

Requirements for the Assembly and Release of Newcastle Disease Virus-Like Particles[▽]

Homer D. Pantua,¹ Lori W. McGinnes,² Mark E. Peebles,³ and Trudy G. Morrison^{1,2*}

*Program in Immunology/Virology¹ and Department of Molecular Genetics and Microbiology,² University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, and
Columbus Children's Research Institute, Center for Vaccines and Immunity,
The Ohio State University, Columbus, Ohio 43205³*

Received 10 April 2006/Accepted 29 August 2006

Paramyxoviruses, such as Newcastle disease virus (NDV), assemble in and bud from plasma membranes of infected cells. To explore the role of each of the NDV structural proteins in virion assembly and release, virus-like particles (VLPs) released from avian cells expressing all possible combinations of the nucleoprotein (NP), membrane or matrix protein (M), an uncleaved fusion protein (F-K115Q), and hemagglutinin-neuraminidase (HN) protein were characterized for densities, protein content, and efficiencies of release. Coexpression of all four proteins resulted in the release of VLPs with densities and efficiencies of release (1.18 to 1.16 g/cm³ and 83.8% ± 1.1%, respectively) similar to those of authentic virions. Expression of M protein alone, but not NP, F-K115Q, or HN protein individually, resulted in efficient VLP release, and expression of all different combinations of proteins in the absence of M protein did not result in particle release. Expression of any combination of proteins that included M protein yielded VLPs, although with different densities and efficiencies of release. To address the roles of NP, F, and HN proteins in VLP assembly, the interactions of proteins in VLPs formed with different combinations of viral proteins were characterized by coimmunoprecipitation. The colocalization of M protein with cell surface F and HN proteins in cells expressing all combinations of viral proteins was characterized. Taken together, the results show that M protein is necessary and sufficient for NDV budding. Furthermore, they suggest that M-HN and M-NP interactions are responsible for incorporation of HN and NP proteins into VLPs and that F protein is incorporated indirectly due to interactions with NP and HN protein.

Paramyxoviruses, such as Newcastle disease virus (NDV), assemble progeny virions at infected cell plasma membranes and release these particles by budding from cell surfaces (20, 43). Paramyxovirus assembly requires the packaging of genomic RNA with the nucleoprotein (NP), as well as phosphoprotein and large polymerase (20), components of the polymerase complex. This ribonucleoprotein core is encased in a host-derived membrane modified by two transmembrane glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins, as well as the matrix or membrane (M) protein, which is associated with the inner surface of the viral membrane (20, 36). The paramyxovirus protein-protein interactions required for particle assembly and the viral and cellular proteins necessary for particle release are not well defined.

The matrix-like proteins of many enveloped RNA viruses play a pivotal role in virus assembly and release (9, 17, 19, 36, 37, 40, 49). These proteins are often sufficient for release of particles. For example, expression of the retroviral Gag precursor protein, in the absence of other viral components, results in the assembly and release of Gag virus-like particles (VLPs) (5, 6, 12, 31). Matrix proteins from Ebola virus (17, 18, 50), vesicular stomatitis virus (19, 22, 38), and influenza virus (14), when expressed alone, are released as VLPs. The human

parainfluenza virus type 1 and the Sendai virus (SV) M proteins expressed alone induced release of VLPs (4, 38, 47, 48). Expression of M protein was also required for simian virus 5 (SV5) VLP formation (42). However, in contrast to the case with PIV1 and SV, the SV5 M protein was not sufficient for VLP release.

Matrix proteins are also often necessary for particle release. For example, M protein-deficient rabies virus generated through reverse genetics was severely impaired in virion formation (29). Measles virus (3) and SV (16) modified by reverse genetics to lack the M protein genes were impaired in budding and release. Moreover, measles virus containing mutant M protein derived from subacute sclerosing panencephalitis virus was also defective in release of particles (34).

The essential role of these matrix proteins in release is due in part to short motifs, called late domains, in these proteins that interact with components of the host vacuolar protein sorting system (2, 17, 25, 31, 35, 40, 45, 49, 51). It is thought that these proteins hijack the host vacuolar protein sorting pathway for use in virus budding (10, 24, 31, 44). A late domain sequence in the SV5 M protein has been defined (41). However, this sequence cannot be sufficient for SV5 release, since the M protein of this virus, when expressed alone, does not direct particle release.

While paramyxovirus M proteins are clearly pivotal in the release of assembled virus, the interactions between M protein and other viral proteins required for the assembly of complete particles are less well defined. Indeed, available information,

* Corresponding author. Mailing address: Dept. of MGM, Rm. S5-250, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. Phone: (508) 856-6592. Fax: (508) 856-5920. E-mail: trudy.morrison@umassmed.edu.

[▽] Published ahead of print on 13 September 2006.

based on properties of VLPs formed after coexpression of different viral proteins with M protein or on colocalization or cofractionation of M protein with other viral proteins, have often led to contradictory conclusions. For example, some reports suggest that M protein binds to F protein, while others suggest a specific interaction with HN protein or both HN and F proteins (1, 13, 39). M protein is also proposed to interact with NP protein in some studies (46).

Thus, general rules for assembly and release of paramyxoviruses are not yet clear. Important questions include (i) the role of each viral protein in virus assembly, (ii) the full definition of paramyxovirus late domains in viral structural proteins, and (iii) the cellular factors involved in the budding process.

Using NDV as a prototype paramyxovirus, we sought to clarify the role of each paramyxovirus protein in assembly and release. We combined 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. We also characterized the colocalization of M protein with the viral glycoproteins in plasma membranes. Our results show that particle assembly involves a network of specific protein-protein interactions and likely correct targeting of proteins to specific cellular domains.

MATERIALS AND METHODS

Cells and viruses. A spontaneously transformed fibroblast cell line derived from the East Lansing strain (ELL-0) of chicken embryos (UMNSAH/DF-1) was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum. NDV, strains Australia-Victoria (AV) and B1, was propagated in embryonated chicken eggs by standard protocols (26). Strain AV was grown in BCL-3 containment.

Plasmids. NDV cDNA sequences encoding NP, M, HN, and uncleaved F (F-K115Q) proteins were subcloned into the expression vector pCAGGS (30, 32) to generate pCAGGS-NP, pCAGGS-M, pCAGGS-HN, and pCAGGS-F-K115Q, respectively. The F-protein cDNA contained a point mutation in the cleavage site sequence at residue 115 (K115Q) which eliminated the furin recognition site (23).

Transfection, infection, and metabolic labeling. Transfections of subconfluent ELL-0 cells were accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. The following amounts of plasmid DNA were used per 35-mm dish: 1.0 μ g pCAGGS-NP, 1.0 μ g pCAGGS-M, 0.75 μ g pCAGGS-F-K115Q, and 1.0 μ g pCAGGS-HN. These amounts were previously determined to yield levels of expression similar to those for cells infected with NDV at a multiplicity of infection of 5. A total of 3.75 μ g 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 μ l of Lipofectamine in OptiMEM medium (Gibco/Invitrogen) was incubated at room temperature for 45 min and then added to cells previously washed with OptiMEM. The cells were incubated for 5 h, the Lipofectamine-DNA complexes were removed, and 2 ml of supplemented DMEM was added. After 36 h, the medium was replaced with 0.7 ml DMEM without cysteine and methionine and supplemented with 100 μ Ci of a [35 S]methionine and [35 S]cysteine mixture (NEG-772 EASYTAG express protein labeling mix, 35 S; Perkin-Elmer Life Sciences, Inc.). After 4 h of pulse-labeling, 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 h of chase, the medium was collected and the cells were lysed in 0.5 ml lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) containing Triton-DOC (1% Triton X-100, 1% sodium deoxycholate) and 1.25 mg *N*-ethylmaleimide. Cells were harvested with a cell scraper and homogenized by passing through a 26-gauge needle 10 to 15 times.

ELL-0 cells were infected at a multiplicity of infection of 5 PFU for 5 h,

labeled with a [35 S]methionine and [35 S]cysteine mixture for 30 min, and chased in nonradioactive medium for 8 h as described above. The cell supernatant was harvested and virions purified as described below. Cells were lysed and homogenized as described above.

Virus and VLP purification. VLPs as well as virions were purified from cell supernatants in protocols previously developed for virus purification (21). The cell supernatants were clarified by centrifugation at 5,000 rpm for 5 min at 4°C, overlaid on top of a step gradient consisting of 3.5 ml 20% and 0.5 ml 65% sucrose solutions (g/ml) in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA), and centrifuged at 40,000 rpm for 12 h at 4°C using an SW50.1 rotor (Beckman). The interface (containing concentrated particles) was collected in a 0.5-ml volume, mixed with 2.0 ml of 80% sucrose, and overlaid on top of a 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. Particles derived from expression of all combinations of proteins were prepared in a single experiment, thus enabling direct comparison of results. The experiment was repeated three times.

