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Mumps Virus Matrix, Fusion, and Nucleocapsid Proteins Cooperate for Efficient Production of Virus-Like Particles[▽]

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Paramyxovirus particles, like other enveloped virus particles, are formed by budding from membranes of infected cells. To define mumps virus (MuV) proteins important for this process, viral proteins were expressed either singly or in combination in mammalian cells to produce virus-like particles (VLPs). Only the MuV matrix (M) protein when expressed by itself was capable of inducing particle release, but the quantity of these M-alone particles was very small. Efficient production of mumps VLPs occurred only when the M protein was coexpressed together with other viral proteins, with maximum production achieved upon coexpression of the viral M, nucleocapsid (NP), and fusion (F) proteins together. Electron microscopy analysis confirmed that VLPs were morphologically similar to MuV virions. The two MuV glycoproteins were not equal contributors to particle formation. The F protein was a major contributor to VLP production, while the hemagglutinin-neuraminidase protein made a smaller contribution. Evidence for the involvement of class E protein machinery in VLP budding was obtained, with mumps VLP production inhibited upon expression of dominant-negative versions of the class E proteins Vps4A and Chmp4b. Disruption of the sequence 24-FPVI-27 within the MuV M protein led to poor VLP production, consistent with findings of earlier studies of a related sequence, FPIV, important for the budding of parainfluenza virus 5. Together, these results demonstrate that different MuV structural proteins cooperate together for efficient particle production and that particle budding likely involves host class E protein machinery.

Mumps virus (MuV) is a paramyxovirus from the *Rubulavirus* genus. Prior to mass vaccination, mumps was a very common childhood illness, with characteristic symptoms including fever, fatigue, and inflammation of the salivary glands. Less frequently, MuV infection results in serious complications including aseptic meningitis and encephalitis (22). Significant outbreaks of mumps have occurred recently in the United Kingdom (6), Canada (40), and the United States (7, 14), highlighting the continued relevance of this disease even in countries where vaccination is widespread. Like other paramyxoviruses, MuV possesses a genome that consists of single-stranded negative-sense RNA, encapsidated by a nucleocapsid (NP) protein and associated with an RNA-dependent RNA polymerase complex composed of large protein and phosphoprotein subunits. This core is linked to the virion membrane by matrix (M) protein. The outer surface of the virion is covered with glycoprotein spikes consisting of the hemagglutinin-neuraminidase (HN) protein, which binds sialic acid to allow virion attachment to cells, and fusion (F) protein, which induces viral and cellular membranes to fuse together during virus entry. Additional components of MuV include the small hydrophobic protein, which prevents infected cells from undergoing apoptosis (67), and V protein, which prevents induction of interferon-induced antiviral responses (29, 30, 62). The late steps of the MuV life cycle that allow for assembly and budding of MuV virions remain for the most part unexplored.

Enveloped virus particles are formed by budding from cellular membranes at specific locations at which viral proteins, and often host factors, have assembled together. For the negative-strand RNA viruses, coordination among the different viral components during virus assembly appears to be directed by the viral matrix proteins, which have the potential to interact with the cytoplasmic tails of the viral glycoproteins and with viral ribonucleoproteins (RNPs) in the cytoplasms of infected cells. M proteins likely assemble as layers beneath the plasma membranes of infected cells and induce other viral components to gather at these locations, from which virus budding occurs (reviewed in references 49 and 57).

For many viruses, it has been possible to achieve assembly and budding of particles from cells that have been transfected to produce one or more viral proteins in the absence of virus infection. These particles often resemble virions morphologically and have been termed virus-like particles (VLPs). VLP production provides a useful means for determining the individual roles of different virus proteins in particle formation, and in some cases the VLPs themselves have shown promise as vaccines (45). For most negative-strand RNA viruses, VLP formation is critically dependent on the presence of the viral matrix proteins (49). Indeed, in the cases of Newcastle disease virus (NDV) (37) and Nipah virus (11, 38), M protein expression is sufficient for highly efficient VLP production, with no apparent need for assistance from any of the other viral structural components, such as the viral glycoproteins or NP proteins. In the case of NDV, incorporation of glycoproteins and

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NP proteins into the budding VLPs requires specific interactions involving the M protein, but these interactions do not appear to facilitate the budding process itself (37).

Although expression of viral matrix protein is sufficient for robust VLP production in the above cases, it has long been thought that additional viral components are also important for efficient budding of many negative-strand RNA viruses. For example, an important role for viral glycoproteins in virus assembly has been established based on studies with recombinant viruses that contain glycoproteins lacking their cytoplasmic tails (4, 17, 26, 34, 35, 48, 52, 66) and analyses of assembly-defective subacute sclerosing panencephalitis measles virus strains (5, 47). In fact, recent evidence suggests that for influenza virus it is the viral glycoproteins (and not viral matrix protein) that are the main drivers of virus budding (9). For other negative-strand RNA viruses, expression of viral glycoproteins together with matrix proteins in some cases significantly enhances the efficiency of VLP release. Ebola VLPs (31), Sendai VLPs (55, 56), and parainfluenza virus 5 (PIV5)-like particles (51) are all produced more efficiently in the presence of viral glycoprotein expression. Ebola virus glycoprotein in some cell types functions during virus release to inhibit the action of tetherin, a cellular protein which functions to prevent the release of enveloped virus particles from infected cells (28). In addition to the viral glycoproteins, other viral components can also enhance the production of VLPs. Production of Ebola VLPs and PIV5-like particles can be further enhanced through expression of the corresponding NP proteins (31, 51), and Sendai VLP production is enhanced through expression of Sendai virus C protein (55). Hence, for these viruses, multiple proteins cooperate with one another to achieve maximum VLP production. The extent to which particle formation actually requires this cooperation differs, however. In the case of PIV5, it is absolutely essential; expression of the M protein alone does not lead to VLP production (51). On the other hand, cooperation among viral proteins is beneficial but not strictly required for the production of Sendai or Ebola VLPs, since expression of the matrix proteins of these viruses is sufficient for VLP production (20, 55, 56, 61).

The late steps of negative-strand RNA virus budding may occur in a way that is analogous to the budding of retroviruses, which employ protein-protein interaction domains called late domains to manipulate host machinery and allow release of virus particles (reviewed in references 1 and 3). Cellular factors recruited by late domains in many cases are class E proteins that are part of the vacuolar protein sorting (Vps) pathway of the cell. Indeed, disruption of the Vps pathway through expression of dominant-negative (DN) versions of the Vps4 ATPase protein blocks the budding of many retroviruses (reviewed in reference 1), as well as the budding of Ebola virus (32), Lassa fever virus (63), and PIV5 (50). However, other negative-strand RNA viruses, such as influenza virus, bud particles in ways that are not substantially affected by disruption of the cellular Vps pathway (reviewed in reference 8).

Here, experiments are described which define MuV proteins important for the assembly and budding of VLPs. Using proteins derived from the 88-1961 wild-type (wt) strain of MuV, optimal production of mumps VLPs is shown to occur upon coexpression of the MuV M, F, and NP proteins together in transiently transfected mammalian cells. Evidence is also provided that supports a role for cellular class E protein machinery in the budding of mumps VLPs.

