A Role for Caveolin 1 in Assembly and Budding of the Paramyxovirus Parainfluenza Virus 5[▽]

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Received 19 May 2010/Accepted 7 July 2010

Caveolin 1 (Cav-1) is an integral membrane protein that forms the coat structure of plasma membrane caveolae and regulates caveola-dependent functions. Caveolae are enriched in cholesterol and sphingolipids and are related to lipid rafts. Many studies implicate rafts as sites of assembly and budding of enveloped virus. We show that Cav-1 colocalizes with the paramyxovirus parainfluenza virus 5 (PIV-5) nucleocapsid (NP), matrix (M), and hemagglutinin-neuraminidase (HN) proteins. Moreover, electron microscopy shows that Cav-1 is clustered at sites of viral budding. HN, M, and F₁/F₂ are associated with detergent-resistant membranes, and these proteins float on sucrose gradients with Cav-1-rich fractions. A complex containing Cav-1 with M, NP, and HN from virus-infected cells and a complex containing Cav-1 and M from M-transfected cells were found on coimmunoprecipitation. A role of Cav-1 in the PIV-5 life cycle was investigated by utilizing MCF-7 human breast cancer cells that stably express Cav-1 (MCF-7/Cav-1). PIV-5 entry into MCF-7 and MCF-7/Cav-1 was found to be Cav-1 independent. However, the interaction between HN and M proteins was dramatically reduced in the Cav-1 null MCF-7 cells, and PIV-5 grown in MCF-7 cells had a reduced infectivity. Similarly, when PIV-5 was grown in MDCK cells that stably expressed dominant negative Cav-1 (MDCK/P132LCav-1), the virus showed a reduced infectivity. Virions lacking Cav-1 were defective and contained high levels of host cellular proteins and reduced levels of HN and M. These data suggest that Cav-1 affects assembly and/or budding, and this is supported by the finding that Cav-1 is incorporated into mature viral particles.

The *Paramyxoviridae* are enveloped negative-strand RNA viruses and include many important human and animal pathogens such as measles virus, mumps virus, Sendai virus, Newcastle disease virus, canine distemper virus, Nipah virus, Hendra virus, and parainfluenza viruses 1 to 4 (PIV-1 to -4). PIV-5 (formerly known as simian virus 5) has been studied as a model system (30). The PIV-5 RNA genome encodes eight known viral proteins and is encapsidated in a helical structure by the nucleocapsid protein (NP) in complex with the viral RNA-dependent RNA polymerase (RNAP). The RNA polymerase is composed of the phosphoprotein (P) and the large (L) protein and, together with NP, is required for transcription and replication of the viral RNA genome. The V protein regulates RNA synthesis (37), but it has the additional functions of blocking the host antiviral interferon response and the induction of apoptosis (31). Blockage of apoptosis in virus-infected cells is also dependent on the small hydrophobic (SH) integral membrane viral protein (31).

Viral entry is mediated by the viral hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins that are expressed on the surfaces of virions. F is responsible for virus-cell fusion at the plasma membrane in a pH-independent manner, and HN is the sialic acid receptor binding attachment protein. The HN neuraminidase (NA) activity is required for releasing the progeny virus from the surfaces of infected cells (32, 67). HN expression is also required for fusion through its fusion promotion activity (32).

The matrix (M) protein is the most abundant protein in the virion, and it plays a central role in virus assembly and budding. Underlying the virion lipid bilayer, M forms a dense layer of homo-oligomers. In addition to its peripheral membrane association, M is also thought to interact with the cytoplasmic tails of the viral glycoproteins F and HN (70, 78). M can also bind with the nucleocapsid protein (NP). The association of M with both the viral glycoprotein and the ribonucleoprotein (RNP) via the NP protein is believed to be a crucial coordinated step leading to ordered assembly and budding of PIV-5 from the plasma membrane (31, 68). From studies using a virus-like particle (VLP) system, it was found that expression of the M protein from cDNA did not initiate budding. Release of VLPs was demonstrated only when M was coexpressed with either HN or F glycoproteins and the nucleocapsid (NP) protein (70). These data indicate that the PIV-5 M protein, as opposed to the M proteins of some other negative-strand viruses such as vesicular stomatitis virus (VSV) and Ebola virus, does not harbor all of the information required for triggering budding, which suggests that other viral proteins are involved. It is also possible that the M protein interacts with host cellular factors during viral assembly and budding events (68). This idea is supported, in part, by previous work in which a protein-protein interaction domain called the “late domain” in the protein sequence of PIV-5 M was identified (69). However, a late-domain-interacting cellular partner(s) of M has yet to be identified, but the host cellular protein angiogenin-like 1 (AmotL1) was shown recently to interact with PIV-5 M (50).

The molecular mechanism of paramyxovirus assembly and the forces driving membrane budding and virus release are not completely understood. Lipid rafts, defined as cholesterol- and sphingolipid-rich microdomains, have been shown to serve as

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[▽] Published ahead of print on 14 July 2010.

sites of assembly and budding for several enveloped viruses, including influenza virus (62, 63, 75), human immunodeficiency virus type 1 (HIV-1) (10, 44, 46, 59), measles virus (39, 76), respiratory syncytial virus (RSV) (8, 9), and Newcastle disease virus (NDV) (29). Interestingly, in the case of HIV-1 (10, 15, 36), RSV (79), and the filoviruses Marburg and Ebola viruses (4), raft microdomains are involved in virus entry as well as virus release.

Caveolae (80) are non-clathrin-coated, small (50- to 100-nm-diameter) flask-shaped invaginations of the plasma membrane (3). Many studies have shown that caveolae mediate multiple cellular functions (47, 54). Caveolae share with lipid rafts a unique lipid composition (e.g., high levels of cholesterol and sphingolipids) and therefore may be considered a subset of lipid rafts. However, the feature that distinguishes caveolar membranes from lipid rafts, in addition to their specific morphology and composition, is the presence of a low-molecular-mass (~22-kDa) protein called caveolin 1 (Cav-1). Cav-1 is an essential structural component of caveolae (22, 57) that has been implicated in caveola-dependent functions such as clathrin-independent endocytosis, cholesterol efflux, and regulation of intracellular signaling pathways (3, 19, 54, 73). The Cav-1 gene belongs to the caveolin gene family together with the Cav-2 and Cav-3 genes, all of which encode 20- to 24-kDa proteins. Cav-1 has been shown to interact directly with Cav-2, forming a high-molecular-mass hetero-oligomeric complex that is targeted to lipid rafts and drives the formation of caveolar structures (61, 64). Cav-1 and Cav-2 are expressed fairly ubiquitously, whereas caveolin 3 is a muscle-specific isoform that functionally substitutes for Cav-1 in skeletal and heart muscle cells (54). Interestingly, it was shown recently that Cav-1 is incorporated into mature particles of NDV (29) and RSV (8), suggesting that it is implicated in assembly and budding of some *Paramyxoviridae*.

