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Influenza Virus Matrix Protein Is the Major Driving Force in Virus Budding

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To get insights into the role played by each of the influenza A virus polypeptides in morphogenesis and virus particle assembly, the generation of virus-like particles (VLPs) has been examined in COS-1 cell cultures expressing, from recombinant plasmids, different combinations of the viral structural proteins. The presence of VLPs was examined biochemically, following centrifugation of the supernatants collected from transfected cells through sucrose cushions and immunoblotting, and by electron-microscopic analysis. It is demonstrated that the matrix (M1) protein is the only viral component which is essential for VLP formation and that the viral ribonucleoproteins are not required for virus particle formation. It is also shown that the M1 protein, when expressed alone, assembles into virus-like budding particles, which are released in the culture medium, and that the recombinant M1 protein accumulates intracellularly, forming tubular structures. All these results are discussed with regard to the roles played by the virus polypeptides during virus assembly.

The final step in the lytic cycle of enveloped viruses involves the budding of the newly formed particles from cellular membranes. Previous to this step, all viral structural components should have been transported, either individually or as preassembled complexes, to the cellular membrane, where viral proteins will drive the budding process.

A number of studies have focused on the assembly and budding processes of viruses (arena-, alpha-, rhabdo-, paramyxo-, orthomyxo-, and retroviruses) that obtain their envelope from the plasma membrane (reviewed in references 2, 13, and 19). For the alphavirus Semliki Forest virus, it has been established that virus budding is strictly dependent on interactions between the transmembrane spike protein and the internal nucleocapsid (46). In retroviruses, however, interactions between the cytoplasmic tail of external virus proteins (Env) and the internal virus components (Gag polyprotein) are not a prerequisite for virus budding since expression of the Gag protein alone is sufficient to drive budding of virus-like particles (VLPs) (7, 14). A different mechanism, which directs the assembly and release of coronavirus particles, which assemble at intracellular membranes, has been described (47). In this case, expression of viral membrane proteins alone is sufficient to drive the assembly and budding of VLPs (47).

It is widely accepted that the matrix protein plays a pivotal role as an assembly organizer for RNA viruses containing a single negative-strand genomic RNA molecule (such as rhabdo- and paramyxoviruses) (reviewed in reference 25). In fact, rabies and measles viruses modified by reverse genetics technology to lack the matrix gene grow poorly, and the released matrix-less particles show drastically altered morphologies (3, 31). Moreover, it has been shown that the M1 proteins of vesicular stomatits virus (VSV) and human parainfluenza virus type 1 have intrinsic budding activity when expressed alone (5, 22, 26), an observation which suggests a certain parallelism with the retrovirus budding model. It has also been established that interactions between the internal viral components and the unique transmembrane protein of rabies and VSV are not an absolute requirement for virus particle formation since spikeless virus particles are released and budded from cells infected with genetically modified viruses deficient in their corresponding transmembrane proteins (30, 38). However, other reports have shown that efficient assembly and budding of these RNA viruses require contacts between the cytoplasmic tails of the transmembrane protein and the internal components (presumably the matrix protein) (4, 29, 30, 44). It should also be mentioned that the glycoproteins of VSV and rabies viruses have some exocytic activity (39), a finding indicating that these viruses incorporate aspects of the budding mechanism used by coronaviruses.

Little is known about the mechanism that governs influenza A virus morphogenesis. The genome of this virus is made up of eight single-stranded negative-sense RNA segments, which direct the synthesis of 10 viral polypeptides in infected cells. Four of these proteins, the nucleoprotein (NP), which encapsidates the viral RNA, and the three subunits of the polymerase (proteins PB1, PB2, and PA) are associated with each of the viral genomic RNAs forming ribonucleoprotein (RNP) complexes. Three of the proteins, the hemagglutinin (HA), neuraminidase (NA), and M2 proteins, are transmembrane polypeptides, and the two other structural components, the matrix (M1) and NS2 (recently renamed nuclear export protein [NEP]) (34) polypeptides, are internal components of the viral particle. NS1 is the only nonstructural protein encoded by the viral genome (all these aspects have been reviewed in reference 24).

The influenza A virus M1 protein has lipid binding properties (16, 40) and interacts tightly with the plasma membrane (9, 11, 18, 23, 53). Biochemical (49, 52) and functional (49, 50, 55) observations indicate that the M1 protein associates with the RNPs and with NEP in the mature virion (51). It has also been demonstrated that influenza viruses lacking the cytoplasmic tail of HA, NA, or both have a reduced infectivity and a lower budding efficiency and that those lacking the cytoplasmic tail of NA show alterations in shape and morphology (12, 20, 21, 33). Thus, it has been proposed that contacts between the cytoplas-

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mic tails of the virus membrane proteins and the virion internal components (most likely M1, but it remains to be formally proven) contribute to formation of the budding particle. Based on these findings, it has been postulated that the M1 protein forms a shell lining the inner surface of the viral envelope. This shell would act as a bridge between the internal components (RNPs and NEP) of the virion and the membrane proteins.

A number of questions remain to be answered regarding the processes of influenza virus assembly and budding. For example, what is the individual role of each protein in virus morphogenesis? are contacts between RNPs and other viral components required for virus assembly? what is the minimum set of viral proteins needed to initiate the bending of the membrane, a process that eventually will lead to the formation of budded virions? what is the major driving force in this process?

We have described a system that allows formation and release of influenza virus VLPs in cells expressing all virus structural polypeptides from recombinant plasmids (15, 32). We have now studied the presence of VLPs in cells expressing different combinations of the influenza virus structural components to get insights into the role played by each viral protein in the process of virus particle formation. It is demonstrated that neither NEP nor the RNPs are needed for formation of VLPs and that M1 is the major virus assembly organizer and the major driving force in the process of bud formation.

MATERIALS AND METHODS

Cells and viruses. COS-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. Influenza virus strain A/Victoria/3/75 (H3N2) was used throughout. Recombinant vaccinia virus vTF7-3 (10) was kindly provided by B. Moss.

Plasmids. Plasmids pGEM-PB1, pGEM-PB2, pGEM-PA, pGEM-NP, pGEM-HA, pGEM-NA, pGEM-M1Δ, pGEM-M2Δ, and pGEM-NS2Δ encoding the influenza virus PB1, PB2, PA, NP, HA, NA, M1, M2, and NS2 polypeptides, respectively, of influenza virus A/Victoria/3/75 have been described (6, 15, 32). In these plasmids, the viral genes are cloned downstream from the T7 promoter of plasmids pGEM-3 and pGEM-4 (Promega). Plasmid pCATCA18 is a pUC18 derivative which contains, in 5′-to-3′ order, the T7 RNA polymerase promoter, the 5′-end noncoding sequences corresponding to influenza virus segment 8, the chloramphenicol acetyltransferase (CAT) gene in negative polarity, the 3′-end noncoding sequences corresponding to influenza virus segment 8, the self-cleaving ribozyme of hepatitis delta virus, and a T7 transcription terminator sequence. The influenza virus and CAT gene sequences present in this plasmid were obtained by PCR from plasmid pIVCAT1/S (37).

