Involvement of the Cytoplasmic Domain of the Hemagglutinin-Neuraminidase Protein in Assembly of the Paramyxovirus Simian Virus 5

ANTHONY P. SCHMITT,1 BIAO HE,1 AND ROBERT A. LAMB1,2*

Howard Hughes Medical Institute1 and Department of Biochemistry, Molecular Biology, and Cell Biology,2 Northwestern University, Evanston, Illinois 60208-3500

Received 20 May 1999/Accepted 7 July 1999

Efficient assembly of enveloped viruses at the plasma membranes of virus-infected cells requires coordination between cytosolic viral components and viral integral membrane glycoproteins. As viral glycoprotein cytoplasmic domains may play a role in this coordination, we have investigated the importance of the hemagglutinin-neuraminidase (HN) protein cytoplasmic domain in the assembly of the nonsegmented negative-strand RNA paramyxovirus simian virus 5 (SV5). By using reverse genetics, recombinant viruses which contain HN with truncated cytoplasmic tails were generated. These viruses were shown to be replication impaired, as judged by small plaque size, reduced replication rate, and low maximum titers when compared to those features of wild-type (wt) SV5. Release of progeny virus particles from cells infected with HN cytoplasmic-tail-truncated viruses was inefficient compared to that of wt virus, but syncytium formation was enhanced. Furthermore, accumulation of viral proteins at presumptive budding sites on the plasma membranes of infected cells was prevented by HN cytoplasmic tail truncations. We interpret these data to indicate that formation of budding complexes, from which efficient release of SV5 particles can occur, depends on the presence of an HN cytoplasmic tail.

Many enveloped viruses bud from the plasma membranes of infected cells. Cytosolic viral components, including encapsidated viral genomes, gather at the cell surface in a coordinated manner with integral membrane glycoprotein “spikes” (reviewed in reference 10). As a result, budding occurs and large numbers of virions containing almost exclusively virally encoded proteins are released. Coordination during virus assembly presumably involves the cytoplasmic tails of glycoproteins, since they have the potential to make contacts with viral components in the interior of the cell. Such contacts might occur directly with the viral nucleocapsid (17, 30) or involve a matrix (M) protein, a peripheral membrane protein which underlies the membrane and possibly acts as a bridge between the glycoprotein cytoplasmic tails and the encapsidated viral genome (23). A role for viral glycoprotein cytoplasmic tails in the specificity of virus assembly has been established for several negative-strand RNA viruses (Mononegavirales). Recombinant rabies viruses possessing a G protein with a truncated cytoplasmic tail contained less G relative to other viral proteins (18), suggesting that specific incorporation of G into virions depends on the presence of the G protein cytoplasmic tail. Recombinant measles viruses containing alterations to the cytoplasmic tails of its spike glycoproteins, hemagglutinin (H), or fusion protein (F) also contained reduced amounts of the altered glycoproteins (7). An increase in the nonspecific incorporation of cellular proteins into these virions was also observed, further supporting the view that the glycoprotein cytoplasmic tails contribute to specificity in virus assembly. For Sendai virus, incorporation of tail-altered hemagglutinin-neuraminidase (HN) glycoproteins expressed in trans into virus particles was found to depend on a specific 5-amino-acid motif, SYWST, in the HN cytoplasmic tail (32). This motif is also found in the cytoplasmic tail of human parainfluenza virus type 1 HN but not in the cytoplasmic tails of other paramyxovirus glycoproteins.

In addition to providing for specificity in the assembly process, glycoprotein cytoplasmic tails have also been shown to promote efficient budding. This is best illustrated for the alphaviruses, which fail to bud when an interaction between the cytoplasmic tail of the E2 glycoprotein and the nucleocapsid core is disrupted (31, 36). Both rabies virus and vesicular stomatitis virus (VSV) recombinants containing deletions of the G protein cytoplasmic tail were found to bud inefficiently, judged by 5- to 10-fold reductions in the amounts of viral proteins released into the supernatants of virus-infected cells (18, 29). An influenza A virus lacking glycoprotein cytoplasmic tails has also been generated, and the budding process is seriously disrupted as judged by gross deformities in the shapes and sizes of released virions (14). These changes were most evident when the cytoplasmic tails of both neuraminidase (NA) and hemagglutinin (HA) were removed. Elimination of the cytoplasmic tail from either HA or NA alone affected virus budding (14). A role for viral glycoprotein cytoplasmic tails in the specificity of virus assembly has been established for several negative-strand RNA viruses (Mononegavirales). Recombinant rabies viruses possessing a G protein with a truncated cytoplasmic tail contained less G relative to other viral proteins (18), suggesting that specific incorporation of G into virions depends on the presence of the G protein cytoplasmic tail. Recombinant measles viruses containing alterations to the cytoplasmic tails of its spike glycoproteins, hemagglutinin (H), or fusion protein (F) also contained reduced amounts of the altered glycoproteins (7). An increase in the nonspecific incorporation of cellular proteins into these virions was also observed, further supporting the view that the glycoprotein cytoplasmic tails contribute to specificity in virus assembly. For Sendai virus, incorporation of tail-altered hemagglutinin-neuraminidase (HN) glycoproteins expressed in trans into virus particles was found to depend on a specific 5-amino-acid motif, SYWST, in the HN cytoplasmic tail (32). This motif is also found in the cytoplasmic tail of human parainfluenza virus type 1 HN but not in the cytoplasmic tails of other paramyxovirus glycoproteins.