Due to their unusual lipid composition, membranes of lipid rafts and caveolae exhibit a liquid-ordered phase behavior (6) that confers upon the membranes insolubility in Triton X-100 (TX-100) at 4°C (also termed detergent-insoluble glycolipid-enriched complexes [DIGs] or detergent-resistant membranes [DRM]) and thus allows their separation from the bulk cellular membrane (7). This separation technique is useful to identify proteins that are associated with DRM. Indeed, DRM association was shown for influenza virus hemagglutinin (HA) and NA (34, 63, 75); Gag and gp160 of HIV (46, 58); F and HN glycoproteins of the paramyxovirus Sendai virus (2, 60); measles virus F and H glycoproteins and also M and N proteins (39, 76); RSV F, G, SH, and M proteins (8, 9, 21, 27, 40, 55); NDV HN and F glycoproteins and NP protein (16); and Nipah virus F glycoprotein (1). Therefore, these viruses may assemble and bud from specialized raft-like microdomains at the plasma membrane. However, in most of these studies no attempt was made to distinguish further between rafts and Cav-1-containing caveolar membranes in relation to the viral infection.

Here, we have investigated the role of the host cellular protein Cav-1 in assembly and budding of PIV-5. Our data show that Cav-1 interacts with the M protein. Moreover, the interaction of Cav-1 with M positively regulates the association of M with HN and causes induction of their association. PIV-5 virions that bud from cells which do not express Cav-1 are significantly defective, as shown by accumulation of host cel-

lular proteins in the mature viral particle, reduced HA activity, and reduced virus titer. The data show that Cav-1 plays an important role in late stages of the viral life cycle, regulating efficient assembly and budding of PIV-5.

MATERIALS AND METHODS

Cells, plasmids, and reagents. Unless otherwise stated, all reagents used were obtained from InvivoGen (San Diego, CA). CV-1 cells were cultured in a monolayer at 37°C, in a humidified atmosphere containing 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), penicillin, and streptomycin (Gemini). BHK-21 cells were grown as described for CV-1 cells and supplemented further with 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO). HeLa-CD4-LTR- β -gal cells were used for their high transfection efficiency and were grown as described for CV-1 cells and supplemented further with 200 μ g/ml G418, 100 μ g/ml hygromycin, and 20 mM HEPES, pH 7.4.

Human breast adenocarcinoma MCF-7 cells stably transfected with full-length Cav-1 cDNA in the pJB20 vector (MCF-7/Cav-1 cells) or pJB20 empty vector control (20, 53) were kindly provided by the lab of the late Mordechai Liscovitch (Weizmann Institute of Science, Rehovot, Israel). MCF-7 and MCF-7/Cav-1 cells, grown as described for CV-1 cells and supplemented further with 400 μ g/ml G418 (20), were transferred to drug-free medium for use in experiments.

To generate mRed-Cav-1 and mRed-P132L-Cav-1 stably transfected MDCK cells (MDCK/mRed-Cav-1 and MDCK/mRed-P132L-Cav-1, respectively), we utilized a monomeric red protein (mRed) fused with the C terminus of full-length Cav-1 or P132L mutant Cav-1 in the pcDNA3.1 vector (72), which was also kindly provided by the lab of the late Mordechai Liscovitch. MDCK cells were transfected with 2 μ g of cDNA plasmids using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The selection for transfected cells was carried out in DMEM containing 731 μ g/ml G418 for at least 4 weeks before an experiment.

pCAGGS plasmids encoding PIV-5 (W3A isolate) M, HN, and HN2-13 and the empty vector control have been described previously (70). Subconfluent monolayers of cells in 6-cm dishes were transfected with a total of 2 μ g of cDNA plasmids using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Antibodies specific for Cav-1 or actin were purchased from BD Biosciences (San Jose, CA). Antibodies specific for HN were polyclonal antibody (PAb) SDS-HN (13) (used for immunoblotting) and monoclonal antibodies (MAb) HN-1b, HN-5a, and HN-4b (52) (used for immunoprecipitation). Antibodies specific for F were PAb 245 (used for immunoblotting) and PAb anti-F2 peptide (used for immunoprecipitation) (13). Antibodies specific for M were MAb M-H and M-F (used for both immunoblotting and immunoprecipitation) (52). Secondary antibodies used were Cy5 conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488, 594, and 680 (Invitrogen) and Alexa Fluor 800 conjugated (Rockland, Gilbertsville, PA).

Viruses. PIV-5 was grown in CV-1, HeLa, MDBK, MCF-7, MCF-7/Cav-1, MDCK/mRed-Cav-1, and MDCK/mRed-P132L-Cav-1 cells. Viral infection, stock propagation, and HA titer assay were as described previously (48, 67). Multistep growth curves were generated by infecting the cells with PIV-5 at a multiplicity of infection (MOI) of 0.01 PFU per cell for 96 h. The culture medium was harvested at 12-h intervals, and virus titers were measured by plaque assay using BHK-21F cells as described previously (48). Plaques were visualized after 4 days by staining with a 0.1% naphthol blue-black solution (Sigma, St. Louis, MO) containing 6% acetic acid and 1.36% sodium acetate. Recombinant PIV-5 harboring the green fluorescent protein (GFP) inserted between the P/V and M genes (rPIV-5-GFP) was generated in our laboratory by Jessica G. Robach as described previously (71).

Virus purification, SDS-PAGE, silver staining, and immunoblotting. Virus purification by sedimentation in sucrose density gradients was done as described previously (48, 67). Briefly, confluent cells were infected with PIV-5 at an MOI of 0.2 PFU/cell, followed by incubation at 37°C. After 4 days, culture medium was harvested by low-speed centrifugation (2,200 $\times$ g for 15 min at 4°C) and the virus was pelleted from the cleared supernatant at 118,000 $\times$ g for 1 h, resuspended in NTE buffer (100 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA), and further purified by centrifugation on a 15 to 60% sucrose gradient at 77,000 $\times$ g for 1 h. The virus bands were collected, diluted in NTE, pelleted at 118,000 $\times$ g for 1 h, resuspended in NTE buffer, and stored at -80°C. Protein concentrations of the purified virus stocks were determined with the BCA protein assay kit (Pierce Chemical, Rockford, IL). Thirty micrograms of the purified sample was boiled in SDS loading buffer and analyzed by SDS-PAGE on a 10%

or 15% polyacrylamide gel. Polypeptides were visualized by silver staining or detected by standard immunoblotting techniques and quantified with a BioImager FLA-5100 and Multi Gauge version 3.0 software (Fuji Medical Systems, Stanford, CT).