Antibodies. Antiserum used to precipitate NP (anti-NDV) was rabbit polyclonal antibody raised against UV-inactivated NDV by standard protocols. Anti-NDV also contained antibodies specific for HN, F, and M proteins. Antiserum used to precipitate F protein were raised against glutathione *S*-transferase fusion proteins that contained the amino acid sequence 130 to 173 (anti-HR1) (27), 470 to 500 (anti-HR2) (7), or 96 to 117 (anti-F₂-96). Antiserum used to precipitate HN protein was raised against HN protein sequences from amino acid 96 to 117 (anti-A) (28). Antiserum used to precipitate M protein was a mouse monoclonal antibody raised against purified M protein (8).

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 with 1% Triton X-100. Samples were incubated with specific antibodies for 16 h at 4°C. Immune complexes were adsorbed to protein A (Pansorbin Cells; CALBIOCHEM) for 2 h at 4°C, pelleted, and then washed three times in immunoprecipitation wash buffer (phosphate-buffered saline containing 0.5% Tween 20 and 0.4% sodium dodecyl sulfate (SDS). Immune complexes were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.4% bromophenol blue) with 1 M β -mercaptoethanol and boiled. Proteins were separated on an 8% polyacrylamide-SDS gel and subjected to autoradiography. Quantification of resulting autoradiographs was accomplished using a Fluor-S MultiImager (Bio-Rad).

Coimmunoprecipitation. Purified VLPs were incubated in ice-cold TNE buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100–2.5 mg/ml *N*-ethylmaleimide for 15 min. 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 and then prewashed in TNE containing 1% Triton X-100 and 1 mg/ml bovine serum albumin, 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 s 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.

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, or 20 μ g of proteinase K per ml of sample and incubating for 30 min on ice. In parallel, VLPs were also brought to a final concentration of 0.5% with respect to Triton X-100 prior to incubation with proteinase K. After digestion, phenylmethylsulfonyl fluoride (0.1 M) was added. For subsequent immunoprecipitation, the reaction mixtures were brought to a final concentration of 1% with respect to Triton X-100 and 0.5% with respect to sodium deoxycholate.

Immunofluorescence microscopy. Avian cells, grown in a 35-mm dish containing glass coverslips, were transfected with different combinations of NDV cDNAs as described above. After 40 h, nuclei were stained with 5 μ g/ml 4',6'-diamidino-2-phenylindole for 30 min at 37°C. Cells were washed twice with ice-cold immunofluorescence (IF) buffer (phosphate-buffered saline containing 1% bovine serum albumin, 0.02% sodium azide, and 5 mM $CaCl_2$), fixed with 2% paraformaldehyde, blocked with IF buffer for 2 h, and incubated for 1 h 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 h, and incubated for 1 h at 4°C in IF buffer containing purified ascites fluids containing anti-M protein monoclonal antibody. Cells were then washed twice with ice-cold buffer, followed by incu-

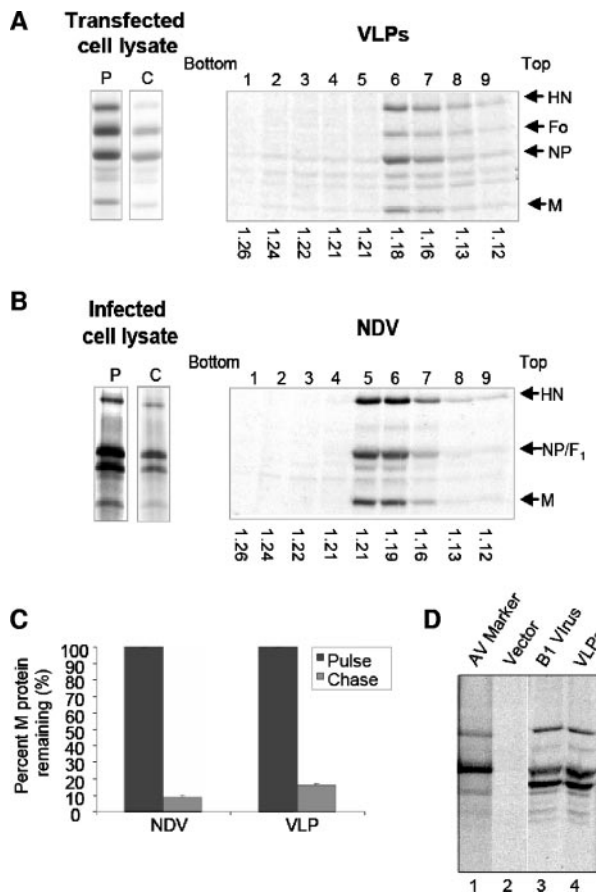


FIG. 1. Coexpression of NP, F, HN, and M proteins resulted in VLP formation and release. Panel A: avian cells, cotransfected with pCAGGS-NP, -M, -F-K155Q, and -HN, were radioactively labeled with [³⁵S]methionine and [³⁵S]cysteine for 4 h (P) and then chased in nonradioactive medium for 8 h (C). Panel B: avian cells, infected with NDV, strain AV, at a multiplicity of infection of 5 for 5 h, were pulse-labeled for 30 min and chased in nonradioactive medium for 8 h. Radioactively labeled proteins in the extracts 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 purified as described in Materials and Methods. After flotation into sucrose gradients (right side of each panel), each gradient fraction was mixed with TNE containing 1% Triton X-100 and immunoprecipitated with the antibody cocktail. The density of each fraction (g/cm³) is shown at the bottom. 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 extracts and is the result of averaging results from three separate experiments. The standard deviation is shown. Panel D shows nonimmunoprecipitated radiolabeled particles released from NDV, strain B1, virus-infected avian cells and cells expressing NP, M, F-K155Q, and HN proteins. Particles were isolated by concentrating onto the 20% and 65% sucrose interface and then floated into a step gradient. Particles in the gradient fractions were then pelleted and resuspended in TNE buffer. HN, hemagglutinin-neuraminidase protein; F₀, uncleaved fusion protein; NP, nucleocapsid protein; F₁, cleaved fusion protein; M, membrane or matrix protein.

bation for 1 h at 4°C in IF buffer containing fluorescein-conjugated goat anti-rabbit immunoglobulin G (Alexa 488) and rhodamine-conjugated goat anti-mouse immunoglobulin G (Alexa 568) (Molecular Probes) secondary antibodies. Cells were washed with ice-cold IF buffer and mounted onto slides using Vectashield mounting medium (Vector Labs, Inc.) for immunofluorescence mi-

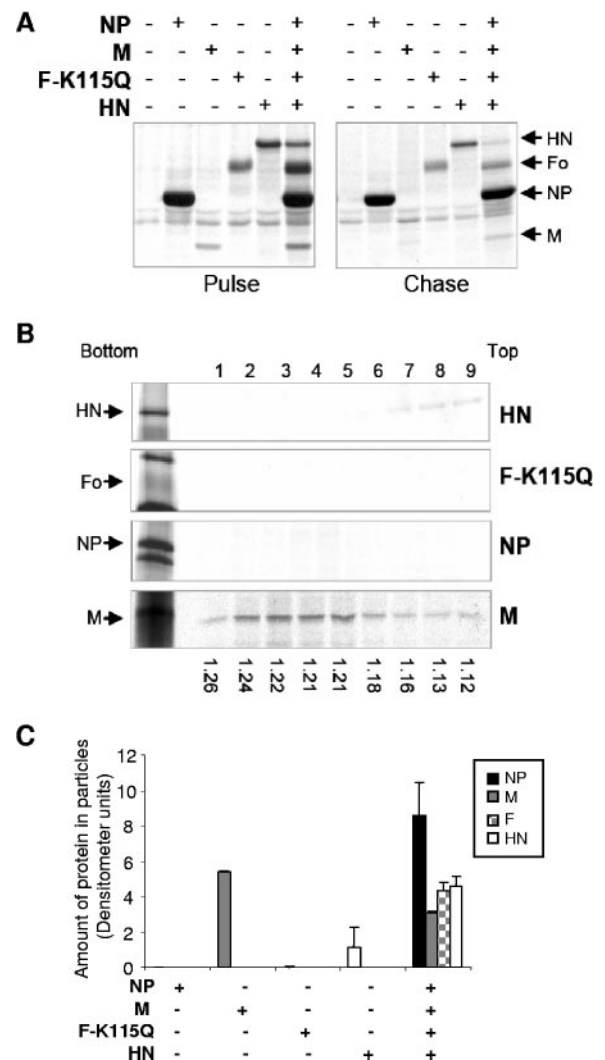


FIG. 2. M protein is sufficient for particle release. Avian cells were transfected with pCAGGS-NP, -M, -F-K115Q, and -HN individually. Panel A shows radioactively labeled proteins in the extracts at time of pulse (left) or chase (right). Particles in the supernatants of avian cells, expressing NP, M, F, and HN individually, were concentrated and floated into sucrose gradients as described in the legend to Fig. 1. Panel B shows the distribution in the gradients of radioactively labeled proteins derived from each supernatant. Densities of gradient fractions are shown at bottom (g/cm³). Quantification of the amounts of proteins in particles present in all fractions is shown in panel C. The results of three separate experiments were averaged, and the standard deviation is shown.