Antibodies and immunoblotting. Polyclonal antisera which recognize the NP and M1 proteins (1, 15) and monoclonal antibodies (MAbs) raised against the A/Victoria/3/75 HA (MAbs HA2-76 and M/234/1/F4) and the M1 protein have been described (28, 42). Goat antiserum against the M2 protein was a gift from Alan Hay. For immunoblotting (Western blotting) analysis, cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to Immobilon-P paper, and developed with the appropriate antibody (antiserum to M2, M1, or NP or MAb HA2-76) by enhanced chemiluminescence (1).

Transfection of COS-1 cells with plasmids encoding the influenza virus proteins and detection of VLPs. The standard protocol has been described in detail by Gómez-Puertas et al. (15). In the experiments reported here the influenza virus-like CAT RNA to be transcribed by the T7 polymerase was generated after transfection of plasmid pCATCA18 instead of transfecting an in vitro-made CAT RNA as done previously. A brief description of the protocol is as follows. Cultures of COS-1 cells (10^6 cells) growing in 35-mm-diameter dishes in the presence of DMEM-Ara (DMEM containing 40 μg of cytosine-β-D-arabinofuranoside per ml) were infected with vTF7-3 (multiplicity of infection of 5) and transfected with a mixture that contained cationic liposomes and plasmids. The amounts of the plasmids included in the transfection mixture were as follows: pGEM-PB1, 0.6 μg; pGEM-PB2, 0.6 μg; pGEM-PA, 0.2 μg; pGEM-NP, 2 μg; pGEM-HA, 0.6 μg; pGEM-NA, 0.6 μg; pGEM-M1Δ, 0.5 μg; pGEM-M2Δ, 0.15 μg; pGEM-NS2Δ, 0.1 μg; pCATCA18, 1 μg. After 5 h of incubation with the plasmid DNAs the medium was replaced with 1 ml of DMEM-Ara, and this washing step was repeated 15 h later. At 72 h postinfection (p.i.), the culture supernatant was collected and centrifuged in a microcentrifuge (~13,000 × *g*) for 20 min at 4°C to remove cell debris. Half of the clarified supernatant (~400 μl) was diluted in 3.5 ml of DMEM, loaded onto a 33% (wt/wt) sucrose cushion (1 ml), and subjected to centrifugation for 55 min at 4°C and 35,000 rpm in an SW55 rotor. The pellet of this centrifugation step was resuspended in 50 μl of SDS sample buffer, and aliquots were analyzed by SDS-PAGE and Western blotting. For detection of CAT RNA in the released VLPs, the pellet obtained from the sucrose centrifugation step was supplemented with 5 μg of tRNA and treated with proteinase K. Following phenol extraction and ethanol precipitation, the sample was resuspended in 10 μl of water and half of this sample was incubated with SuperScript RT (GIBCO-BRL) and with a 20-mer oligodeoxynucleotide (CGTCTAGCCAATCCCTGGG) which hybridizes with the negative-sense CAT RNA and which yields a 258-nucleotide-long cDNA product. After incubation at 37°C for 1 h, 1/20 of the sample was subjected to PCR (30 cycles) using primers GGACAACTTCTTCGCCCCCG (corresponding to the CAT gene) and CAAGGGTGTTTTTTCAGATC (corresponding to the influenza virus noncoding sequences present in the CAT RNA) to amplify a DNA fragment of 200 bp. The products of this reaction were analyzed by electrophoresis in a 2% agarose gel.

Detection of particles formed on expression of combinations of HA, NA, and M1 proteins. COS-1 (3×10^6 cells) cells growing in 60-mm-diameter dishes were infected and transfected as indicated above. The amounts of plasmids pGEM-HA, pGEM-NA, and pGEM-M1Δ in the transfection mixture were 15 μg each. Cell supernatants (~4 ml) were collected at 60 h p.i., clarified by low-speed centrifugation, and split into four aliquots, which were independently loaded onto 1-ml sucrose cushions of 25, 33, 41, and 49% (wt/wt). These samples were centrifuged and analyzed by immunoblotting as described above.

Electron microscopy (EM). Routinely, the transfected cells were prepared in 60-mm-diameter dishes. The protocol followed to prepare ultrathin sections from transfected cultures has been detailed previously (15). Briefly, the cell cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate (pH 7.4) buffer and postfixed with 1% osmium tetroxide. The samples were then dehydrated and embedded in epoxy resin EPON 812. Sections were made with a LKB Ultratome IV, stained in 1% aqueous uranyl acetate-lead citrate, and visualized in a Philips 400T electron microscope at 80 kV. When detection of HA was needed, the transfected cultures were incubated, previous to the glutaraldehyde fixation step, with anti-HA MAb M/234/1/F4 and with a secondary antibody (10-nm gold particle-labeled goat anti-mouse immunoglobulin G [IgG]; Auroprobe EM GAM IgG G10; Amersham). For detection of the M1 protein, the ultrathin sections were sequentially incubated with a mixture of anti-M1 MAbs and 10-nm gold particle-labeled goat anti-mouse IgG according to the procedure described by Vivo et al. (48).

The procedure used for negative staining has been detailed previously (32). Briefly, the material pelleted following centrifugation through a 33% sucrose cushion was resuspended in 50 μl of TNE (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA), and a drop of the resuspended material was adsorbed onto carbon-coated grids, which were incubated sequentially with MAb M234/1/F4 (anti-HA) and with 10-nm gold particle-labeled goat anti-mouse IgG. The preparations were then negatively stained with 2% phosphotungstic acid, pH 7.2, and examined in a Phillips 400T electron microscope at 80 kV.

Equilibrium gradient centrifugation. COS-1-transfected cultures were harvested and fractionated as described by Zhao et al. (54). Briefly, 3×10^6 cells were rinsed with phosphate-buffered saline and scraped into 1 ml of a buffer containing 10% (wt/vol) sucrose and 10 mM Tris-HCl (pH 7.5). Cells were broken by passing the suspension 30 times through a 25-gauge needle, and the mixture was then centrifuged at 2,000 rpm for 5 min at 4°C in an Eppendorf microcentrifuge. The resulting supernatant was made 80% (wt/vol) sucrose (final volume, 5 ml), laid at the bottom of a Beckman SW41 centrifuge tube, and overlaid with 5 ml of 65% (wt/vol) sucrose and 2.5 ml of 10% (wt/vol) sucrose. Following centrifugation in an SW41 rotor at 35,000 rpm for 18 h at 4°C, 12 fractions (1 ml) were collected from the top and samples of these fractions (25 μl) were analyzed by immunoblotting.

