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Measles Virus Assembly within Membrane Rafts

SÉVERINE VINCENT, DENIS GERLIER,* AND SERGE N. MANIÉ

Immunité & Infections Virales, VPV, CNRS-UCBL UMR 5537, Faculté de Médecine Lyon RTH Laennec, 69372 Lyon Cedex 08, France

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During measles virus (MV) replication, approximately half of the internal M and N proteins, together with envelope H and F glycoproteins, are selectively enriched in microdomains rich in cholesterol and sphingolipids called membrane rafts. Rafts isolated from MV-infected cells after cold Triton X-100 solubilization and flotation in a sucrose gradient contain all MV components and are infectious. Furthermore, the H and F glycoproteins from released virus are also partly in membrane rafts (S. N. Manié et al., J. Virol. 74:305–311, 2000). When expressed alone, the M but not N protein shows a low partitioning (around 10%) into rafts; this distribution is unchanged when all of the internal proteins, M, N, P, and L, are coexpressed. After infection with MGV, a chimeric MV where both H and F proteins have been replaced by vesicular stomatitis virus G protein, both the M and N proteins were found enriched in membrane rafts, whereas the G protein was not. These data suggest that assembly of internal MV proteins into rafts requires the presence of the MV genome. The F but not H glycoprotein has the intrinsic ability to be localized in rafts. When coexpressed with F, the H glycoprotein is dragged into the rafts. This is not observed following coexpression of either the M or N protein. We propose a model for MV assembly into membrane rafts where the virus envelope and the ribonucleoparticle colocalize and associate.

The replication cycle of the morbillivirus measles virus (MV) occurs within the cytosol of target cells. After transcribing the genome to allow virus protein synthesis, the MV RNA-polymerase L associated with its cofactor, the phosphoprotein P, replicates the genome, which is simultaneously encapsidated by the nucleoprotein N (14). Together with the L and P proteins, the RNA-protein complex is assembled into the replication unit or ribonucleoparticle (RNP). The last step of MV assembly involves the wrapping of the RNP into an envelope derived from cell membrane regions enriched in the cytosolic M protein and two integral membrane glycoproteins, the hemagglutinin (H) and fusion (F) proteins (14). A current model favors an organizer role for the M protein, which lines the inner surface of the virus envelope. The M protein, which can interact with the cytoplasmic tail of the F protein (28), appears to act by concentrating the F and H proteins, as well as the RNP, at the site of virus assembly (3). Ultrastructural studies have shown that the final MV assembly likely occurs at the plasma membrane (6, 21). Alteration of MV assembly, including abrogation of M protein function, is likely responsible for a rare persistent infection of the central nervous system by MV, subacute sclerosis panencephalitis (3).

Recently, we proposed that specific regions of the plasma membrane called rafts may provide a site for the assembly of MV (19). Rafts are cholesterol- and glycosphingolipid-rich microdomains originally characterized by their insolubility at 4°C in nonionic detergent such as Triton X-100 (2). Recent reports favor the concept of rafts in living cells (9, 30). Glycosphingolipids have the properties of self-association and association with cholesterol (12) to constitute liquid-ordered membrane domains which are resistant to cold solubilization by nonionic detergents (1, 25). Such nonsolubilized membranes can be isolated from low-density fractions after flotation in a sucrose gradient (2). Some proteins, such as those anchored by a glycosylphosphatidylinositol moiety, are resident in rafts (2). Membrane rafts from MV-infected human cells are enriched in MV structural proteins since they contain 30 to 50% of the viral proteins and less than 5% of total cell proteins (19). The mature cleaved form of the F glycoprotein (F1F2 disulfide-linked heterodimer) but not the uncleaved F0 precursor associates with rafts (19), in agreement with the raft assembly process from the Golgi (26). Furthermore, isolated rafts contain all components required to create a functional virus, as revealed by their ability to infect cells. Finally, MV components from membrane rafts are incorporated into released MV particles. This work was undertaken to determine which MV component(s) can localize and assemble into rafts.

MATERIALS AND METHODS

Cells. Human epithelial (HeLa) cells were grown in Dulbecco's modified Eagle's medium supplemented with 6% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM glutamine, and 10 μg of gentamicin per ml at 37°C in the presence of 7% CO_2.

Viruses. The Edmonston strain of MV (23) and the chimeric virus MGV, where the reading frames of MV envelope glycoproteins H and F (MV-H and -F) were replaced by a single reading frame encoding the vesicular stomatitis virus G glycoprotein (VSV-G) (28), were amplified on Vero cells. The recombinant vaccinia viruses vvH, vvF, vvHF, vvN, vvNF (31), vvNP, vvNPL (15), and vvM (32) were amplified on BHK-21 cells. After one cycle of freezing/thawing of infected cells, clarified supernatants were collected and used as virus stocks.