MATERIALS AND METHODS

Plasmids. MuV cDNAs corresponding to the M, NP, HN, and F genes were obtained by reverse transcription and PCR amplification of RNA isolated from cells infected with MuV strain 88-1961 (GenBank accession no. AF467767) and were subcloned into the eukaryotic expression vector pCAGGS (36) to generate the plasmids pCAGGS-MuV M, pCAGGS-MuV NP, pCAGGS-MuV HN, and pCAGGS-MuV F, respectively. cDNA encoding the MuV RW strain M protein with a C-terminal hemagglutinin tag, a kind gift of Takemasa Sakaguchi (Hiroshima University, Japan), was subcloned into the pCAGGS vector to generate the plasmid pCAGGS-MuV M-HA (RW). Site-directed mutants of the MuV M (strain 88-1961) cDNA sequence were made by PCR mutagenesis, and their identities were confirmed by DNA sequencing of the entire M gene (Macrogen Inc., South Korea). The plasmids pCAGGS-PIV5 M, pCAGGS-PIV5 NP, pCAGGS-PIV5 HN, and pCAGGS-PIV5 F (51) were kind gifts of Robert Lamb (Northwestern University, Evanston, IL). The plasmids pGFP-Vps4A and pGFP-Vps4A E228Q (18) were kind gifts of Wesley Sundquist (University of Utah, Salt Lake City). The plasmid pEGFP-CHMP4B (24) was a kind gift of Takemasa Sakaguchi (Hiroshima University, Japan). cDNAs corresponding to the Nipah virus M and N genes, kind gifts of Paul Rota (Centers for Disease Control and Prevention, Atlanta, GA), were subcloned into pCAGGS vectors to generate the plasmids pCAGGS-NiV M and pCAGGS-NiV N.

Antibodies. Polyclonal antibodies specific to the MuV M, NP, F, and HN proteins were generated by immunizing rabbits with the following peptides: M protein amino acids (aa) 110 to 123 (DQTDIRVRKTASDK); NP protein aa 460 to 473 (ARQGGQNDFRAQPL); F protein aa 523 to 537 (HINTISSSVDDL IR); and HN protein aa 1 to 14 (MEPSKLFTISDNAT). Peptide synthesis and antibody production were carried out by GenScript Corp. (Piscataway, NJ). Monoclonal antibodies M-h, HN1b, and NPa, specific to the PIV5 M, HN, and NP proteins (44), respectively, were kind gifts of Richard Randall (St. Andrews University, St. Andrews, Scotland, United Kingdom). Polyclonal antibody Fsol, specific to the PIV5 F protein, was a kind gift of Robert Lamb (Northwestern University, Evanston, IL). To generate antibodies specific to the Nipah virus M and N proteins, the corresponding full-length cDNAs were subcloned into pRSETB (Invitrogen, Carlsbad, CA) and the proteins were expressed in *Escherichia coli* by autoinduction (54). The resulting His-tagged recombinant proteins were purified by metal affinity chromatography and sent to Harlan Bioproducts for Science (Indianapolis, IN) for rabbit immunization and polyclonal antibody production. Polyclonal antibody specific to green fluorescent protein (GFP) was purchased from Clontech (Mountain View, CA).

Measurements of VLP production and virion production. 293T cells in 10-cm-diameter dishes (70 to 80% confluent) grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum were either infected with MuV strain 88-1961 (a kind gift of Steve Rubin) with a multiplicity of infection (MOI) of 0.1 PFU/cell or transfected with plasmids encoding MuV, Nipah virus, and/or PIV5 proteins to generate VLPs. Transfections were carried out in Opti-MEM using Lipofectamine-Plus reagents (Invitrogen, Carlsbad, CA). Plasmid quantities per dish were as follows unless otherwise indicated: pCAGGS-MuV M and derivatives, 1.5 μg; pCAGGS-MuV NP, 250 ng; pCAGGS-MuV F, 1.5 μg; pCAGGS-MuV HN, 1.0 μg; pCAGGS-NiV M, 1.2 μg; pCAGGS-PIV5 M, 800 ng; pCAGGS-PIV5 NP, 140 ng; pCAGSS-PIV5 F, 3.0 μg; pCAGGS-PIV5 HN, 3.0 μg; pGFP-VPS4A, 200 ng; pGFP-VPS4A E228Q, 200 ng. Transfections were supplemented with a pCAGGS plasmid lacking an insert when necessary to equalize total plasmid DNA quantities.

At 48 h postinfection or 24 h posttransfection, the culture medium was replaced with Dulbecco's modified Eagle medium containing 1/10 the normal amount of methionine and cysteine and 40 μCi of [^{35}S]Promix/ml (Perkin Elmer, Waltham, MA). After an additional 10 h, the cells and media were harvested for protein expression and VLP production analysis.

To analyze the VLPs or virions released from cells, culture media were centrifuged at 7,500 × *g* for 2 min to remove cell debris and then layered onto 20% sucrose cushions (10 ml in NTE [0.1 M NaCl; 0.01 M Tris-HCl, pH 7.4; 0.001 M EDTA]). Samples were centrifuged at 140,000 × *g* for 1.5 h. Pellets were resuspended in 0.5 ml of NTE and mixed with 1.3 ml of 80% sucrose in NTE. Layers containing 50% sucrose (1.8 ml) and 10% sucrose (0.6 ml) in NTE were applied to the tops of the samples, which were then centrifuged at 110,000 × *g* for 3 h. A 2.1-ml volume was collected from the top of each gradient, and VLPs/virions contained within this floated fraction were pelleted by centrifugation at 140,000 × *g* for 1.5 h. VLP/virion pellets were resuspended in sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 2.5% (wt/vol) dithiothreitol.

Cell lysate preparation and immunoprecipitation of proteins was performed as described previously (51). The precipitated proteins and VLPs were analyzed by SDS-PAGE using 10% gels. Detection and quantification were performed using a Storm 860 imaging system (GE Healthcare, Piscataway, NJ). Budding efficiency was calculated as the M-protein-specific counts in culture media divided by the M-protein-specific counts in the corresponding cell lysates and normalized relative to values obtained in control experiments.

Electron microscopy. To generate virions and VLPs for electron microscopy, 293T cells in 10-cm-diameter dishes (five dishes per group) were infected with MuV (strain 88-1961) at a MOI of 0.1 PFU/cell or transfected with plasmids to produce MuV M, NP, F, and HN proteins, MuV M, NP, and F proteins, or MuV M and F proteins. At 64 h postinfection or 40 h posttransfection, the culture medium was harvested. Virions/VLPs were purified by centrifugation through sucrose cushions and flotation on sucrose gradients as described above. Virion and VLP pellets were resuspended in 75 μl of NTE by passaging 10 times through a 26-gauge needle. Insoluble material was removed by centrifugation at 14,000 × *g* for 5 min. Ten microliters of virion/VLP preparation was adsorbed onto carbon-coated Parlodion copper grids and stained with 1% uranyl acetate. Samples were examined using a Jeol JEM 1200 EX II electron microscope operated at 80 kV.

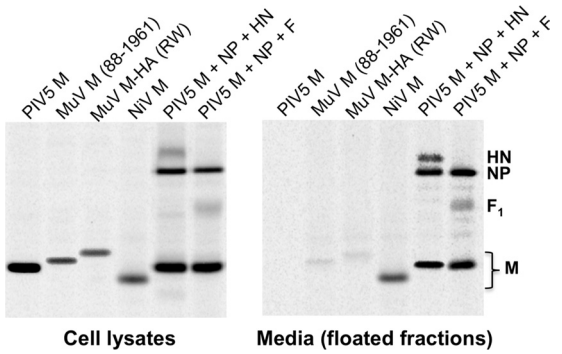

FIG. 1. Paramyxoviruses differ from one another in the requirements for VLP production. 293T cells were transfected to produce the indicated viral proteins. Radiolabeled viral proteins from cell lysates were collected by immunoprecipitation. Particles from culture supernatants were purified by centrifugation through sucrose cushions and flotation on sucrose gradients. Viral proteins from both cell lysates and purified particles were fractionated by SDS-PAGE and detected using a phosphorimager. The result shown is a representative example of three independent experiments.