RESULTS

Formation and characterization of VLPs in cell cultures expressing different combinations of the influenza virus structural proteins. We have demonstrated previously that influenza virus VLPs are released from COS-1 cells infected with vaccinia virus vTF7-3, which expresses the T7 RNA polymerase, and subsequently transfected with pGEM-derived plasmids which encode the nine influenza virus structural proteins (15, 32). In our previous experiments, an influenza virus-like CAT RNA was always cotransfected into the COS-1 cells. Routinely, the supernatant collected from transfected cells was added to fresh MDCK cultures that were subsequently superinfected with a wild-type influenza virus helper virus to provide the proteins needed to amplify the CAT RNA delivered by the VLPs. Thus, detection of CAT activity in MDCK cells indicated the presence of VLPs which contained the CAT RNA. Using this system, we demonstrated that CAT activity transmission to MDCK cells was not observed if any of the plasmids

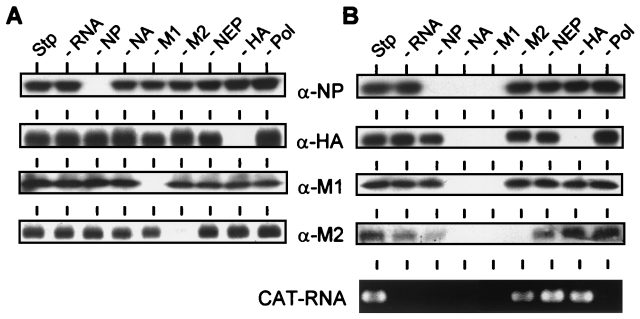

FIG. 1. Identification of VLPs by biochemical assays. COS-1 cells were infected with vTF7-3 and transfected with the nine pGEM-derived plasmids encoding all the viral structural polypeptides (Stp, −RNA) or with only eight plasmids (−NP, −NA, −M1, −M2, −NEP, −HA; the gene omitted in each sample encodes the indicated protein) or with a DNA mixture that lacked the plasmids encoding the three polymerase subunits (−Pol). All transfection mixtures, except sample −RNA, contained also plasmid pCATCA18, which drives the expression of a synthetic influenza virus-like CAT RNA. Supernatants from the transfected cultures were collected at 72 h p.i., clarified by low-speed centrifugation, and centrifuged through a 33% sucrose cushion as detailed in Materials and Methods. Extracts from the transfected COS-1 cells (A) and from the material pelleted following centrifugation of the supernatants through the 33% sucrose cushion (B) were analyzed by immunoblotting using antibodies to the NP, HA, M1, and M2 proteins as indicated. Another aliquot of the pelleted material was phenol extracted and analyzed for the presence of CAT RNA by RT-PCR as detailed in Materials and Methods (B, bottom).

encoding the viral structural components was omitted (15, 32). One explanation to account for this lack of CAT transmission is that VLPs were not formed in the absence of a particular viral gene product. Alternatively, the VLPs were formed but they were not competent to deliver the CAT RNA to the MDCK cultures. To distinguish between these possibilities, we tested whether VLPs were formed in cultures expressing different combinations of the viral proteins by EM analysis of transfected cultures and by biochemical detection of VLPs.

Thus, COS-1 cell cultures were infected with vTF7-3 and transfected with DNA mixtures containing the nine plasmids coding for all structural proteins (samples Stp and −RNA) or mixtures that lacked one of the plasmids (samples −NP, −NA, −M1, −M2, −NEP, and −HA) or that lacked the plasmids encoding the three polymerase subunits (sample −Pol) (Fig. 1). All these cultures, except sample −RNA, were also transfected with plasmid pCATCA18, which contains an influenza virus CAT gene flanked by a T7 RNA polymerase promoter and the hepatitis delta virus ribozyme. This plasmid yields in vTF7-3-infected cells a synthetic negative-sense CAT RNA which is amplified and expressed by the influenza virus recombinant polymerase in the same manner as we had previously reported when transfecting an in vitro-made CAT RNA (15) (data not shown). To determine biochemically the presence of VLPs in the transfected cultures, the supernatants of the COS-1 cells were collected, clarified by low-speed centrifugation, and centrifuged through a 33% sucrose cushion. The pellet from this centrifugation was expected to contain the VLPs since authentic influenza virus virions sediment through this sucrose cushion (see Fig. 3) (41). The pellet was analyzed for the presence of the NP, M1, HA, and M2 proteins by SDS-PAGE and immunoblotting. Detection of the NA and NEP polypeptides could not be carried out due to the poor quality of the antisera which we had available (not shown).

As shown in Fig. 1A, the four proteins analyzed accumulated to similar levels in all transfected cultures, indicating that omission of a particular recombinant plasmid in the transfection mixture did not affect significantly the accumulation of other recombinant proteins. Of course, NP, HA, M1, and M2 proteins were not detected in cultures transfected with DNA mixtures lacking the corresponding plasmid (samples −NP, −HA, −M1, −M2, respectively, Fig. 1A). All four viral proteins tested were detected in the supernatant from COS-1 cells expressing all virus structural proteins (Stp and −RNA, Fig. 1B), whereas none of these proteins was found in the supernatants from cells not expressing either the NA or the M1 protein. This result therefore suggested that the proteins detected in the supernatants of samples Stp and −RNA corresponded to viral polypeptides incorporated into VLPs and not to soluble proteins and that expression of M1 and NA was required for biochemical detection of VLPs. According to this interpretation, neither P proteins (−Pol) nor CAT RNA (−RNA) nor NEP (−NEP) is required for formation of VLPs (Fig. 1B). Moreover, the data shown in Fig. 1 indicated that NP, M2, and HA could be individually removed without significantly affecting generation of VLPs (−NP, −M2, and −HA, respectively, Fig. 1B).

To determine whether the VLPs contained the CAT RNA, the pellet harvested from the sucrose cushion centrifugation was analyzed by reverse transcription-PCR (RT-PCR) using primers designed to amplify the negative-sense CAT RNA (Fig. 1B, bottom). An amplified DNA band (with the expected size) was detected in the supernatant from cells expressing all viral structural proteins and transfected with plasmid pCATCA18 (Stp) but not in cultures in which this plasmid was omitted from the transfection mixture (−RNA). The band observed in sample Stp was in fact derived from CAT RNA molecules since it was not detected on omission of the RT reaction (data not shown). Importantly, the amplified DNA band was not detected in the supernatants from samples in which VLPs were not found (−M1 and −NA), indicating that the amplified band corresponded to RNA molecules incorporated within VLPs. Moreover, detection of the CAT RNA required expression of NP and the polymerase protein (samples −NP and −Pol, respectively), indicating that the CAT RNA incorporated into the VLPs was in the form of RNPs and not a naked RNA. Interestingly, it was observed that particles lacking M2, NEP, or HA contained the CAT RNA, demonstrating that none of these proteins were essential for packaging CAT RNPs.