In addition to providing for specificity in the assembly process, glycoprotein cytoplasmic tails have also been shown to promote efficient budding. This is best illustrated for the alphaviruses, which fail to bud when an interaction between the cytoplasmic tail of the E2 glycoprotein and the nucleocapsid core is disrupted (31, 36). Both rabies virus and vesicular stomatitis virus (VSV) recombinants containing deletions of the G protein cytoplasmic tail were found to bud inefficiently, judged by 5- to 10-fold reductions in the amounts of viral proteins released into the supernatants of virus-infected cells (18, 29). An influenza A virus lacking glycoprotein cytoplasmic tails has also been generated, and the budding process is seriously disrupted as judged by gross deformities in the shapes and sizes of released virions (14). These changes were most evident when the cytoplasmic tails of both neuraminidase (NA) and hemagglutinin (HA) were removed. Elimination of the cytoplasmic tail from either HA or NA alone affected virus morphology to a lesser extent. This suggests some degree of redundancy in the functions of the influenza virus NA and HA cytoplasmic tails in budding (14).

Simian virus 5 (SV5) is a member of the Rubulavirus genus within the Paramyxoviridae family of nonsegmented negative-strand RNA viruses (16). SV5 encodes three integral membrane proteins, F, HN, and small-hydrophobic protein (SH). SH mediates virus attachment to sialic acid-containing molecules on target cells and also facilitates release of progeny virions from virus-infected cells by catalyzing the removal of sialic acid from complex carbohydrate chains (reviewed in reference 16). F protein is involved in viral entry into cells by mediating membrane fusion at neutral pH (reviewed in reference 15). Unlike most paramyxovirus fusion proteins, SV5 F

* Corresponding author. Mailing address: Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 North Campus Dr., Evanston, IL 60208-3500. Phone: (847) 491-5433. Fax: (847) 491-2467. E-mail: ralamb@nwu.edu.
protein does not require coexpression of its homotypic HN protein in order to cause cell-cell fusion (2). SV5 F and HN proteins contain predicted cytoplasmic tails of 20 and 17 amino acids, respectively, and the cytoplasmic tail of the F protein has been found to be required for normal fusion activity (3). SH is predicted to have an 18-amino-acid cytoplasmic tail, but it seems unlikely that the SH cytoplasmic tail plays a critical role in virus assembly, as the entire SH gene is dispensable for normal growth of SV5 in cultured cells (12).

A reverse-genetics system was established recently for SV5, allowing the recovery of recombinant viruses from cloned DNA (13). We report here the use of this system to generate recovered SV5 (rSV5) viruses with truncations in the cytoplasmic tail of HN. We find that the presence of an HN cytoplasmic tail is necessary for efficient concentration of viral components into patches at the surfaces of SV5-infected cells and that in the absence of the HN cytoplasmic tail, release of progeny virus particles is inefficient.

MATERIALS AND METHODS

Plasmid construction and oligonucleotide-directed mutagenesis. Recombinant DNA techniques were performed according to standard procedures (1). The HN DNA sequence from rSV5 genomic clone pBH276 (GenBank accession no. AF052755 [13]) was subcloned into pGemMNN, a derivative of pGEM3 containing Necl and NcoMI restriction sites, to generate pGEMMNN-HN. This plasmid was used as a template for oligonucleotide-directed unique-site elimination mutagenesis (Pharmacia Biotech, Piscataway, N.J.) to generate N-termi nal deletions of HN. Each deletion removed an even multiple of 6 nucleotides from the HN DNA sequence. The nuclease sequence of the entire HN gene was confirmed for each mutant with an ABI Prism 310 genetic analyzer (Applied Biosystems, Inc., Foster City, Calif.). Transected HN genes were subcloned into the rSV5 genomic clone derivative pBH352 (pBH276 lacking the HN gene) by using the unique Necl and NcoMI restriction sites to generate HN-truncated SV5 genome plasmids.