RESULTS

MuV proteins cooperate with one another to direct efficient particle production. To determine if the MuV M protein alone has the ability to elicit particle formation and release, this protein was expressed by itself in 293T cells via transient transfection. For comparison, M proteins of other paramyxoviruses, PIV5 and Nipah virus, were expressed separately. In each case, the amount of plasmid DNA used for transfection was selected to maximize particle production, as determined in initial titration experiments which are not shown. Cells were metabolically labeled for 10 h, after which the cells and culture media were harvested. Proteins from cell lysates were collected by immunoprecipitation. Note that expression levels of the different M proteins should not be directly compared in this experiment because each was detected using a different antibody. Particles collected from the culture media were ultracentrifuged through 20% sucrose cushions and further purified by flotation on sucrose gradients. Particles in the floated fractions were pelleted, resuspended, and loaded directly on SDS gels (with no immunoprecipitation). Consistent with earlier findings (51), no particles could be detected upon expression of the PIV5 M protein by itself; particles could be detected only if PIV5 M protein was coexpressed with other PIV5 proteins (Fig. 1). In contrast, Nipah virus M protein expressed by itself led to efficient production and release of particles (Fig. 1), a result that is in agreement with earlier findings (11, 39). Thus, these paramyxoviruses differ substantially in the requirements for efficient particle production. When the MuV M protein was expressed, a low level of particle production could be detected (Fig. 1). M proteins from two different strains of MuV (88-1961 strain and RW strain) were expressed, and similar results were obtained in each case. These results indicate that the MuV M protein can elicit only very weak particle production when it is expressed alone in 293T cells.

We next performed a quantitative analysis of the particle production that results from expression of different combinations of MuV proteins in cells (M, NP, F, and HN proteins, all from strain 88-1961) (Fig. 2A and B). All plasmid amounts were optimized in titration experiments to maximize particle production (data not shown). Of the different combinations tested, M+NP+F led to the most efficient particle production. In this case, particles were made with an efficiency that was, on average, more than 20 times greater than that observed when the M protein was expressed by itself (Fig. 2B). Particle production efficiency was quantified on the basis of M protein signal detected in purified particles divided by the amount of M protein signal detected in the corresponding cell lysate fraction. When expressed individually, only the M protein led to weak but detectable production of particles; the NP, F, and HN proteins all failed to produce detectable levels of particles when expressed alone.

The MuV F protein was a major contributor to particle production efficiency, while the MuV HN protein was, at best, a minor contributor. Inclusion of the F protein together with the M and NP proteins led to particle production at a level that was about 10 times greater than that observed without F protein coexpression (Fig. 2A and B). On the other hand, inclusion of the HN protein together with the M and NP proteins led to particle production at a level that on average was only 2.5-fold greater than that observed without HN protein coexpression. Interestingly, inclusion of both the F and HN proteins together with the M and NP proteins reproducibly led to a level of particle production that was reduced from the level observed with just M+NP+F (Fig. 2B). Similarly, M+F+HN particles were produced at a reduced level compared with that for M+F particles (data not shown). Together, these results indicate that the MuV F protein makes a large, positive contribution to particle production while the MuV HN protein (in the absence of F protein expression) makes a smaller, positive contribution to particle production. These two activities are not additive, however, with the presence of the HN protein seemingly causing an impairment in the ability of the F protein to enhance particle formation.

MuV NP protein expression was necessary to achieve maximum particle production. Inclusion of the NP protein together with the M and F proteins led to particle production at a level that was on average 4.3-fold higher than that observed without NP protein expression (Fig. 2A and B). This positive effect of

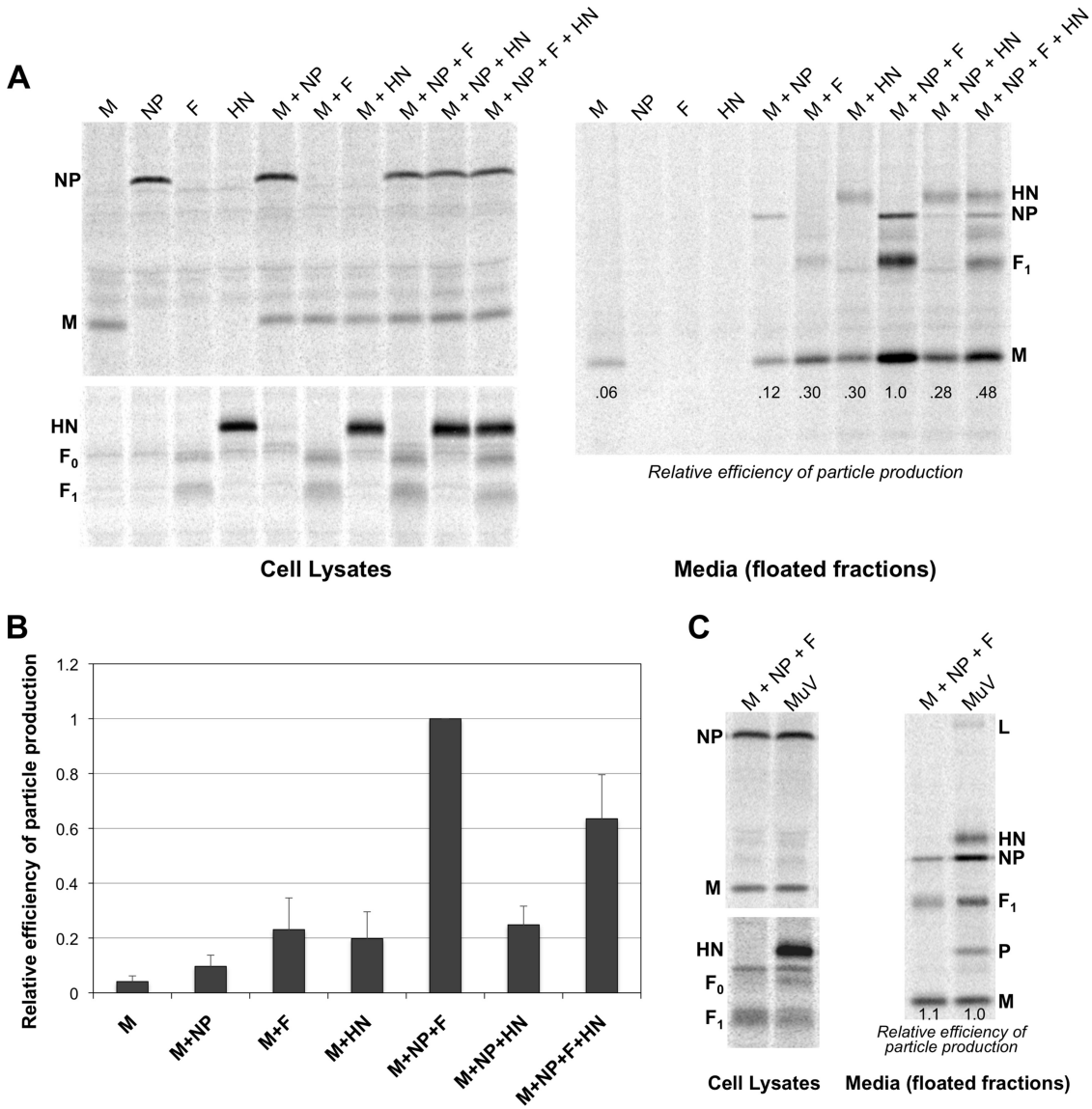

FIG. 2. MuV proteins cooperate with one another to direct efficient particle production. (A) 293T cells were transfected to produce the indicated MuV proteins for VLP production. Efficiency of particle production was calculated as the amount of M protein detected in the culture medium divided by the amount of M protein detected in the corresponding cell lysate and was normalized relative to the value obtained upon M+NP+F transfection. (B) Relative particle production efficiency values were calculated from three independent experiments performed as for panel A, with standard deviations indicated by error bars. (C) 293T cells were transfected to produce MuV M+NP+F VLPs or were infected with MuV. VLPs/virions were purified and quantified, with values normalized to the particle production efficiency that was obtained upon MuV infection.