Infections. After incubation overnight, HeLa cells were infected at the indicated MOI (multiplicity of infection). After 1 h at 37°C, the cells were washed once with fresh medium and incubated at 37°C in the presence or absence of the fusion-inhibitory peptide Z-D-Phe-L-Phe-Gly (24) at 20 μg/ml. The cells were collected 24 h after MV infection and 7 days after MGV infection in order to obtain virus protein expression in most cells at the onset of massive virus release. Infection or coinfection with recombinant vaccinia virus lasted 24 h prior to membrane raft analysis.

Antibodies. The following antibodies were used: rabbit anti-MV-F cytoplasmic tail, produced as reported by Cathomen et al. (4); anti-MV-H, BH195 (8); anti-MV-N, C125 (10); anti-MV-P, a mouse anti-MV serum; anti-MV-M (Chemicon, Temecula, Calif.); anti-CD55, 12A12 (17); anti-CD71 (Zymed Laboratories, San Francisco, Calif.); and anti-VSV-G, P5D4 (Sigma, St. Louis, Mo.).

Isolation of membrane rafts after Triton X-100 extraction at 4°C and flotation on sucrose gradients. Infected HeLa cells (3×10^6), washed in ice-cold phosphate-buffered saline and harvested in phosphate-buffered saline–EDTA, were lysed on ice for 20 min in TNE (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM

* Corresponding author. Mailing address: Immunité & Infections Virales, VPV, CNRS-UCBL UMR 5537, Faculté de Médecine Lyon RTH Laennec, 69372 Lyon Cedex 08, France. Phone: 33 4 78 77 86 18. Fax: 33 4 78 77 87 54. E-mail: gerlier@laennec.univ-lyon1.fr.