To get information on the size and morphology of the VLPs assembled in COS-1 cells, the transfected cultures were incubated with an anti-HA MAb and with a gold-labeled antimouse serum and then visualized by transmission EM. We used gold decoration as a major criterion for a particle to be considered a true influenza virus VLP, and thus only samples which were transfected with the HA plasmid were included in the analysis. As previously demonstrated (15), filamentous gold-decorated particles which contained a fuzzy coat were observed in cultures expressing all virus structural polypeptides (Fig. 2, Stp). These particles were indistinguishable, in terms of size and general morphology, from the virions observed in COS-1 cells which had been infected with the viral strain A/Victoria/3/75 (Fig. 2, FLU). Similar filamentous structures were readily detected in cultures −RNA, −NP, −M2, and −NEP (Fig. 2 and data not shown), but they were never observed in cultures not expressing the M1 protein (not shown). Besides, and in agreement with previous data (15), particles morphologically indistinguishable from true virions were observed in cultures lacking NA, but in this case the particles were associated with cell surfaces and/or formed large aggregates (Fig. 2, −NA). Based on all these data it was concluded that the filamentous particles decorated by the anti-HA MAb corresponded to authentic influenza virus VLPs. Importantly, the particles do not correspond to microvilli containing HA since the gold-decorated

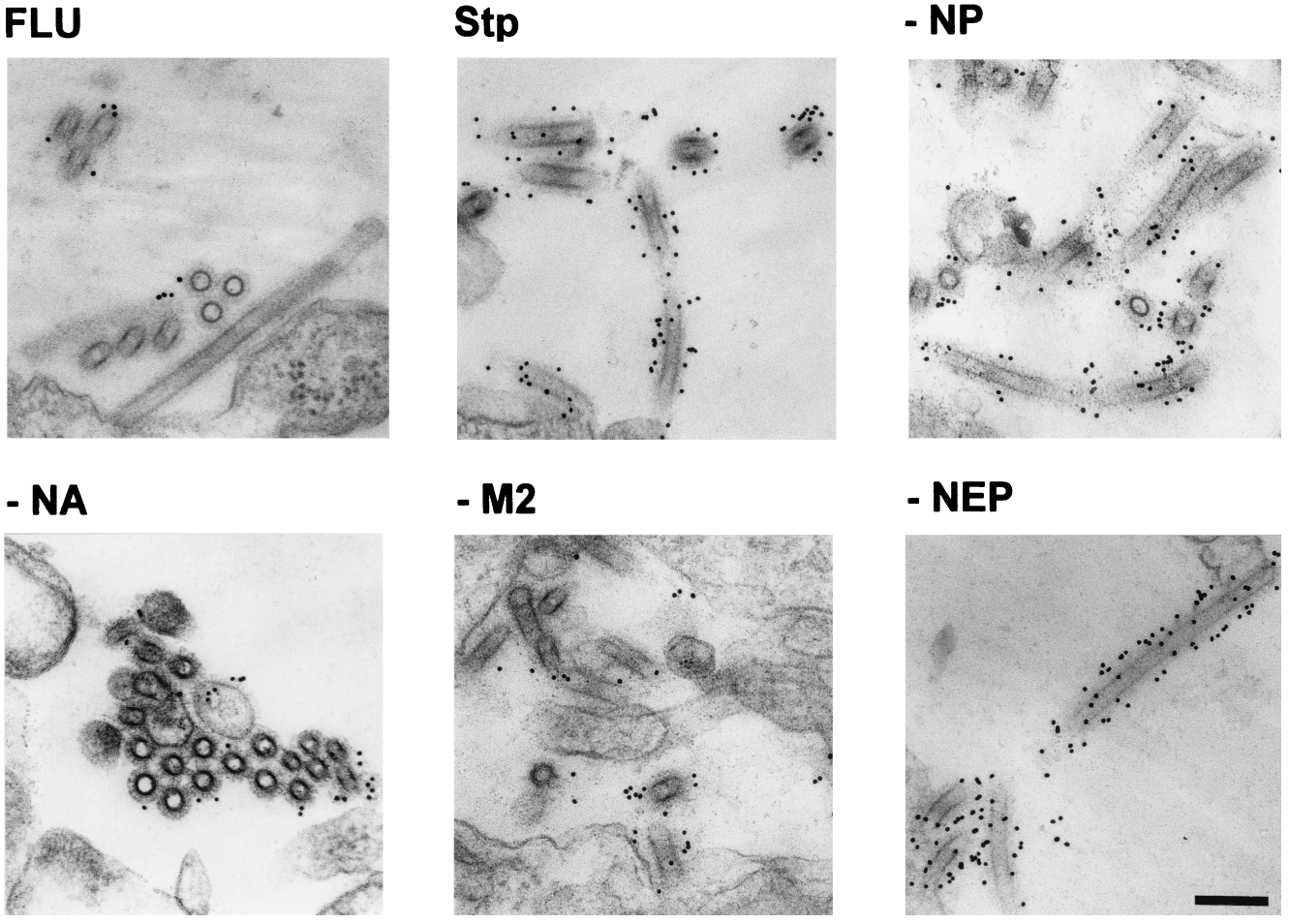

FIG. 2. Visualization of VLPs in transfected COS-1 cell cultures. COS-1 cell cultures were infected with vTF7-3 and transfected with plasmids as described in the legend to Fig. 1. At 60 h p.i., cells were sequentially incubated with an anti-HA MAb (M234/1/F4) and decorated with a gold-labeled antiserum before fixation and analysis by EM (details are given in Materials and Methods). Sample FLU corresponds to COS-1 cell cultures infected with influenza virus strain A/Victoria/3/75. Bar, 200 nm (all pictures are shown at the same magnification).

particles were never observed in the absence of M1 expression and since microvilli exhibit a variable diameter along their lengths whereas the decorated structures observed in the EM pictures (Fig. 2) are homogenous in diameter.