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

Electron microscopy. Particles were purified as described above except that after flotation they were concentrated by centrifugation into a pellet (40,000 rpm for 4 h at 4°C) and resuspended in TNE buffer. Virus and particles were adsorbed to Formvar carbon-coated nickel grids, treated with 2% glutaraldehyde, negatively stained with 4% uranyl acetate, and examined by transmission electron microscopy.

RESULTS

Cells expressing NDV NP, M, F-K115Q, and HN proteins released VLPs. To determine if VLPs could be released from

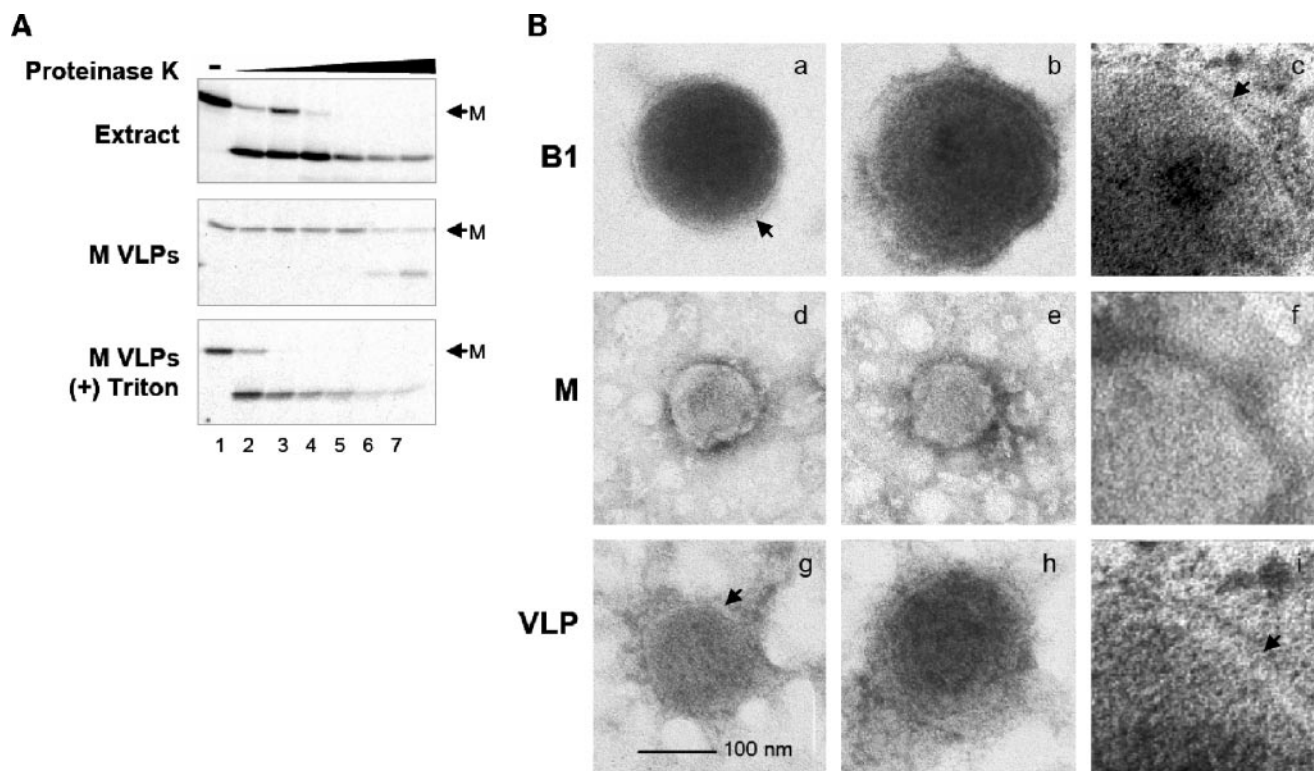


FIG. 3. M proteins are encased in membranous particles. Panel A: Avian cells were transfected with pCAGGS-M, and radioactively labeled VLPs were isolated and purified as described in Materials and Methods. Extract (upper panel) and VLPs, which were concentrated onto a 20% to 65% sucrose interface (middle panel), were treated with different concentrations (0.25, 0.5, 1, 5, 10, or 20 μ g/ml; lanes 2 to 7, respectively) of proteinase K for 30 min on ice. In parallel, particles 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 phenylmethylsulfonyl fluoride. M proteins were immunoprecipitated as described in Materials and Methods. Panel B: Electron microscopy of particles released from NDV-infected cells (B1), M protein-expressing cells (M), or cells expressing NP, M, F-K115Q, and HN proteins (VLP). Particles were purified as described in Materials and Methods. Middle and left panels show two representative particles from each preparation. Right panels show enlargements of the edges of typical particles.

cells coexpressing the major structural proteins of NDV, radioactively labeled particles released from a chicken fibroblast cell line coexpressing NP, M, F, and HN proteins were purified by sucrose density ultracentrifugation. We used an uncleaved version of F protein to eliminate any potential effects of cell-to-cell fusion on particle release. Cells were cotransfected with plasmids at concentrations of DNA we previously determined would result in expression levels and ratios of proteins comparable to those of infected cells (data not shown). Cells were pulse-labeled with [35 S]methionine and [35 S]cysteine and then chased for 8 h, a time which we determined in preliminary experiments to result in maximal particle release (data not shown).

Coexpression of NP, M, F, and HN proteins resulted in the release of VLPs with a density of 1.18 to 1.16 g/cm³ (Fig. 1A). Virus particles purified in parallel from NDV (strain AV)-infected cells had a density of 1.21 to 1.19 g/cm³ (Fig. 1B). The slightly lighter density of VLPs than of authentic virus was likely due to the absence of the virion RNA within the VLPs. The efficiencies of VLP and virus release were calculated as the percentages of M protein remaining in the cell extracts after the chase relative to the amount of protein in the pulse. By this criterion, the efficiency of VLP release was 83.8% $\pm$ 1.1%, while the efficiency of NDV release was 91% $\pm$ 1.4% (Fig. 1C).

It is assumed that M protein lost in the chase was incorporated into VLPs. However, it is possible that the decrease during the chase may also be due to some degradation, although we have not seen evidence for degradation of a mutant M protein that is retained within cells (unpublished data). These results demonstrate that NDV VLPs are efficiently assembled and released from avian cells expressing the four major structural proteins.

To determine the purity of released particles, we compared, without immunoprecipitation, the protein content of radio-labeled particles released from cells expressing NP, M, F-K115Q, and HN proteins with that of radiolabeled tissue culture-grown virus. The NDV B1 strain was used in this experiment for two reasons. First, this strain packages only an uncleaved F protein like the VLPs. Second, B1 infection results in less inhibition of host protein synthesis than infection with the more virulent NDV strain AV (unpublished observation). Thus, any host proteins that are incorporated into virus are more likely to be detected in B1 virus. Figure 1D shows that the profile of proteins present in purified VLPs is similar to that in virions (compare virus and VLP lanes). This result showed that particles released from cells expressing the four major structural proteins are as pure as virions released from

infected cells. The identity of the major band below NP for both VLPs and virions is unknown.

M protein was sufficient for release of membranous particles. To determine the minimum protein requirements for particle release, we asked if any of the NDV proteins was individually capable of directing particle release. Cells expressing each of the viral proteins individually were radioactively labeled in a pulse-chase protocol, and particles were isolated as described above. Figure 2B shows that particles were released only from cells expressing the M protein. Furthermore, almost no M protein could be detected in cell extracts after the 8-h chase (Fig. 2A, right panel), indicating that much of the pulse-labeled protein was released from cells. By comparing the levels of M protein in the pulse-labeled extract and the chase extract, the efficiency of release was calculated to be $90\% \pm 3.0\%$. In contrast, most of the pulse-labeled NP, F, and HN proteins remained in extracts after the chase (Fig. 2A). This result correlated with the lack of significant amounts of particles detected in the corresponding cell supernatant (Fig. 2B), although there was a trace of very light-density material released from HN protein-expressing cells.

Figure 2C 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 particles had a very heterogeneous density, with values ranging from 1.24 to 1.12 g/cm³ (Fig. 2B). These results show that M protein is sufficient for the release of particles.

The observation that M protein, released from cells, floated into a sucrose gradient indicates that the protein was associated with the membrane. To confirm that the M protein-containing particles were membrane-bound particles, these particles were incubated with protease. In this assay, VLPs and cell extracts were either left untreated (Fig. 3A, 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 (Fig. 3A, extract 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 particles were largely protected from protease digestion (Fig. 3A, VLP panel). In contrast, disruption of the particle membrane with detergent resulted in digestion of the M protein (Fig. 3A, VLP with Triton X-100 panel). Taken together, these results demonstrated that the M protein particles are membrane-bound particles.