Recovery of rSV5 containing HN cytoplasmic tail truncations. Cultures of A549, MDBK, CV-1, and BHK-21F cells were maintained as described previously (12). A549 cells in 3.5-cm-diameter wells (90% confluent) were infected with modified vaccinia virus Ankara (MVA) expressing bacteriophage T7 RNA polymerase (35) at a multiplicity of infection (MOI) of 3 PFU/cell. After 1 h, HN cytoplasmic tail-truncated genome plasmids, as well as helper plasmids bearing the genes encoding viral proteins NP, P, and L, were transfected into cells with Lipofectin (Gibco-BRL, Rockville, Md.). Plasmid amounts were as follows: 3.0 mg of SV5 genome plasmid, 1.2 mg of pUC19-NP3A (20), 0.3 mg of pGEM-P (33), and 1.5 mg of pGEM-L (21). After 24 h, the transfection medium was removed and the cells were overlaid with fresh CV-1 cells in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and the cultures were grown for an additional 42 h at 37°C. The transfection supernatant was replaced with DMEM supplemented with 10% fetal calf serum, and the cultures were grown for an additional 48 h at 37°C. For the HHN virus, the cells were washed three times with PBS, and cultures were grown in 0.5 ml of DMEM supplemented with 1% fetal calf serum for various periods (6, 12, 24, 48, 72, and 96 h) at 37°C. Medium was then harvested from the cultures, and virus titers were determined on confluent MDBK cells as described above. Purification and analysis of SV5 virions. Confluent MDBK cells were infected at an MOI of 0.01 PFU/cell with rSV5 (3.2 × 10^6 cells), rSV5 HNΔ29 (3.2 × 10^6 cells), or rSV5 HNΔ13 (3.2 × 10^6 cells). Seven days postinfection (p.i.), medium was harvested and clarified by low-speed centrifugation, and virus particles were pelleted in a Beckman type 19 rotor at 18,000 rpm for 1 h. Viral-containing pellets were lysed in PBS (0.1 M NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA), layered onto a 15 to 60% sucrose gradient, and centrifuged in a Beckman SW41 rotor at 24,000 rpm for 1 h. Thirty-six equal fractions were collected from the top of the gradient and assayed for the presence of viral nucleocapsid protein by dot blotting followed by immunodetection with an NP-specific polyclonal primary antibody and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Detection and quantification was performed with a STORM 860 imaging system (Molecular Dynamics, Sunnyvale, Calif.). Fractions containing detectable NP protein were pooled (fractions 20 to 29 in each case), diluted to 20 ml with NTE, and centrifuged in a Beckman T70 rotor (40,000 rpm, 1 h). Pellets were suspended in NTE and centrifuged through a second 15 to 60% sucrose gradient, and fractions were assayed for NP protein as described above. Fraction 25 from each gradient was analyzed by SDS-PAGE on a 10% polyacrylamide gel, and polypeptides were visualized by silver staining.

Fluorescence microscopy. CV-1 cells grown on glass coverslips were infected with rSV5, rSV5 HNΔ29, or rSV5 HNΔ13 at an MOI of 0.2 PFU/cell. At 16 h.p.i. monolayers were fixed with 1% methanol-free formaldehyde for 15 min and blocked with 1% bovine serum albumin in PBS. In further steps with cells to be stained, 2% goat anti-mouse immunoglobulin G (H+L) was used as the primary antibody, followed by 1:500 dilution of a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. Cells were washed six times with PBS-F, removed from dishes with PBS containing 50 mM EDTA, and fixed in suspension by addition of 0.2 to a final concentration of 0.5%. The fluorescence of 10,000 cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.).
washed six times with PBS, and fluorescence was visualized with a model LSM 400 confocal microscope (Zeiss, Inc., Thornwood, N.Y.).

**RESULTS**

Expression and intracellular transport of HN cytoplasmic tail-truncated proteins. To assess the importance of the HN cytoplasmic tail in SV5 assembly, we constructed a series of HN proteins truncated in their cytoplasmic tails. As illustrated in Fig. 1, each truncation progressively removed 2 amino acids from the N terminus of HN. This led ultimately to the complete removal of the predicted cytoplasmic domain from the protein in the case of HNΔ2-17. The use of variants differing in length from wt HN by 2n amino acids was advantageous in that rSV5 genomes harboring the truncations remained as even multiples of 6 nucleotides, thus abiding by the so-called rule of six found for many paramyxovirus genomes (5).

A potential concern with the use of altered HN proteins was that the alterations might cause misfolding and/or lack of proper transport of the proteins to the cell surface. Thus, the recovery of viruses containing these HN proteins would be unlikely, given the seemingly critical roles of HN in paramyxovirus attachment and release. For this reason, we first tested each individual HN protein for proper intracellular transport using the vaccinia virus T7 expression system (9). Transport of these proteins through the Golgi complex was assessed by endo H digestion. The cDNAs encoding wt and cytoplasmic tail-altered HN proteins were expressed in CV-1 cells, and HN-expressing cells were incubated with 35S-labeled amino acids for 15 min and then incubated in a nonradioactive chase medium for various times. HN was immunoprecipitated from cell lysates with MAb HN5a, and half of each immune complex was hydrolyzed addition and that the population of HN confirmed the presence of the desired truncation in each case (not shown).