NP protein expression on particle production could also be observed independently of the viral F protein, since M+NP particles were produced at a level that was on average 2.5-fold higher than that observed for M-alone particles. Inclusion of NP protein expression did not substantially affect the production of M+HN particles, however. Overall, these results demonstrate that optimal production of MuV particles, similar to the optimal production of PIV5-like particles (51) and Ebola VLPs (31), requires NP protein expression.

The transfection-based MuV particle production system was evaluated further by comparing particle production to the level of MuV virions produced after MuV infection. Equivalent amounts of 293T cells either were transfected to produce M+NP+F particles or were infected with MuV (MOI = 0.1). Cells were metabolically labeled for 10 h, starting at 24 h posttransfection or at 48 h postinfection (conditions that maximize particle production in each case). Proteins from cell lysates were immunoprecipitated and fractionated on SDS gels. As shown in Fig. 2C, the amounts of mumps virus M, NP, and F proteins produced by the transfected cells were roughly equivalent to the amounts of these proteins that were produced by virus-infected cells at the time of harvest. Particles were collected from the culture medium of transfected or infected cells, purified as before, and loaded directly on SDS

gels. The amount of M+NP+F particles produced from transfected cells was nearly the same as the amount of MuV virions produced from infected cells, as judged by M protein content (Fig. 2C). Hence, expression of MuV proteins in transfected cells can give rise to particle production that is efficient even compared to authentic MuV infection. However, it should be noted that these experiments cannot measure particle production on a per-cell basis. Thus, it is not clear whether all transfected cells or just a subset of those cells contributes to particle production in a meaningful way.

Although significant quantities of particles were produced from transfected cells, the polypeptide composition of these particles was noticeably different from that of virions. Particles produced by transfection incorporated slightly less F protein than that observed in virions (ratio of F to M signals was 0.49 on average for transfection versus 0.57 in virions; $n = 3$ experiments), although there was substantial variation in this ratio from experiment to experiment. Particles produced by transfection also incorporated substantially less NP protein than was the case with virions (the ratio of NP signal to M signal was 0.39 on average for transfection versus 1.71 for virions; $n = 3$ experiments). It should be noted that the NP protein incorporated into virions is generally in the form of full-length viral RNPs while the NP protein incorporated into particles produced by transfection is likely to be in the form of encapsidated cellular RNAs that are on average much shorter than RNPs.

MuV particles released from transfected cells are VLPs that resemble virions when viewed by electron microscopy. To characterize the morphology of particles produced in transfected 293T cells, particles were pelleted through 20% sucrose cushions, further purified using sucrose flotation gradients, concentrated, and viewed by electron microscopy with negative staining (Fig. 3). Three different configurations of particles were examined (M+NP+F+HN, M+NP+F, and M+F), and these were compared with authentic MuV virions produced from infected 293T cells. In all three cases, observations of the particles revealed them to be VLPs, since they were similar to virions with respect to particle size, particle shape, and the presence of an outer spike layer. Mumps VLPs ranged in size from approximately 100 nm in diameter at the smallest to approximately 200 nm, with an average size of approximately 150 nm. Authentic MuV virions had an average diameter that was slightly larger than that of VLPs, between 150 nm and 200 nm. The virions were heterogeneous in size and shape, as described in previous reports (15, 21), ranging in size from approximately 150 nm at the smallest to more than 300 nm. VLP shape was heterogeneous for all configurations, with the majority of particles being roughly spherical. VLPs possessed clearly visible spike layers with overall spike morphologies that are consistent with those of paramyxovirus virions. However, the VLP spikes generally did not appear to be packed together quite as densely as the spikes observed in virions. This finding is consistent with the polypeptide composition analysis (Fig. 2C) and suggests that mumps VLPs generated using the conditions optimized for this study incorporate glycoproteins with less efficiency than MuV virions. Attempts were made to observe M-alone mumps VLPs by electron microscopy, but these purified VLP preparations were not readily distinguishable from the vesicles and membrane debris purified from mock-transfected 293T cells (data not shown), a result that could be attributed to the very low efficiency of M-alone VLP production.

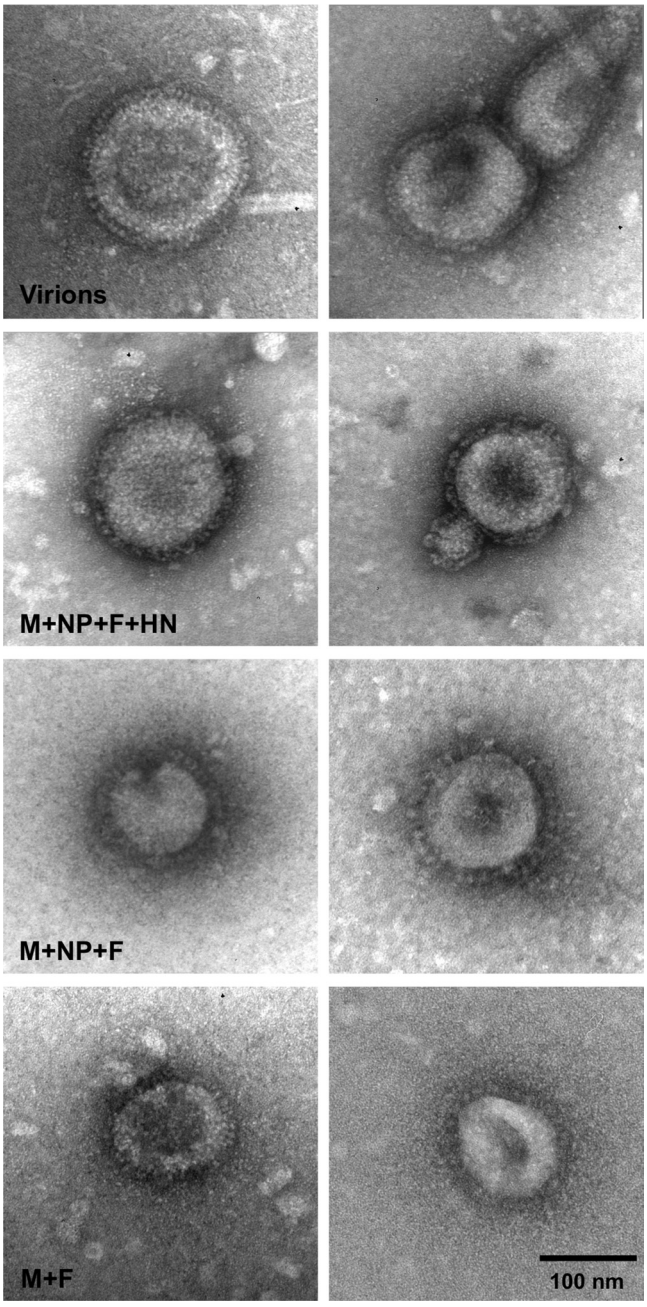

FIG. 3. Mumps VLPs produced from transfected cells resemble MuV virions morphologically. 293T cells were infected with MuV strain 88-1961 or were transfected to produce the indicated MuV proteins for VLP production. Virions/VLPs were purified by centrifugation through sucrose cushions and flotation on sucrose gradients as described in Materials and Methods. Virions/VLPs were adsorbed onto carbon-coated grids and visualized by transmission electron microscopy after negative staining.