Particles visualized by electron microscopy. Visualization of particles released from cells expressing M protein only (Fig. 3B, M panel), as well as from cells expressing all four proteins (panel B, VLP panel) or purified tissue culture virions (panel B, B1 panel) was accomplished using electron microscopy. Particles formed with all four proteins looked very similar to authentic virus. Both B1 virions and VLPs released (B1 and VLP panels) from cells expressing all four proteins have membrane structures that were suggestive of spike glycoproteins in the envelope (see arrows). M protein particles were more variable in size and had no evidence of glycoprotein spike structures.

M protein was required for VLP release. Since the M protein is sufficient for particle release, we next asked whether the

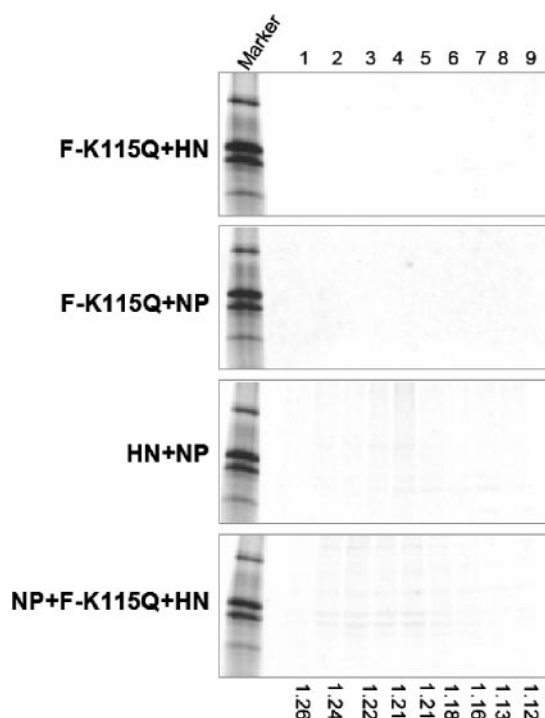


FIG. 4. M protein is required for particle release. Avian cells were transfected with all possible combinations of cDNAs in the pCAGGS vector encoding NP, F, and HN proteins in the absence of M cDNA (F-K115Q+HN, F-K115Q+NP, HN+NP, NP+F-K115Q+HN). Particles in cell supernatants were purified as described in the legend to Fig. 1. 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/cm³). The control for this experiment is shown in Fig. 1, panel A.

M protein is required for the release. To answer this question, 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 particles (Fig. 4). Furthermore, in the absence of M protein, NP, F, and HN proteins expressed in pairwise combinations were retained in cell extracts after the 8-h chase (Fig. 5A, lanes 2, 4, and 5). These results strongly suggest that M protein is required for particle release.

Minimal amounts of glycoprotein or NP were incorporated into VLPs after pairwise expression with M protein. To determine the contribution of NP, F, or HN protein to M protein-driven particle release, particles released from cells expressing all possible combinations of two proteins were isolated and characterized as described above. Pairwise expression of NP, F, or HN protein with M protein resulted in the release of particles containing both proteins (Fig. 5B). Intriguingly, however, particles contained only trace amounts of NP, F, or HN protein, while M protein was the predominant protein (Fig. 5B). The distribution of NP, F, or HN protein in the gradients was identical to that of M protein (Fig. 5B). In addition, the particle densities were very heterogeneous and were much like that of M protein-only particles. Surprisingly, the amounts of M protein-containing particles were decreased upon coexpres-

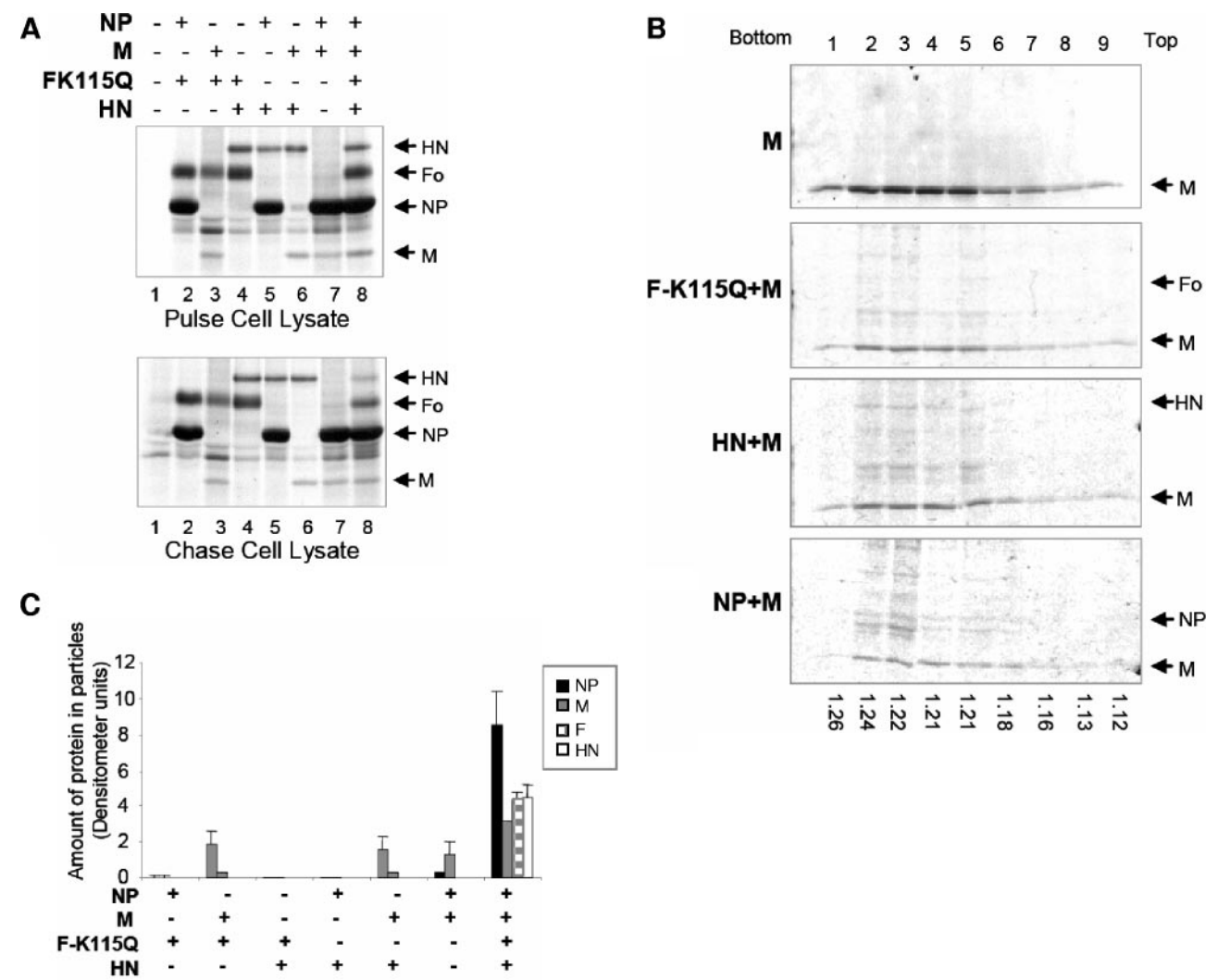


FIG. 5. Effect of expressing NP, F, or HN protein with M protein on particle release. Avian cells, transfected with cDNAs encoding F-K115Q plus M, HN plus M, or NP plus M structural protein genes, as well as all four cDNAs, were labeled by using a pulse-chase protocol as described in the legend to Fig. 1. Particles present in the supernatants were concentrated and then floated into sucrose gradients as described in Materials and Methods. Panel A shows labeled proteins in cell extracts at end of pulse (top) or chase (bottom). Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail. Densities (g/cm³) of the fractions are shown at the bottom. Panel C shows the quantification of each protein in particles in all fractions. Autoradiographs of gradient fractions from F-K115Q plus NP-, F-K115Q plus HN-, or HN plus NP-expressing cells did not contain any protein and are shown with gels in Fig. 4. Results are the averages for three experiments, and the standard deviation is shown.

sion of M protein with particularly NP but also with F or HN protein (Fig. 5C).