Sequence alignment and analysis. To determine the PIV-5 M gene sequence, the viral RNA was isolated with a QIAamp kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions and reverse transcriptase PCR amplification was performed as described previously (78). Sequencing was performed with a 3100-Avant automated DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The nucleotide sequence analysis showed a change from that originally recorded at nucleotide 2850, T to G, causing an amino acid change of a valine to a glycine at position 362 of the PIV-5 M protein (GenBank accession number AF052755). This corrected PIV-5 M amino acid sequence, together with the M protein sequences from *Rubulavirus* genus members mumps virus (accession number AAL83742) and human parainfluenza virus type 2 (accession number BAE00053) and type 4a (accession number BAA01086), was subjected to alignment analysis using Gene Inspector for Macintosh (Textco BioSoftware, Inc., NH). The conserved cytoplasmic tail region is shown in Fig. 5B.

Virus entry assay. Confluent monolayers (5×10^4 cells per well) of MCF-7 and MCF-7/Cav-1 cells grown in 96-well plates (black walled, clear bottomed; Greiner Bio-One, NC) were infected with a serially diluted rPIV-5-GFP (2-fold dilution per step with an initial MOI of 1.5×10^5 PFU per well) or left uninfected as controls. At 16 h postinfection (p.i.) the cells were washed with phosphate-buffered saline (PBS) and analyzed for GFP fluorescence intensity using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Optimal instrument settings selected and used for all experiments were as follows: excitation λ , 488 nm; emission λ , 525 nm; emission cutoff filter, 515 nm.

Isolation of caveolin-rich membrane domains in sucrose density gradients. Low-density TX-100-insoluble membrane domains were purified from cultured cells as described previously (38). Briefly, cell monolayers were washed twice with Ca- and Mg-deficient PBS and scraped in 1 ml of ice-cold lysis buffer containing 1% TX-100, 25 mM MES (morpholineethanesulfonic acid; pH 6.5), 150 mM NaCl, and protease inhibitors (48). After homogenization, cell extracts were adjusted to 40% sucrose by mixing with an equal volume of the lysis buffer (minus TX-100 and protease inhibitors) containing 80% (wt/vol) sucrose and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the lysate by adding 6 ml of 30% sucrose solution and 4 ml of 5% sucrose solution. Samples were centrifuged at $190,000 \times g$ for 16 to 20 h at 4°C. Fractions (1 ml) were collected from the top of the gradient. The pellet was resuspended in 1 ml of lysis buffer. Aliquots of each sucrose density gradient fraction were resolved by SDS-PAGE and analyzed by immunoblotting.

Metabolic labeling, immunoprecipitation, and TX-100 solubility. HeLa cells grown in 6-cm dishes were either uninfected or infected with PIV-5 at an MOI of 5 PFU/cell. At 18 h p.i. the cells were starved with cysteine (Cys)- and methionine (Met)-deficient DMEM for 30 min, then metabolically labeled with 100 μ Ci of 35 S-labeled Promix (Amersham Pharmacia Biotech, Piscataway, NJ) in 1 ml of Cys- and Met-deficient DMEM for 1 h and chased with DMEM supplemented with 10% FBS for 2 h as described previously (12).

To evaluate the TX-100 solubility of HN, F, and M viral proteins, the metabolically labeled cells were homogenized in Dounce buffer, containing 10 mM HEPES, 10 mM NaCl, and protease inhibitors, and extracted with ice-cold NTE buffer containing 1% (final concentration) TX-100. Soluble and insoluble fractions were separated by centrifugation at $14,000 \times g$ for 30 min. Prior to immunoprecipitation, the pellet containing TX-100-insoluble material was resuspended in 1 $\times$ radioimmunoprecipitation assay (RIPA) buffer (1% deoxycholic acid, 1% TX-100, 0.1% SDS, 10 mM Tris-hydrochloride, pH 7.4) containing 150 mM NaCl, sonicated for 20 s, clarified by ultracentrifugation at $100,000 \times g$ for 10 min, and resuspended in 1 $\times$ RIPA buffer plus 150 mM NaCl as described previously (12).

For protein immunoprecipitation, the soluble and insoluble fractions were aliquoted and antibodies (20 μ l of anti-Cav-1, 20 μ l of M-H, or 20 μ l of HN-1b, 20 μ l of HN-4b, and 10 μ l of HN-5a) were added. After 2 h at 4°C immune complexes were adsorbed onto 30 μ l of protein A-Sepharose for 1 h, washed three times with RIPA buffer containing 300 mM NaCl, twice with RIPA buffer containing 150 mM NaCl, and once with 50 mM Tris-hydrochloride buffer, pH 7.4, containing 150 mM NaCl and 2.5 mM EDTA as described previously (67). Immunoprecipitated radiolabeled polypeptides were analyzed by SDS-PAGE on 10% gels, then visualized and quantified by a BioImager FLA-5100 and Multi Gauge version 3.0 software (Fuji Medical Systems).

Coimmunoprecipitation. Cells were grown in 6-cm dishes and were either infected with PIV-5 or transfected with plasmids. At 18 h p.i. and posttransfection (p.t.), cells were lysed in 0.5 ml ice-cold immunoprecipitation buffer con-

taining 1% TX-100, 60 mM *N*-octylglucoside, 50 mM Tris-hydrochloride (pH 7.4), 150 mM NaCl, 1 mM EDTA, and protease inhibitors. Cellular debris was removed by centrifugation at $12,000 \times g$ for 15 min at 4°C. The lysates were immunoprecipitated with antibodies, followed by adsorption onto protein A-Sepharose as described above, and then collected by centrifugation and washed extensively with 1 ml of washing buffer (50 mM Tris-hydrochloride, pH 7.4, 150 mM NaCl, 1 mM EDTA). Immunoprecipitated proteins were solubilized by boiling in alkaline SDS loading buffer (note that alkaline SDS loading buffer is required to disrupt caveolin oligomers [43]) and subjected to SDS-PAGE, and polypeptides were analyzed by immunoblotting. Proteins were detected and quantified with an Odyssey infrared imaging system and Odyssey version 3.0 software (Li-Cor Biosciences, Lincoln, NE).

