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Influenza Virus Hemagglutinin and Neuraminidase, but Not the Matrix Protein, Are Required for Assembly and Budding of Plasmid-Derived Virus-Like Particles▽

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For influenza virus, we developed an efficient, noncytotoxic, plasmid-based virus-like particle (VLP) system to reflect authentic virus particles. This system was characterized biochemically by analysis of VLP protein composition, morphologically by electron microscopy, and functionally with a VLP infectivity assay. The VLP system was used to address the identity of the minimal set of viral proteins required for budding. Combinations of viral proteins were expressed in cells, and the polypeptide composition of the particles released into the culture media was analyzed. Contrary to previous findings in which matrix (M1) protein was considered to be the driving force of budding because M1 was found to be released copiously into the culture medium when M1 was expressed by using the vaccinia virus T7 RNA polymerase-driven overexpression system, in our noncytotoxic VLP system M1 was not released efficiently into the culture medium. Additionally, hemagglutinin (HA), when treated with exogenous neuraminidase (NA) or coexpressed with viral NA, could be released from cells independently of M1. Incorporation of M1 into VLPs required HA expression, although when M1 was omitted from VLPs, particles with morphologies similar to those of wild-type VLPs or viruses were observed. Furthermore, when HA and NA cytoplasmic tail mutants were included in the VLPs, M1 failed to be efficiently incorporated into VLPs, consistent with a model in which the glycoproteins control virus budding by sorting to lipid raft microdomains and recruiting the internal viral core components. VLP formation also occurred independently of the function of Vps4 in the multivesicular body pathway, as dominant-negative Vps4 proteins failed to inhibit influenza VLP budding.

Viruses that derive their lipid envelope by budding from the plasma membrane undergo a complex, multistep assembly process involving the proper organization of viral proteins at the cell membrane and formation of the viral particle by pinching off from the cell surface (53). For influenza virus, an enveloped, negative-stranded, segmented-RNA virus, assembly requires the coalescence of hemagglutinin (HA), neuraminidase (NA), and the M2 ion channel protein (viral membrane proteins) and matrix protein (M1) and ribonucleoprotein (RNP) complexes (soluble viral components) (54). Influenza viruses bud from discrete lipid raft microdomains (52), and the concentration of HA and NA into lipid raft microdomains is both an intrinsic property of the surface glycoproteins (32) and a requirement for efficient virus replication (2, 59). Proper virion assembly is thought to involve a series of protein-protein interactions between the glycoproteins and the internal soluble components. The prevailing model of influenza virus assembly suggests that M1 underlies the lipid bilayer and coordinates assembly between the surface glycoproteins through interactions with their cytoplasmic tails, concomitantly forming a bridge to the internal RNP complexes making up the viral core (3, 54).

Studies concerning the roles of the cytoplasmic tails of HA, NA, and M2 suggest that assembly interactions occur between M1 and the cytoplasmic tails of these proteins. Although M1 expressed from cDNA has been shown to underlie the lipid bilayer (51), biochemical assays have shown that the cytoplasmic tails of HA and NA promote membrane and lipid raft association of M1 (1, 10, 66). Viruses generated by reverse genetics containing mutant HA and NA proteins have demonstrated a role for the HA and NA cytoplasmic tails in controlling virus morphology (28), virus assembly (66), and genome packaging (65). Furthermore, mutagenesis studies have identified specific residues in the cytoplasmic domains of HA (e.g., palmitoylation sites [7]) and NA (e.g., a critical proline [5, 40]) that contribute to efficient virus replication, presumably due to interactions between the glycoproteins and M1 during virus assembly. In the case of M2, the cytoplasmic tail has also been shown to play a role in genome packaging (37), and distinct domains of the M2 cytoplasmic tail have been suggested to mediate binding of M2 to M1 to facilitate virus assembly (36).

The observation that virus budding is severely impaired when the cytoplasmic tails of the viral integral membrane proteins are mutated suggests that the M1 protein is not sufficient to overcome these assembly defects and may not provide the major driving force for influenza virus budding, as has been proposed (18). The property whereby matrix proteins drive enveloped-virus budding differs among viruses. The discovery of late (L) domains in the major structural proteins of many enveloped viruses, including human immunodeficiency virus type 1 (HIV-1) and Ebola virus, suggests that these proteins are sufficient to recruit the necessary cellular machinery re-

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quired for virus budding (4, 13). Overexpression of HIV-1 gag (12), Ebola virus VP40 (22, 61), vesicular stomatitis virus (VSV) matrix protein (23, 29), or the matrix proteins of paramyxoviruses human parainfluenza virus type 1 (hPIV-1) (9), Nipah virus (8), and Sendai virus (57) has been shown to be sufficient for the release of membrane-bound vesicles resembling virus-like particles (VLPs) that bud from the plasma membrane. In contrast, budding of the alphaviruses Semliki Forest virus and Sindbis virus requires coexpression of, and specific interactions between, both nucleocapsid cores and envelope glycoproteins (11, 35, 58). Additionally, analysis of the requirements for the budding of the paramyxovirus PIV-5 VLPs demonstrated a requirement for the glycoproteins to promote VLP formation (56). In contrast to that for other paramyxoviruses, expression of the matrix protein of PIV-5 alone did not result in the release of detectable VLPs, despite the presence of a unique late domain (55). Therefore, it appears that the matrix proteins of enveloped viruses differ in their abilities to direct virus assembly and recruit cellular machinery during the budding process.

For influenza virus, previous studies for the production of VLPs have used either recombinant baculoviruses (31) or vaccinia virus-based systems (18, 19, 39). These studies indicated that overexpression of M1 alone resulted in the release of particles; however, by relying on overexpression of proteins without comparison to virus-infected cells, these studies may have overlooked critical aspects of the requirements for VLP assembly and budding. To avoid the complications arising from the more than 80 proteins expressed by vaccinia virus, a plasmid-driven VLP system was also developed (44). Whereas the functionality of the VLPs produced by using the plasmid-based system was characterized carefully by measuring the transfer of a green fluorescent protein (GFP)-expressing pseudogene reporter construct, biochemical and morphological characterization of the VLPs was not addressed. Thus, a quantitative study detailing the viral requirements for influenza virus budding in a VLP system that reflects a biological infection remains to be performed.

In this study, we have characterized a plasmid-based VLP system that uses cDNAs derived from the A/Udorn/72 (H3N2) strain of human influenza virus. By using this system, we demonstrate that the VLPs reflect influenza virions biochemically, morphologically, and functionally. Furthermore, we show through the expression of various combinations of viral proteins that particle budding is not driven by M1 but rather is dependent on the expression of HA and NA and, in particular, the cytoplasmic tails of HA and NA.

MATERIALS AND METHODS

Plasmids. DNA sequences corresponding to the influenza A/Udorn/72 (H3N2) virus nucleoprotein (NP), M1, HA, NA, M2, and NS2 (NEP) proteins and the VSV G protein were subcloned into the eukaryotic expression vector pCAGGS (45). M1 and HA were also subcloned into pGEM for T7 RNA polymerase-driven expression. DNA sequences for the PB1, PB2, and PA proteins were subcloned into the eukaryotic expression vector pcDNA3.1. A reporter pseudogene construct was made by inserting the coding sequence of GFP flanked by the 5′ and 3′ noncoding regions of influenza virus genome segment 7 behind the polI promoter of genome-expression plasmid pHH21 (43) in the negative sense. The mutant influenza virus HA protein HA-SSS contains cysteine-to-serine mutations at positions C555, C562, and C565 and has been described previously (7). The HA tail mutant protein HAt− is truncated at the transmembrane domain-cytoplasmic tail border after residue 556 and has been characterized previously (28). The NA tail mutant protein NAt− contains a deletion of the N-terminal 5 amino acids after the initiating methionine residue, and this NA cytoplasmic tail deletion mutant has been characterized previously (28). Plasmids expressing dominant-negative Vps4A ATPase point mutants E228Q (Vps4A-EQ) and K173Q (Vps4A-KQ) and Vps4B mutant K180Q (Vps4B-KQ) were a gift from W. Sundquist (University of Utah) and have been described previously (17, 62).

Cells and viruses. 293T, HeLa T4, Vero, COS-7, and CV1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. Influenza A/Udorn/72 virus was propagated as described previously (48). Recombinant vaccinia virus vTF7-3, which expresses bacteriophage T7 RNA polymerase, was a gift from B. Moss (National Institutes of Health) and was propagated as described previously (14). VSV was a gift from J. Rose (Yale University) and was propagated in BHK-21 cells.

Antibodies. Goat serum raised to purified and detergent-disrupted influenza A/Udorn/72 virus (goat anti-Ud) was used to detect HA, NP, and M1 proteins by immunoprecipitation and immunoblotting. Polyclonal goat antiserum raised against influenza A/Singapore/1/57 virus NA protein (N2 subtype; National Institute of Allergy and Infectious Diseases Repository V308-541-157) was used to detect NA protein by immunoblotting. Monoclonal mouse antibody 14C2 (64) was used to detect M2 protein by immunoblotting. Monoclonal mouse anti-VSV-G antibody (clone P5D4; Sigma-Aldrich [Sigma], St. Louis, MO) was used to detect VSV G protein. Rabbit anti-GFP peptide antibody (BD Biosciences, San Jose, CA) was used to detect Vps4A dominant-negative proteins fused to GFP. Donkey anti-goat immunoglobulin G (IgG) and goat anti-rabbit IgG conjugated to AlexaFluor 680 (Invitrogen, Carlsbad, CA) and donkey anti-mouse IgG conjugated to IR800 (Rockland Immunochemicals, Gilbertsville, PA) were used as secondary antibodies.