Efficient incorporation of other viral proteins into VLPs required the expression of M protein and at least two of the other proteins. To examine the effects of coexpression of three viral proteins on particle release, cells were transfected with all possible combinations of three cDNAs (Fig. 6A). In contrast to the expression of a single glycoprotein with the M protein, coexpression of both F and HN glycoproteins with M protein resulted in significantly increased incorporation of both glycoproteins into particles (Fig. 6B and C). The F and HN proteins were detected in the same gradient fractions as M protein. Furthermore, the densities of the particles were more homogeneous than those generated from cells expressing M protein alone (compare Fig. 6B and Fig. 2B) 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 and the glycoprotein into particles (Fig. 6B and C). The distribution of NP protein-containing particles in the gradient was similar to that of particles released from cells expressing all four structural proteins (Fig. 6B). Importantly, the densities of these particles were more homogeneous than those of particles released from cells expressing M protein alone and were analogous to the density of the authentic virus or complete VLPs (compare Fig. 6B and Fig. 1B). Overall, these results indicate that M protein is necessary and sufficient for particle release and that expression of M protein

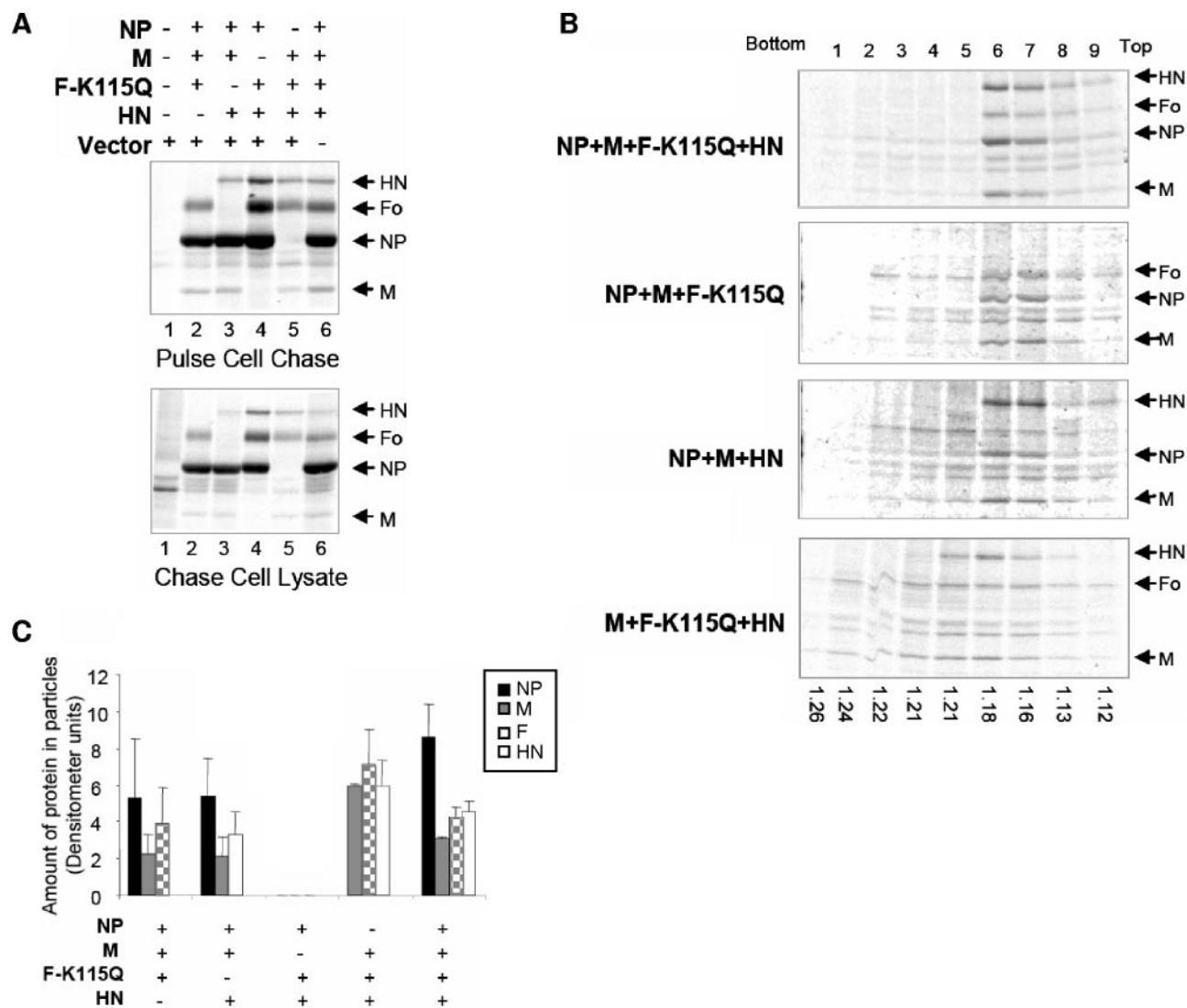


FIG. 6. Effect of expressing all combinations of three viral proteins on particle release. Avian cells, transfected with all possible combinations of three NDV structural protein genes including M cDNA, were labeled in a pulse-chase protocol, and particles in the supernatant were concentrated and floated into a sucrose gradient as for Fig. 1. The proteins in the cell extracts were immunoprecipitated with the antibody cocktail. Panel A shows labeled proteins in cell extracts at end of pulse (top) or chase (bottom). Panel B shows the proteins present in each gradient fraction after immunoprecipitation of each fraction with an antibody cocktail. Densities (g/cm³) of the fractions are shown at the bottom. Panel C shows quantification of the amounts of each protein in particles in all fractions. Autoradiograph of particles released from NP-, F-, and HN-protein-expressing cells is shown in Fig. 4.

with at least two other proteins is required for efficient incorporation of other proteins into particles.

M protein colocalized with viral glycoproteins. To explore further the role played by each protein in VLP assembly, we characterized, by immunofluorescence, the plasma membrane localization of M, F, and HN proteins after their expression individually or after expression of combinations of NP, M, F, and HN proteins. 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. Figure 7A shows vector-transfected control cells and cells expressing individually M, F-K115Q, or HN protein. The

F-K115Q and HN proteins were diffusely distributed on the surfaces of the cells (F-K115Q and HN panels). M protein exhibited diffuse cytoplasmic staining as well as punctate structures of various sizes (Fig. 7A, anti-M and merged images). Coexpression of either F or HN proteins with M protein (panel B) had little effect on the distribution of M protein, F protein, or HN protein (Fig. 7B, anti-M, anti-F, and anti-HN images), and little to no colocalization of F or HN glycoproteins with M protein was observed (Fig. 7B, merged image). This finding correlates with the very low level of incorporation of F or HN proteins into M protein-containing particles after pairwise co-expression.

Coexpression of M protein with at least two other proteins slightly changed the distribution of M protein (Fig. 7C, anti-M