Immunoelectron microscopy. HeLa cells were grown in 3.5-cm dishes and infected with PIV-5 at an MOI of 5 PFU/cell. At 18 h p.i. cells were fixed for 20 min at room temperature in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.1, and fixation was continued overnight at 4°C in 2% formaldehyde. Cell monolayers were washed in several changes of phosphate buffer, treated for 10 min with freshly prepared 0.1% NaBH₄ in 0.1 M phosphate buffer, pH 7.1, and then permeabilized with 0.05% saponin in phosphate buffer. Nonspecific binding was blocked by incubation in phosphate buffer containing 1% bovine serum albumin (BSA), 0.1% cold water fish gelatin (electron microscopy [EM] grade) (Electron Microscopy Sciences, Hatfield, PA), and 0.05% saponin. Cells were reacted for 2 h with HN- and Cav-1-specific antibodies diluted in blocking buffer. Monolayers were washed extensively in multiple changes of blocking buffer over 60 min. Primary antibody binding was visualized by sequential reaction with host-specific 0.6-nm ultrasmall-gold-conjugated F(ab')₂ fragments (Aurion; Electron Microscopy Sciences, Hatfield, PA) and washed by two cycles of silver enhancement to generate two populations of size markers as described previously (81). Cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 30 min, washed, and then postfixed with 0.5% osmium tetroxide in 0.1 M phosphate buffer for 15 min. Finally, cells were washed, dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon resin. Sections 60 to 70 nm thick were lightly stained with aqueous 1% uranyl acetate so that the small markers would be easily visible. Mock-infected cells were processed in parallel as controls.

Immunofluorescence. CV-1 cells were grown at low density on glass coverslips. Cells were infected with PIV-5 at an MOI of 5 PFU/cell. At 18 h p.i. cells were fixed for 15 min with 4% formaldehyde in PBS at room temperature. Nonspecific binding was blocked by a 30-min incubation in PBS containing 0.2% BSA, 0.1% cold water fish gelatin (Sigma, St. Louis, MO), 0.1% normal donkey serum, and 0.05% saponin. PIV-5 M and Cav-1 were detected by simultaneously staining cells with specific antibodies diluted in block buffer. Cells were then washed, and antibody binding was detected by host-specific Alexa Fluor-conjugated secondary IgGs (Invitrogen, Eugene, OR). PIV-5 HN and Cav-1 were stained sequentially; HN was stained first in a block buffer lacking saponin so that only surface-expressed HN would be stained, and then Cav-1 was stained intracellularly using block buffer containing saponin to permeabilize the cells. Coverslips were mounted on slides using Prolong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Eugene, OR) and examined on a 200 M inverted microscope equipped with an Apotome optical sectioning device (Zeiss, Thornwood, NY). Mock-infected cells were similarly stained as controls. Colocalization of HN and M with caveolin 1 was quantified using Velocity software (Improvision, Waltham, MA) on unedited image files of fluorescently stained infected cells. Images were compared to a threshold, and Pearson's *R* coefficient was calculated over 50 cells, with over- and underexposed cells excluded from analysis.

RESULTS

Cav-1 colocalizes with matrix (M) and hemagglutinin-neuraminidase (HN) proteins. To examine if Cav-1 colocalizes with PIV-5 proteins in virus-infected CV-1 cells, we used dual-color fluorescence staining. As shown in Fig. 1, Cav-1 demonstrated a punctuate staining pattern mainly localized to the plasma membrane, a pattern which is consistent with its known localization in clustered caveolar microdomains (24, 57). Staining for surface HN protein (Fig. 1A) and intracellular M protein (Fig. 1B) showed that these proteins are concentrated in patches on or at the plasma membranes of the virus-infected cells. Many filamentous virion particles were observed. The

FIG. 1. Cav-1 colocalizes with M and HN in PIV-5-infected cells. CV-1 cells grown on coverslips were either uninfected (Con.) or infected (Inf.) with PIV-5 at an MOI of 5 PFU/cell. At 18 h p.i. the cells were fixed with formaldehyde. HN (A) or M (B) and Cav-1 were stained as described in Materials and Methods. Bound IgG was detected with host-specific secondary antibodies conjugated to either Alexa Fluor 488 or 594. Nuclei were stained with DAPI. Cells were imaged on a Zeiss 200 M inverted microscope equipped with an Apotome optical sectioning device. M background staining (not shown) was similar to HN staining of uninfected cells.

organized filaments and patches may represent specific assembly sites where viral budding events occur. In addition to the punctate pattern Cav-1 staining was found to colocalize with the patches containing HN and M staining, as shown in the merge image (yellow) (Fig. 1). Pearson coefficient values calculated for the yellow signals were 0.65 ± 0.12 and 0.52 ± 0.09 for HN-Cav-1 and M-Cav-1 complexes, respectively, confirming significant overlap of fluorescence signals. These data suggest that Cav-1 colocalizes with the viral proteins HN and M and that this interaction may take place at viral assembly and budding sites.

Cav-1 is clustered at sites of viral budding. To investigate further the colocalization of Cav-1 with viral budding sites, Cav-1 and HN were immunogold stained in thin sections of PIV-5-infected cells (Fig. 2). To observe Cav-1 labeling, cells were permeabilized with saponin and Cav-1 was stained with 0.6-nm gold particles, followed by silver enhancement (see Materials and Methods). Heavily labeled HN on both spherical and filament structures was observed, and these structures are thought to be budding virus (Fig. 2). Significant amounts of Cav-1 were found to be clustered at the inner leaflets of the plasma membrane regions, in presumed caveolar microdomains (Fig. 2A to C, black arrows), and the Cav-1 labeling was found to be in very close proximity to HN labeling. Moreover, labeling of Cav-1 in virus particles was also observed (Fig. 2 A to C, white arrows). These data indicate that Cav-1 is concentrated at the sites of budding and is incorporated into mature

viral particles. As described below, immunoblotting analysis of purified PIV-5 particles showed that Cav-1 was incorporated into virions (see Fig. 6A, lane 3), suggesting that Cav-1 may be involved in the assembly and budding processes of PIV-5.