VLP and virion preparation and analysis. 293T or HeLa T4 cells (1×10^6 cells) were seeded in 6-cm-diameter dishes and grown in DMEM-10% FBS supplemented with 0.1 mM nonessential amino acids (Invitrogen). To generate influenza VLPs, 24 h later the cells were transfected with the appropriate plasmid DNA by using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions. Complete, or wild-type (wt), VLPs were produced with the following plasmid quantities: pcDNA-PB1, 1.0 μg; pcDNA-PB2, 1.0 μg; pcDNA-PA, 0.2 μg; pCAGGS-NP, 2.0 μg; pCAGGS-HA, 0.5 μg; pCAGGS-NA, 0.5 μg; pCAGGS-M1, 1.0 μg; pCAGGS-M2, 0.25 μg; pCAGGS-NS2, 0.1 μg; pHH21-vGFP, 1.0 μg. Empty vector was used in place of omitted plasmids where appropriate. At 5 h posttransfection (p.t.), the transfection medium was replaced with DMEM-10% FBS supplemented with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate (Invitrogen). If required, exogenous bacterial NA (*Clostridium perfringens*; Sigma) was added to the replacement medium to a final concentration of 100 mU/ml. At 48 h p.t., the culture medium was harvested and cellular debris was pelleted by centrifugation at $2{,}000 \times g$ for 10 min. The culture medium was then layered onto a 30% sucrose-NTE (100 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA) (wt/vol) cushion and centrifuged at $200{,}000 \times g$ for 2 h at 4°C in a Beckman Ti70.1 rotor (Beckman Coulter, Fullerton, CA). The pellet was resuspended in sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 15% polyacrylamide gel. To prepare the cell lysate, transfected cells were lysed in SDS loading buffer, sonicated for ~20 s, boiled for 5 min, and analyzed by SDS-PAGE on a 15% polyacrylamide gel. Polypeptides were detected by standard immunoblotting techniques and quantified with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Virions were prepared by infecting 293T cells (1×10^6 cells) in 6-cm-diameter dishes at a multiplicity of infection of 3 PFU/cell for 1 h at 37°C. The virus inoculum was replaced with DMEM-10% FBS. At 20 h postinfection (p.i.), the culture medium and infected cells were prepared and analyzed as described for VLPs.

The procedure for analyzing virions or VLPs by sucrose gradient density flotation has been described previously (7).

For experiments involving protein expression by using vaccinia virus that expresses T7 RNA polymerase, the protocol of Gómez-Puertas et al. was followed essentially as described by them (18). CV1 or HeLa T4 cells (3×10^6 cells) grown in 6-cm-diameter dishes were infected with vTF7-3 at a multiplicity of infection of 5 for 1 h at 37°C. After removal of the virus inoculum, the cells were transfected with plasmids by using Lipofectamine 2000 (Invitrogen). At 5 h p.t., the transfection medium was replaced with DMEM-10% FBS supplemented with 40 μg/ml cytosine-β-D-arabinofuranoside (ara-C; Sigma). At 20 h p.t., the medium was again replaced with DMEM–10% FBS–ara-C and exogenous bacterial NA (100 mU/ml) was added where indicated. At 60 h p.t., the culture

medium and infected/transfected cells were analyzed as described above. Fifteen micrograms of pGEM-HA and pGEM-M1 was used as described previously (18).

Budding assays for HIV-1 (17, 62), PIV-5 VLPs (55), and VSV (25) with dominant-negative Vps4 proteins were performed as described previously.

VLP and virus budding efficiency. To measure the budding efficiencies of VLPs compared to those of virions, VLPs were prepared by transfection as described above. At 60 h p.t., the cells were starved for 30 min at 37°C in DMEM deficient in methionine and cysteine (DMEM-MetCys−) supplemented with 10% FBS and then metabolically labeled with 200 μCi Redivue Promix L-^{35}S in vitro cell-labeling mixture (^{35}S-labeled Promix; Amersham Biosciences, Piscataway, NJ) in DMEM-MetCys−-10% FBS for 1 h at 37°C. After the labeling, the medium was replaced with DMEM-10% FBS. After 20 h, the culture medium was harvested and pelleted by ultracentrifugation through a 30% sucrose-NTE cushion as described above. The pellet was resuspended in radioimmunoprecipitation assay buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 10 mM Tris [pH 7.4], 0.15 M NaCl, 1× protease inhibitor cocktail [Sigma], 1 mM phenylmethylsulfonyl fluoride [Sigma]), and the viral proteins were immunoprecipitated with goat anti-Ud antibody and protein A/G-conjugated Sepharose beads (Amersham Biosciences) as described previously (7). Proteins were analyzed by SDS-PAGE on a 15% polyacrylamide gel, and quantification of radiolabeled polypeptides was performed by using a Fuji BioImager FLA-5100 and Multi Gauge v3.0 software (Fuji Medical Systems, Stanford, CT).

For virions, infections were performed as described above, except at 5 h p.i., the culture medium was removed and the cells were starved and metabolically labeled as described for VLPs. At 20 h p.i., the culture medium and cells were harvested as described above, viral proteins were immunoprecipitated, and samples were analyzed as described above.

Electron microscopy (EM). VLPs or virions were prepared by transfecting or infecting, respectively, 293T cells as described above. VLPs or virions were pelleted from culture medium through a 30% sucrose-NTE cushion at 200,000 × *g* for 2 h at 4°C. The pellet was resuspended in NTE and homogenized by passage through a 23-G needle. VLPs or virions were absorbed onto freshly glow-discharged, carbon- and Formvar-coated, 300-mesh nickel grids for 2 min. VLPs were stained using a mouse anti-HA (H3) antibody and a secondary antibody labeled with 15-nm colloidal gold essentially as described previously (49). Samples were examined with a JEOL 1230 electron microscope (EM; JEOL Ltd., Tokyo, Japan).

VLP infectivity assay. VLPs were prepared by transfecting 293T cells as described above. After the culture medium was harvested, VLPs were treated with 5 μg/ml *N*-acetyl-trypsin (Sigma) for 15 min at 37°C to cleave and activate HA. Soybean trypsin inhibitor (0.1 mg/ml; Sigma) was then added to inhibit trypsin activity. 293T cells (5×10^5 cells) grown in 6-cm-diameter dishes were transfected with pcDNA-PB1 (1.0 μg), pcDNA-PB2 (1.0 μg), pcDNA-PA (0.2 μg), and pCAGGS-NP (1.0 μg) by using Lipofectamine and Plus reagents. At 6 h p.t., the transfection mixture was removed from the target cells and replaced with trypsin-treated VLPs. Twenty hours after VLP addition, the cells were washed with phosphate-buffered saline, scraped into phosphate-buffered saline plus 50 mM EDTA, and fixed in 0.5% formaldehyde. GFP fluorescence in fixed cells was quantified by using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

RESULTS

Viral protein expression levels and particle budding efficiencies from influenza virus protein-expressing and virus-infected cells are comparable. To generate influenza VLPs, nine viral proteins (PB2, PB1, PA, M1, HA, NA, NP, M2, and NS2 [NEP]) were transiently coexpressed in human 293T cells by transfection with plasmid DNA. An artificial pseudogene reporter construct encoding GFP in the negative sense flanked by segment 7 noncoding regions (vGFP) was also included. The amounts of plasmid DNA transfected encoding HA, NA, NP, M1, and M2 were optimized such that the level of protein expression for each construct was comparable to the level of protein expressed in virus-infected 293T cells (Fig. 1A, lane 1, and B, lane 1). The amounts of plasmid DNA encoding PB2, PB1, PA, and vGFP used in the transfection were optimized, along with that for NP, to maximize GFP expression in transfected 293T cells (data not shown). The amounts of M2- and NS2-encoding plasmid DNA transfected were optimized to maximize the vGFP transfer to target cells in a VLP infectivity assay (data not shown). The optimal amounts of each plasmid DNA used are described in Materials and Methods.

To analyze the particles released into the culture medium from transfected or infected cells, the culture medium was harvested, the particles were pelleted through a 30% sucrose cushion, and the protein composition of the pellet was analyzed by SDS-PAGE and immunoblotting (Fig. 1A, lane 2, and B, lane 2). To further analyze the composition of the particles, the material pelleted through the 30% sucrose cushion was floated through a sucrose density gradient, fractionated, repelleted, and analyzed by SDS-PAGE and immunoblotting (Fig. 1A, lanes 3 to 8, and B, lanes 3 to 8).

When the protein composition of VLPs was compared to that of virions, the levels of HA, NP, and M1 in the 30% sucrose pellet were found to be comparable, suggesting that the VLPs have a protein composition similar to that of authentic virions. VLPs also had a sucrose gradient flotation distribution similar to that of virions, with HA and M1 floating predominantly to fractions 2 to 4 (Fig. 1A, lanes 4 to 6, and B, lanes 4 to 6), although VLPs appeared to be slightly more dense than virions in this assay. Less NP appeared to be incorporated into the VLPs, potentially due to inefficient assembly in the absence of authentic genome segments, as has been suggested previously (15, 42). The amounts of NA and M2 incorporated into VLPs were also greater than those found in virions, and this may reflect overexpression of NA and M2.