Inhibition of mumps VLP production through expression of DN class E proteins. To investigate the importance of class E protein machinery in the budding of MuV particles, MuV proteins were coexpressed in 293T cells together with either

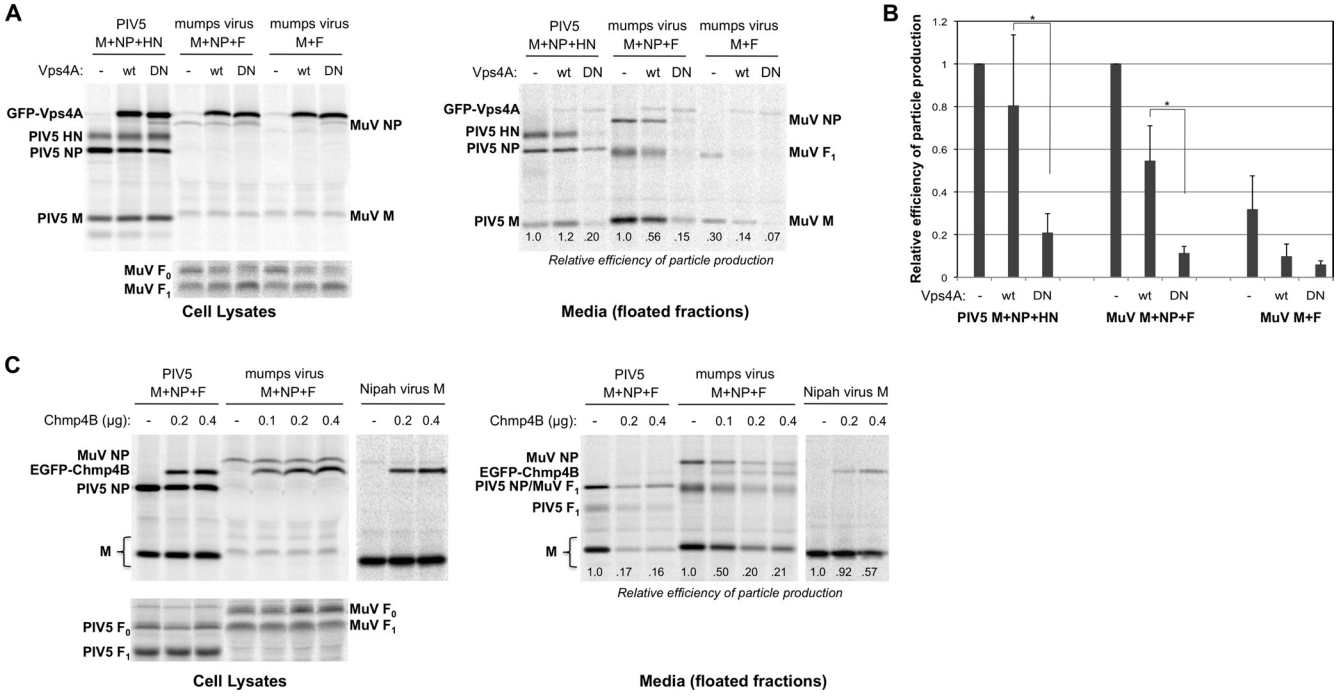

FIG. 4. Mumps VLP production is inhibited upon expression of DN versions of class E proteins. (A) 293T cells were transfected to produce PIV5 proteins or MuV proteins together with the GFP-Vps4A wt protein or the GFP-Vps4A DN protein that harbors the E228Q mutation, as indicated. VLP production efficiency was measured, and values were normalized to those obtained in the absence of Vps4A protein expression. (B) Relative particle production efficiency values were calculated from three independent experiments performed as for panel A, with standard deviations indicated by error bars. Differences between pairs were assessed for statistical significance using Student's *t* test, with *P* values of <0.05 denoted by an asterisk. (C) 293T cells were transfected to produce PIV5 proteins, MuV proteins, or Nipah virus proteins together with the EGFP-Chmp4B protein, as indicated. VLP production efficiency was measured, and values were normalized to those obtained in the absence of EGFP-Chmp4B protein expression.

the wt Vps4A protein or the DN Vps4A E228Q protein. Control experiments were performed in which these Vps4A proteins were coexpressed together with PIV5 proteins. Consistent with previous results (50), expression of the DN Vps4A protein reduced PIV5-like particle production (M+NP+HN configuration) to levels that were approximately 20% of control levels (Fig. 4A and B). Similar effects were observed for the production of PIV5 M+NP+F VLPs and PIV5 M+NP+F+HN VLPs (data not shown). Expression of the wt Vps4A protein generally had a minor effect on PIV5 VLP production, although substantial experiment-to-experiment variation was noted in this case (Fig. 4B). Mumps VLP production (M+NP+F configuration) was inhibited by DN Vps4A protein coexpression, with particle levels falling to approximately 15% of control levels and statistically significant differences observed between wt Vps4A and DN Vps4A protein expression ($P < 0.05$) (Fig. 4A and B). Similar results were obtained with MuV M+NP+F+HN VLPs (data not shown). The effect of DN Vps4A protein expression on production of MuV M+F VLPs was also investigated. Although production of M+F VLPs is inefficient compared with production of M+NP+F VLPs, it was clear that even this reduced level of VLP production was substantially inhibited upon expression of the DN Vps4A protein (Fig. 4A and B). However, coexpression of the wt Vps4A protein also reduced M+F VLP production substantially, and so the difference between VLP production in the presence of the wt Vps4A protein and VLP production in the presence of the DN Vps4A protein in this case was relatively small and statistically insignificant.

Further experiments were conducted to assess the effect of a DN enhanced green fluorescent protein (EGFP)-fused version of the Chmp4B protein (an ESCRT III component) on mumps VLP production. Production of MuV M+NP+F VLPs was reduced to approximately 20% of control levels upon coexpression of the DN Chmp4B protein (0.2 μg of plasmid DNA), and production of PIV5 M+NP+F VLPs was reduced to approximately 17% of control levels (Fig. 4C). Here, Nipah virus M-alone VLP production, previously shown to occur even after disruption of the cellular Vps pathway (39), was used as a control. DN Chmp4B protein expression (0.2 μg of plasmid DNA) barely affected Nipah virus M-alone VLP production, validating this experimental system. However, when 0.4 μg of the DN Chmp4B plasmid DNA was used, even Nipah virus M-alone VLP production was reduced to approximately 60% of control levels, suggesting the possibility of a nonspecific effect on VLP production when the DN Chmp4B protein is expressed at an inappropriately high level. Taken together, these experiments demonstrate that production of mumps VLPs and PIV5-like particles can be inhibited through expression of at least two distinct DN class E proteins.

Mutation of 24-FPVI-27 within the MuV M protein inhibits VLP production. A short sequence near the amino-terminal end of the PIV5 M protein, 20-FPIV-24, was identified in an earlier study as being necessary for budding function, with