Several conclusions can be drawn when the results of Fig. 1 and 2 are analyzed together. All viral structural proteins, except M1, can be removed individually without compromising the formation and budding of VLPs which morphologically resemble wild-type virions. Moreover, we have confirmed that NA expression is required for release of assembled virions from the producing cells (15), a result which agrees with previous studies using NA-deficient viruses (27, 35). The explanation for this aggregation phenotype is that the HA incorporated into the NA-deficient particles binds to but does not later dissociate from, as in the NA-containing particles, sialic acid residues present in other cell membrane surfaces. The fact that particles formed in the absence of NA could not be detected biochemically (Fig. 1, −NA) is a consequence of the budded VLPs not being freed into the cell medium.

RNPs are not required for assembly of influenza virus VLPs. The results shown in Fig. 1 and 2 suggested that interactions between RNPs (and NEP) with other virus structural components are not needed for virus particle assembly. However, since the various RNP components were removed individually, it may be postulated that the internal virus components contained redundant signals required for virion assembly so that the absence of one of them was being compensated for by signals present in the others. To conclusively clarify this issue, COS-1 cell cultures were transfected with plasmids encoding the M1, HA, and NA proteins or with only the two plasmids encoding the virus glycoproteins (HA and NA). To determine the formation of VLPs, the supernatants collected from the transfected cultures were split into four aliquots, which were centrifuged individually through four sucrose cushions (25, 33, 41, and 49%), and the material pelleted was then analyzed by immunoblotting. Under these conditions, the virus particles produced in a lytic influenza virus infection sedimented through the 25 and 33% sucrose gradients, whereas practically no virions went through the 41 and 49% sucrose cushions (Fig. 3A). Similarly, the supernatant collected from cells coexpressing HA, NA, and M1 contained particles which went through the 25 and 33% sucrose cushions (Fig. 3B), whereas in the supernatant from cells expressing the two glycoproteins, only a minor HA signal was found in the pellet obtained after centrifugation through the 25% sucrose cushion (Fig. 3C). The material found in the pellet of the 33% sucrose cushion from both the influenza virus-infected cells and the cultures expressing the three viral proteins was analyzed by negative staining and EM; this analysis revealed that it consisted, in both sam-

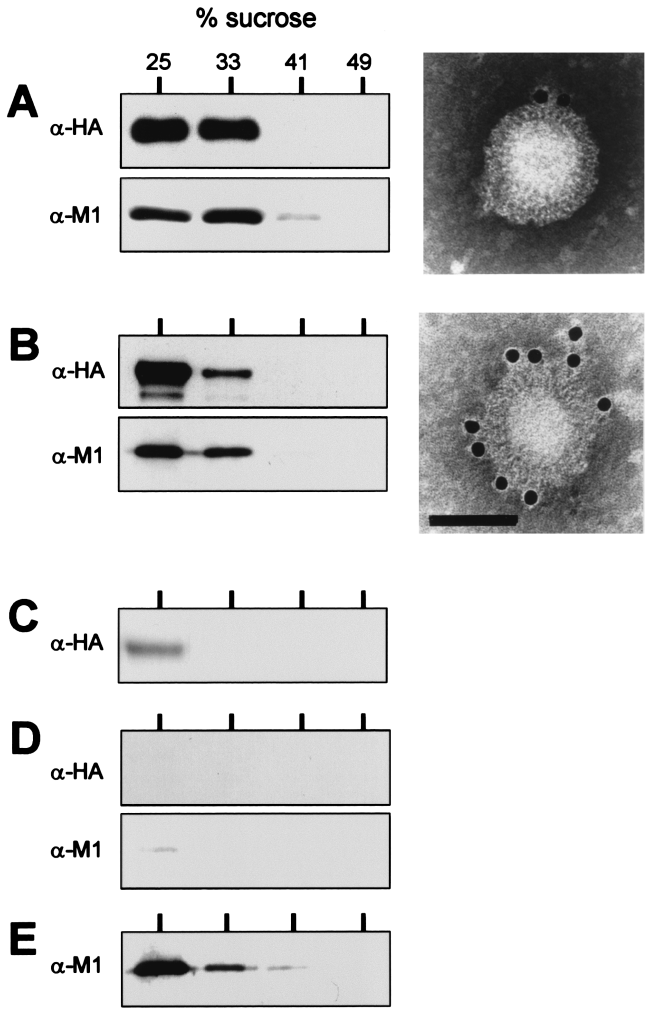

FIG. 3. Analysis by sucrose gradient centrifugation of the particles formed in cells expressing M1 and the viral glycoproteins. (A) COS-1 cells were infected with influenza virus A/Victoria/3/75, and the supernatant from this culture was collected and split into four aliquots, which were independently centrifuged through sucrose cushions of 25, 33, 41, and 49%. The material pelleted in each case was resolved by SDS-PAGE and analyzed by immunoblotting using antibodies to the HA and M1 proteins, as indicated. An aliquot of the material that sedimented through the 33% sucrose cushion was adsorbed onto carbon-coated grids, immunolabeled with an anti-HA antibody, negatively stained, and visualized by EM (right). (B to E) COS-1 cells were infected with vTF7-3 and transfected with different combinations of plasmids, and the supernatants were analyzed as described for panel A. The plasmids transfected were those encoding M1, HA, and NA (B); HA and NA (C); M1 and HA (D); or M1 alone (E). An aliquot of the panel B sample was also analyzed by EM as described for panel A. Bar, 100 nm.

ples, of pleomorphic membranous structures that contained HA (Fig. 3A and B, right).

The transfected cultures that coexpressed M1, HA, and NA were also examined by transmission EM on thin sections (Fig. 4A). Long filamentous particles, indistinguishable from virions produced in infected cells, were observed. The filamentous particles contained a layer of spikes on their surfaces and were labeled with an anti-HA MAb (Fig. 4A). No such structures were observed in cells coexpressing the two glycoproteins (data not shown).

Taken together, the results from the sedimentation and EM analyses indicated that particles indistinguishable from true virions in density and general morphology were formed upon coexpression of just three viral proteins (M1, HA, and NA). Therefore, it is demonstrated that neither the RNPs nor NEP is needed for the formation, budding, and release of VLPs.

The M1 protein drives formation of budded particles. To further delineate the contribution of M1, HA, and NA proteins to the budding process, we looked for the presence of VLPs in cultures expressing the M1 protein alone or in cells that coexpressed the M1 and HA proteins. Cultures coexpressing the M1 and NA proteins were not included in the analysis, because of the lack of a good antibody to detect the NA polypeptide.

VLPs were easily detected in thin sections of cultures transfected with plasmids expressing the M1 and HA proteins (Fig. 4B). As expected from the results described above (Fig. 1 and 2, −NA), these particles were aggregated or bound to cell surfaces and they were not detected in the culture supernatants following centrifugation and Western blotting analysis (Fig. 3D).

