. The figure shows the proteins in purified VLPs detected by Western blotting (WB) using an antibody cocktail (described in the legend to Fig. 7) (top panel) or anti-HA antibody (bottom panel). M, marker NDV-infected cell extract.

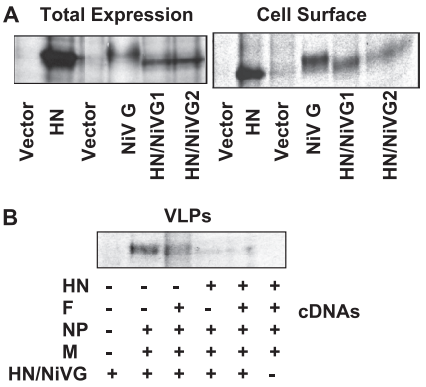

FIG. 10. HN/NiVG expression and assembly into ND VLPs. (A) The first through the sixth lanes show the total protein immunoprecipitated from radioactively labeled extracts of cells expressing the HN protein, the NiV G protein, or one of the two chimeric proteins. HN protein was precipitated with anti-NDV antibodies (first and second lanes). The NiV G protein and the chimeric proteins were precipitated with anti-NiV antibody (third through sixth lanes). The 7th through the 12th lanes show biotinylated surface-expressed protein levels in cells transfected with the HN protein, NiV G protein, or chimeric protein cDNAs. Biotinylated proteins were sequentially precipitated with neutravidin-agarose and then anti-NDV antibodies (seventh and eighth lanes) or anti-NiV G protein antibody (9th through 12th lanes). (B) Radioactively labeled NiV G protein sequences in VLPs prepared from cells transfected with the cDNAs indicated at the bottom of the panel for each lane. Purified VLPs were lysed, and the proteins precipitated with anti-NiV G protein antibody and detected by autoradiography.

NDV HN protein are both type 2 glycoproteins, the ectodomain sequence of the NiV G protein was fused to the TM and CT domain sequences of the HN protein as diagramed in Fig. 9. Two different chimeric proteins were constructed, as shown in Fig. 9.

To determine if the resulting HN-NiV G chimeric proteins were expressed, avian cells were transfected with cDNAs encoding the chimeric proteins, as well as the wild-type G protein and the wild-type HN protein. Figure 10, panel A, first to sixth lanes, shows the precipitation of radioactively labeled proteins from cell extracts using anti-NDV antibody (first and second lanes) or anti-Nipah virus antibody (third to sixth lanes). The chimeric proteins were expressed at levels similar to the amount of wild-type G protein. To determine if the chimeric proteins were expressed on cell surfaces, the surfaces of radioactively labeled cells expressing these proteins were biotinylated. Following cell lysis, the biotinylated proteins were sequentially precipitated with neutravidin-agarose and then with either anti-NDV or anti-Nipah virus G protein antibody. Figure 10, panel A, 7th to 12th lanes, shows the proteins in the final precipitates. Clearly, both of the chimeric proteins were expressed on cell surfaces at levels comparable to the amount of wild-type protein.

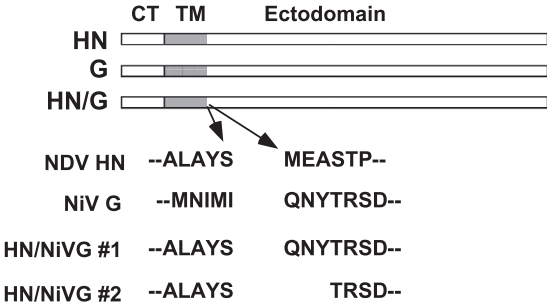

FIG. 9. Construction of HN-NiV G chimeric protein. The diagram shows the locations of the cytoplasmic domain (CT), transmembrane domain (TM), and ectodomain of the NDV HN protein and the NiV G protein and the domains present in two NDV HN-NiV G chimeric proteins. Below the bars, the sequences at the junctions of the TM and ectodomains of the two wild-type proteins, as well as those of two different chimeric proteins, HN/NiVG#1 and HN/NiVG#2, are shown.

To determine if a chimeric protein could be incorporated into ND VLPs, cells were transfected with the cDNA encoding the HN/NiVG#1 chimera and the cDNAs encoding the NDV NP and the NDV M proteins. Another set of cells was transfected with the NiVG#1 chimera's cDNA only. In addition, to determine whether the inclusion of one or both NDV glycoproteins had any effect on the assembly of the HN-NiV G chimeric protein into VLPs, cDNAs encoding the F protein, the HN protein, or both glycoproteins were included in parallel transfections. VLPs were purified from radioactively labeled cells and solubilized in lysis buffer, and the chimeric protein present in the VLPs was immunoprecipitated with anti-Nipah virus G protein antibody. Any particles in the supernatant of cells transfected with the chimeric protein alone were purified, and associated proteins immunoprecipitated in parallel. Figure 10, panel B, shows that the chimeric protein was incorporated into particles in the presence of NP and M proteins (second

lane). No chimeric protein was detected in the particles purified from cells expressing only the chimeric protein (first lane). The inclusion of F protein slightly inhibited the incorporation of the chimeric protein (third lane). The inclusion of HN protein in either the absence or presence of F protein inhibited the incorporation of the chimeric protein (fourth and fifth lanes). Thus, the entire ectodomain of a foreign glycoprotein can be incorporated into ND VLPs in the presence of the NDV NP and M protein.