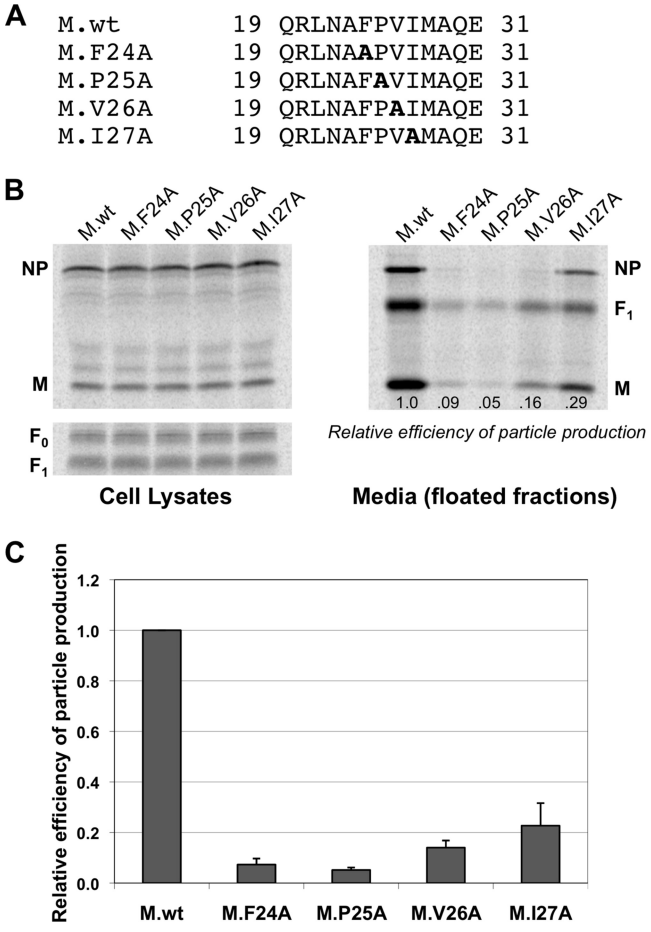

FIG. 5. Mutation of 24-FPVI-27 within the MuV M protein inhibits VLP production. (A) Schematic illustrating the amino acid positions within the MuV M protein that were targeted by alanine substitution mutagenesis. (B) 293T cells were transfected to produce the MuV NP and F proteins together with either the wt MuV M protein or the indicated alanine substitution mutant M proteins for VLP production. VLP production efficiency was measured, and values were normalized to those obtained with the wt MuV M protein. (C) Relative VLP production efficiency values were calculated from two independent experiments performed as for panel A, with standard deviations indicated by error bars.

residues F20 and P21 being the most critical (50). The MuV M protein contains a similar sequence, 24-FPVI-27. To test the importance of these residues for MuV M protein function and to validate the utility of the mumps VLP production system as a screening tool for mutagenesis studies, four alanine single-substitution mutant proteins targeting these residues were generated (Fig. 5A). Each of the altered M proteins was coexpressed together with the MuV NP and F proteins in 293T cells for VLP production. All of the mutant M proteins were stably expressed in cells and were detected in cell lysates at levels that were similar to that of the wt M protein, as judged by immunoprecipitation (Fig. 5B). Mumps VLP production was reduced severely when either F24 or P25 was changed to alanine (Fig. 5B and C). Mutations of residues V26 and I27 led to defects in VLP production that were less severe (Fig. 5B and C). Thus, the FPVI sequence within the MuV M protein is critical to VLP production, with residues F24 and P25 playing more important roles than residues V26 and I27. Taken together with earlier studies, these results demonstrate that FPIV-like sequences are important for the budding functions of multiple paramyxovirus M proteins.

MuV proteins can cooperate with PIV5 proteins for efficient VLP production in some cases. The PIV5 HN protein is a major contributor to PIV5-like particle production (Fig. 1) (51), while the MuV HN protein is, at best, a minor contributor to mumps VLP production (Fig. 2). Here, an experiment was conducted to test if the MuV HN protein could act as a major contributor to PIV5-like particle production. The PIV5 M and PIV5 NP proteins were coexpressed with increasing amounts of either the PIV5 HN protein or the MuV HN protein, and VLP production was analyzed. PIV5-like particles were abundantly produced no matter which HN protein was expressed (Fig. 6A; Table 1). In both cases, particle production was about fivefold more efficient than that observed in the absence of HN protein expression. Hence, either the PIV5 HN protein or the MuV HN protein can function to allow efficient PIV5-like particle production. Although these two HN proteins were interchangeable in this case, previous experiments have demonstrated a degree of specificity in the glycoprotein requirement, since it was not possible to generate PIV5-like particles efficiently using either the influenza virus hemagglutinin protein or the vesicular stomatitis virus (VSV) G protein in place of the PIV5 glycoprotein (51).

The converse experiment was also performed to test if the PIV5 HN protein could contribute to mumps VLP production. Consistent with the results shown in Fig. 2, coexpression of the MuV HN protein together with the MuV M and NP proteins led to only a small enhancement in VLP production. Replacement of the MuV HN protein with the PIV5 HN protein did not change this result substantially (Fig. 6B). Thus, neither the MuV HN protein nor the PIV5 HN protein could significantly affect the efficiency of mumps VLP release.

The PIV5 F protein is a major contributor to PIV5-like particle production (Fig. 1) (51), and likewise the MuV F protein is a major contributor to mumps VLP production (Fig. 2). Here, the PIV5 M and PIV5 NP proteins were coexpressed with either the PIV5 F protein or the MuV F protein, and VLP production was measured. PIV5-like particles were produced with almost equal efficiencies regardless of which F protein was expressed (Fig. 6C). Thus, either the PIV5 F protein or the MuV F protein can function to allow efficient production of PIV5-like particles.

In the converse experiment, the MuV M and MuV NP proteins were coexpressed together with either the MuV F protein or the PIV5 F protein. The PIV5 F protein was unable to completely replace the function of the MuV F protein in this case, since this substitution reduced particle production by more than fourfold (Fig. 6D). However, the PIV5 F protein did have an overall positive effect on mumps VLP production. VLP production was more than threefold higher in the presence of PIV5 F protein coexpression than it was when glycoprotein was omitted completely (Fig. 6D). This result indicates that the PIV5 F protein can partially function in place of the MuV F protein to allow production of mumps VLPs.

The PIV5 NP protein is required for optimal production of PIV5 VLPs (51), and the MuV NP protein is required for optimal production of mumps VLPs (Fig. 2), but the Nipah

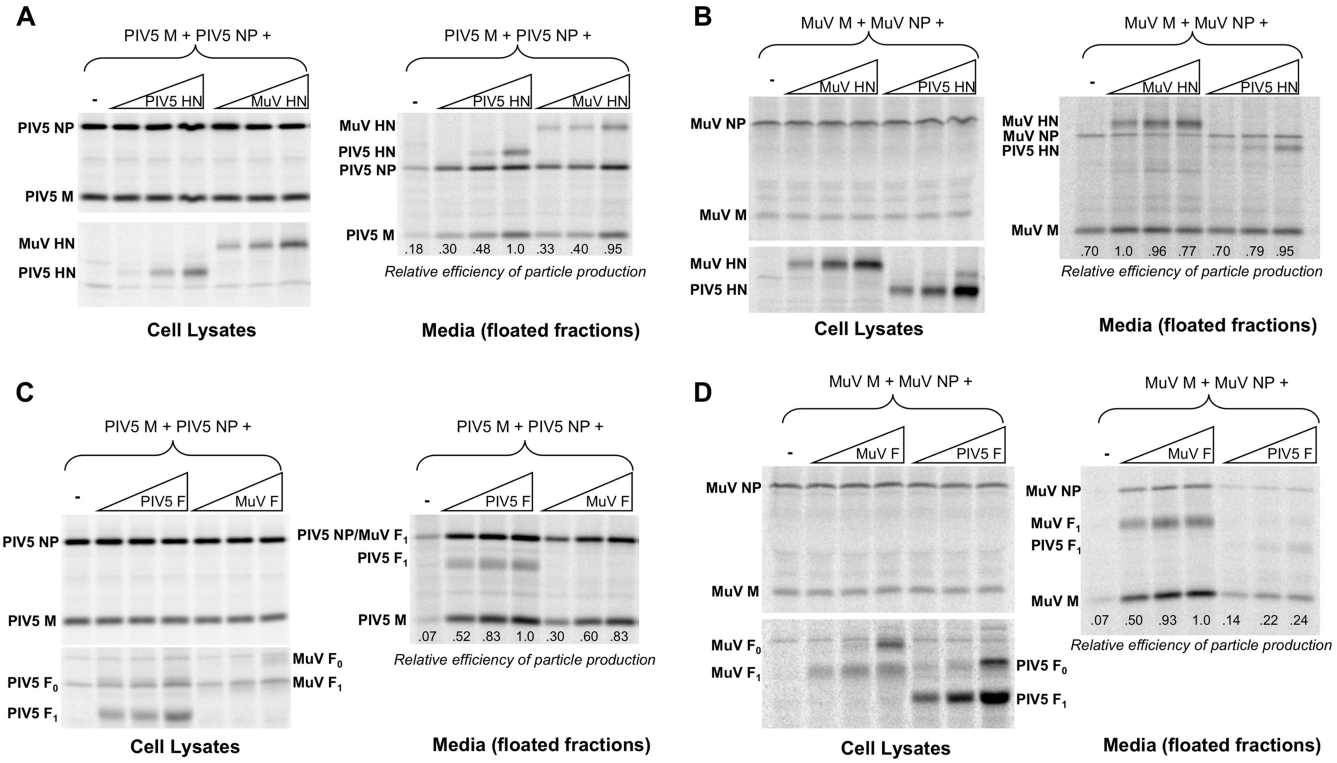

FIG. 6. Production of PIV5-like particles with MuV glycoproteins and production of mumps VLPs with PIV5 glycoproteins. 293T cells were transfected to produce the indicated viral proteins for VLP production. Plasmid quantities are as indicated in Materials and Methods, with the exception of the increasing HN and F plasmid amounts, which represent 0.75, 1.5, and 3.0 μg of plasmid DNA. VLP production efficiency was measured, and values were normalized to the maximum value obtained in each experiment. Each result shown is representative of at least two independent experiments.