In establishing a biologically relevant VLP system, the efficiency of VLP release needs to be comparable to that of virus release. To measure budding efficiency, cells were metabolically labeled at 16 h p.t. for VLPs or 6 h p.i. for virions, and the culture media and cells were harvested 20 h later and particles pelleted through a 30% sucrose cushion. Samples were then immunoprecipitated and analyzed by SDS-PAGE, and the amount of M1 released into the media and pelleted through 30% sucrose compared to the total amount of M1 present was calculated. Approximately 8% of the total M1 was released in the VLP system, compared to 10% released from virus-infected cells, indicating that the efficiency of VLP budding from transfected cells was comparable to that of virus budding from infected cells (Fig. 1C). Additional time points were also tested, and comparable amounts of released VLP and virus were detected in the culture media (data not shown), indicating similar rates of VLP and virus formation.

HA is released into the media independently of other viral proteins, and HA enhances release of NA and M2. As the glycoprotein cytoplasmic tails play an important role in virus assembly (66), the possibility that the glycoproteins are able to drive budding and virus particle formation exists. The abilities of HA, NA, and M2 to be released from cells into the culture media without any internal viral proteins were tested by expressing these proteins from plasmid DNA in various combinations, pelleting the culture media 48 h p.t. through a 30% sucrose cushion, and analyzing the pellet for released proteins (Fig. 2). When HA was expressed alone, no HA was detected in the 30% sucrose pellet (Fig. 2, lane 1). However, when HA-expressing cells were treated with exogenous bacterial NA, HA was released efficiently from cells into the culture medium in a form that could be pelleted through a 30% sucrose cushion

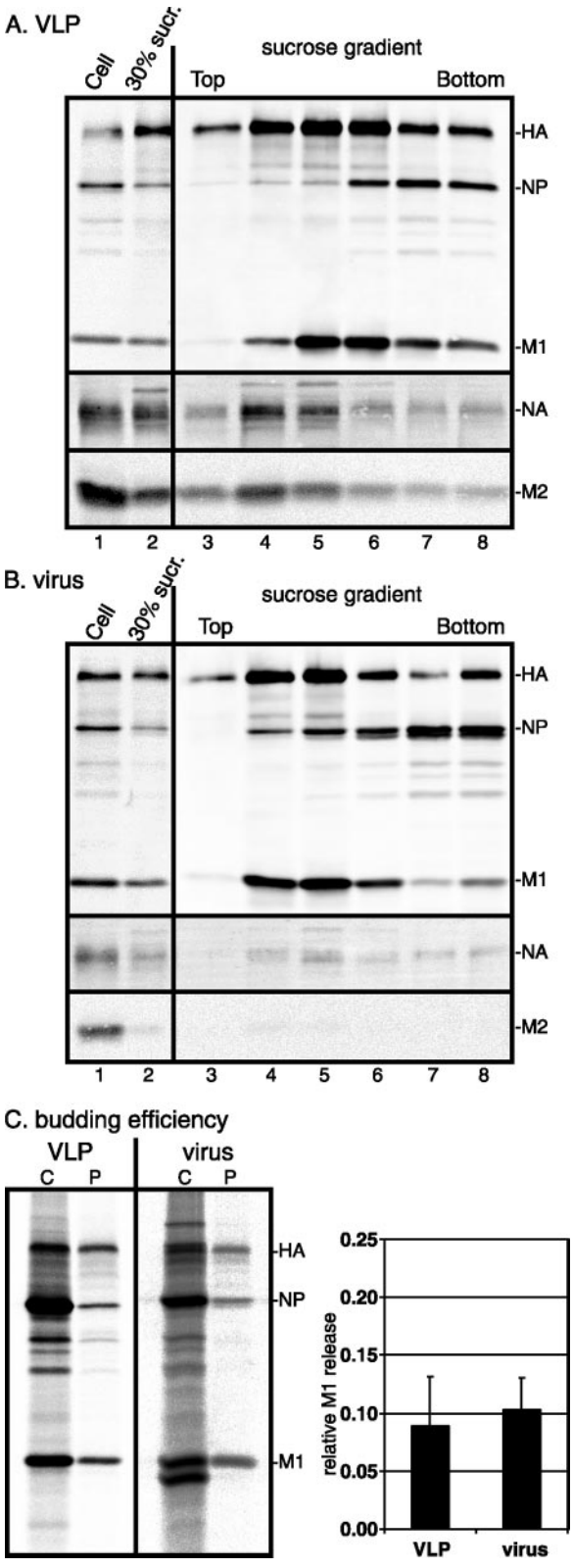

FIG. 1. Protein expression levels and budding efficiencies of virus and VLPs. VLPs (A) or virions (B) were prepared by transfecting or infecting 293T cells as described in Materials and Methods. At 48 h p.t. or 20 h p.i., the culture media and cells were harvested. The cell lysate (cell, lane 1), material pelleted through a 30% sucrose cushion (30% sucr., lane 2), and fractions collected following flotation of VLPs or virions through a sucrose density gradient (lanes 3 to 8) were analyzed by SDS-PAGE followed by immunoblotting to detect viral proteins. NP appears as a doublet, which probably reflects the known proteolytic cleavage of NP in infected cells (68). (C) The budding efficiencies of VLPs were compared to those of virus by metabolically labeling transfected or infected cells with ^{35}S-Promix and harvesting the culture media after 20 h. The amount of radiolabeled M1 released into the culture media and pelleted through a 30% sucrose cushion was quantified by using Image Gauge software. Relative M1 release was determined by dividing the amount of M1 present in the 30% sucrose pellet (P) by the total amount of M1 present in the pellet and cell lysate (C). Only the full-length M1 band, and not the clipped form present in the infected cells (unpublished observation), was used for quantification. Error bars represent standard deviations from the averages for three experiments.

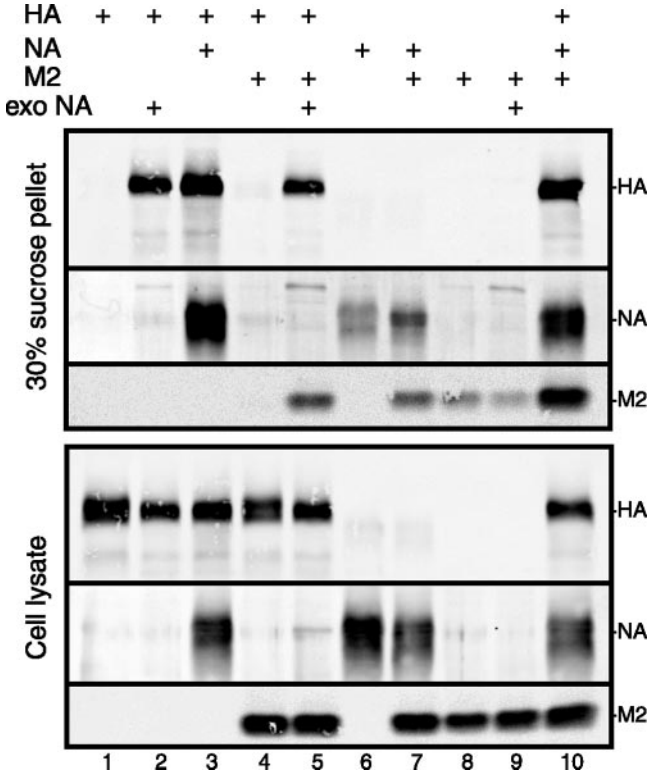

FIG. 2. Glycoprotein release from 293T cells. HA, NA, and M2 proteins were expressed in 293T cells in combinations as indicated. The culture medium was harvested at 48 h p.t. and pelleted through a 30% sucrose cushion, and cells were prepared as described in Materials and Methods. Samples were analyzed by SDS-PAGE followed by immunoblotting to detect viral proteins. Where indicated, exogenous bacterial NA (exo NA) was added to the replacement media following transfection.