Association of HN, F, and M with detergent-resistant membrane microdomains. Caveolar and raft membrane microdomains, enriched with sphingolipids and cholesterol, can be isolated by their insolubility in TX-100 at 4°C (7, 18). The distribution of PIV-5 proteins among membrane fractions differentially solubilized by Triton X-100 was analyzed. Approximately 50% of HN, 66% of M, and 50% of F₁ were found to be insoluble in 1% TX-100 at 4°C (Fig. 3, lanes I). In contrast, the uncleaved F₀ precursor was found to be 82% soluble in TX-100 and was largely excluded from these microdomains (Fig. 3). Proteolytic cleavage of F₀ by the host cell protease furin occurs in the *trans*-Golgi network, and sorting of proteins into microdomains also occurs in the *trans*-Golgi network; hence, it is consistent that F₀ is soluble whereas cleaved F₁/F₂ is insoluble in TX-100.

To analyze further the fractions of viral proteins that associate with caveolar membranes, a sucrose density flotation gradient assay was performed on PIV-5-infected cells after solubilization with TX-100. Cav-1 was used as a marker for caveolae. As shown in Fig. 4, Cav-1 is highly enriched in fractions 4 to 6, which correspond to the interphase between 5% and 30% sucrose, and this fractionation pattern is consistent with the known sedimentation properties of caveolar mem-

FIG. 2. Cav-1 is clustered at the sites of viral budding in PIV-5-infected HeLa cells. HeLa cells were infected with PIV-5 at an MOI of 5 PFU/cell. At 18 h p.i. cells were fixed with formaldehyde and permeabilized with saponin. HN and Cav-1 were stained using ultrasmall gold reagents followed by sequential silver enhancement to generate markers of two different sizes as described in Materials and Methods. HN is identified by the small, approximately 5-nm gold-silver particles and Cav-1 by the larger, approximately 15-nm-diameter markers. Cav-1 can be seen clustered at the plasma membrane in a distribution consistent with caveolae (black arrows); however, Cav-1 (white arrows) is also found in budding PIV-5 virions, which are identified by morphology and the density of HN on their surfaces. Additional fields of caveolin 1 clusters at sites of budding spherical (B) and filamentous (C) virions are shown with magnification. Bars = 500 nm.

branes (38). In addition, a fraction of HN, M, and F₁/F₂ float into these Cav-1-rich fractions, indicating their localization to lipid rafts and caveolar membranes. As expected, F₀ remained at the bottom of the gradient in the nonraft fractions. The amount of the viral proteins found in the caveolin 1-rich fractions in Fig. 4 is considerably lower than that observed in the TX-100 insolubility assay (Fig. 3). We interpret this as being a consequence of the fact that different protein isolation conditions as well as protein detection techniques (metabolic labeling and immunoprecipitation versus immunoblotting) were used.

Cav-1 interacts with the PIV-5 M protein. The association of the viral proteins with the detergent-resistant microdomains and the localization of Cav-1 to sites of viral budding suggest that Cav-1 interacts with viral proteins. To investigate an interaction between Cav-1 and the PIV-5 proteins further, co-immunoprecipitation experiments were performed. A complex containing Cav-1, M, and HN in PIV-5-infected cells (Fig. 5A)

FIG. 3. TX-100 solubility of HN, F, and M. PIV-5-infected HeLa cells at 16 h p.i. were metabolically labeled with a ³⁵S translabel for 1 h and incubated in unlabeled medium for a further 2 h. Cells were extracted with 1% TX-100 at 4°C, and soluble (S) and insoluble (I) fractions were prepared by centrifugation. Proteins were immunoprecipitated (IP), and polypeptides were analyzed by SDS-PAGE.

was observed. In addition, as the antisera used to detect HN also detects NP, we identified NP in a complex with M. This result confirms previous data demonstrating an interaction between M and NP (49) and suggested its importance for assembly and budding of Sendai virus (74), RSV (23, 56), and PIV-5 VLP formation (70).

Cav-1 has been shown to interact with and modulate the action of many different proteins via a scaffolding domain (CSD) that binds short sequence motifs rich in aromatic amino acids (ΦXΦXXXXΦ, ΦXXXXΦXXΦ, or the combined motif ΦXΦXXXXΦXXΦ, where Φ is any aromatic amino acid, [W, F, or Y]) (14, 35). A search for these motifs in the sequences of PIV-5 M and HN indicated that both proteins harbor a hypothetical Cav-1 binding motif. For HN, the motif was found at residues 394 to 401 (YXYXXXXY), but as this site is in the HN ectodomain, it is topologically unable to interact with Cav-1. Furthermore, in cells transiently expressing Cav-1 and HN, these proteins could not be colocalized or coimmunoprecipitated (data not shown). Therefore, the complex of Cav-1

FIG. 4. Analysis of DIGs on sucrose gradients. PIV-5-infected HeLa cells at 16 h p.i. were extracted with 1% TX-100 at 4°C. The lysate was then loaded at the bottom of a flotation sucrose density gradient and subjected to equilibrium centrifugation. The gradient was fractionated from the top, and polypeptides were analyzed by SDS-PAGE and immunoblotting.

FIG. 5. PIV-5 M interacts with Cav-1 and HN. (A) Coimmunoprecipitation of Cav-1 with HN, NP, and M was from lysates of PIV-5-infected HeLa cells as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with Cav-1-, M-, and HN-specific antibodies as indicated. (B) The C-terminal amino acid sequence of the PIV-5 M protein is shown aligned with those of members of the *Rubulavirus* genus in the subfamily *Paramyxovirinae*: mumps virus, human PIV-2 (HPIV-2), HPIV-4a, and NDV. Alignment analysis was carried out using Gene Inspector for Macintosh (Textco BioSoftware, Inc., NH) with the database accession numbers listed in Materials and Methods. Direct nucleotide sequencing of the PIV-5 RNA genome indicated a glycine at position 362 of the PIV-5 M protein (highlighted in green) and not valine, as appears in the protein sequence database. The conserved aromatic acids within the hypothetical Cav-1 scaffolding domains are shown in red. (C) Colocalization of Cav-1 with PIV-5 M protein was examined in HeLa cells grown on coverslips and transfected with 2 μ g pCAGGS M plasmid.

and HN observed in virus-infected cells by fluorescence staining (Fig. 1A) is not due to direct binding of these proteins. However, a binding domain was identified in the PIV-5 M protein (residues 355 to 363; FXXXXWXXF) that is conserved within the *Rubulavirus* genus (Fig. 5B). Strong colocalization of Cav-1 with PIV-5 M was found in HeLa cells transiently expressing the M protein (Fig. 5C) (Pearson coefficient of 0.72 ± 0.15). In addition, in transiently expressing cells, M is coimmunoprecipitated by antibodies specific for Cav-1 (Fig. 5D). It has been inferred previously that M protein associates with the cytoplasmic tail of HN and that this interaction is important for efficient assembly and budding of PIV-5 (67, 70). However, this interaction was not tested previously in coimmunoprecipitation experiments. To test this further, cells were transfected to express M and HN or M and HN Δ 2-13, the latter version of HN containing a deletion within the HN cytoplasmic tail (residues 2 to 13), and lysates were prepared for immunoprecipitation. It was found that, whereas M could coimmunoprecipitate with HN, it was not able to coimmunoprecipitate with HN Δ 2-13 (Fig. 5E). Taken together, these data suggest that in PIV-5-infected cells the M protein initiates a protein complex by binding to both Cav-1 (possibly through its CSD) and the HN cytoplasmic tail. However, it should be noted that the interaction of Cav-1 with M could be indirect, as we cannot rule out other host cell proteins in the complex.