virus N protein does not affect the efficiency of Nipah VLP production (38). To test if the MuV NP protein can function to allow efficient production of PIV5 VLPs, the PIV5 M and F proteins were coexpressed together with either the PIV5 NP protein or the MuV NP protein, and VLP release was quantified (Fig. 7A). The MuV NP protein was able to partially function in place of the PIV5 NP protein for VLP production, since VLP production was more than fourfold greater than that observed in the absence of NP protein expression but fivefold less than that observed in the presence of the PIV5 NP protein. Coexpression of the Nipah virus N protein, on the other hand, failed to increase PIV5-like particle production beyond the level observed in the absence of NP protein expression (data not shown). These results, summarized in Table 2, indicate that only the PIV5 NP protein, and not the other NP or N proteins tested here, allows optimal production of PIV5 VLPs, although the MuV NP protein can facilitate PIV5-like particle production to a limited extent.

In a related experiment, the MuV M and F proteins were coexpressed together with either the MuV NP protein, the PIV5 NP protein, or the Nipah virus N protein. Analysis of VLP release indicated that neither the PIV5 NP protein nor the Nipah virus N protein was able to function in place of the MuV NP protein for efficient VLP production (Fig. 7B; Table 2). VLP production increased by only a slight amount (less than twofold) in the presence of PIV5 NP protein, and was essentially unaffected by the presence of the Nipah virus N protein. Hence, only the MuV NP protein and not the other NP or N proteins tested here allowed optimal production of mumps VLPs.

TABLE 1. Production of VLPs with heterologous glycoproteins[a]

Viral protein	Effect on VLP production when coexpressed with:	
	PIV5 M and PIV5 NP	MuV M and MuV NP
PIV5 HN	+++	+
MuV HN	+++	+
PIV5 F	+++	++
MuV F	+++	+++

[a] "+" denotes increase ranging from 1.4-fold to 2-fold; "++" denotes increase ranging from 3-fold to 5-fold; "+++" denotes increase greater than 5-fold.

DISCUSSION

We have defined here a set of MuV proteins that are necessary and sufficient for efficient particle formation. Optimal production of MuV particles occurred upon coexpression of the MuV M, NP, and F proteins together in transfected 293T cells. This led to particle production in quantities comparable to those observed in MuV-infected cells. Particles produced from the transfected cells were VLPs exhibiting a morphology that was very similar to that of authentic virions as judged by

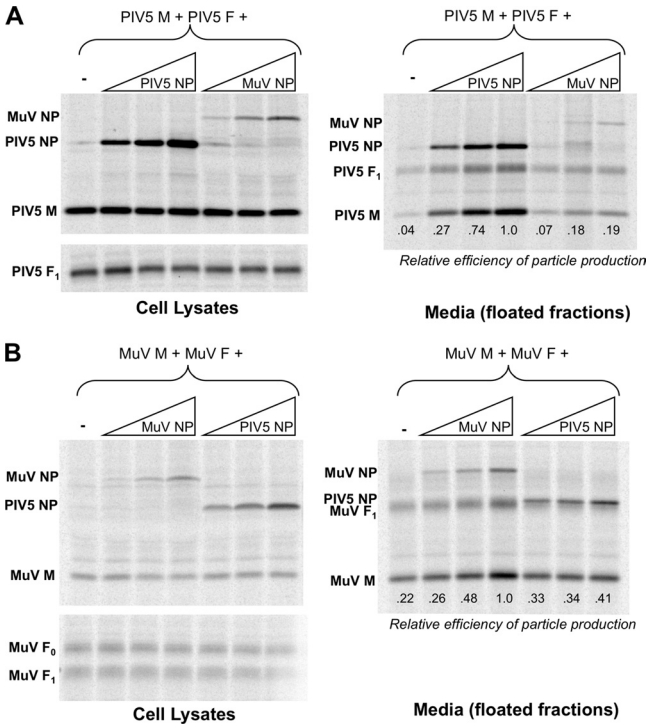

FIG. 7. Production of PIV5-like particles with the MuV NP protein and production of mumps VLPs with the PIV5 NP protein. 293T cells were transfected to produce the indicated viral proteins for VLP production. Plasmid quantities are as indicated in Materials and Methods, with the exception of the increasing NP plasmid amounts, which represent 70, 140, and 280 ng of plasmid DNA. VLP production efficiency was measured, and values were normalized to the maximum values obtained in each experiment. Each result shown is representative of at least two independent experiments.

electron microscopy. Mumps VLP production was critically dependent upon expression of the viral M protein, a result that is consonant with the M protein's role as the principal organizer of virus assembly and one that is consistent with findings made with a variety of other negative-strand RNA viruses including VSV (27), respiratory syncytial virus (60), human parainfluenza virus type 1 (12), Ebola virus (20, 61), Sendai virus (55, 56), PIV5 (51), lymphocytic choriomeningitis virus (41), Lassa fever virus (41), NDV (37), Nipah virus (11, 38), and measles virus (42).

Mumps VLP production was highly dependent on cooperation among the different MuV proteins. Of the four MuV structural proteins examined (M, NP, F, and HN), only the M protein when expressed by itself was capable of inducing detectable levels of particle release, but this quantity of M-alone particles was very small (approximately 25-fold less than that observed upon M+NP+F coexpression). Thus, efficient production of mumps VLPs occurred only when the M protein was coexpressed together with other viral proteins. This dependence on viral protein coexpression appeared to be less strict, however, than that observed with PIV5, since PIV5 M-alone particle release cannot be observed (51) while a small amount of MuV M-alone particle release can be observed reproducibly.

The two MuV glycoproteins were not equal contributors to particle formation. The MuV F protein was a major contributor to VLP production, while the contribution made by the MuV HN protein was much smaller. These results differ from those obtained with the closely related PIV5, in which the two glycoproteins were found to have redundant and nearly equivalent activities necessary for efficient budding of VLPs and virions (51, 66). The results obtained with MuV more closely resemble those obtained with the Sendai virus glycoproteins. For Sendai virus, studies with temperature-sensitive viruses and recombinant viruses have demonstrated that the viral HN protein is largely dispensable for particle formation while the F protein is very important for this process (17, 43). Sendai VLP production was enhanced by F protein expression but was inhibited by HN protein expression (55). For MuV, production of VLPs was strongly enhanced by F protein expression, but this enhancement was diminished when the HN protein was expressed together with the F protein (Fig. 2B). It is possible that interaction between the F and HN glycoproteins impairs the ability of the F protein to function for particle release or that the effect of the F protein on particle release is concentration dependent, with the relatively ineffective HN protein competing with the F protein for space at VLP budding sites, diluting the effective concentration of the F protein and reducing VLP production efficiency.