(Fig. 2, lane 2). When NA or M2 was expressed on its own, a small amount of NA or M2 was detected in the 30% sucrose pellet (Fig. 2, lanes 6 and 8). However, when NA was coexpressed with HA, NA release into the culture medium was greatly enhanced (Fig. 2, lane 3). Coexpression of HA and M2, without exogenous NA or viral NA, resulted in a reduction in M2 release (Fig. 2, lane 4), possibly due to sequestration of M2 in HA-containing vesicles. Treatment of cells expressing HA and M2 with exogenous NA resulted in release of particles

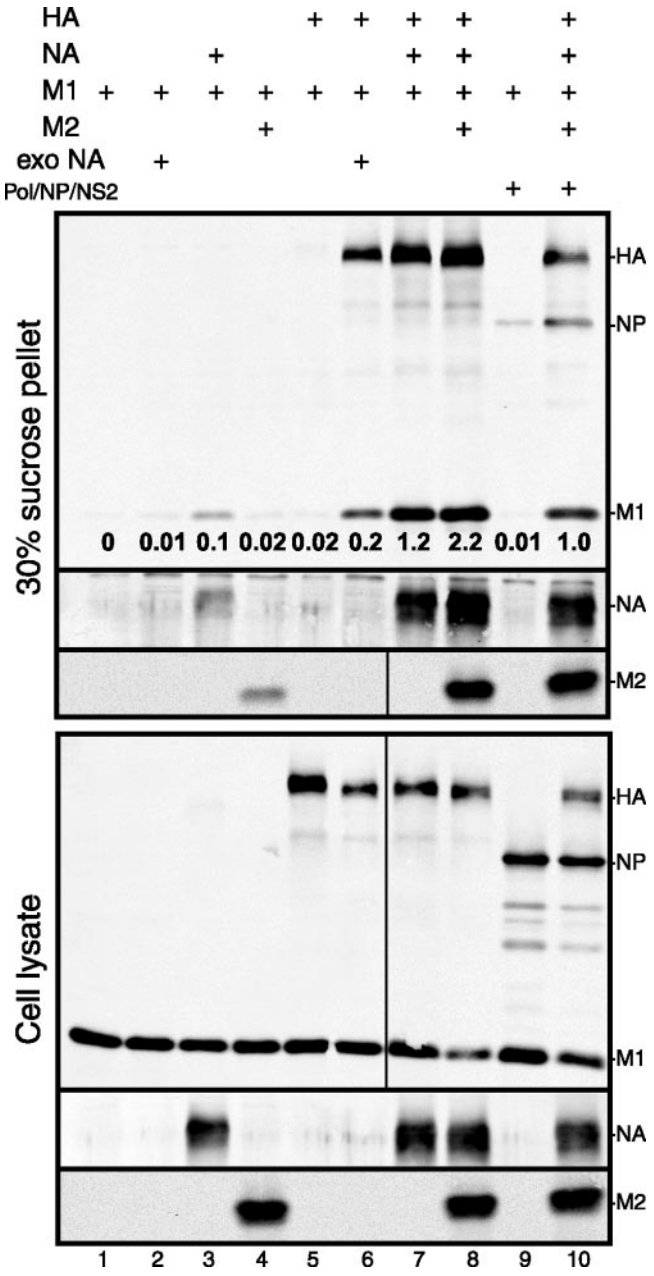

FIG. 3. M1 protein release from 293T cells driven by glycoprotein expression. HA, NA, M1, M2, polymerase proteins (Pol; PB1, PB2, and PA), NP, and NS2 (NEP) were expressed in 293T cells in combinations as indicated. The culture medium was harvested at 48 h p.t. and pelleted through a 30% sucrose cushion, and cells were prepared as described in Materials and Methods. Samples were analyzed by SDS-PAGE followed by immunoblotting to detect viral proteins. Where indicated, exogenous bacterial NA (exo NA) was added to the replacement media following transfection. M1 protein in the 30% sucrose pellet was quantified by using the Odyssey infrared imaging system and normalized to the amount of M1 protein found in the cell lysate. The amount of M1 released from cells expressing all VLP proteins (lane 10) was set to 1.0.

containing HA and M2 (Fig. 2, lane 5); nonetheless, M2 release was greatest when M2 was coexpressed with both HA and NA (Fig. 2, lane 10). Thus, HA can be released from cells efficiently in the presence of either viral or exogenous NA activity, and HA can enhance the release of the other surface integral membrane proteins in the absence of any internal viral proteins.

M1 release from cells requires coexpression of HA and NA activity. Expression of the major internal structural proteins of several enveloped viruses (e.g., retrovirus gag [12] and Ebola virus VP40 [61]) results in the budding of particles, suggesting that these structural proteins are able to drive virus budding. The detection of these structural proteins in the culture medium has been taken to reflect the release of properly assembled VLPs. It was shown previously that influenza virus M1 protein, when overexpressed by recombinant baculoviruses (31) or by vaccinia virus T7 RNA polymerase-driven systems (18), could be released from cells. In contrast, with a Semliki Forest virus-derived expression system, overexpression of M1 and NP did not result in the release of M1 into the culture media (67). To test whether M1 could drive VLP budding in our plasmid expression system, M1 was expressed alone or in combination with the other viral proteins. Culture media were harvested at 48 h p.t. and pelleted through a 30% sucrose cushion. The amount of M1 found in the 30% sucrose pellet was quantified, normalized to the amount of M1 present in the cell lysate, and compared to the amount of M1 released when all VLP proteins were present (Fig. 3, lane 10).

When M1 was expressed alone in 293T cells, M1 was not detected in the 30% sucrose pellet (Fig. 3, lane 1). Transfecting increasing amounts of M1 expression plasmid or pelleting the culture media through 10% or 20% sucrose cushions also did not result in detection of released M1 protein (data not shown). However, when M1 was coexpressed with HA and either treated with exogenous bacterial NA or coexpressed with NA, M1 was detected in the 30% sucrose pellet at high levels, suggesting that M1 release into the media requires HA release (Fig. 3, lanes 6 and 7).

When M1 was coexpressed with only the internal viral components, PB2, PB1, PA, NP, NS2 (NEP), and vGFP, M1 was not released efficiently into the culture medium (Fig. 3, lane 9). Additionally, when M1 was coexpressed with only M2, very little M1 was released into the medium (Fig. 3, lane 4), reflecting the observation that M2 is released poorly without HA and NA expression (Fig. 2). Similarly, a small fraction of M1 (10% of the control amount) was detected in the 30% sucrose cushion when M1 was coexpressed with only NA (Fig. 3, lane 3), consistent with the poor release of NA in the absence of HA (Fig. 2).

As previous studies using vaccinia virus-expressed T7 RNA polymerase to drive expression of VLPs showed that M1 could be released from cells, we sought to replicate those experiments to explain the discrepancy with the current study. CV1 cells were infected with vTF7-3 and then transfected with plasmids expressing HA or M1 driven by the T7 RNA promoter, following closely the protocol described by Gómez-Puertas et al. (18). When M1 alone was expressed in this manner and the culture medium was pelleted through a 30% sucrose cushion, M1 was detected at high levels (Fig. 4A, lane 6). With this protocol, a large percentage of cells were observed to be necrotic and had detached from the dish after the 60-h incubation, possibly accounting for the large amount of M1 released into the culture medium. Analysis of the culture media pelleted through 30% sucrose from earlier time points prior to

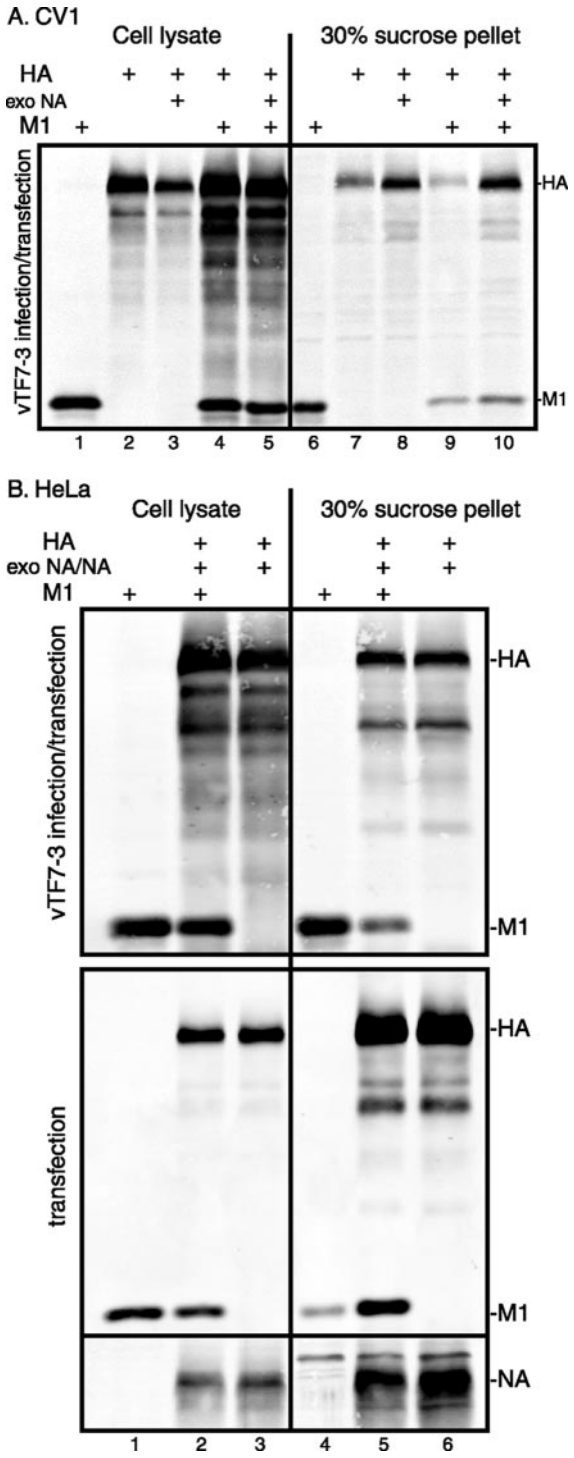

FIG. 4. Release of HA and M1 proteins expressed by vTF7-3 infection/transfection in CV1 and HeLa T4 cells. HA and M1 were expressed in CV1 (A) or HeLa T4 (B, upper) cells by vTF7-3-expressed, T7-RNA polymerase-driven expression, according to the protocol of Gómez-Puertas et al. (18). At 60 h p.t., the culture medium was harvested and pelleted through a 30% sucrose cushion and cells were prepared as described in Materials and Methods. Where indicated, exogenous bacterial NA (exo NA) was added to the replacement medium at 20 h p.t. HeLa T4 cells (B, lower) were also transfected with HA, NA, and M1 protein expression plasmids, and the culture media and cells were analyzed as described above. Samples were analyzed by SDS-PAGE followed by immunoblotting to detect viral proteins.

extensive cell death (i.e., at 24 or 36 h p.t.) revealed greatly reduced levels of M1 released into the media (data not shown).