Expression of truncated HN proteins in virus-infected cells was confirmed by analyzing HN expressed in CV-1 cells in infected with rSV5 harboring tail truncations was successful for HNΔ2-3, HNΔ2-9, HNΔ2-11, and HNΔ2-13. In each case rescue was achieved within three attempts. Recovery of infectious virus was recovered in 11 of 12 attempts as judged either by the observation of syncytia in BHK-21F cells infected with transfection supernatants or by the detection by immunofluorescence microscopy of viral proteins expressed in infected CV-1 cells. Rescue of rSV5 harboring tail truncations was successful for HNΔ2-3, HNΔ2-9, HNΔ2-11, and HNΔ2-13. In each case rescue was achieved within three attempts. Recovery of infectious virus failed for HNΔ2-5 (four attempts), HNΔ2-7 (eight attempts), and HNΔ2-15 (two attempts).

Rescued viruses were passaged once in CV-1 cells (a cell line refractive to MVA replication) and then plaque purified on BHK-21F cells. The identities of rescued viruses were confirmed by isolating total RNA from CV-1 cells infected with plaque-purified virus stocks and performing RT-PCR with genomic viral RNA as the template. Sequence analysis of the 5′ portion of HN confirmed the presence of the desired truncation in each case (not shown).

Expression of truncated HN proteins in virus-infected cells was confirmed by analyzing HN expressed in CV-1 cells infected with wt HN and the cytoplasmic tail-altered HN proteins were in-
polyacrylamide gels, samples were treated with peptide-N glycosidase F to remove carbohydrate groups prior to analysis. As shown in Fig. 3, the HN cytoplasmic tail-altered proteins expressed by rSV5 HNΔ2-9 and rSV5 HNΔ2-13 migrated slightly faster than HN expressed by wt rSV5 whereas the NP proteins of these viruses comigrated. These data are consistent with the RT-PCR–sequencing analysis, confirming that the recombinant viruses encode HN containing deletions in its cytoplasmic tail.

Replication of HN cytoplasmic tail-truncated viruses in cultured cells. Plaque-purified viruses were amplified in MDBK cells so that virus stocks of the highest possible titer could be obtained. rSV5 HNΔ2-9, rSV5 HNΔ2-11, and rSV5 HNΔ2-13 each reached a maximum titer of about 10⁶ PFU/ml in plaque assays. wt rSV5 replicated to a titer of approximately 10⁸ PFU/ml, and rSV5 HNΔ2-3 reached a similar titer. Plaques formed by HN cytoplasmic tail-truncated viruses were noticeably smaller than those formed by rSV5 (Fig. 4A), except those formed by rSV5 HNΔ2-3, which were similar in size to wt SV5 plaques (not shown). To investigate further the replication of SV5 recombinants, a growth curve experiment was performed for rSV5 HNΔ2-9 and rSV5 HNΔ2-13. MDBK cells were infected with viruses at an MOI of 1.0 PFU/cell. This was the highest possible MOI, given the titers of the HN tail-truncated virus stocks. At various times p.i. the culture media were harvested and virus titers were determined by plaque assay. As shown in Fig. 4B, there were substantial decreases in the amounts of infectious virus produced by cells infected with rSV5 HNΔ2-9 and rSV5 HNΔ2-13 relative to the amount produced by the rSV5 control virus. For these HN cytoplasmic tail-truncated viruses, most of the increase in virus titer occurred between 24 and 48 h p.i., whereas for rSV5, most virus replication occurred between 6 and 12 h p.i. Furthermore, the final titer achieved was 10- to 100-fold lower for the HN cytoplasmic tail-deletion viruses. These results indicate that truncations to the HN cytoplasmic tail result in substantial decreases in infectious virus production.

Physical characteristics of HN cytoplasmic tail-truncated virus particles. To determine whether total virus particle production was impaired by the HN cytoplasmic tail truncations, particles released from MDBK cells infected with rSV5 HNΔ2-9 and rSV5 HNΔ2-13 were purified and characterized. Medium was harvested from infected cells 7 days p.i., and virus particles were pelleted by ultracentrifugation. Particles were then purified by centrifugation through sucrose gradients, and fractions were collected. Virus particles were detected in gradient fractions by using a quantitative dot blot assay and an NP-specific serum. Sedimentation profiles for the different

---

**TABLE 1. Surface expression of HN cytoplasmic tail-truncated HN proteins**

<table>
<thead>
<tr>
<th>Protein expressed</th>
<th>Surface expression efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of positive cells</td>
</tr>
<tr>
<td>HN</td>
<td>93.0</td>
</tr>
<tr>
<td>HNΔ2-3</td>
<td>94.1</td>
</tr>
<tr>
<td>HNΔ2-5</td>
<td>94.7</td>
</tr>
<tr>
<td>HNΔ2-7</td>
<td>92.6</td>
</tr>
<tr>
<td>HNΔ2-9</td>
<td>94.3</td>
</tr>
<tr>
<td>HNΔ2-11</td>
<td>94.0</td>
</tr>
<tr>
<td>HNΔ2-13</td>
<td>94.1</td>
</tr>
<tr>
<td>HNΔ2-15</td>
<td>93.8</td>
</tr>
<tr>
<td>HNΔ2-17</td>
<td>25.5</td>
</tr>
<tr>
<td>None (mock transfected cells)</td>
<td>93.8</td>
</tr>
</tbody>
</table>

*HN proteins were synthesized in CV-1 cells with the vaccinia virus T7 expression system. Surface expression of HN was determined by flow cytometry at 16 h posttransfection with a cocktail of two HN-specific MAbs followed by a fluorescein isothiocyanate-conjugated secondary antibody. Values shown are averages of results from three experiments.