Extracellular membranous spikeless particles, which had an external diameter (~55 nm) similar to that of true virions, were observed in the cells expressing exclusively the M1 protein (Fig. 4C). Moreover, the sedimentation analysis demonstrated the presence of the M1 protein in the pellet fractions of the 25, 33, and 41% sucrose cushions (Fig. 3E), indicating that extracellular M1-containing particles are produced in these cultures. From the results shown in Fig. 3 and 4, it was concluded that the M1 protein has all the structural information to induce the formation of VLPs which bud from cell membranes.

Influence of viral glycoproteins on properties of M1 in transfected cells. In addition to budded particles, long electron-dense filamentous structures were observed in both the nuclei and cytoplasm of cells expressing exclusively the M1 protein (Fig. 5). The structures were not homogeneous in diameter but had enlarged portions at different levels along the filament. Some of the filaments were observed in cross sections as circular structures with an empty core, whereas others appeared as solid circles. The external diameters of the structures ranged from 35 to 90 nm, and the wall thickness was not constant but appeared to vary in size increments of ~8 nm, suggesting that these structures were composed of successive layers, each of them made up of the same components. These intracellular structures were not observed in cells infected with vTF7-3 and transfected with the plasmid encoding HA (not shown). Moreover, the structures detected in the M1-expressing cultures could be gold decorated when a mixture of anti-M1 MAbs was used (Fig. 5C) but not when an unrelated MAb was used (data not shown). Therefore, it was concluded that the intracellular structures contained the M1 protein.

These intracellular M1-containing filaments were observed in more than 30% of the cells transfected with the M1 plasmid alone and only sporadically in cells cotransfected with plasmids encoding M1 and HA. This result suggested that an interaction between M1 and the HA glycoprotein prevented formation of M1-containing intracellular aggregates. To get additional evidence on the influence of glycoprotein expression on the M1 properties, we decided to study, by a membrane flotation analysis, the membrane association properties of the recombinant M1 protein expressed in the presence and absence of the viral glycoproteins. Thus, cell lysates were prepared in a buffer containing 80% sucrose and placed at the bottom of a centrifuge tube, which was then overlaid with layers of 65 and 10% sucrose. Following centrifugation to equilibrium the membranes and the membrane-associated proteins move to the interface between the 10 and 65% sucrose, whereas the non-membrane-associated proteins remain at the bottom of the gradient (9, 23, 53).

The results of this analyses are shown in Fig. 6. As described previously by other groups (9, 23, 53), it was observed that

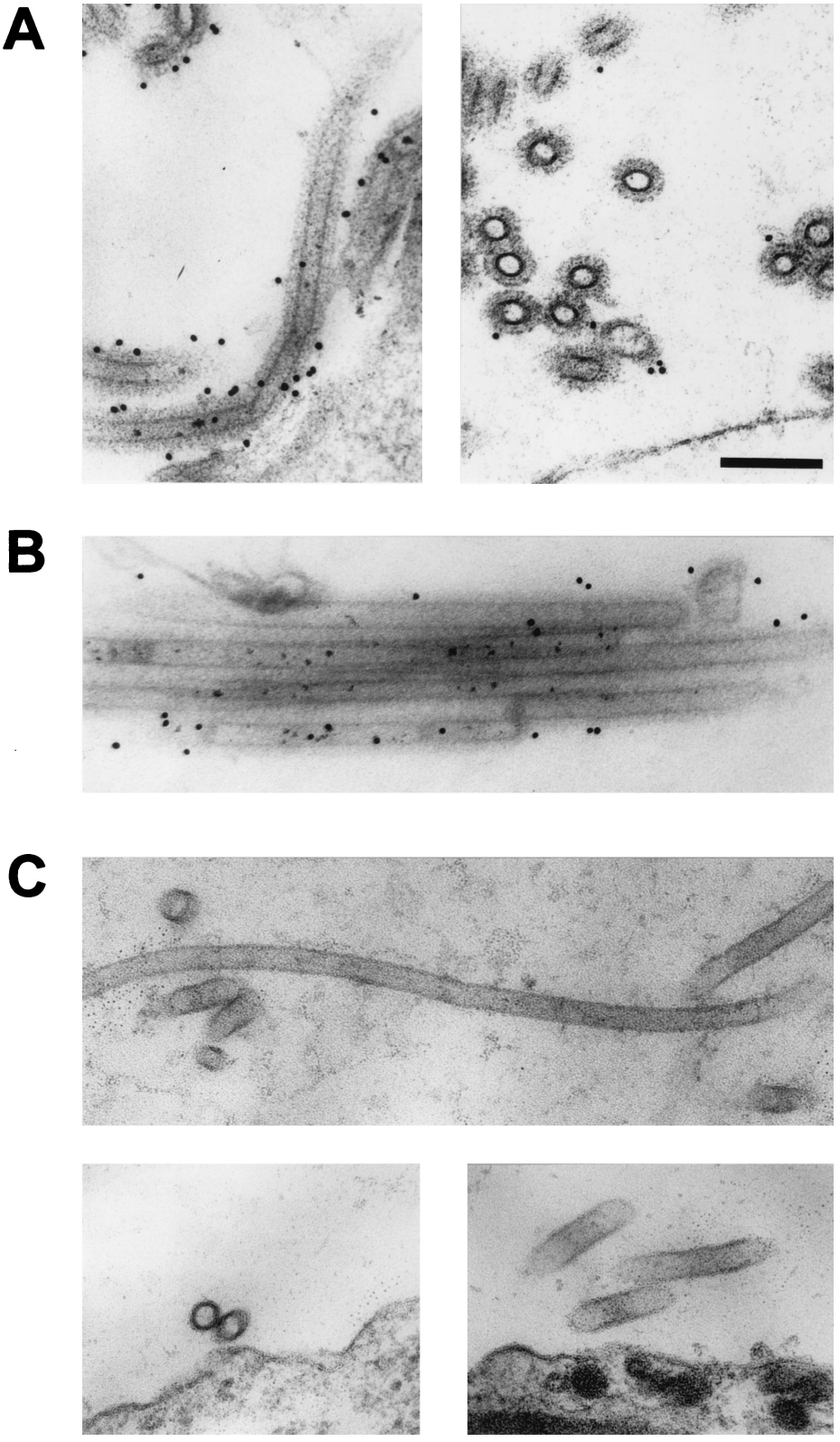

FIG. 4. Visualization of the particles formed on expression of M1 and the viral glycoproteins. COS-1 cells were infected with vTF7-3, transfected with different plasmids, and analyzed by EM. (A and B) Cells were previously immunostained with an anti-HA MAb as described in the legend to Fig. 2. The samples analyzed were cultures that coexpressed M1, HA, and NA (A); M1 and HA (B); or M1 alone (C). Bar, 200 nm (all pictures are shown at the same magnification).