To demonstrate that the difference in M1 release observed between use of a vaccinia virus infection/transfection protocol and a plasmid transfection-only protocol was not cell type specific, parallel experiments were performed with HeLa T4 (Fig. 4B) and Vero (data not shown) cells. As found for CV1 cells, when M1 was expressed in HeLa T4 cells or Vero cells by vTF7-3 infection followed by plasmid transfection, M1 was detected at high levels in the 30% sucrose pellet (Fig. 4B, upper, and data not shown). Both HeLa T4 cells and Vero cells became partially detached and necrotic following this protocol, as observed with CV1 cells. Consistent with the observations made for 293T cells, M1 expressed alone by transfection was released at a low level into the culture medium from HeLa T4 cells (Fig. 4B, lower), Vero cells, and COS-7 cells (data not shown). However, upon coexpression of HA and NA, M1 release from HeLa T4 cells increased approximately 10-fold compared to the small amount of M1 released when M1 was expressed alone (Fig. 4B, lower). Similar results were observed in Vero and COS-7 cells (data not shown), and these findings support a requirement for HA in VLP assembly and budding.

VLP release and composition depend on expression of glycoproteins. To examine which viral proteins are required for VLP release in the context of the other viral proteins, single proteins were omitted from the VLP system (Fig. 5). The amount of M1 protein released into the culture medium and pelleted through 30% sucrose was quantified, normalized to the amount of M1 present in the cell lysate, and compared to the amount of M1 released into wt VLPs (Fig. 5, lane 1). Most striking was the impact of deletion of HA from the VLP system. Omission of HA resulted in a drastic decrease in M1 release from cells to about 10% compared to the wt level as well as decreases in NA, M2, and NP release (Fig. 5, lane 6). When vGFP or the polymerase proteins were omitted, there was a small but reproducible reduction in the amount of M1 released (Fig. 5, lanes 2 and 3). The amount of NP released into the culture medium, however, did not appear to be affected. Omission of NP or NS2 (NEP) appeared to have no effect on the amount of M1 released into the culture medium (Fig. 5, lanes 4 and 10). However, omission of NA drastically affected the amount of M1 released (Fig. 5, lane 7), as anticipated due to the need for sialidase activity to release particles tethered to the cell surface. As the sialidase activity of NA was found to be required for HA and M1 release (Fig. 2), exogenous bacterial NA treatment of cells was tested and was shown to largely restore M1 release (Fig. 5, lane 8), although never to wt VLP levels, despite attempts to titrate the optimal concentration and time for addition of exogenous bacterial NA treatment (data not shown). When M2 was omitted from the VLP system, the amount of M1 release was consistently decreased to about 50% of the wt level (Fig. 5, lane 9), a finding that supports a role for M2 in assembly as suggested previously (26, 36). Notably, omission of M1, HA, NA, M2, or NS2 (NEP) had a negative impact on the amount of NP detected in the 30% sucrose pellet (Fig. 5, lanes 5 to 10), suggesting a defect in the packaging of NP into VLP particles in the absence of these proteins.

Morphologies of VLPs, and VLPs lacking M1, resemble those of influenza virions as shown by EM. Influenza virus

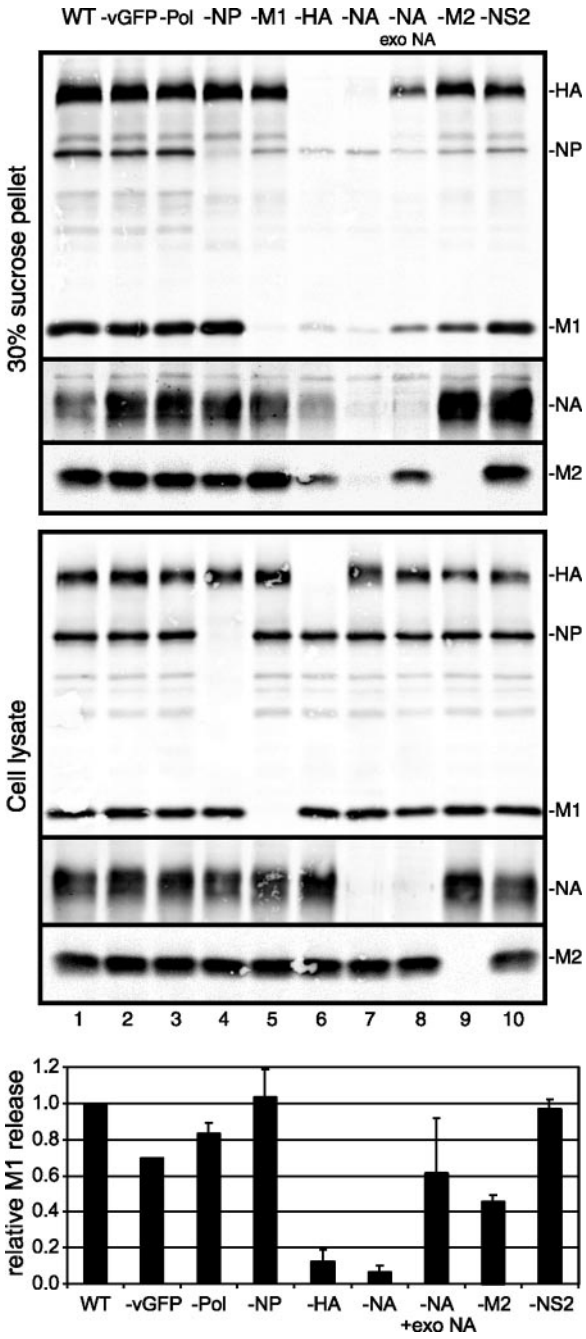

FIG. 5. Budding of VLPs with protein omissions. VLPs were prepared by transfecting 293T cells as described in Materials and Methods. Where indicated, plasmids encoding individual proteins were omitted from the transfection, the plasmids encoding the three polymerase proteins (PB1, PB2, and PA) were omitted (Pol−), or exogenous bacterial NA (exo NA) was added to the replacement media following transfection. The culture medium was harvested at 48 h p.t. and pelleted through a 30% sucrose cushion, and cells were prepared as described in Materials and Methods. Samples were analyzed by SDS-PAGE followed by immunoblotting to detect viral proteins. M1 protein in the 30% sucrose pellet was quantified by using the Odyssey infrared imaging system and normalized to the amount of M1 protein found in the cell lysate. The amount of M1 released from cells expressing all VLP proteins (WT, lane 1) was set to 1.0. Error bars represent standard deviations from the averages for three experiments.

grown in 293T cells forms irregular to spherical particles with an average diameter of around 100 nm (Fig. 6). To determine whether the morphologies of particles produced in our VLP system resembled those of authentic virions, particles concentrated from culture media of transfected cells were examined by negative staining and EM (Fig. 6). wt VLPs produced by transfecting PB2, PB1, PA, vGFP, NP, M1, HA, NA, M2, and NS2 (NEP) were found to resemble typical influenza virions. These VLPs had diameters between 100 and 150 nm and were irregular to spherical in morphology, similar to typical influenza virions. Glycoprotein spikes were clearly visible in the lipid envelopes of the VLPs, and HA could be labeled with an anti-HA monoclonal antibody and a secondary antibody labeled with 15-nm colloidal gold. Interestingly, VLPs produced in the absence of M1 protein were very similar to complete VLPs. These M1-omitted VLPs retained a distinct lipid envelope with clear glycoprotein spikes that could be labeled with the anti-HA antibody.

HA and NA cytoplasmic tails influence the release of M1 during VLP budding. It was demonstrated previously that the cytoplasmic tails of HA and NA play important roles in controlling virus morphology (28), genome packaging (65), and virus assembly (66). In the case of HA, it was further shown that the three palmitoylation sites present in the HA cytoplasmic tail are required for virus replication and, in particular, are required for proper virus assembly (7). Furthermore, the requirement for HA and NA to be expressed to form a VLP (Fig. 5) suggested that packaging of M1 into VLPs may be influenced by the cytoplasmic tails of HA and NA. To test the importance of the HA and NA cytoplasmic tails in VLP budding, tail-deleted constructs of HA and NA were coexpressed with the other viral proteins to produce VLPs. An HA palmitoylation mutant, HA-SSS, in which the three C-terminal cysteine residues were changed to serine to block addition of palmitate, was also tested (7). The culture medium from transfected cells was pelleted through a 30% sucrose cushion, and the composition of the VLPs was analyzed by SDS-PAGE and immunoblotting (Fig. 7A). The amount of M1 found in the 30% sucrose pellet was quantified, normalized to the amount of M1 present in the cell lysate, and compared to the amount of M1 released into wt VLPs (Fig. 7A, lane 6).