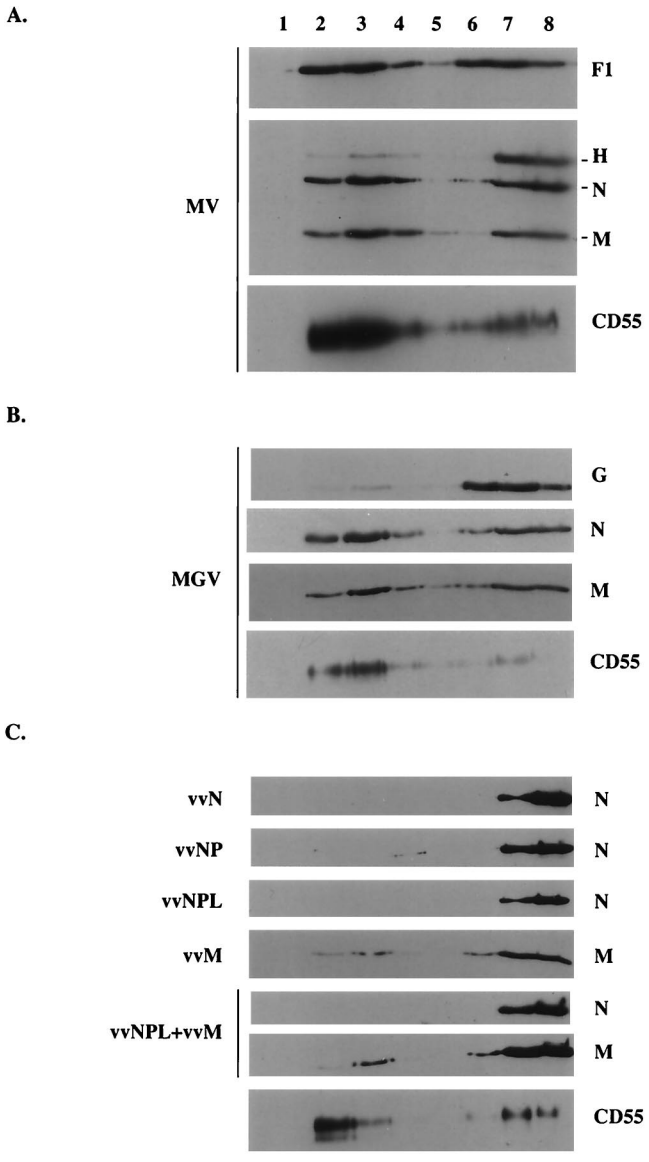

FIG. 1. Only coexpression of the MV genome allows the N and M proteins to localize into membrane rafts. HeLa cells were infected for 2 days at an MOI of 1 with MV tag (A), for 7 days with MGV (MOI of 2) (B), or for 24 h with recombinant vaccinia virus (MOI of 5) encoding one, two, three, or four of the MV internal proteins (C). Cells were then lysed with 0.5% Triton X-100 at 4°C, and the membrane rafts were separated by flotation on a discontinuous sucrose gradient. Immunoblot analyses of MV proteins from each fraction of the gradient (fraction 1 represents the top of the gradient) were performed with specific antibodies. The distributions of CD55, a resident of rafts, and of G protein, which is not located in rafts, are shown as controls.

EDTA) containing 0.5% Triton X-100 and a cocktail of protease inhibitors (Complete; Boehringer Mannheim). The cell lysate volume was made up to 800 μl with TNE and sucrose 60% (wt/wt) to obtain a 45% sucrose concentration in a 6-ml centrifuge tube. The lysate was sequentially overlaid with 2.5 ml of 30% sucrose and 1 ml of 2.5% sucrose in TNE. The gradient was centrifuged at 200,000 × *g* for 16 h in an SW50.1 rotor (Beckman) and fractionated from the top of the tube in eight fractions. The membrane rafts are resistant to solubilization by Triton X-100 at 4°C, and their flotation allows their recovery from the low-density fractions (fractions 2, 3, and 4) of the discontinuous sucrose gradient.

Western blot analysis. An aliquot of each fraction was used for immunoblotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. Membranes were saturated with 5% nonfat dried milk in TBS-T (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) and incubated for 1 h with specific antibodies in TBS-T–1% milk. Immunoreactive bands were visualized by using secondary horseradish peroxidase-conjugated antibodies (Promega, Madison, Wis.) and enhanced chemiluminescence (Boehringer Mannheim). Quantification of the autoradiograms was performed after scanning, using the ImageQuant software (Molecular Dynamics).

RESULTS

The internal MV proteins associate with rafts only when coexpressed with the MV genome. Membrane rafts were isolated from MV-infected HeLa cells after cold solubilization with 0.5% Triton X-100 and flotation in a sucrose gradient. The low-density fractions 2, 3, and 4 contained the membrane rafts, as shown by the predominant localization of the glycolipid-anchored CD55, a protein resident of rafts (Fig. 1A). A significant proportion of the MV proteins (~16% of H, ~59% of F, ~42% of N, and ~45% of M) was found to be associated with the rafts (Fig. 1A; Table 1). To verify the ability of internal MV proteins to localize within the rafts in the absence of the envelope H and F transmembrane proteins as previously observed in another cell line (19), the HeLa cells were infected with MGV (see Materials and Methods) (2). Similar proportions of MV-N and -M (~63 and 44%, respectively; Table 1) were found to be associated with rafts, which contain ~95% of the CD55 raft marker but less than 4% of VSV-G (Fig. 1B). To evaluate the intrinsic ability of internal MV proteins to localize into the rafts, the M and N proteins were expressed alone or in association with other MV internal proteins after infection with recombinant vaccinia virus. When expressed alone, only trace amounts of the N protein (~2%) and a low but constant amount of M (~20%) could associate with the rafts (Fig. 1C; Table 1). The coexpression of N with P, N with P and L, or the four MV internal proteins N, P, L, and M did not result in any change in the distribution of N or M within the sucrose gradients. Taken together, these results indicate that the M but not the N protein has an intrinsic, albeit low, ability to associate with the membrane rafts and that a major redistribution of the M and N proteins into rafts requires the presence of the MV genome.

MV-F can drag MV-H into rafts. The ability of the envelope glycoproteins to associate with membrane rafts was then investigated. When expressed alone using recombinant vaccinia virus, ~47% of the F1 protein and ~1% of the H protein were found to localize with the rafts characterized by their high CD55 contents. As previously reported (33), the CD71 molecule did not cosediment with rafts (Fig. 2A and B). However, when coexpressed with the F protein, ~29% of the H protein was found to associate with the raft fractions (Fig. 2C). This increase in raft localization of the H protein was specific since the CD71 molecule remained nonassociated with the rafts. Thus, MV-F has the intrinsic ability to associate with the membrane rafts and can drag into them the H glycoprotein as well.