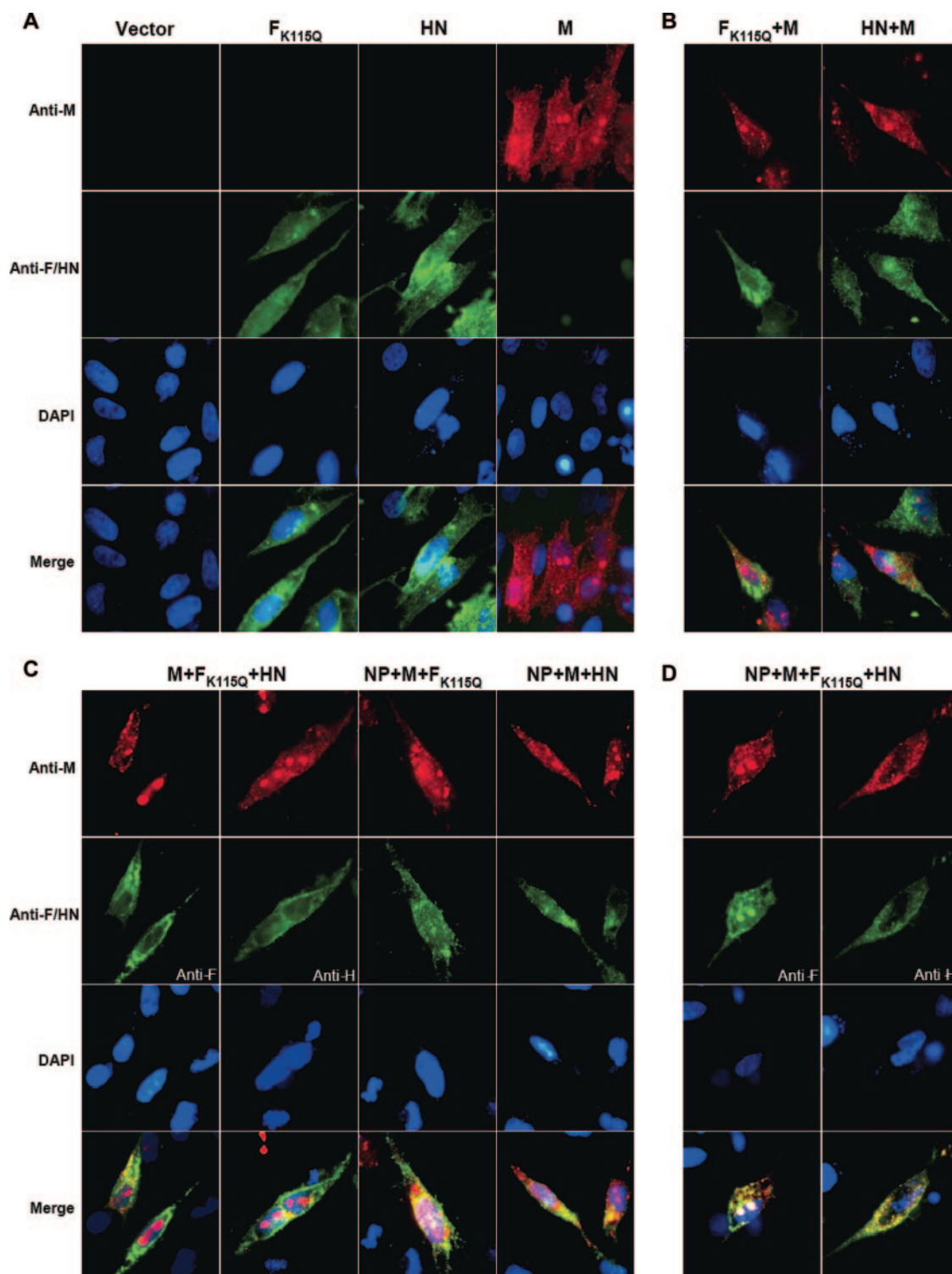


FIG. 7. Colocalization of M protein with F and HN proteins. The cell surface localization of NDV F and HN proteins and the cellular localization of M proteins were analyzed by immunofluorescence microscopy. Avian cells were transfected either individually (A) or with F-K115Q plus M or HN plus M (B), with NP plus M plus F-K115Q, with NP plus M plus HN, with M plus F-K115Q plus HN (C), or with all 4 cDNAs (D). Nuclei were stained with 4',6'-diamidino-2-phenylindole (blue) 40 h posttransfection. 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 taken at magnification $\times 60$ and were merged using Adobe Photoshop. Results shown are representative of all cells in the slide.

images) and F and HN proteins (Fig. 7C, anti-F and anti-HN images) and increased the colocalization of M protein with either F or HN protein (Fig. 7C, merged images). This result is consistent with increased incorporation of HN, F, or NP protein when two proteins were coexpressed with M protein.

When all four proteins were coexpressed, the distribution of M protein was changed to more punctuate structures distributed mostly along the edges of the cells (Fig. 7D, anti-M images). Importantly, most of the F or HN protein signal colocalized with the M protein (Fig. 7D, merged images). This result is consistent with the more ordered assembly of VLPs when all four proteins are coexpressed.

Altogether, these results suggest that colocalization of viral proteins is detected with expression of three proteins and is most dramatic when NP, M, F, and HN proteins are coexpressed. These results also suggest that there are specific protein-protein interactions involved in assembling particles.

There are specific viral protein interactions in VLPs. To identify the specific protein interactions involved in VLP assembly, radioactively labeled particles formed with different combinations of proteins were solubilized in 1% Triton X-100, and proteins present were precipitated, separately, with cocktails of monospecific antibodies for M, HN, or F protein. Proteins were also precipitated with a mix of antibodies with specificities for all proteins in order to precipitate total 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 (Fig. 8A). 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 (lane 3) but M protein very inefficiently (lane 3). This result suggests that there may be a direct link between F protein and NP but not F protein and M protein.

The interactions in VLPs were more clearly defined by precipitation of proteins from particles formed with all combinations of three proteins. When particles released from cells expressing the M, F-K115Q, and HN proteins were used in a similar coimmunoprecipitation procedure (Fig. 8B), anti-F protein precipitated only F protein and traces of HN protein (Fig. 8B, lane 3). This result indicates that the F protein does not directly complex with the M protein. Immunoprecipitation with anti-HN protein antibody precipitated M protein with HN protein (Fig. 8B, lane 4), and immunoprecipitation with anti-M protein antibody brought down HN protein with M protein (Fig. 8B, lane 5). These results strongly suggest that the M protein interacts with HN protein but not with the F protein.

When particles containing NP, M, and F-K115Q were used in a similar immunoprecipitation protocol, complexes formed with anti-F protein antibody contained NP as well as F protein but not M protein (Fig. 8C, lane 3). Complexes formed with anti-M protein antibody contained NP as well as M protein but no F protein (Fig. 8C, lane 4). These observations indicate that M protein directly interacts with NP and that the F protein interacts with NP and confirm the lack of F and M protein interactions. Anti-M protein antibody does not indirectly pre-

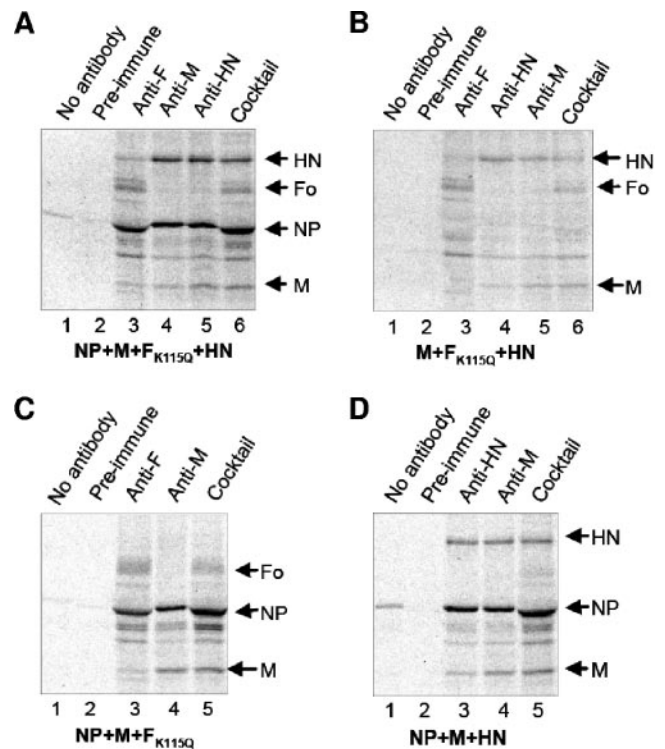


FIG. 8. Coimmunoprecipitation of viral proteins in particles. Radioactively labeled particles released from cells expressing NP plus M plus F-K115Q plus HN (A), M plus F-K115Q plus HN (B), NP plus M plus F-K115Q (C), or NP plus M plus HN (D) were purified into a 20% to 65% sucrose interface. Particles were solubilized in TNE buffer containing 1% Triton X-100 at 4°C for 15 min. Solubilized particles 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 a cocktail of NDV-specific antibodies overnight at 4°C. No antibody as well as preimmune 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 coimmunoprecipitation were accomplished at 4°C. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Materials and Methods. Results show one of three independent experiments, all with identical results.

cipitate detectable amounts of F protein. This result may be due to inefficient precipitation of NP protein, decreasing the amounts of F protein precipitated to very low levels. Alternatively, the NP-NP interactions required to precipitate F protein with anti-M protein antibody may be disrupted by particle lysis.

When particles containing NP, M, and HN were used, complexes formed with anti-HN protein antibody contained NP and M proteins as well as HN protein (Fig. 8D, lane 3). In addition, anti-M protein antibody precipitated NP and HN proteins (Fig. 8D, lane 4). These observations are consistent with the conclusion that the M protein interacts with both NP and HN proteins. These results cannot rule out an interaction between HN protein and NP.

Overall, results of coimmunoprecipitation of proteins in particles as well as results of cellular colocalization 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.

DISCUSSION

Paramyxoviruses spread through cell monolayers or through an organ by two mechanisms, successive rounds of virus infection and cell-cell fusion. Thus, infected cell surfaces produce virus particles as well as fusing with adjacent cells. In order to begin to understand the relationships between these two processes in NDV-infected cells, the protein requirements for assembly and release of virus-like particles were characterized. Avian cells, expressing the viral NP, M, HN, and F proteins, released VLPs nearly as efficiently as virus. These released particles had protein ratios similar to those of infectious virus, and their densities were homogeneous and only slightly less than that of authentic virus. The efficiencies of release of VLPs produced in three other paramyxovirus systems were 10% (SV), 34% (SV5), and 70% (SV) (42, 47, 48). All previously described paramyxovirus systems have utilized human 293T cells for expression of viral proteins. We have found that NDV VLP release from avian cells, the natural host cell for NDV, had an efficiency of nearly 84%, but the efficiency of NDV VLP release from 293T or COS-7 cells was approximately 50% (unpublished observations). Therefore, differences between the efficiencies of VLP formation in the NDV system reported here and the efficiencies reported for other systems may be due to a cell type-dependent effect. Thus, NDV VLP assembly in avian cells represents an ideal system for exploring protein requirements for assembly and release of virus particles.