When the HA cytoplasmic tail was truncated at the border of the transmembrane and cytoplasmic domains, M1 release was decreased to about 20% (Fig. 7A, lane 7) compared to the amount of M1 in wt VLPs (Fig. 7A, lane 6). When the NA cytoplasmic tail was truncated, a modest reduction (30%) in M1 release was observed (Fig. 7A, lane 8). Combining both truncated glycoproteins resulted in a reduction in M1 release to less than 10%, suggesting that the cytoplasmic tails serve redundant functions in virus assembly (Fig. 7A, lane 9), although truncation of the HA cytoplasmic tail alone had a greater effect on VLP budding. Consistent with the important role of HA palmitoylation in virus assembly and replication, the HA-SSS mutant also failed to efficiently incorporate M1 into VLPs (Fig. 7A, lane 10).

In a previous study that tested the functionality of VLPs generated from transfected cells, it was shown that the VSV G glycoprotein could replace HA and NA to produce functional VLPs (44). However, the protein composition of these VLPs was not examined. To examine the composition of VSV G-

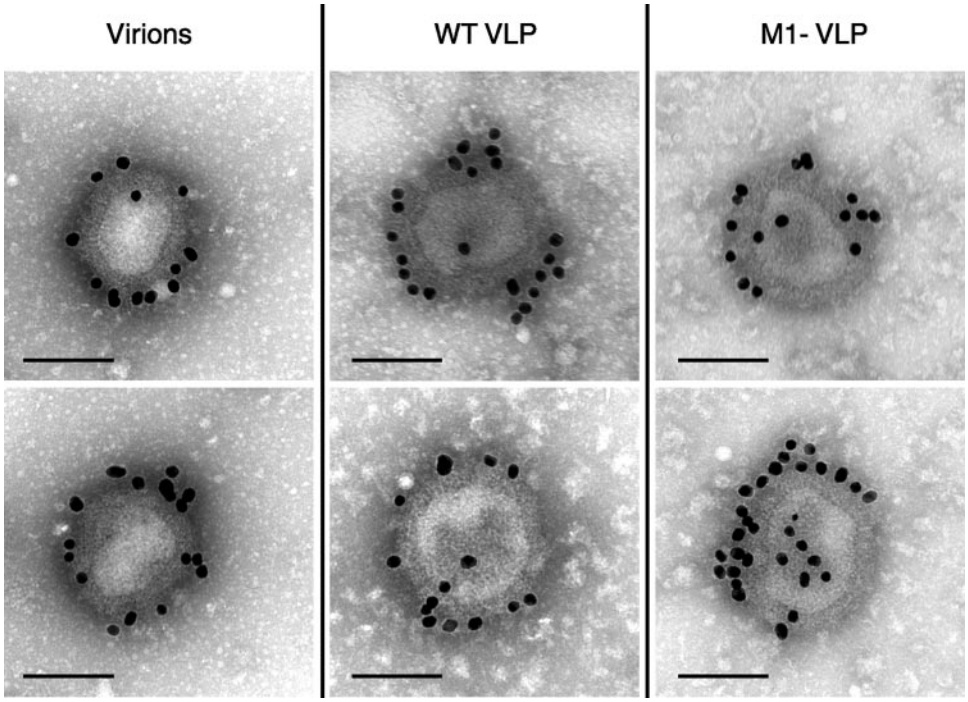

FIG. 6. Morphologies of VLPs as shown by EM. Virions or VLPs containing all VLP proteins (WT VLP) or lacking M1 protein (M1− VLP) were prepared by infecting or transfecting 293T cells as described in Materials and Methods. The culture medium was harvested at 20 h p.i. or 48 h p.t. and pelleted through a 30% sucrose cushion. The pellet was resuspended in NTE and prepared for EM. Virions and VLPs were immunostained with a monoclonal anti-HA antibody followed by IgG conjugated to 15-nm gold and then negative stained. Bar, 100 nm.

containing VLPs using our VLP system, HA- and NA-expressing plasmids were omitted from the VLP transfection and replaced by increasing amounts of VSV G-expressing plasmid (Fig. 7B). When HA and NA were omitted from the VLPs, M1 release was reduced to approximately 10% (Fig. 7B, lane 7) compared to the amount of M1 in wt VLPs (Fig. 7B, lane 6). With increasing amounts of VSV G, the amount of released M1 increased to only 40% (Fig. 7B, lanes 8 to 10) of the control amount, suggesting that the VSV G glycoprotein cannot completely replace the function of the HA and NA cytoplasmic tails during VLP assembly and budding. The amount of NP released into the medium, however, increased with increasing amounts of VSV G, a finding that may explain the observation that these VSV G-pseudotyped VLPs could still transfer a reporter plasmid to target cells in a VLP infectivity assay (44).

Functional VLPs require expression of M1. As it was observed by both biochemical and morphological examination that VLPs could be formed in the absence of M1 protein, we tested whether these M1-deleted VLPs were functional as infectious VLPs. To test VLP infectivity, VLPs were prepared by transfecting 293T cells as before. The culture media were harvested and treated with trypsin to activate HA and then overlaid onto target 293T cells that had been transfected with PB2, PB1, PA, and NP expression plasmids. wt VLPs were able to transfer the vGFP reporter construct into target cells, leading to transcription of the pseudogene by the polymerase proteins and expression of GFP that was quantified by flow cytometry (Fig. 8). VLPs that were not treated with trypsin failed to efficiently transfer the vGFP reporter construct to target cells, demonstrating that HA-mediated fusion was required for VLP infectivity. VLPs that lacked either the polymerase proteins, NP, or NS2 (NEP) also failed to efficiently transfer the reporter construct to target cells, as anticipated due to the role in genome replication and RNP packaging played by these proteins (30, 47). Importantly, M1-deleted VLPs also failed to transfer the vGFP reporter construct into target cells, as observed previously (44), presumably due to a failure to properly package the vGFP pseudogene into the VLP. Thus, while VLPs that resemble authentic virions can form in the absence of M1 (Fig. 6), functional VLPs require the inclusion of M1.

Influenza VLP budding is not inhibited by dominant-negative Vps4 proteins. Budding of many enveloped viruses proceeds by hijacking host proteins involved with the multivesicular body (MVB) formation pathway (41, 53). The activity of the downstream protein Vps4 is required for the final pinching-off process during MVB formation and has been shown to be required for budding of HIV-1 (17, 62) and PIV-5 (55) but, interestingly, not for VSV budding (25). To test whether influenza VLP budding required the activity of Vps4, we compared HIV-1, PIV-5 VLP, and VSV budding to influenza VLP budding in the presence of catalytically inactive, dominant-negative forms of the two mammalian isoforms of Vps4, Vps4A and Vps4B. Whereas HIV-1 budding was nearly completely inhibited in the presence of Vps4A-KQ or Vps4B-KQ proteins (Fig. 9A), budding of influenza VLPs was unaffected by coexpressed Vps4A-KQ, Vps4B-KQ, or Vps4A-EQ dominant-negative proteins (Fig. 9B, C, and E). Cotransfection of increasing amounts of plasmid expressing Vps4A-EQ did not inhibit influenza VLP budding, as determined both by immunoblot analysis of the particles released into the media and

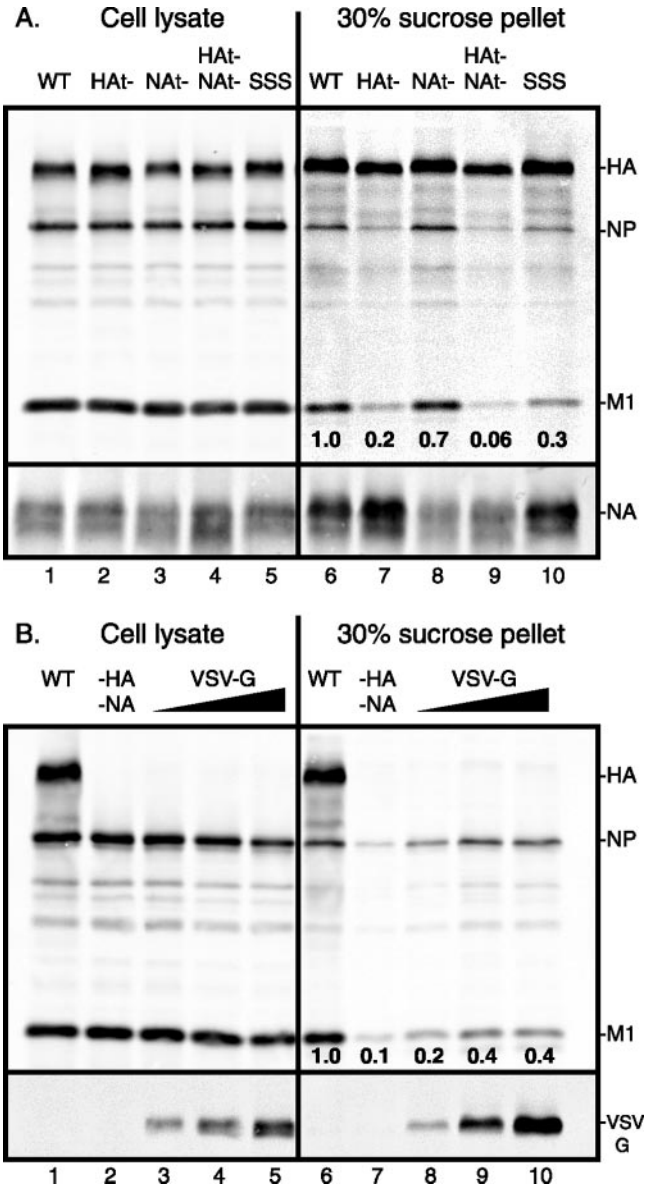

FIG. 7. Release of VLPs with mutant glycoproteins or VSV G protein. VLPs were prepared by transfecting 293T cells as described in Materials and Methods. Where indicated, plasmids encoding wt HA or wt NA were replaced by plasmids encoding HAt−, NAt−, or HA-SSS mutant proteins (A), or plasmids encoding HA and NA protein were omitted and replaced by increasing amounts (0.5 μg, 1.0 μg, and 2.0 μg) of plasmid encoding VSV G protein (B). The culture medium was harvested at 48 h p.t. and pelleted through a 30% sucrose cushion, and cells were prepared as described in Materials and Methods. Samples were resolved by SDS-PAGE followed by immunoblotting to detect viral proteins. M1 protein in the 30% sucrose pellet was quantified by using the Odyssey infrared imaging system and normalized to the amount of M1 protein found in the cell lysate. The amount of M1 released from cells expressing wt VLP proteins (WT, lane 6) was set to 1.0.