DISCUSSION

The results presented here describe the characterization of ND VLPs as a vaccine and as a vaccine platform for the presentation of sequences from other pathogens. The results showed, first, that ND VLPs could be quantitatively prepared from cells transiently transfected with cDNAs encoding the NDV HN, F, NP, and M proteins. Second, VLP-associated glycoproteins retained their functional activities of attachment and fusion. Third, immunization of mice with these VLPs stimulated antibody and T cell responses comparable to those stimulated by virus. Fourth, foreign sequences, including the entire ectodomain of a foreign glycoprotein, could be incorporated into these VLPs, raising the possibility that these VLPs can serve as a platform for the construction of vaccines for a number of pathogens.

VLPs as vaccines. Virus-like particles have a great deal of potential as viral vaccines (reviewed in references 11, 15, and 34), since this type of vaccine may offer significant advantages over more commonly used vaccines. Two VLP vaccines have been licensed for use in humans, but no VLP vaccine is currently used in animals (11). The hepatitis B vaccine is a particle produced in yeast and is composed of membrane and the HBV surface antigen (22). VLPs composed of virus capsid proteins have been approved as a papillomavirus vaccine (13). VLPs composed of structural proteins of numerous other viruses have been reported (reviewed in reference 34). Many of these VLPs have been tested as vaccines in animal model systems with generally positive results (summarized in references 15 and 34). VLPs based on paramyxovirus structural proteins have been reported (3, 5, 37, 39, 42, 45, 46), but none has been explored as a potential vaccine.

ND VLPs as an NDV vaccine. The consideration of ND VLPs as a vaccine candidate for Newcastle disease illustrates the potential of VLP vaccines. Newcastle disease virus (NDV), or avian paramyxovirus 1 (APMV-1), is an avian pathogen with a significant economic impact worldwide (1). There are many different strains of NDV, which are grouped according to their virulence in birds (1, 43). Most commercially raised chickens in the United States are vaccinated with an infectious, avirulent strain of NDV. The currently used vaccines are not, however, ideal. First, live virus vaccines often cause mild respiratory or gastrointestinal disease (1). As a result, vaccinated birds have lower body weights, lower egg production, and greater susceptibility to other pathogens than unvaccinated birds (1). A second problem is the failure of these vaccines to stimulate an immune response that is protective against all strains of virus. Flocks of vaccinated chickens can be susceptible to virulent or "exotic" strains, resulting in outbreaks of the disease in the United States (35, 36). A third potential problem with live virus vaccination is the reported recombination between vaccine viruses and circulating wild viruses (40). A fourth problem with the currently used vaccines, either live virus or inactivated virus, is the difficulty in discriminating between birds that have been vaccinated and those that have been infected with a wild virus, a distinction that is important in monitoring outbreaks of the disease in vaccinated flocks.

ND VLPs as a vaccine for Newcastle disease could overcome most of the problems with currently used NDV vaccines. ND VLPs are incapable of spreading infection, thereby eliminating this negative effect of live virus immunization. ND VLPs have no genome, eliminating the possibility of recombination with endemic NDV strains. We also demonstrated that VLPs could be constructed with glycoproteins from two different strains of NDV, raising the possibility that broad-spectrum VLP vaccines can be developed. The assembly of the NDV glycoproteins into VLPs requires the cytoplasmic domains of the proteins, probably for specific interactions with the viral NP and/or M protein (L. W. McGinnes, K. A. Gravel, and T. G. Morrison, unpublished data). In addition, the specific transmembrane domain of the glycoproteins is critical for their assembly into ND VLPs (McGinnes et al., unpublished). There are minor sequence differences between the CT and TM domains of different strains of NDV. For example, the HN proteins from the B1 and AV viruses vary by 3 and 6 residues in the CT and TM domains, respectively. While the assembly of the B1 HN protein into ND VLPs composed of AV NP, F, and M proteins is less efficient than assembly of the AV HN protein, this reduction can be completely accounted for by the reduced expression of the B1 HN protein in cells. The F proteins of the B1 and AV strains of NDV vary by 4 residues and 1 residue in the TM and CT domains, respectively. These residues had no effect on the assembly of the B1 F protein into VLPs. Thus, the minor sequence differences between the TM and CT domains of proteins from different strains of virus have minimal effects on their assembly into VLPs, suggesting that these ND VLPs can be constructed with glycoproteins from different strains of NDV. We also showed here that VLPs can be sequence tagged, which would facilitate discrimination between vaccinated and previously infected birds.