Using avian cells, we found that the NDV M protein, and only M protein, was sufficient for particle release. Expression of M protein alone resulted in release of M protein-containing particles with an efficiency comparable to that observed when all four proteins were expressed, suggesting that no other protein is required for efficient release. Particles released from cells expressing NDV M protein alone, however, were very heterogeneous with respect to density. While the reasons for this finding are unclear, it is possible that budding of M protein particles occurred indiscriminately from different cell membranes with differing densities. Alternatively, it is possible that particles contained different lipid-to-protein ratios due to variable oligomerization of the M protein. M proteins of other negative-stranded RNA viruses are reported to form oligomeric structures (11, 15, 33). Particles formed from monomer M protein may have a higher lipid-to-protein ratio than particles formed from M protein in an oligomeric state. While M proteins of SV and human parainfluenza virus type 1 were also shown to be sufficient for particle release (4, 47, 48), the SV5 M protein was not sufficient (42).

NDV M protein was also necessary for particle release. In the absence of M protein expression, no other viral protein or combination of proteins resulted in significant particle release. By contrast, previous studies by two different groups reported that SV F protein exhibited an autonomous exocytosis activity demonstrated by the release of vesicles containing only the F protein (47, 48), although the level of release was very low. We found that cells expressing the NDV F protein alone did not release F protein-containing material, results similar to those reported by Schmitt et al. for the SV-5 system (42). We did

observe a trace amount of very light-density material that contained HN protein when this protein was expressed alone, but no HN protein was released when it was coexpressed with combinations of NP and F-K115Q.

Although all studies agreed upon the central role played by the M protein in virus release, specific interactions of other viral proteins with M protein required for the assembly of complete VLPs are still poorly understood. To define these interactions required for NDV assembly, we used three approaches. First, we determined the requirements for efficient incorporation of NP, F, and HN proteins into particles by expressing all combinations of these proteins with M protein. Second, the protein interactions in particles formed with all combinations of three and four proteins were defined by co-immunoprecipitation. Last, the colocalization of cell surface HN and F proteins with M protein when expressed in different combinations with M and NP proteins were characterized.

Pairwise expression of NP, HN, or F protein with M protein resulted in only trace amounts of NP, HN, or F protein incorporated into M-containing particles. In addition, expression of NP, F, or HN protein with M protein did not change the heterogeneous density of M protein-containing particles. In contrast, coexpression of M protein with two other proteins significantly increased the incorporation of NP, HN, or F protein into particles. The released particles had more-homogeneous density, similar to that of particles containing all four proteins, a result that suggested that necessary and specific interactions between the three proteins resulted in both efficient incorporation of NP or glycoproteins and more-ordered particles. Furthermore, coexpression of two proteins with M protein also significantly increased the colocalization of M protein with either HN or F protein in the plasma membrane, indicating increased interactions with M protein.

To define these protein-protein interactions, particles 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 protein. 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. 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.

The protein-protein interactions were more clearly defined by immunoprecipitation of proteins from particles 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 (diagramed in Fig. 9). There is no evidence for a direct interaction between F protein and M protein. There is likely a weak interaction between F and HN proteins, since anti-F protein antibodies precipitated HN protein from particles containing M, HN, and F proteins. In addition, since there is no interaction between F and M proteins, incorporation of F protein into these particles must be accomplished by interactions with HN protein. Our results cannot rule out an interaction between HN protein and NP.

Thus, when all four proteins are coexpressed, NP and HN

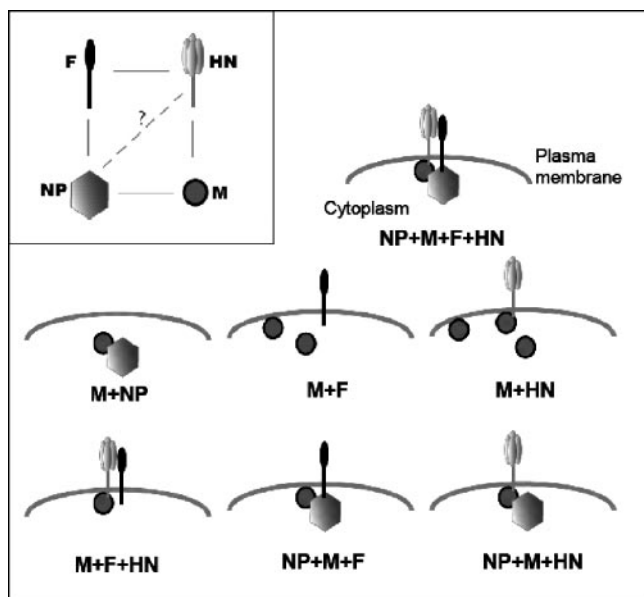


FIG. 9. Protein-protein interactions in particles. Inset shows viral protein-protein interactions detected by coimmunoprecipitation of proteins in particles. Also shown are interactions proposed to result in assembly of particles formed by coexpression of all combinations of NP, F, and HN proteins with M protein.

protein are incorporated into VLPs by a direct interaction with M protein (Fig. 9). F protein is likely incorporated indirectly due to interactions with NP and HN protein. An ordered complex of the four proteins is supported by the dramatic colocalization of M protein with F protein and M protein with HN protein in the plasma membrane when all four proteins are coexpressed.

However, when only F is expressed with M protein, F protein is likely not significantly incorporated into particles because there is no direct interaction between the two proteins (Fig. 9). Supporting this conclusion is the observation that there was no colocalization 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 particles when NP and M proteins were coexpressed in the pairwise combination. Previous reports that show that the M protein of Sendai virus is recruited in the cytoplasm by the viral nucleocapsid (46). Perhaps NP causes the retargeting of M protein to this compartment. Indeed, coexpression of M protein with NP resulted in a 2.5-fold suppression of M protein-containing particle release, a result also consistent with retention of M protein in cells by NP protein.

Although precipitations of particles 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 particles when HN and M proteins were coexpressed in a pairwise combination. Furthermore, there was little colocalization of the two proteins in the plasma membrane. Perhaps in the absence of other proteins, HN and M proteins are never localized in the same regions of the cell, preventing their association. 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 reason for the 50% reduction of M protein particles upon coexpression of HN protein with M protein is unclear but has been previously reported for the Sendai virus system (47).

Particles formed with NP, M, and F proteins are likely due to interactions between M and NP and interactions between F and NP (Fig. 9). F protein may relocate NP to the plasma membrane, drawing M to specific domains containing F protein. Indeed, addition of NP increases the colocalization of M protein with F protein in the plasma membrane. Particles formed with NP, M, and HN proteins likely form due to interactions of both HN protein and NP with M protein (Fig. 9). Expression of NP with HN and M proteins certainly increases the colocalization of M and HN proteins in the plasma membrane. Perhaps NP-M protein interactions alter the conformation of M, facilitating its interaction with HN protein. Indeed, surface HN protein in the presence of NP appears more punctuate along the cell edges.

The network of interactions proposed here could account for the conclusions of Schmitt et al. that the cytoplasmic domains (CT) of the HN and F proteins have redundant functions (42). 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 proposed interaction of M protein and NP is supported by studies of Stricker et al. of Sendai virus (46). Interaction of HN protein with M 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 (1, 40). Indeed, it has been reported that the respiratory syncytial virus G protein specifically interacts with M protein (13). 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 here are consistent with the proposal that the NDV M protein buds indiscriminately from different cellular membranes in the absence of other viral proteins. When both glycoproteins are present in the plasma membrane, the M protein association with the plasma membrane may stabilize. NP association with F and M protein may also further stabilize and organize the network of interactions within the assembling particle.

In summary, we have established a VLP production system for NDV. We also showed that the M protein is sufficient and is required for NDV particle budding. Moreover, there are specific protein-protein interactions in VLPs involved in the ordered assembly of particles. Interactions identified between M and HN or F and NP may play a role in targeting M and NP into assembly sites in the plasma membrane.

ACKNOWLEDGMENTS

We thank Julie Reitter, Jason Laliberte, Surbhi Jain, and Maria Genevieve Hernandez for helpful discussions and critical reviews of the manuscript.

This work was supported by a grant from the National Institutes of Health, AI 30572, and by the Massachusetts Technology Transfer Center.