TABLE 1. Cumulative data from several experiments showing the distribution of MV proteins within membrane rafts

HeLa cells infected by:	Mean % of MV protein localized in raft ± SD			
	N	M	F	H
MV	42 ± 12	45 ± 21	59 ± 3	16 ± 5
MGV	63	44		
vvN	2 ± 2			
vvM		20 ± 6		
vvF			47 ± 14	
vvH				1 ± 2
vvHF			44 ± 1	29 ± 8

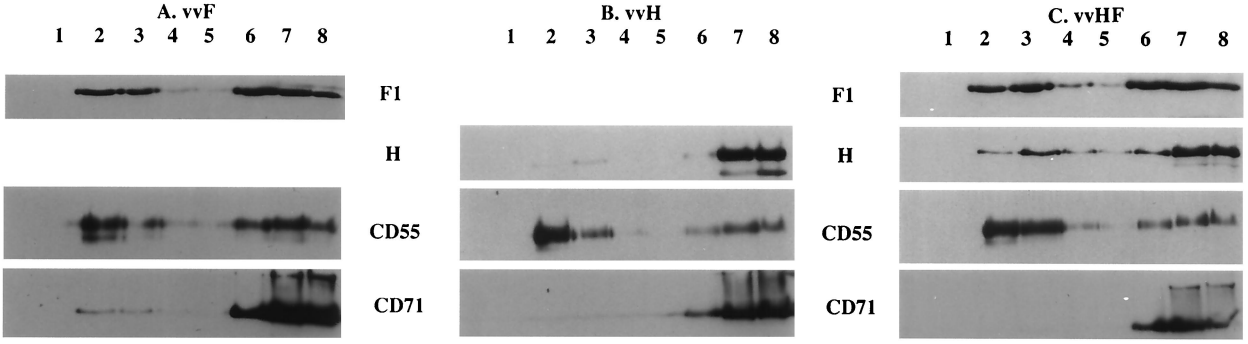

FIG. 2. MV-F drags MV-H into membrane rafts. HeLa cells were infected for 24 h with recombinant vaccinia virus (MOI of 5) encoding MV-F (A), MV-H (B), or both (C). Cells were then subjected to extraction by Triton X-100 at 4°C and flotation. The distribution in the gradient of MV proteins together with CD55 and CD71 as control proteins was assayed by Western blotting.

Inability of MV-F to drag internal MV proteins. Coexpression of the envelope F glycoprotein with the internal M or N protein after infection with recombinant vvNF or both vvM and vvF did not result in any change in the distribution of the M and N proteins throughout the sucrose gradient (Fig. 3A). Indeed, although ~40% of F1 colocalized with most of the CD55 in the light fractions, only trace amounts of the N and ~10% of the M protein were found associated with these fractions, as observed in the absence of the F protein.

Inability of the MV RNP to drag H protein into rafts. Likewise, coexpression of the MV RNP with the H glycoprotein, after super infection of MGV-infected HeLa cells with vvH, did not allow the H glycoprotein to significantly associate with the rafts (Fig. 3B). Indeed, ~32% of the N and M proteins and only trace amounts of the G and H proteins cosedimented with ~82% of the CD55.

DISCUSSION

When MV-H, -F, -M, and -N were expressed alone using recombinant vaccinia virus, only the F and M proteins exhibited high and low, respectively, intrinsic abilities to localize into the cell membrane rafts. A possible interference of the vaccinia virus infection in the distribution of MV proteins in rafts was excluded because (i) the distribution of MV proteins in infected cells remained unchanged after coinfection with a vaccinia virus (unpublished data; see also the similar distributions of MV-M and -N after infection with MGV [Fig. 1B] and coinfection with vaccinia virus [Fig. 3B]) and (ii) similar results were obtained when these proteins were transiently expressed using eukaryotic vectors (unpublished).

Coexpression of the RNP proteins N, P, and L did not result in a significant redistribution of the N protein into the rafts. This could be due neither to the lack of N polymerization, because coexpression of the N and P proteins spontaneously results in the formation of nucleocapsid-like structures likely wrapping cellular RNAs (7, 27), nor to the use of recombinant vaccinia virus, because similar results were obtained for the 293-3-46 cell line (23), which constitutively expresses both N and P proteins (not shown). Likewise, an unbalanced proportion of N, P, and L proteins was unlikely, since the three proteins were expressed in proportion suitable for the transcription and replication of an MV CAT-DI minigenome (reference 15 and data not shown). When the M protein was coexpressed with the N, P, and L proteins, the N protein did not redistribute to the rafts despite the known interaction of the RNP with the M protein (13, 29). Only the presence of the MV genome resulted in the strong targeting of both M and N proteins into the membrane rafts. We propose that only the MV genome RNA could allow the formation of the RNP scaffold for wrapping into an M protein lattice. This lattice would in turn be more efficiently targeted to (or more stably associated with) the rafts than isolated M proteins.