NP protein expression was necessary for optimal production of mumps VLPs. Much of the NP protein that was present in the cell during VLP formation was likely in the form of nonspecifically encapsidated cellular RNAs, since it is well established that the NP proteins of VSV and paramyxoviruses, when expressed in the absence of the corresponding viral P proteins, nonspecifically bind and encapsidate cellular RNAs to form short NP-like structures with herringbone morphologies that resemble viral RNPs (2, 12, 16, 33, 51, 53). The viral P proteins act as chaperones that interact with NP proteins via specific domains to form soluble complexes, reducing the nonspecific encapsidation of cellular RNAs (10, 13, 16, 33). This is thought to confer specificity to the encapsidation process in virus-infected cells, so that viral RNA genomes are encapsidated preferentially over cellular RNAs (10, 33). Roles for the NP protein have now been defined in the production of PIV5-like particles (51), Ebola VLPs (31), and mumps VLPs (this study). For these viruses, a requirement for the NP protein during virus assembly might be beneficial to avoid the production of noninfectious particles that lack viral genomes. If so, this strategy has not been universally adopted among the paramyxoviruses, since Sendai virus, NDV, and Nipah virus all bud VLPs in a way that is not enhanced upon NP protein expression (37,

TABLE 2. Production of VLPs with heterologous NP proteins[a]

Viral protein	Effect on VLP production when coexpressed with:	
	PIV5 M and PIV5 F	MuV M and MuV F
PIV5 NP	+++	+
MuV NP	++	++
NiV N	–	–

[a] "–" denotes increase smaller than 1.4-fold; "+" denotes increase ranging from 1.4-fold to 2-fold; "++" denotes increase ranging from 3-fold to 5-fold; "+++" denotes increase greater than 5-fold. NiV, Nipah virus.

38, 55), leaving open the questions of whether and how these viruses are able to avoid the budding of empty particles. The amount of the NP protein in mumps VLPs (M+NP+F configuration), judged by the NP-to-M-protein ratio, was less than the amount found in MuV virions. This result was somewhat unexpected in light of previous work with PIV5 and Sendai virus in which the opposite was found, i.e., the ratio of NP to M proteins was higher in the VLPs than in the virions (51, 55). Thus, on average, mumps VLPs incorporated less NP protein than PIV5-like particles and Sendai VLPs. It should be pointed out that all of these VLP populations are unlikely to be completely uniform and the NP protein content could vary substantially from one particle to the next.

MuV glycoproteins were able to cooperate together with the PIV5 M and NP proteins for efficient production of chimeric VLPs. Conversely, PIV5 glycoproteins expressed together with the MuV M and NP proteins were less effective for the production of chimeric VLPs. Although MuV and PIV5 are closely related viruses, both from the *Rubulavirus* genus, the amino acid sequences of the HN and F protein cytoplasmic tails of MuV bear little resemblance to those of PIV5. Some studies have suggested sequence-specific functions for viral glycoproteins in virus assembly. For example, assembly-defective subacute sclerosing panencephalitis measles virus strains often harbor hypermutated F protein cytoplasmic tail sequences (5, 47). Other studies have instead suggested a requirement for a nonspecific glycoprotein cytoplasmic tail in virus assembly (52). These contrasting observations underscore the difficulties in predicting, a priori, if a given viral glycoprotein will function together with a heterologous matrix protein for virus assembly. Nonetheless, such knowledge could be useful, for example, during the design of recombinant virus vaccine vectors harboring new viral glycoproteins in place of the biological ones. For instance, during the development of vaccine vectors based on recombinant PIV3, Tao and coworkers were able to recover recombinant viruses harboring heterologous glycoproteins when those glycoproteins were derived from PIV1 but not when they were derived from PIV2. Chimeric PIV3 with PIV2 glycoproteins could be recovered only when the PIV2 glycoproteins were altered to contain PIV3 cytoplasmic tail sequences (58, 59). A strategy employing VLP production could have predictive value in some instances. For example, our results suggest that PIV5 harboring MuV glycoproteins in place of its own glycoproteins is potentially viable, at least from a virus assembly standpoint. On the other hand, in the case of recombinant MuV harboring PIV5 glycoproteins, the prediction is that the virus may suffer from assembly defects.

Alterations in the MuV M protein that disrupted the sequence 24-FPVI-27 inhibited VLP production. This sequence is similar to a sequence within the PIV5 M protein, 20-FPIV-24, which is known to be critical for PIV5 budding (50). The FPIV sequence of PIV5 likely functions as a late domain to recruit host factors for budding, since it was identified based on its ability to restore budding function to the human immunodeficiency virus type 1 Gag protein lacking its natural late domain sequence PTAP (50). Our results with mumps VLPs extend these findings by demonstrating that FPIV-like sequences are important for proper budding of multiple paramyxoviruses. Not all paramyxovirus M proteins contain FPIV-like sequences in their amino-terminal regions, however. While the M proteins of PIV5, MuV, and NDV all contain FPIV-like sequences near their amino-terminal ends, other paramyxovirus M proteins, including the Nipah virus and Sendai virus M proteins, lack any similar sequences near their amino-terminal ends (50). In the case of the Nipah virus M protein, the alternative sequences YMYL (11) and YPLGVG (39) exist, which are critical for budding activity and may function to recruit host proteins. The Sendai virus M protein contains the sequence YLDL, which has been shown to bind to the cellular protein Aip1/Alix (25). In addition, the C-terminal portion of the Sendai virus C protein may provide a similar function, since it binds independently to Aip1/Alix (46), although conflicting data exist regarding the importance of Aip1/Alix in the context of Sendai virus infection (19, 24, 46). Since the FPIV sequence within the PIV5 M protein is likely to function as a protein-protein interaction domain, we have recently carried out additional experiments with the PIV5 M protein, searching for cellular M-interacting partner proteins. Among the proteins we have identified is angiomotin-like 1, a tight-junction-localized, PDZ-binding-motif-containing protein that harbors two PPXY sequences within its N-terminal region (Z. Pei and A. P. Schmitt, unpublished result). However, the M-interacting proteins we have identified to date, including angiomotin-like 1, do not bind to the M protein in a FPIV-dependent way. Thus, binding partners recruited via FPIV-like sequences to facilitate the budding of paramyxoviruses remain to be identified.

Mumps VLP production was inhibited upon expression of DN versions of the class E proteins Vps4A and Chmp4b. These findings suggest that class E protein machinery is important for the budding of MuV, as it is for the budding of many retroviruses (1). In the case of human immunodeficiency virus type 1, expression of the DN Vps4A and DN Chmp4b proteins arrests virus budding at the very late pinching-off step (65). In addition to MuV, other negative-strand RNA viruses have also been shown to bud poorly in the presence of DN class E protein expression, including Ebola virus (32), Lassa fever virus (63), and PIV5 (50). In the case of Sendai virus, DN Vps4 protein expression was recently shown to affect only the accelerated release of VLPs that occurs in the presence of the viral C protein and not M-alone VLP production (24), although conflicting reports have been published regarding the effect of DN Vps4 protein expression on the budding of Sendai virions from infected cells (19, 24, 46). It is becoming clear that not all viruses depend on the same ESCRT machinery for budding, since VSV, influenza virus, respiratory syncytial virus, and Nipah virus all bud particles efficiently even when ESCRT machinery is functionally disabled through expression of DN Vps4 proteins (9, 23, 39, 64). Hence, these viruses apparently possess alternative strategies for budding, either in place of or in addition to the strategy of class E protein recruitment. In the case of MuV, our results suggest that class E protein recruitment is likely to be very important for virus budding. However, the mechanism for this recruitment does not appear to involve classical late domains, since these are not found within the MuV M protein. Instead, alternative sequences may be used to recruit host factors to MuV assembly sites for budding.

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