For use as a vaccine, ND VLPs must be quantitatively and efficiently produced. Indeed, quantitative amounts of ND VLPs could be produced from 2×10^8 to 6×10^8 avian cells. They could be purified using protocols utilized for virus purification, and the purified VLPs showed minimal cell protein contamination. Furthermore, we found previously (37) and confirmed here that the ratios of viral proteins were similar to those in virus particles. Most importantly, in order for VLPs to be an effective immunogen, the proteins, and particularly the glycoproteins, assembled into the particles must be in an authentic conformation. The most stringent test of the conformation of a glycoprotein is the retention of the biological activities typical of glycoproteins associated with virus. Indeed, we showed that the ND VLPs' HN protein mediated cell binding and possessed neuraminidase activity. F protein in these particles could direct the fusion of the VLP membrane with red blood cell membranes.

The effectiveness of VLPs as an immunogen was demonstrated in a mouse model. The immune responses were compared to those stimulated by a vaccine strain of NDV. The

soluble antibodies, characterized by ELISA, resulting from ND VLP immunization were at least as good as those resulting from immunization with inactivated virus. The neutralizing antibody responses were also as good as the responses to vaccine virus. Furthermore, ND VLPs stimulated T cell responses at levels slightly better than those stimulated by a vaccine virus. Thus, ND VLPs stimulated immune responses in an animal model that have all the characteristics of an effective vaccine. Their effectiveness in generating protective immune responses in chickens will be tested in future experiments.

ND VLPs as a vaccine platform. Because of the efficient ND VLP preparation and the potent immune responses that they stimulated, ND VLPs have the potential to be utilized as a platform for the construction of vaccines for other viruses, including human viruses. This hypothesis was tested in several ways. First, the incorporation of peptide sequences into ND VLPs could expand their use as vaccines. For example, if a small domain of a protein has been identified as a domain that stimulates neutralizing antibody responses, the incorporation of this domain into the VLPs could stimulate these antibodies. The incorporation of T cell epitopes could enhance the ability of the VLPs to stimulate cell-mediated immune responses to specific pathogens. To test the feasibility of incorporation of foreign sequences into ND VLPs, the HA sequence tag was added to the amino terminus or the carboxyl terminus of the NDV NP protein or to the carboxyl terminus of the HN protein. All three approaches resulted in the successful incorporation of the sequence tag into VLPs, suggesting that these approaches could be utilized to expand the scope of immune responses to the ND VLPs. In contrast, a sequence tag fused to the carboxyl terminus of the F protein inhibited its incorporation into particles. This sequence had no effect on F protein fusion activity or surface expression. This result is consistent with an important role of the F protein CT domain in assembly and suggests that extra sequences at the end of this F protein domain can interfere with necessary interactions required for F protein assembly.

In an alternative approach to extending the use of ND VLPs as vaccines, we hypothesized that specific, efficient incorporation of foreign proteins into ND VLPs could be achieved by constructing chimeric protein genes composed of foreign proteins fused to the transmembrane (TM) domain and cytoplasmic (CT) domain of the appropriate NDV glycoprotein. The NDV glycoprotein TM and CT domains should specifically interact with the NDV M and NP proteins, resulting in efficient incorporation of the chimeric protein into VLPs. To test this hypothesis, we have reported that a chimeric protein resulting from the fusion of the respiratory syncytial virus G protein ectodomain with the HN protein CT and TM domains can be incorporated into ND VLPs (32). Furthermore, we demonstrated that these VLPs stimulated anti-RSV immune responses, responses that were protective upon RSV challenge (32). Here we have demonstrated that the sequence encoding the Nipah virus G protein ectodomain, fused to the CT and TM domains of the NDV HN protein, could also be incorporated into ND VLPs, demonstrating that RSV G protein is not a special case and that ectodomains of different type 2 glycoproteins could be incorporated into these VLPs using similar approaches. This same approach could also be used to assemble into VLPs the ectodomain of a type 1 glycoprotein by fusing the ectodomain to the TM and CT domains of the NDV F protein, a type 1 glycoprotein. Indeed, such an approach has been used to incorporate the influenza HA protein ectodomain into NDV virions (38). Using this approach, the respiratory syncytial virus F protein ectodomain has been assembled into ND VLPs (McGinnes, et al., unpublished). Thus, ND VLPs have potential for the development of vaccines for other paramyxoviruses and, indeed, for other human pathogens.

ACKNOWLEDGMENTS

This work was supported in part by grants AI30572 (T.M.) and U19AI057319 (awarded to UMass Center for Translational Research on Human Immunology and Biodefense) from the National Institutes of Health and by grants from the Massachusetts Technology Transfer Center and the Worcester Foundation.

We thank Paul Rota for cDNA and antibody specific to the NiV G protein and Mark Peeples for anti-M protein antibody.

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