The effect of Cav-1 expression on the interaction of HN and M in PIV-5-infected cells and incorporation of Cav-1 into virions. The formation of a complex containing M, HN, and Cav-1 proteins in PIV-5-infected cells suggests that Cav-1 could be incorporated into virions. To examine the incorporation of Cav-1 into mature viral particles, sucrose gradient-purified virus samples were analyzed by SDS-PAGE and immunoblotting with Cav-1 antibodies. Cav-1 was detected in PIV-5 virions (Fig. 6A, lane 3) as well as in the antibody control HeLa cell lysates (Fig. 6A, lane 4). Two additional low-molecular-weight bands were detected in the virions by the Cav-1 antibodies. These bands also appeared in lysates of the MDBK cells used to grow the PIV-5 (data not shown), but not in HeLa cell lysates (lane 4). The identities of these bands have not been investigated, but the bands may result from partial protein degradation in a cell-type-dependent process.

To examine the effect of Cav-1 expression on the PIV-5 assembly process, human breast adenocarcinoma MCF-7 cells that stably express human Cav-1 (MCF-7/Cav-1) were used (20). In wild-type (wt) MCF-7 cells Cav-1 expression is shut down by methylation of a CpG island in the 5' promoter region of the Cav-1 gene (17, 28). Immunoblotting experiments confirmed that, in contrast to MCF-7/Cav-1 cells, the wt cell line does not express detectable levels of Cav-1 (Fig. 6A, lanes 2

After 24 h, the cells were fixed with formaldehyde and permeabilized with 0.1% saponin, and intracellular localization of M and Cav-1 was detected by fluorescence microscopy as described in the legend to Fig. 1. The yellow overlay represents colocalization of M with Cav-1. (D and E) Coimmunoprecipitation experiments were carried out as described for panel A using lysates of HeLa cells transfected with pCAGGS empty vector plasmid (con.), pCAGGS plasmid encoding PIV-5 M (M), or PIV-5 HN (HN) (D) or cotransfected with PIV-5 M and PIV-5 HN (M,HN) or PIV-5 M and HN Δ 2-13 (M, Δ HN) proteins (E).

FIG. 6. Effect of Cav-1 expression on the interaction of HN and M in PIV-5-infected cells and its incorporation into virions. (A) PIV-5 viral particles grown in MDBK cells infected at an MOI of 0.2 PFU/cell for 4 days were purified from supernatants by centrifugation through sucrose gradients as described in Materials and Methods. Thirty micrograms of total protein of purified virus fractions (lane 3) and lysates of MCF-7 (lane 1), MCF-7/Cav-1 (lane 2), and HeLa cells (lane 4) were analyzed by SDS-PAGE and immunoblotting with actin- and Cav-1-specific antibodies as indicated. (B to D) Coimmunoprecipitation of Cav-1 (B), HN (C), and M (D) proteins was performed using lysates of PIV-5-infected MCF-7 or MCF-7/Cav-1 cells as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with Cav-1-, M-, and HN-specific antibodies as indicated. Uninfected cell lysate was used as the control (con.).

and 1, respectively). Using coimmunoprecipitation of Cav-1 in MCF-7/Cav-1 cells (Fig. 6B), we detected Cav-1 in a complex with HN and M proteins, a finding similar to that made using PIV-5-infected HeLa cells (Fig. 5A). To test the effect of Cav-1 expression on the interaction of HN and M proteins, MCF-7 cells were infected with PIV-5 and coimmunoprecipitation experiments were performed. It was found that there was a large reduction in the amount of M protein that was associated with HN in PIV-5-infected MCF-7 cells compared to that in MCF-7/Cav-1 cells (Fig. 6C). Likewise, in the reciprocal coimmunoprecipitation, M protein antibodies only weakly precipitated HN from MCF-7 cells (Fig. 6D). Interestingly, the amounts of NP detected by these antibodies were not affected by the expression of Cav-1 and were similar for both MCF-7 and MCF-7/Cav-1 cells (Fig. 6D), indicating that the NP and M interaction is Cav-1 independent. We conclude that the interaction of M with HN, in contrast to that with NP, is regulated by Cav-1 expression and that in the absence of Cav-1 expression the M protein is no longer tightly bound with the cytoplasmic tail of HN.

PIV-5 cell entry and replication are Cav-1 independent.

Entry of several viruses into cells, including the paramyxovirus RSV (79), has been shown to be dependent on the caveolar

FIG. 7. Cav-1-independent entry and replication of PIV-5. MCF-7 and MCF-7/Cav-1 cells were infected with various PFU of rPIV-5-GFP as indicated. GFP fluorescence intensity (EM525) was measured at 16 h p.i. as described in Materials and Methods. The data shown represent the values of fluorescence intensity as percentages of the samples infected with the highest number of PFU (1.5×10^5 PFU) for MCF-7 and MCF-7/Cav-1.

endocytic pathway (41). To determine if the uptake of PIV-5 is via caveola-dependent endocytosis, we infected MCF-7 and MCF-7/Cav-1 cells with a recombinant PIV-5 that expresses green fluorescent protein (rPIV-5-GFP) (71). Cells were infected with rPIV-5-GFP over a range of multiplicities of infection (MOI) (0 to 1.5×10^5 PFU), and GFP fluorescence intensity was measured at 16 h p.i. (Fig. 7). The extent of rPIV-5-GFP infection in MCF-7 cells was very similar to that for MCF-7/Cav-1-infected cells, indicating that Cav-1 expression has no effect or very little effect on the early stages of the viral life cycle (e.g., entry and replication).