pelleted through 30% sucrose (Fig. 9C, lanes 6 to 8) and by the infectivity assay of the released VLPs (Fig. 9E). In contrast, PIV-5 VLP budding was inhibited by Vps4A-EQ (Fig. 9D, upper), whereas VSV budding was not inhibited by Vps4A-EQ (Fig. 9D, lower), as reported previously (25, 55).

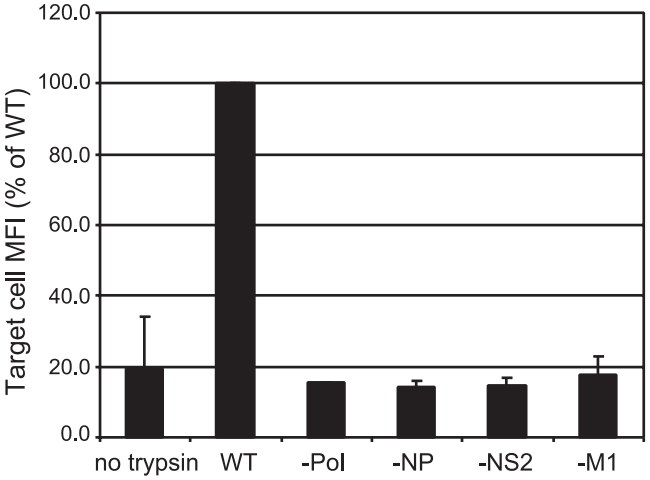

FIG. 8. Infectivities of VLPs with protein omissions. VLPs were prepared by transfecting 293T cells as described in Materials and Methods. Where indicated, plasmids encoding polymerase proteins (PB1, PB2, and PA), NP, NS2 (NEP), or M1 were omitted. The culture medium containing VLPs was harvested at 48 h p.t. and treated with trypsin. VLPs that were not treated with trypsin were used as a negative control. VLPs were transferred to target 293T cells expressing PB1, PB2, PA, and NP. Twenty-four hours later, the target cells were fixed and GFP mean fluorescence intensity (MFI) was quantified by flow cytometry. The MFI of target cells overlaid with complete VLPs (WT) was set to 100%. Error bars represent standard deviations from the averages for three experiments.

DISCUSSION

In this study, we have characterized a plasmid-driven influenza virus VLP system that generates VLPs that bud with an efficiency comparable to that of virus budding (Fig. 1C). Viral proteins in this VLP system are expressed in cells at levels similar to those for viral proteins expressed in a virus-infected cell, and the VLPs released into the culture medium have a protein composition and a sucrose gradient flotation profile similar to those of authentic virions (Fig. 1A and 1B). Furthermore, the VLPs are found to resemble influenza virions morphologically when examined by EM (Fig. 6) and are functional in an infectivity assay (Fig. 8). Thus, the data indicate that the VLPs generated by this transfection-based system will be useful in defining the requirements for influenza virus assembly and budding.

Previous studies have suggested that the matrix protein of influenza virus provides the driving force for virus budding (18, 19, 31, 39). This conclusion was based largely on the observation that M1 was released from cells that overexpressed M1. Here, we have reproduced this observation in three cell types by closely replicating the previously described experimental protocol involving infection with vaccinia virus expressing T7 RNA polymerase, followed by transfection with plasmid DNA (Fig. 4 and data not shown). Whereas it was found previously that M1 protein was released from cells under these conditions, we observed extensive cell necrosis that may have caused nonspecific release of M1 protein from dying cells. Alternatively, M1 release may be a consequence of expression of vaccinia virus-specific proteins, although, as these experiments were performed in the presence of ara-C, such proteins would include only the early vaccinia virus gene products. These pos-

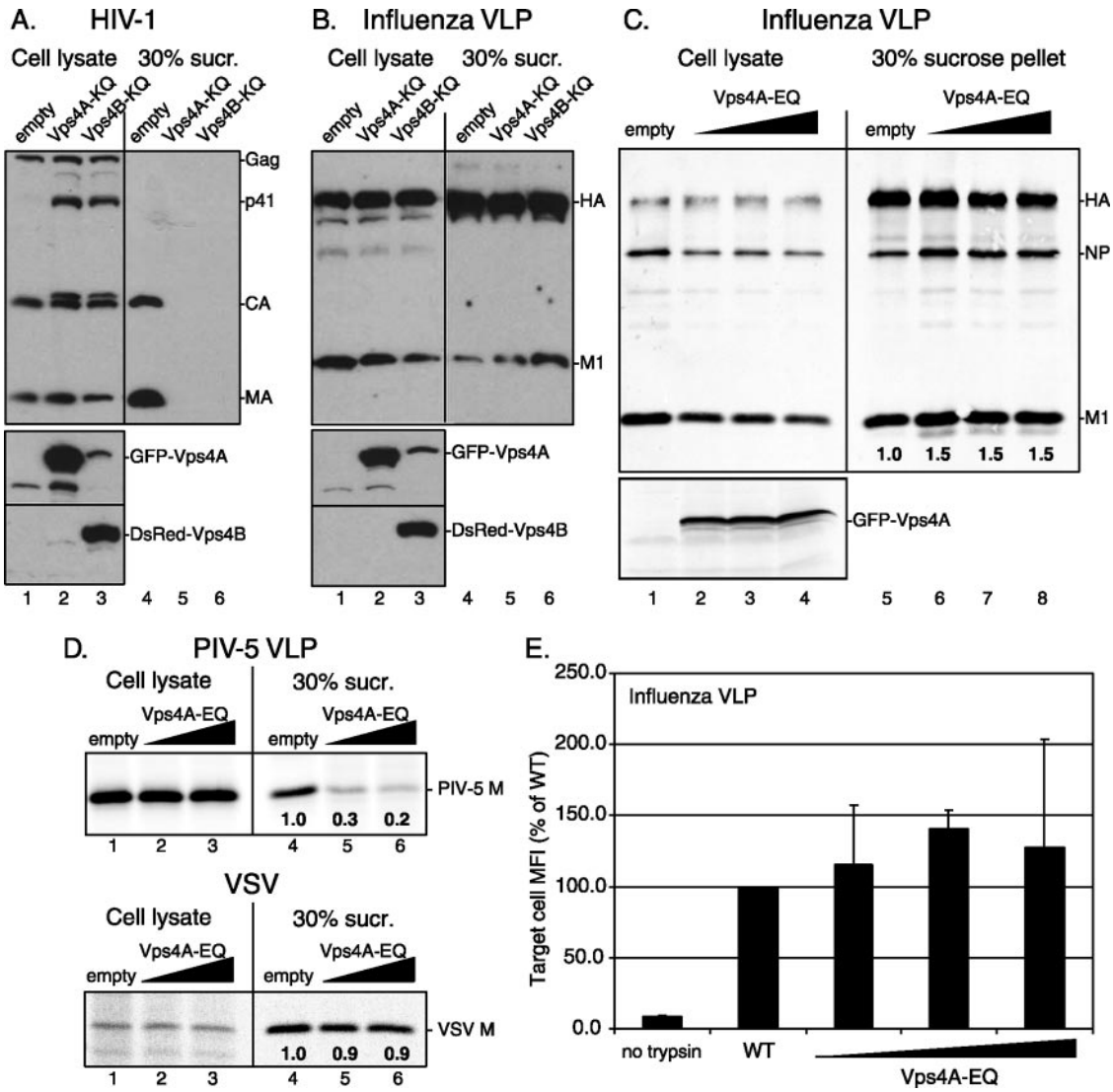

FIG. 9. Effect of dominant-negative Vps4 proteins on influenza VLP, HIV-1, PIV-5 VLP, and VSV budding. (A) HIV-1 budding in the presence of Vps4A-KQ or Vps4B-KQ proteins was examined as described previously (17, 62). (B and C) Influenza VLPs were prepared by transfecting 293T cells as described in Materials and Methods. Where indicated, plasmids encoding Vps4A-KQ (B) or Vps4B-KQ (B) or increasing amounts (0.5 μg, 1.0 μg, and 2.0 μg) of plasmid encoding Vps4A-EQ (C) were included in the transfection. The culture medium containing VLPs was harvested at 48 h p.t. and pelleted through a 30% sucrose cushion, and cells were prepared as described in Materials and Methods. Samples were resolved by SDS-PAGE followed by immunoblotting to detect viral proteins and Vps4 proteins. M1 protein in the 30% sucrose pellet was quantified by using the Odyssey infrared imaging system and normalized to the amount of M1 protein found in the cell lysate. The amount of M1 released from cells transfected with VLP plasmids and empty vector (lane 5) was set to 1.0. (D) PIV-5 VLP budding and VSV budding in the presence of increasing amounts (0.5 μg and 1.0 μg) of plasmid encoding Vps4A-EQ were performed essentially as described previously (25, 55). The relative amounts of PIV-5 and VSV M proteins released into the media and detected in the 30% sucrose pellet were quantified as for panel C. (E) Infectivities of influenza VLPs prepared as for panel C were analyzed as described in the legend to Fig. 8 and Materials and Methods.