---

**FIG. 2. Intracellular transport of HN cytoplasmic tail-truncated proteins.** HN proteins were synthesized in CV-1 cells with the vaccinia virus T7 expression system. Cells were radiolabeled with 35S-Promix for 15 min and then incubated in chase medium for the indicated times. Mock-transfected cells (lane M) were processed with no chase. HN was immunoprecipitated from cell lysates, and half of each immune complex was incubated with (+) and without (−) endo H. Polypeptides were analyzed by SDS-PAGE on 10% gels (A). R and S denote the migrations of endo H-resistant and endo H-sensitive HN proteins, respectively. (B) The radioactivities in the endo H-sensitive and -resistant HN species were quantitated with a BioImager.
SV5 recombinants are shown in Fig. 5. wt SV5 particles are known to be heterogeneous in size and shape, and as a result, particles distribute across a fairly broad density range (Fig. 5A). No substantial differences in sedimentation profiles were observed for rSV5 HN\(\Delta 2-9\) and rSV5 HN\(\Delta 2-13\), suggesting that particle size and shape were not grossly affected by the HN cytoplasmic tail truncations (Fig. 5B and C).

The total yield of particles released for each virus was calculated by summing the amounts of NP detected across all sucrose gradient fractions shown in Fig. 5 and taking into account differences in the numbers of cells that were infected with each virus. The yield of released particles per cell infected was reduced 7-fold for rSV5 HN\(\Delta 2-9\) and 12-fold for rSV5 HN\(\Delta 2-13\) (Table 2). Thus, the cytoplasmic tail of HN is important for the efficient budding of progeny virions.

To determine if truncations of the HN cytoplasmic tail affected HN incorporation into virus particles, gradient fractions were analyzed by SDS-PAGE (Fig. 6). wt SV5 preparations consisted predominantly of the known SV5 structural proteins, together with cellular actin, which has previously been identified as a cellular component of purified SV5 particles (24, 34). As seen in Fig. 6 and confirmed by the quantitation of stained polypeptides (not shown), HN cytoplasmic tail-truncated virus preparations were not substantially defective in HN protein incorporation, as the ratios of HN to NP and HN to M were similar in wt rSV5 and HN cytoplasmic tail-truncated virus preparations. No defects in the incorporation of other virus-encoded proteins into virions, including the F1, M, and P proteins, were detected. However, for rSV5 HN\(\Delta 2-9\) and rSV5 HN\(\Delta 2-13\) there were dramatic increases in the amounts of nonviral proteins contained in the virus preparations, with actin being particularly abundant. To assess whether these nonviral proteins were incorporated into virions or whether the viral preparations were contaminated with microvesicles, samples were analyzed by electron microscopy. Virions were observed in all preparations, and as expected, their morphology was pleomorphic even for wt rSV5. No obvious differences in size or shape were observed between SV5 and HN cytoplasmic tail-truncated recombinants. The amounts of virus particles relative to amounts of lipid contaminants were found to be roughly similar between rSV5 and HN cytoplasmic tail-truncated virus preparations (not shown). Thus, it is unlikely that the increases in cellular proteins relative to the amounts of

FIG. 3. Expression of HN cytoplasmic tail-truncated proteins in recovered-virus-infected cells. CV-1 cells were mock infected or infected with the indicated recovered recombinant viruses and radiolabeled with \(^{35}\)S-Promix at 48 h p.i. SV5 HN and NP proteins were immunoprecipitated from cell lysates, and immune complexes were digested with peptide N-glycosidase F (PNGase). Polypeptides were then analyzed by SDS-PAGE on 8% gels. The positions of NP and unglycosylated HN are indicated.

FIG. 4. Growth curve analysis of HN cytoplasmic tail-truncated rSV5. MDK cells were infected with the indicated viruses at an MOI of 1.0 PFU/cell, and the culture medium was harvested at the indicated times. Virus titers were determined by plaque assay on BHK-21F cells. Plaques (A) and growth curves (B) of wt rSV5, rSV5 HN\(\Delta 2-9\), and rSV5 HN\(\Delta 2-13\) are shown. Values plotted represent averages of results from two experiments.
viral proteins in the HN cytoplasmic tail-truncated virus preparations can be accounted for by contamination with cellular microvesicles. These data suggest that HN cytoplasmic tail-truncated viruses have a defect in the exclusion of cellular host proteins from progeny virions.