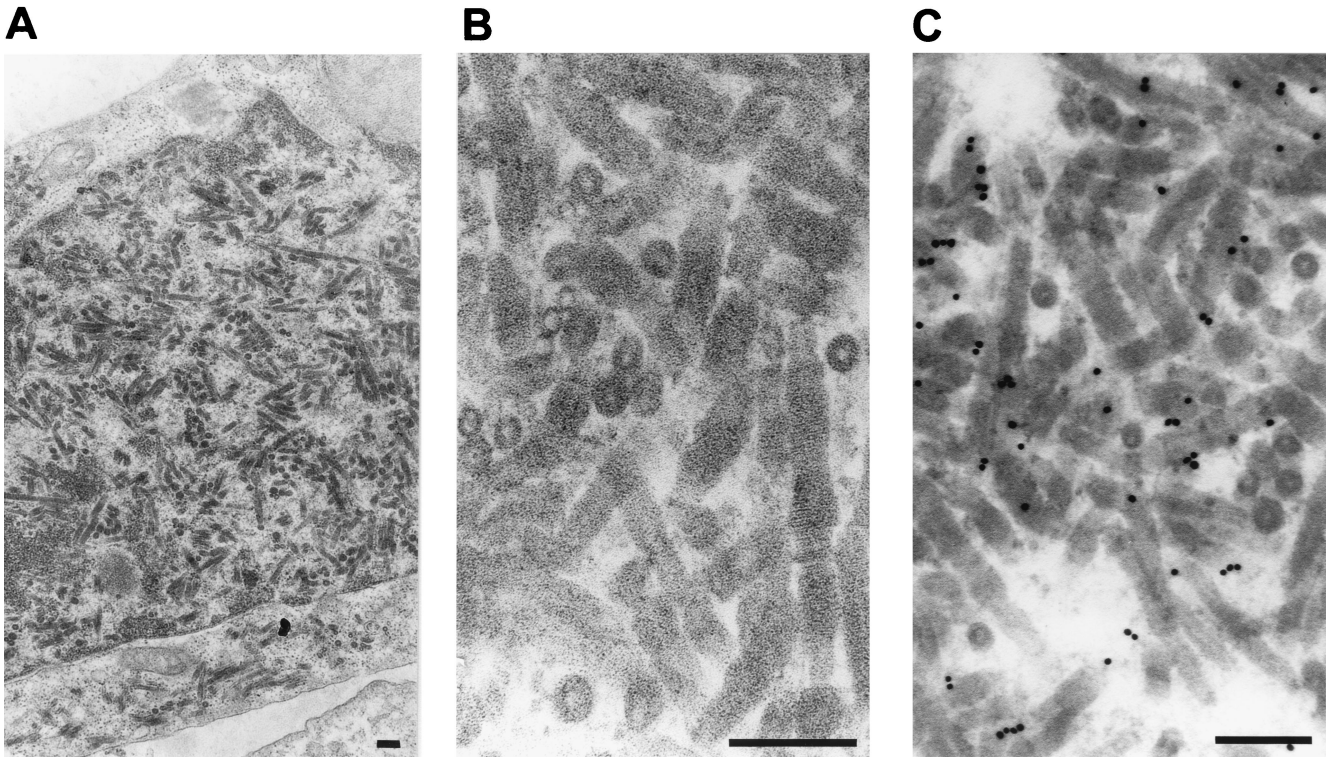

FIG. 5. Intracellular aggregates observed in cells expressing the M1 protein. (A and B) COS-1 cells were infected with vTF7-3 and transfected with plasmid pGEM-M1Δ. At 60 h p.i., thin sections of the transfected cells were processed for EM analysis as detailed in Materials and Methods. (C) The embedded thin sections were immunogold labeled with a mixture of anti-M1 MAbs. Bar, 200 nm.

when M1 was expressed alone, the protein was distributed in fractions containing membrane-associated proteins and in fractions containing soluble proteins (Fig. 6A). Under our experimental conditions, 25% of the expressed M1 protein was found in fractions 2, 3, and 4 (Fig. 6D), which contained the membrane-associated proteins (see the distribution of HA in Fig. 6B and C). However, on coexpression of the M1 protein with either HA and NA or HA alone, the amount of M1 which bound to cell membranes increased up to 60% (Fig. 6B to D). Therefore coexpression of HA stimulated binding of M1 to cell membranes.

DISCUSSION

M1 as the key element in influenza virus particle formation. We have demonstrated here that the M1 protein is the only viral structural component whose omission abrogated formation of VLPs, a result consistent with M1 playing a major role in virus particle assembly. Furthermore, we have shown that expression of the M1 protein in the absence of other influenza virus proteins is sufficient to drive the formation of vesicles, which resemble spikeless virions and which are released into the culture supernatant. Based on these findings, it is concluded that the M1 protein of influenza virus has all the structural information needed for self-assembly, interaction with cell membranes, and accomplishment of the budding process. However, our studies do not preclude the possibility that other viral proteins could also contribute to the budding process. As indicated in the introduction, expression of the M protein of VSV has been shown to induce the formation and budding of vesicles (22, 26). These VSV M-containing liposomes were ring-like and did not show the characteristic bullet-shaped structure of rhabdoviruses. Thus, we think that the situation presented here is more similar to that described for cells expressing the Gag component of retroviruses, where the released particles resemble true virions (7, 14). In this regard it is worth mentioning that structural similarities between the influenza virus M1 and the human immunodeficiency virus matrix protein have been observed (17).

Long M1-containing filamentous structures accumulated in the nuclei and cytoplasm of cells expressing exclusively the M1 protein, a feature also shared with cells that express the Gag protein from a cDNA (7, 14). It is known that the M1 protein forms homo-oligomers when expressed from a cDNA (54). Moreover, the three-dimensional structure of an amino-terminal fragment of the M1 protein predicts that this protein can form dimers which could self-assemble into large polymers (45). Therefore, we postulate that the intracellular tubular aggregates detected in transfected cells are exclusively made up of M1 proteins. We are aware that amorphous M1-containing electron-dense bodies have been detected in influenza virus-infected cells (36), but it should be mentioned that these aggregates do not bear any resemblance to the tubular structures described here. Moreover, to our knowledge, filamentous structures have never been described in cells expressing a recombinant influenza virus M1 protein.