Cav-1 expression increases the infectivity of PIV-5. The role of Cav-1 in late stages of PIV-5 infection (e.g., assembly and budding) was investigated. The multistep growth curves of PIV-5 grown in MCF-7 cells and MCF-7/Cav-1 cells were compared (Fig. 8A). As shown in Fig. 8A, after 24 h p.i. PIV-5 grew to a higher titer in MCF-7/Cav-1 cells (1 to 1.5 log units) than in MCF-7 cells, suggesting that Cav-1 serves as a positive regulator of PIV-5 growth.

Samples from the multistep growth curve were also examined for hemagglutinating (HA) activity, a property of the HN protein and a measure of virus particles released from cells. As shown in Fig. 8B, the HA titer of PIV-5 grown in MCF-7/Cav-1 cells was 11- to 14-fold greater than that of PIV-5 grown in MCF-7 cells.

To test the effect of Cav-1 on virus production in a second cell line, MDCK cells were stably transfected with a mutant P132L Cav-1 tagged with mRed at its N terminus. The P132L mutation in Cav-1 was identified in 16% of primary human breast cancer cells (26), and, when expressed in COS-7 and NIH 3T3 fibroblasts, the mutant was shown to behave in a dominant negative fashion by causing missorting of normal Cav-1 (33). These results were confirmed by fluorescence microscopy. Whereas wt mRed-tagged Cav-1 demonstrated a typical punctate membrane staining pattern, mRed-P132L-Cav-1 expression caused its retention at a perinuclear compartment (Fig. 9A). The control mRed-expressing cells showed a diffuse cytoplasmic staining distributed over the whole cell (Fig. 9A).

FIG. 8. Reduced infectivity of PIV-5 grown in MCF-7 cells. MCF-7 and MCF-7/Cav-1 cells were infected with PIV-5 at an MOI of 0.01 PFU/cell. The culture medium was harvested at 12-h intervals, and virus titer (A) and HA activity (B) were determined as described in Materials and Methods.

The MDCK cells stably transfected with mRed-Cav-1 (MDCK/mRed-Cav-1) and the mutant Cav-1 mRed-P132L-Cav-1 (MDCK/mRed-P132L-Cav-1) were used to examine the growth rate of PIV-5. As shown in Fig. 9B, PIV-5 grown in the MDCK cells expressing dominant negative mRed-P132L-Cav-1 grew to a titer of 1 log lower than that of virus grown in the control MDCK cells expressing mRed-Cav-1. Furthermore, PIV-5 grown in MDCK cells expressing mRed-P132-Cav-1 had a lower HA titer than virus grown in control mRed-Cav-1 (Fig. 9C).

Accumulation of host cellular proteins in virions grown in Cav-1 null cells. To determine if the reduced association of M with HN (Fig. 6C and D) caused by the lack of Cav-1 expression affected incorporation of proteins into mature virions and to examine if the lower HA titer of virus grown in MCF-7 cells was due to reduced HN incorporation, the polypeptide compositions of virions purified from MCF-7 and MCF-7/Cav-1 cells were compared. As shown in Fig. 10A, both of the virion preparations contained the known proteins encoded by PIV-5 (e.g., L, HN, NP, F₁/F₂, P, A, and M). It was observed that virions grown in MCF-7 cells incorporated many more proteins of host cell origin than virions grown in MCF-7/Cav-1 cells (Fig. 10A). To compare the levels of virion proteins in the two virus preparations, an immunoblotting analysis was performed (Fig. 10B). As expected Cav-1 was incorporated only into virions purified from MCF-7/Cav-1 cells. In addition, similar levels of actin, a cellular protein known to be found in PIV-5 (51, 77), and F were found in the virions. In contrast, the levels of HN and M were significantly lower in the particles purified from

FIG. 9. Reduced infectivity of PIV-5 grown in MDCK cells stably expressing a dominant negative Cav-1 mutation. (A) HeLa cells were grown on coverslips and transfected with 2 μ g pcDNA3 mRed-Cav-1, mRed-P132L-Cav-1, or mRed plasmids. After 24 h, the cells were fixed with formaldehyde and permeabilized with 0.1% saponin. Nuclei were stained with DAPI. Cells were imaged on a Zeiss 200 M inverted microscope equipped with an Apotome optical sectioning device. Intracellular localization of Cav-1 was detected using settings designed for mRed. (B and C) MDCK/mRed-Cav-1 and MDCK/mRed-P132L-Cav-1 cells were infected with PIV-5 at an MOI of 0.01 PFU/cell. The culture medium was harvested at 12-h intervals, and virus titer (B) and HA activity (C) were determined as described in Materials and Methods.

MCF-7 cells (approximately 1.8- and 1.5-fold, respectively) than in those purified from MCF-7/Cav-1 cells, indicating a defect in their incorporation into mature viral particles that was due to the lack of Cav-1 expression.

FIG. 10. Protein composition of PIV-5 virions purified from MCF-7 and MCF-7/Cav-1 cells. PIV-5 viral particles were grown in MCF-7 and MCF-7/Cav-1 cells infected at an MOI of 0.2 PFU/cell and incubated for 96 h. Virions were purified from supernatants by centrifugation through sucrose gradients as described in Materials and Methods. Purified virus was analyzed by SDS-PAGE, and the protein pattern was visualized by silver staining, where the migration of viral proteins and cellular actin are indicated (A), or immunoblotting with HN-, F-, M-, Cav-1-, and actin-specific antibodies, as indicated by arrowheads (B).

DISCUSSION

Assembly of negative-strand RNA viruses requires concentration of the viral components at specific regions of the plasma membrane. The assembled components are later packaged during budding within a lipid envelope derived from the host cell membrane. Although these processes are not fully understood, an accumulating number of studies suggest that lipid raft microdomains serve as a platform for assembly and budding of several enveloped viruses, including *Paramyxoviridae* (11, 42, 68). We examined the role of Cav-1, the major structured protein of caveolae, in assembly and budding of PIV-5. Our data show that Cav-1 interacts, directly or indirectly, with the PIV-5 M protein, and we identified a complex containing Cav-1, M, and HN at the plasma membranes of PIV-5-infected cells. As a result, Cav-1 is incorporated into viral particles. Virion production and infectivity were significantly reduced in host cells lacking wt Cav-1, and virions that bud from Cav-1 null cells accumulated high levels of host cellular proteins. Thus, Cav-1 is important for PIV-5 budding, which we speculate occurs from caveolae.