Redistribution of viral proteins at the surfaces of cells infected with HN cytoplasmic tail-truncated viruses. One key step in the budding of enveloped viruses is thought to be the coalescence of both cytosolic and membrane-bound viral components into patches at the plasma membranes of virus-infected cells, thus allowing production of progeny virions that are highly concentrated with viral proteins but from which cellular components are excluded (8). To examine the localization of viral proteins, CV-1 cells were infected with rSV5 HN\textsubscript{D2-9} and rSV5 HN\textsubscript{D2-13}, and viral proteins were collected and assayed for NP protein by dot blotting. The density and amount of NP (arbitrary units) for each fraction are shown.

**FIG. 5.** Density gradient purification of HN cytoplasmic tail-truncated SV5 virions. MDBK cells were infected with wt rSV5 (A), rSV5 HN\textsubscript{D2-9} (B), and rSV5 HN\textsubscript{D2-13} (C) at an MOI of 0.01 PFU/cell, and culture medium was harvested at 7 days p.i. Virus particles were pelleted by ultracentrifugation, resuspended, and centrifuged through sucrose gradients. Thirty-six equal fractions were taken from the top of the gradient, and fractions were assayed for NP protein by dot blotting. NP-containing fractions (fractions 20 to 29) were pooled, virus particles were pelleted by ultracentrifugation, and samples were further purified by centrifugation through a second sucrose gradient. Thirty-six fractions were collected and assayed for NP protein by dot blotting. The density and amount of NP (arbitrary units) for each fraction are shown.

**FIG. 6.** Polypeptide composition of HN cytoplasmic tail-truncated SV5 virions. wt rSV5, rSV5 HN\textsubscript{D2-9}, and rSV5 HN\textsubscript{D2-13} were grown in MDBK cells, and virions were purified by centrifugation through two sequential sucrose gradients. Polypeptides from purified gradient fractions were fractionated by SDS-PAGE on 10% gels and visualized by silver staining. The positions of viral proteins, as well as cellular actin, are indicated.
gest that the bulk of the cytoplasmic tail of HN is required for efficient organization and concentration of HN into the presumptive budding sites.

To investigate whether the distribution of other viral proteins is affected by deletion of the bulk of the HN cytoplasmic tail, the localization of the M and F proteins was examined. M protein staining of saponin-permeabilized cells suggested that in rSV5-infected cells, the M protein is organized into patches on the cytosolic face of the plasma membrane in a distribution that is similar to that of the HN protein. However, in cells infected by the cytoplasmic-tail-truncated viruses rSV5 HNΔ2-9 and rSV5 HNΔ2-13, M protein was found distributed randomly throughout the cytoplasm. Double-label confocal microscopy staining for HN and M was not performed due to the lack of appropriate antibody reagents. Nonetheless, the similarity of HN and M staining patterns suggests that targeting of M protein into presumptive budding sites at the cell surface depends on the presence of an HN cytoplasmic tail.

In rSV5-infected cells, the distribution of F protein staining was found to be more heterogeneous than that of the HN or M protein. Patches of F protein staining were often observed to be superimposed on a background of evenly distributed staining. There was also significant cell-to-cell variation in F protein staining. Patches of F staining were identified clearly only in approximately 50% of positive cells. The other cells showed fairly uniform staining for F protein. In cells infected with the HN cytoplasmic tail-truncated viruses rSV5 HNΔ2-9 and rSV5 HNΔ2-13, F protein localization into patches was observed in less than 5% of positive cells. Thus, while the localization of the F protein seemed to be affected in HN cytoplasmic tail-altered viruses, the differences were less obvious in comparison to those observed for the distributions of the HN and M proteins.

**Figure 7.** Localization of viral proteins in cells infected with HN cytoplasmic tail-truncated viruses. CV-1 cells grown on glass coverslips were infected with wt rSV5, rSV5 HNΔ2-9, and rSV5 HNΔ2-13. At 16 h p.i. cells were fixed with formaldehyde (and for M protein staining, they were permeabilized with 0.1% saponin) and bound with MAbs specific to the SV5 HN, M, or F protein and then with fluorescein isothiocyanate-conjugated secondary antibodies. Fluorescence was examined with a Zeiss LSM 400 confocal microscope with a 1-μm-thick optical section.
of 0.002 PFU/cell and at various times p.i. cells were fixed, stained, and examined by phase-contrast microscopy. As shown in Fig. 8, at 24 h p.i. few syncytia were observed in rSV5-infected cells but large syncytia had already formed in cells infected with rSV5 HNΔ2-13. At 72 h p.i. large numbers of distinct syncytia were seen in the rSV5-infected monolayer, but these were moderately sized. In contrast, in cells infected with rSV5 HNΔ2-9 and rSV5 HNΔ2-13 almost the entire monolayer of cells consisted of large aggregates of nuclei, indicating that extensive cell-cell fusion had occurred. At 96 h p.i., more than 90% of these cells had detached from the monolayer whereas the rSV5-infected monolayer remained intact and still contained distinct, moderately sized syncytia (not shown). Thus, virus-mediated syncytium formation was more rapid and extensive in CV-1 cells infected with the HN cytoplasmic tail-truncated viruses rSV5 HNΔ2-9 and rSV5 HNΔ2-13 than in cells infected with wt rSV5.