M1 interactions involved in virus morphogenesis. We have demonstrated that coexpression of HA has two effects on the M1 protein: it reduces M1's tendency to form intracellular tubular structures, and it stimulates M1 binding to cell membranes. The enhanced membrane association of the M1 protein on coexpression of HA was also observed by Enami and Enami (9). However, other studies (23, 53) have failed to detect this effect. The reasons for these discrepancies are not known, and we can only speculate that the cell line used and/or

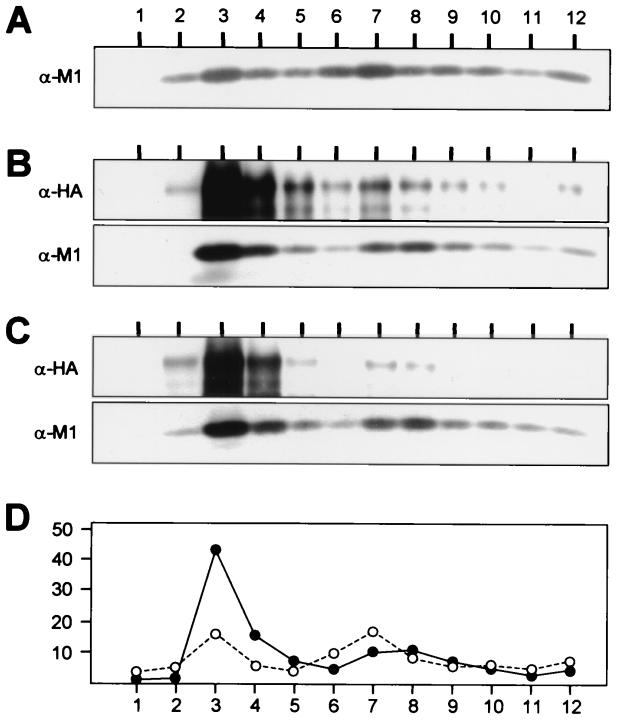

FIG. 6. Flotation analysis of transiently expressed influenza virus proteins. COS-1 cells were infected with vTF7-3 and transfected with different plasmids, and cell extracts were prepared and made 80% sucrose. These extracts were laid at the bottom of a centrifuge tube, which was overlaid with layers of 65 and 10% sucrose. Following centrifugation, fractions were collected from the top and analyzed by immunoblotting with either anti-HA or anti-M1 antibodies as indicated. The transfected cultures expressed M1 alone (A); M1 and HA (B); or M1, HA, and NA (C). (D) The M1 signals detected in the films shown in panels A (open circles) and B (filled circles) were quantitated by scanning and expressed as percentages of the total amount of M1 protein.

the origin of the M1 gene may contribute to the differences observed.

Our results suggest that there is an interaction between M1 and the cytoplasmic tail of HA, as previously indicated by other studies (see the introduction). We propose that a major contribution of this M1-HA interaction to the virus assembly process is to target the M1 protein to cell membranes, which are the site for virus assembly. As an indirect consequence of this interaction, the intracellular concentration of the non-membrane-bound M1 protein would be reduced, and thus formation of nonproductive intracellular tubular aggregates would be prevented.

We have demonstrated that interactions between the M1 protein and the RNPs are not crucial for formation of VLPs since VLPs can be formed in the absence of RNPs. However, the results of Fig. 1 indicate that if NP is present, it becomes packaged into the released VLPs, suggesting that there are contacts between M1 and RNPs which govern the packaging of the encapsidated RNAs into infectious virus particles. Further experiments are needed to analyze the molecular mechanism involved in this process.

We postulate that the M1 protein can be viewed as a brick containing lateral faces involved in M1 homo-oligomerization and two other faces (front and back), which contact the membrane and the inner components of the virion, respectively. The front and back faces would contain pockets or grooves for interaction with the glycoproteins of the membrane (external face) and the RNPs (inner face). Importantly, none of these heteromeric contacts are essential for the M1 protein to form homo-oligomers or drive the budding process.

For an influenza virus particle to be infectious it must contain all viral structural components. Therefore, all these elements should move to the proximity of the cell membrane for the assembly of virus particles. The membrane-spanning proteins are transported to the site of assembly through the secretory pathway of the cell, whereas the pathways used by the other structural elements to reach the point of assembly are ill defined. It has been shown for Sendai virus that the matrix protein binds to viral glycoproteins while they are in transit through the secretory pathway (43). It is thus tempting to speculate that a similar situation occurs in influenza virus-infected cells so that the glycoproteins would interact with M1 before reaching the cell membrane. At some point during this transit, the RNP complexes, either as preassembled RNP-M1 hetero-oligomers or as free RNPs, would bind to the glycoprotein-bound M1 protein. Once at the cell membrane the M1 protein present in the complexes would drive the budding process to allow formation of the infectious virion.

Individual roles of the virus structural proteins in formation of VLPs competent to deliver a CAT RNA. We showed previously that all virus structural proteins were required for formation of VLPs capable of transmitting an enclosed CAT RNA to MDCK cells (15, 32). Taking into account the results shown here, we can now provide an explanation for the lack of CAT transmission when only eight of the viral structural proteins were expressed. It is clear that lack of CAT transmission when protein M1 was not expressed was due to the absence of VLPs in the supernatant from transfected cells. When the NA gene was absent, VLPs were formed but they were not released into the supernatant fluids. On omission of HA, the released VLPs lacked the HA receptor binding activity needed for the VLPs to bind to the target MDCK cells. The situation when either the NEP or the M2 gene was not present in the transfection mixture is intriguing, since in both cases VLPs which apparently contain all viral structural proteins and the CAT RNA were formed. The fact that these particles do not transmit the CAT RNA to fresh MDCK cells is interpreted as suggesting that both M2 and NEP play a role during early stages in viral entry (i.e., the binding, uncoating, and unpackaging of the RNPs, etc.). In fact, a role for M2 ion channel activity during viral entry has been proposed (50), whereas no such activity has ever been assigned to the NEP polypeptide.

NEP and M1 are involved in nuclear export of RNPs later in infection (34, 50). Although it is shown here that VLPs formed in the absence of NEP contain the CAT RNA, we consider that this result should not be interpreted as suggesting that NEP is dispensable for nuclear export of RNPs. It should be noted that the cells that produce the VLPs are infected with vaccinia virus and that this infection causes important alterations in the cell metabolism. In fact, it has been observed that when NP alone is overexpressed using the vacinia virus T7 transient expression system (8), NP is found both in the nuclei and cytoplasm of the cells, indicating that the normal mechanism that retains NP, until late in the infectious cycle, in the nuclei of influenza virus-infected cells does not operate properly in the vaccinia virus-infected cells.

In summary, we have studied here the individual contribution of the influenza virus structural proteins to the process of virus particle formation and we have demonstrated the pivotal role played by the M1 protein in this process. In fact, we have shown that the M1 protein, in the absence of other viral polypeptides, can assemble into virus-like budding particles which are released into the culture medium. Moreover, we

have presented evidence that suggests that coexpression of the HA glycoprotein modulates the self-association and membrane-binding properties of the M1 polypeptide.

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