REFERENCES

- Ali, A., and D. P. Nayak. 2000. 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.
- Bieniasz, P. D. 2006. Late budding domains and host proteins in enveloped virus release. *Virology* **344**:55–63.
- Cathomen, T., B. Mrkic, D. Spehner, R. Drillien, R. Naef, J. Pavlovic, A. Aguzzi, M. A. Billeter, and R. Cattaneo. 1998. A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. *EMBO J.* **17**:3899–3908.
- Coronel, E. C., K. G. Murti, T. Takimoto, and A. Portner. 1999. Human parainfluenza virus type 1 matrix and nucleoprotein genes transiently expressed in mammalian cells induce the release of virus-like particles containing nucleocapsid-like structures. *J. Virol.* **73**:7035–7038.
- Delchambre, M., D. Gheysen, D. Thines, C. Thiriart, E. Jacobs, E. Verdin, M. Horth, A. Burny, and B. Fex. 1989. The GAG precursor of simian immunodeficiency virus assembles into virus-like particles. *EMBO J.* **8**:2653–2660.
- Demirov, D. G., and E. O. Freed. 2004. Retrovirus budding. *Virus Res.* **106**:87–102.
- Dolganiuc, V., L. McGinnes, E. J. Luna, and T. G. Morrison. 2003. Role of the cytoplasmic domain of the Newcastle disease virus fusion protein in association with lipid rafts. *J. Virol.* **77**:12968–12979.
- Faeborg, K., and M. Peeples. 1988. Strain variation and nuclear association of NDV Matrix protein. *J. Virol.* **62**:586–593.
- Freed, E. O. 2003. The HIV-TSG101 interface: recent advances in a budding field. *Trends Microbiol.* **11**:56–59.
- Freed, E. O. 2002. Viral late domains. *J. Virol.* **76**:4679–4687.
- Garoff, H., R. Hewson, and D. J. Opstelten. 1998. Virus maturation by budding. *Microbiol. Mol. Biol. Rev.* **62**:1171–1190.
- Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde. 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**:103–112.
- Ghildyal, R., D. Li, I. Peroulis, B. Shields, P. G. Bardin, M. N. Teng, P. L. Collins, J. Meanger, and J. Mills. 2005. Interaction between the respiratory syncytial virus G glycoprotein cytoplasmic domain and the matrix protein. *J. Gen. Virol.* **86**:1879–1884.
- Gomez-Puertas, P., C. Albo, E. Perez-Pastrana, A. Vivo, and A. Portela. 2000. Influenza virus matrix protein is the major driving force in virus budding. *J. Virol.* **74**:11538–11547.
- Hewitt, J. A., and M. V. Nermut. 1977. A morphological study of the M protein of Sendai virus. *J. Gen. Virol.* **34**:127–136.
- Inoue, M., Y. Tokusumi, H. Ban, T. Kanaya, M. Shirakura, T. Tokusumi, T. Hirata, Y. Nagai, A. Iida, and M. Hasegawa. 2003. 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**:6419–6429.
- Jasenosky, L. D., and Y. Kawaoka. 2004. Filovirus budding. *Virus Res.* **106**:181–188.
- Jasenosky, L. D., G. Neumann, I. Lukashevich, and Y. Kawaoka. 2001. Ebola virus VP40-induced particle formation and association with the lipid bilayer. *J. Virol.* **75**:5205–5214.
- Jayakar, H. R., E. Jeetendra, and M. A. Whitt. 2004. Rhabdovirus assembly and budding. *Virus Res.* **106**:117–132.
- Lamb, R. A., and D. Kolakofsky. 2001. Paramyxoviridae: the viruses and their replication, 3rd ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Levinson, W., and H. Rubin. 1966. Radiation studies of avian tumor viruses and Newcastle disease virus. *Virology* **28**:533–542.
- Li, Y., L. Luo, M. Schubert, R. R. Wagner, and C. Y. Kang. 1993. Viral liposomes released from insect cells infected with recombinant baculovirus expressing the matrix protein of vesicular stomatitis virus. *J. Virol.* **67**:4415–4420.
- Li, Z., T. Sergel, E. Razvi, and T. Morrison. 1998. Effect of cleavage mutants on syncytium formation directed by the wild-type fusion protein of Newcastle disease virus. *J. Virol.* **72**:3789–3795.
- Martindale, D. 2003. Budding viral hijackers co-opt the endocytic machinery to make a getaway. *J. Biol.* **3**:2.
- Martin-Serrano, J., T. Zang, and P. D. Bieniasz. 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat. Med.* **7**:1313–1319.
- McGinnes, L., J. N. Reitter, H. Pantua, and T. Morrison. 2006. Newcastle disease virus: propagation, quantification and storage. In R. Coico, T. Kowalik, J. Quarles, B. Stevenson, and R. Taylor (ed.), *Current protocols in microbiology*, vol. 1. John Wiley & Sons, Inc., Hoboken, N.J.
- McGinnes, L. W., K. Gravel, and T. G. Morrison. 2002. Newcastle disease virus HN protein alters the conformation of the F protein at cell surfaces. *J. Virol.* **76**:12622–12633.
- McGinnes, L. W., and T. G. Morrison. 1998. 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.
- Mebatsion, T., F. Weiland, and K. K. Conzelmann. 1999. 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**:242–250.
- Miyazaki, J., S. Takaki, K. Araki, F. Tashiro, A. Tominaga, K. Takatsu, and K. Yamamura. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* **79**:269–277.
- Morita, E., and W. I. Sundquist. 2004. Retrovirus budding. *Annu. Rev. Cell Dev. Biol.* **20**:395–425.
- Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193–199.
- Panchal, R. G., G. Ruthel, T. A. Kenny, G. H. Kallstrom, D. Lane, S. S. Badie, L. Li, S. Bavari, and M. J. Aman. 2003. In vivo oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding. *Proc. Natl. Acad. Sci. USA* **100**:15936–15941.
- Patterson, J. B., T. I. Cornu, J. Redwine, S. Dales, H. Lewicki, A. Holz, D. Thomas, M. A. Billeter, and M. B. Oldstone. 2001. Evidence that the hypermutated M protein of a subacute sclerosing panencephalitis measles virus actively contributes to the chronic progressive CNS disease. *Virology* **291**:215–225.
- Patton, G. S., S. A. Morris, W. Chung, P. D. Bieniasz, and M. O. McClure. 2005. Identification of domains in Gag important for prototypic foamy virus egress. *J. Virol.* **79**:6392–6399.
- Peeples, M. E. 1991. Paramyxovirus M proteins: pulling it all together and taking it on the road, p. 427–456. In D. W. Kingsbury (ed.), *The paramyxoviruses*. Plenum, New York, N.Y.
- Pornillos, O., J. E. Garrus, and W. I. Sundquist. 2002. Mechanisms of enveloped RNA virus budding. *Trends Cell Biol.* **12**:569–579.
- Sakaguchi, T., T. Uchiyama, Y. Fujii, K. Kiyotani, A. Kato, Y. Nagai, A. Kawai, and T. Yoshida. 1999. Double-layered membrane vesicles released from mammalian cells infected with Sendai virus expressing the matrix protein of vesicular stomatitis virus. *Virology* **263**:230–243.
- Sanderson, C. M., H. H. Wu, and D. P. Nayak. 1994. Sendai virus M protein binds independently to either the F or the HN glycoprotein in vivo. *J. Virol.* **68**:69–76.
- Schmitt, A. P., and R. A. Lamb. 2004. Escaping from the cell: assembly and budding of negative-strand RNA viruses. *Curr. Top. Microbiol. Immunol.* **283**:145–196.
- Schmitt, A. P., G. P. Leser, E. Morita, W. I. Sundquist, and R. A. Lamb. 2005. Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus. *J. Virol.* **79**:2988–2997.
- Schmitt, A. P., G. P. Leser, D. L. Waning, and R. A. Lamb. 2002. Requirements for budding of paramyxovirus simian virus 5 virus-like particles. *J. Virol.* **76**:3952–3964.
- Simons, K., and H. Garoff. 1980. The budding mechanisms of enveloped animal viruses. *J. Gen. Virol.* **50**:1–21.
- Strack, B., A. Calistri, S. Craig, E. Popova, and H. G. Gottlinger. 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAP p9 functioning in virus budding. *Cell* **114**:689–699.
- Strack, B., A. Calistri, and H. G. Gottlinger. 2002. Late assembly domain function can exhibit context dependence and involves ubiquitin residues implicated in endocytosis. *J. Virol.* **76**:5472–5479.
- Stricker, R., G. Mottet, and L. Roux. 1994. 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**:1031–1042.
- Sugahara, F., T. Uchiyama, H. Watanabe, Y. Shimazu, M. Kuwayama, Y. Fujii, K. Kiyotani, A. Adachi, N. Kohno, T. Yoshida, and T. Sakaguchi. 2004. Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein. *Virology* **325**:1–10.
- Takimoto, T., K. G. Murti, T. Bousse, R. A. Scroggs, and A. Portner. 2001. Role of matrix and fusion proteins in budding of Sendai virus. *J. Virol.* **75**:11384–11391.
- Takimoto, T., and A. Portner. 2004. Molecular mechanism of paramyxovirus budding. *Virus Res.* **106**:133–145.
- Timmins, J., S. Scianimanico, G. Schoehn, and W. Weissenhorn. 2001. Vesicular release of Ebola virus matrix protein VP40. *Virology* **283**:1–6.
- von Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbara, A. Scott, H. G. Krausslich, J. Kaplan, S. G. Morham, and W. I. Sundquist. 2003. The protein network of HIV budding. *Cell* **114**:701–713.