DISCUSSION

The paramyxovirus assembly pathway involves the encapsidation of viral genomic RNAs, the movement of both cytosolic and membrane-bound viral components to budding sites, envelopment of nucleocapsids by cellular membranes containing the spike glycoproteins, and the release of virus particles. The coalescence of viral components at budding sites has been presumed to be facilitated by the interaction of the cytoplasmic tails of the glycoproteins and the viral M proteins. However, direct biochemical and biophysical evidence to support this notion has been very difficult to obtain, in part because of the poor solubility of the M proteins at physiological salt concentrations. Here, we used a genetic approach and found that upon removal of most of the residues of the HN cytoplasmic tail (8 or 12 of 17 amino acids), movement of HN and M proteins to the presumptive budding sites in SV5-infected cells did not occur. It is expected that if the association of viral components depended on interactions of the cytoplasmic tail with the M protein, then a similar glycoprotein redistribution would be observed in viruses lacking an M protein. Although we have been unsuccessful to date in our attempts at recovering rSV5 lacking an M gene (11a), generation of measles virus lacking an M protein (and M gene) was reported recently (6). Unlike with wt measles virus, where the H and P proteins were found localized in patches in the virus-infected cell, with measles virus containing the M gene knockout, the H and P proteins were found distributed more homogeneously throughout the infected cell. Thus, redistribution of viral components from an organized to a random distribution has now been observed for paramyxoviruses deficient for either M protein or a glycoprotein cytoplasmic tail. Surprisingly, no such redistribution was observed in measles virus recombinants containing F or H proteins with altered cytoplasmic tails. Neither removal of 14 of 34 amino acids from the H protein cytoplasmic tail nor replacement of the F protein cytoplasmic tail sequence with that of Sendai virus, or both, resulted in the redistribution of measles virus proteins in virus-infected cells (7). For measles...
virus it was suggested that other forces for assembly may exist in addition to those involving the glycoprotein cytoplasmic tails, e.g., interactions involving the glycoprotein transmembrane domains or indirect associations involving membrane rafts (7). With SV5, it does not appear that such interactions outside the glycoprotein cytoplasmic tails are sufficient for organization of viral proteins into the presumptive budding sites, as viral proteins were randomly distributed upon truncation of the HN cytoplasmic tail. One caveat needs to be added concerning interpretation of the data obtained with measles virus because budding even for wt measles virus is poor (19) and much of the infectious material is cell associated; sonication of infected cells increases the virus yield. Thus, for measles virus lacking an M protein the infectivity titer is so low (3.6 \times 10^3 50% tissue culture infective doses [TCID_{50}]/ml versus 8 \times 10^4 TCID_{50}/ml for wt measles virus [6]) that it is difficult to distinguish between reduced active virus budding and the endogenous rate of passive, non-M protein-driven vesiculation of the plasma membrane, with the vesicles containing viral glycoproteins and a nucleocapsid. This situation is analogous to that with the VSV G protein-containing vesicles that envelope an RNA replicon expressing the G protein (27). Therefore, there needs to be a means of distinguishing bona fide viruses from “gollum” viruses, infectious material that has been passively assembled that lacks a genetic “soul” necessary for efficient budding.

It is thought that the purpose of coalescing viral components into budding sites is to ensure that progeny virions are highly concentrated in viral proteins and possibly to facilitate the budding process itself (8). rSV5 containing deletions in the HN cytoplasmic tail in addition to displaying altered localization of HN and M also showed a reduction in virus particle production. Thus, for SV5, the cytoplasmic tail of HN is important for forming budding complexes from which efficient release of particles can occur, possibly because interactions between HN and M have been impaired. However, another possibility that cannot be excluded is that HN-M-RNP complexes form normally in the absence of the HN cytoplasmic tail but that they redistribute to sites that are not competent for budding. Our results suggest that failure of HN and M to coalesce at budding sites on the cell surface leads to fewer budding events, although at least some particles are released that are infectious and morphologically similar to wt particles.

Purified preparations of HN cytoplasmic tail-truncated SV5 particles had a much greater content of cellular proteins than wt virions. A similar result was observed for measles virus with altered H or F cytoplasmic tails (7). Although it is tempting to speculate that this is due to a failure to exclude host proteins from virions, it is difficult to rule out the possibility that the cellular proteins were supplied by contaminating microvesicles. With human immunodeficiency virus type 1, it was shown that in many cases purified virus preparations are contaminated with microvesicles that coexist with virions on sucrose gradients (4, 11). Thus, the presence of cellular proteins in gradient-purified virus preparations does not demonstrate that these proteins are physically associated with virus particles. This finding is particularly relevant in cases where the yield of virus particles is low, as a relatively large number of cells have to be infected to obtain a sufficient number of particles for analysis. Therefore, we cannot rule out the possibility that some of the additional cellular proteins contained in HN cytoplasmic tail-truncated SV5 preparations were supplied by microvesicles. However, we did examine the preparations by electron microscopy and we found no gross changes in purity or the occurrence of large numbers of empty particles. Thus, we favor the interpretation that host protein exclusion into progeny virions is impaired as a result of HN cytoplasmic tail truncations.