sibilities had not been ruled out in the previous studies. In contrast, by using a noncytotoxic expression system, we found that M1 protein expressed alone was not released into the culture medium at appreciable levels from at least four different cell types (Fig. 3 and 4 and data not shown). In addition, in the context of understanding the oligomeric nature of M1 and NP interactions in BHK-21 cells, it was shown that overexpressed M1 associated with cellular membranes but did not result in membrane budding or vesicularization (67). We observed that the efficient release of M1 and VLPs required the presence of HA and sialidase activity provided by NA or exogenous NA (Fig. 3, 4, and 5). Additionally, HA, when expressed alone in cells and in the presence of sialidase activity, was released from cells independently of the expression of other viral proteins (Fig. 2). Coexpression of HA with NA and M2 enhanced the release of these proteins (Fig. 2). Taken together, these data suggest a model in which the surface glycoproteins HA and, to a lesser extent, NA, after partitioning into lipid raft microdomains (32, 52), recruit M2 and the internal viral core components into the budding viral particle at the viral budozone (54). There have been few previously reported cases of viral membrane proteins that organize vesicu-

lar budding structures, although the assembly and budding of alphaviruses are a notable example in which the interaction between the spike glycoprotein E2 and nucleocapsid is required for organization of an icosahedral capsid (11, 35, 58).

Considerable evidence from previous studies has shown a role for the cytoplasmic tails of the glycoproteins in influenza virus assembly. In particular, truncations to the highly conserved cytoplasmic tails of HA and NA resulted in viruses with aberrant morphologies (28) and reduced genome-to-protein content, resulting in reduced infectivity (65). Despite NA having a cytoplasmic tail of only 6 amino acids, mutagenesis studies of the NA cytoplasmic tail demonstrated a critical role in virus replication of a proline residue conserved among influenza A and B viruses, suggesting the importance of the secondary structure of the NA cytoplasmic tail (5, 40). In the case of HA, mutagenesis of the conserved palmitoylated residues in the cytoplasmic tail was detrimental to virus replication, presumably due to an assembly defect (7). A possible role for palmitoylation could be to maintain the HA cytoplasmic tail in a specific conformation necessary for proper virus assembly to occur. A similar requirement was shown for the alphavirus Sindbis virus, for which biochemical data showed that binding of nucleocapsid to a synthetic, palmitoylated E2 cytoplasmic domain peptide was not sequence specific but was palmitate dependent, suggesting recognition of palmitoylated E2 during virus assembly (63). Consistent with earlier studies of HA and NA mutants with altered cytoplasmic tails (7, 27, 28, 65, 66), we have shown here that truncation of the HA and NA cytoplasmic tails or mutation of the HA palmitoylation sites negatively affects M1 release from cells (Fig. 7A). Whereas truncation of the NA cytoplasmic tail had a moderate effect on M1 release, the combination of both HAt− and NAt− appeared to have a synergistic effect, reducing M1 release to less than 10% compared to levels for VLPs containing wt HA and wt NA. Evidence from several studies has shown that deletion of either the HA (27) or the NA (16, 40) cytoplasmic tail results in a mild defect in virus replication, whereas deletion of both cytoplasmic tails has a more substantial effect on virus assembly and replication (28). Although we observed a decrease in M1 release with VLPs containing both HAt− and NAt−, we did not observe gross morphological differences when these VLPs were examined by EM (data not shown). This may be due to different cell types used as predominantly spherical particles are released from nonpolarized cells, such as 293T cells, and the observation of filamentous or aberrant VLPs may require expression in polarized cells (50). Interestingly, despite lacking a cytoplasmic tail, the HAt− mutant protein was released from cells at a level similar to that for wt HA, suggesting that other regions of the HA protein may be responsible for directing vesicle formation.

Truncations to the M2 cytoplasmic tail affect virus replication as well, presumably through a defect in genome packaging and perhaps due to disrupted M2-M1 binding interactions (26, 36, 37). The mechanism by which M2 is recruited to areas of virus budding remains unclear, particularly as M2 is excluded from lipid raft microdomains (32). However, inclusion of small numbers (15 to 25) of M2 tetramers in virions is essential for efficient virus replication (60).

We observed that when HA and NA glycoproteins were replaced with the VSV G glycoprotein, M1 was not released efficiently (Fig. 7B). This contrasts with a previous study that showed that VSV G could functionally replace HA and NA to produce infectious VLPs (44). However, we did observe an increase in the amount of NP present in VLPs with VSV G protein. Therefore, the infectivities of particles containing VSV G may be due to their having incorporated NP and the vGFP reporter construct nonspecifically. Thus, proper influenza virus VLP assembly appears to require specific recognition of M1 by HA and NA, whereas VSV G is able to nonspecifically and inefficiently package M1 and NP into budding particles.

Our previous observations have suggested that M1 proteins from different influenza virus subtypes may differ in their abilities to support virus assembly. This notion was based on the disparate viability of recombinant viruses containing a palmitoylation-deficient HA protein and either an A/Udorn/72- or an A/WSN/33-derived M1 protein (7). Whereas we were able to demonstrate a decrease in budding efficiency when HA-SSS was used in the VLP system (Fig. 7A), budding of VLPs containing WSN M1 in place of Udorn M1 did not appear to increase significantly (data not shown). This finding indicates that there may be some limitations to the VLP system, such as one in which virus budding requires factors specifically regulated during a virus infection that cannot be reproduced by using VLPs. Notably, we observed that the flotation pattern of VLPs was subtly different from that of virions and that the amount of NP packaged into our VLPs was not as high as the amount of NP found in virions (Fig. 1A and B). We also observed a slight decrease in M1 budding when the pseudogene vGFP or the polymerase proteins were omitted from VLPs (Fig. 5). This may reflect a requirement for each of the eight RNA genome segments to be present and packaged into RNPs in order to promote efficient virus assembly, as has been suggested by studies examining genome packaging signals (33, 42, 46). Additionally, it has been reported that the strength of association between M1 and RNPs differs among M1 proteins from different virus subtypes (34). Thus, the interaction between M1 and the RNPs, and its role in virus budding, needs to be examined further.

Matrixless recombinant influenza virus has not yet been recovered efficiently by reverse genetics (D. Jackson and R. A. Lamb, unpublished observation), although recombinant rabies (38) and measles (6) viruses lacking matrix protein have been recovered, but, in both cases, the viruses were drastically impaired. Based on our results showing that M1 is required to produce an infectious VLP (Fig. 8), the requirement for M1 may be predominantly to package the genome. Although other roles for M1 cannot be ruled out, it is interesting to note that M1 was not required to produce a VLP that is morphologically very similar to authentic virions (Fig. 6). Recently, a cryoelectron tomography analysis of influenza virus morphology identified regions of virions that lacked M1 associated with the membrane, including virions that lacked M1 entirely (20), providing additional evidence that M1 may not be required for virus budding.

The major structural proteins of many enveloped viruses are, in some cases, homologous in structure (21), and they have been shown to be sufficient for driving VLP budding. The prevailing view is that budding occurs through hijacking host proteins, and much work has centered on defining viral L

domains and their interactions with host proteins involved in the MVB formation pathway. For influenza virus, two studies characterizing a potential L domain within M1 and its interactions with cellular proteins were recently retracted from the scientific literature (24). Whereas we show that M1 does not appear to drive influenza VLP budding, this finding does not preclude an interaction between M1 and cellular proteins during the budding process. Indeed, PIV-5 matrix protein does not bud from cells when expressed alone (56), yet a putative L domain has been identified (55). Similarly, some viruses (e.g., VSV [25]) possess matrix proteins that bud on their own and have L domains yet appear not to require Vps4 activity in the MVB pathway. Although dominant-negative mutants of Vps4 can inhibit release of viruses containing all currently known L domains presumably by preventing the final step of vesicle formation, the finding that influenza VLP budding, like VSV budding, was not inhibited by dominant-negative Vps4 mutants (Fig. 9) raises the possibility that alternative, Vps4-independent pathways exist for enveloped-virus budding from the plasma membrane. Alternatively, L domains may reside in the cytoplasmic tails or other regions of the viral surface integral membrane proteins, which may represent a new mechanism for recruiting cellular machinery for virus budding.

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