Cav-1 was first identified as an ~22-kDa tyrosine-phosphorylated protein in Rous sarcoma virus-transformed cells (24). Later it was shown to be required for the formation of caveola-like structures (57, 73). In most cells Cav-1 is targeted to caveolae (25, 57, 66); however, it is also localized in a cell-type-specific manner in endocytic vesicles called caveosomes, the Golgi apparatus, the endoplasmic reticulum (ER), and lipid droplets (54). In addition to its structural role in caveolar formation, Cav-1 also serves as a scaffolding protein that organizes and sequesters within caveolar membranes specific lipids (e.g., cholesterol and glycosphingolipids) and signaling mol-

ecules (e.g., growth factor receptors, src-like kinases, G proteins, and H-Ras) (45, 73). Cav-1 regulates caveola-dependent functions, and interaction of cytoplasmic proteins with Cav-1 was shown to be sufficient for targeting these proteins to caveolar and raft microdomains (5). In PIV-5-infected epithelial cells, we found using fluorescence microscopy that Cav-1 is colocalized to the plasma membrane with the viral proteins M and HN (Fig. 1). Moreover, thin section electron microscopy showed that PIV-5 buds from, or is in close proximity to, structures at the plasma membrane that are heavily stained by Cav-1 antibody labeled with gold particles and thus may indicate clusters of Cav-1 oligomers in caveolae. In addition, Cav-1 staining was observed within newly budded virions (Fig. 2). We have confirmed the presence of Cav-1 in purified virions grown in MDBK (Fig. 6A) and MCF-7/Cav-1 cells (Fig. 10B). In the latter case both the full-length Cav-1 α and Cav-1 β (which lacks 31 N-terminal residues) (65) isoforms were detected. The exact pathway leading to the incorporation of Cav-1 into PIV-5 virions has yet to be established. Our data suggest that this may be due to a Cav-1 interaction with the PIV-5 matrix protein, as demonstrated by coimmunoprecipitation experiments in both PIV-5-infected and M-transfected cells (Fig. 5A and D). In M-transfected cells, the Cav-1/M complex was visualized at the plasma membrane by fluorescence microscopy (Fig. 5C); thus, Cav-1 interaction and targeting of M to caveolar membrane are independent of other viral proteins. The importance of the Cav-1/M protein interaction in the viral life cycle is supported by the fact that the caveolin-binding motif found in the M sequence is conserved within the *Rubulavirus* genus (Fig. 5B).

It has been observed previously that Cav-1 is incorporated into NDV (29) and RSV (8) virions and also the avian retrovirus murine leukemia virus (MLV) (82). A viral protein interacting with Cav-1 for RSV and NDV has not been reported. To test whether their M proteins could interact with Cav-1, we searched for Cav-1 binding motifs in the M protein sequences. Both sequences, although enriched with aromatic amino acids, do not contain a full Cav-1 binding motif. However, the NDV M protein has two partial motifs located at amino acid residues 48 to 56 (FXXXXXFXF) and at residues 359 to 362 (YXXF). Interestingly, the motif in NDV at residues 48 to 56 aligns with the conserved motif identified for the *Rubulavirus* genus (Fig. 5B).

It is possible that the interaction of Cav-1 with PIV-5 M and the subsequent targeting to caveolar membranes constitute an important regulatory step of PIV-5 assembly and budding. We speculated that, during viral assembly, HN and M complexes bind with Cav-1, which in turn forms oligomers. This dynamic process of Cav-1 oligomerization may cause enhanced clustering of the viral complexes at the plasma membrane and trigger viral budding from caveolae, leading to incorporation of Cav-1 into the virions. Our data provide several lines of evidence supporting this hypothesis. First, if PIV-5 budding occurs at the caveolar membrane, then the viral proteins would be expected to concentrate at these sites. We observed that high levels of the viral proteins HN, M, and F₁/F₂ are found in TX-100-insoluble detergent-resistant membranes and are also present in Cav-1-rich fractions of sucrose density gradients. Second, consistent with this hypothesis is that HN associates with M and Cav-1, provided HN has a cytoplasmic tail. Third, the findings that virus entry is not affected by ablation of Cav-1

expression and that the absence of Cav-1 causes a decrease in virus infectivity support a role for Cav-1 in late stages of viral infection. The failure to exclude host proteins from the virions grown in cells lacking Cav-1 expression is consistent with an assembly defect. The caveat has to be added that, as reported previously for the commonly used virus purification technique, we cannot completely rule out the presence of contaminating microvesicles in the purified virus preparations (67). However, the high yields of virions produced, as well as the clean viral polypeptide pattern for the control virions that bud from Cav-1-expressing cells, support our conclusion. In addition, we also observed a specific reduction in the amounts of HN and M but not F or the host protein actin in the virions. The reduced incorporation of HN and M proteins into virions may be the outcome of the reduced interaction of M with HN, as observed in coimmunoprecipitation experiments in infected cells. It is possible that clustering of M and HN with Cav-1 has a positive effect on their concentration at budding sites and their efficient assembly into progeny virions. Interestingly, a large number of host proteins were also reported in purified samples of PIV-5 particles that contained an HN with a cytoplasmic tail deletion (67). Thus, reduced interactions caused by either an absence of Cav-1 expression or the lack of an HN cytoplasmic tail may lead to reduced association of HN with M, which has a severe effect on viral infectivity.

Taken together, the above data indicate that Cav-1 interacts with PIV-5 M most likely through a conserved Cav-1 binding motif, resulting in Cav-1 incorporation into virions. Cav-1 interactions with M induce its binding with HN, target the viral components to caveolar membrane microdomains, and result in incorporation of Cav-1 into virions. Cav-1 knockout in cells or expression of dominant negative Cav-1 significantly reduces viral infectivity, indicating that Cav-1 is important for efficient PIV-5 assembly and budding. It is predicted that the multifunctional protein Cav-1 will be implicated in several cellular pathways positively affecting late stages of PIV-5 infection.

ACKNOWLEDGMENTS

We thank Reay G. Paterson, Richard F. Gaber, Jeremy S. Rossman, and Sarah A. Connolly for helpful discussions. We are grateful to the lab of the late Mordechai Liscovitch (the Weizmann Institute, Israel) for providing cell lines and vectors. The electron microscopy was performed in the Northwestern University Biological Imaging Facility (Evanston campus).

This work was supported in part by a Public Health Service grant AI-23173 from the National Institute of Allergy and Infectious Diseases. D.R. is an Associate and R.A.L. is an Investigator of the Howard Hughes Medical Institute.

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