Rapid and extensive cell-cell fusion was induced by infection with HN cytoplasmic tail-truncated SV5. Similar fusion phenotypes were also observed for measles viruses with F or H proteins containing altered cytoplasmic tails and for measles virus lacking an M protein (6, 7). One possible explanation for the increase in cell-cell fusion is overaccumulation of F protein at the surfaces of infected cells, possibly due to lack of budding. We observed that in MDBK cells infected with rSV5 HN tail-truncated viruses, approximately twofold more F protein accumulated at the cell surface than in rSV5-infected cells at 72 h p.i. as measured by flow cytometry (our unpublished observation). Another explanation for increased fusion activity originally suggested by Cathomen and colleagues is that an interaction of M protein with the cytoplasmic tails of the glycoproteins modulates fusion activity (6, 7).

The SV5 HN cytoplasmic tail-truncated viruses constitute the first step towards defining the amino acids within the SV5 HN cytoplasmic tail which contribute to virus assembly in a sequence-specific manner. As rSV5 HNΔ2-3 was phenotypically indistinguishable from wt virus but rSV5 HNΔ2-9 was assembly defective, it can be inferred that the specific residues of the cytoplasmic tail spanning amino acids 4 through 9 (EDAPVR) are necessary for normalSV5 assembly. We had originally hoped to define with even greater precision amino acids within the HN cytoplasmic tail that are important for SV5 assembly by rescuing a complete set of viruses containing progressive deletions to HN of 2n amino acids. However, although we rescued SV5 containing the HNΔ2-3, HNΔ2-9, HNΔ2-11, and HNΔ2-13 cytoplasmic tail deletions, we failed in several attempts to recover virus containing the HNΔ2-5 and HNΔ2-7 cytoplasmic tail deletions, which would have allowed more precise mapping of the assembly phenotypes to specific amino acid residues. These results suggest that the HNΔ2-5 and HNΔ2-7 truncations are particularly detrimental to SV5 replication, although lack of rescue itself does not demonstrate lack of viability. It is possible that for these deletions, the protein structure formed by the residual cytoplasmic tail residues was inhibitory for virus assembly or other aspects of virus replication. 

It is not known whether the EDAPVR amino acid sequence in the cytoplasmic tail of HN is itself important for SV5 assembly or whether there is a nonspecific requirement for a cytoplasmic tail. With VSV, it was found that cytoplasmic and transmembrane sequences of glycoprotein G could be replaced by the corresponding, unrelated sequences from the human CD4 protein with relatively mild consequences for budding but that deletion of the G cytoplasmic tail severely impaired virus budding (29). Furthermore, a revertant to the cytoplasmic tail-deletion virus was selected and found to encode an 8-amino-acid cytoplasmic tail unrelated in sequence to the normal G cytoplasmic tail, and this short cytoplasmic tail was sufficient to promote normal VSV budding (29). By analogy, it is possible that assembly phenotypes observed here upon truncation of the SV5 HN cytoplasmic tail result not from the elimination of a specific assembly signal but from a nonspecific requirement for a cytoplasmic tail. Sequence-specific assembly motifs have been identified in the cytoplasmic tails of other viruses, however. For example, a specific tyrosine-containing motif that is critical for budding has been identified in the cytoplasmic tail of the E2 glycoprotein of Semliki Forest virus (36). Also, it was recently shown that a specific motif within the cytoplasmic tail of the Sendai virus HN protein (SYWST) is important for HN incorporation into virions (32). This motif is also found in the HN cytoplasmic tail of the related paramyxovirus, human para-
influenza virus type 1, and two other paramyxoviruses (human para-influenza virus type 3 and bovine para-influenza virus type 3) contain a portion of this motif (YW). The 17-amino-acid cytoplasmic tail of SV5 HN, however, completely lacks both tyrosine and tryptophan residues and shows poor homology to the HN cytoplasmic tails of other paramyxoviruses, suggesting that if specific sequence requirements exist, different paramyxoviruses can contain their own unique signals for efficient virus particle assembly. The generation of additional SV5 re-combinants containing amino acid substitutions within this region of the HN cytoplasmic tail should provide further insight into the specific requirements for cytoplasmic tail-dependent assembly events.

ACKNOWLEDGMENTS

We thank George Leser for performing the electron microscopy on the virus preparations and Andrew Pekosz for helpful discussions. This work was supported in part by research grant AI-23173 from the National Institute of Allergy and Infectious Diseases. A.P.S. and B.H. are associates and R.A.L. is an investigator of the Howard Hughes Medical Institute.

REFERENCES