The F protein allows the H glycoprotein to associate with the membrane rafts in a proportion similar to that observed after MV infection. This correlates with the known propensity of

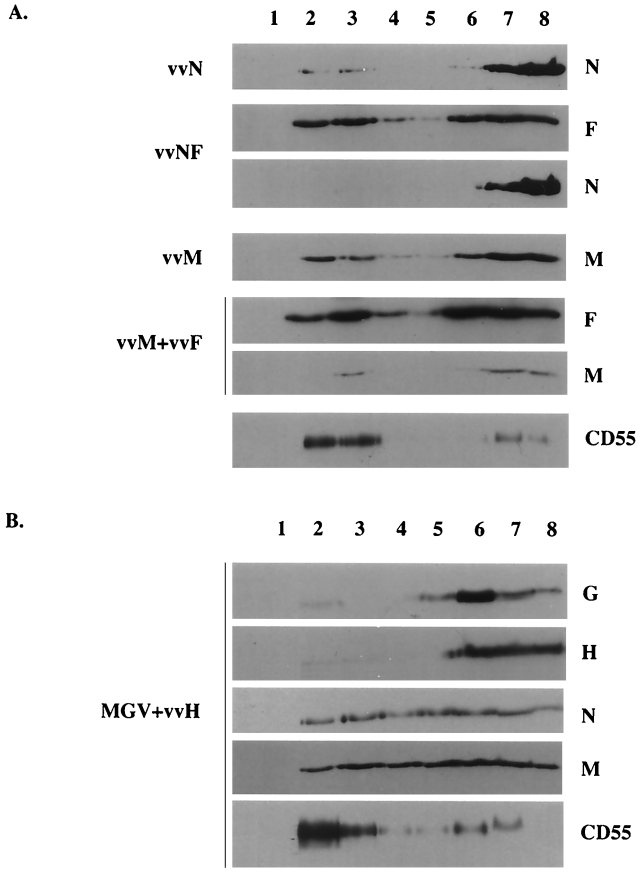

FIG. 3. Neither MV-F (A) nor the MV RNP (B) can drag into membrane rafts the internal MV proteins (A) and MV-H (B). (A) HeLa cells infected with the recombinant vaccinia viruses (MOI of 5) indicated at the left; (B) cells coinfected with MGV (MOI of 2) and with vaccinia virus encoding MV-H. Cells were analyzed as described for previous figures.

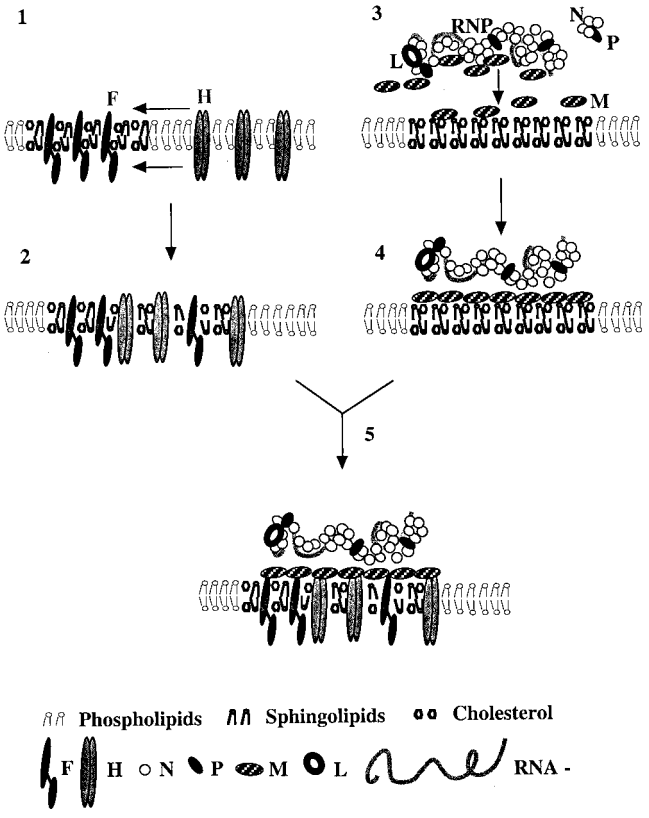

FIG. 4. Assembly model for MV. The F glycoprotein is spontaneously targeted to the rafts (step 1) and drags the H protein into the rafts (step 2). Which of these two steps come first will depend on the ability of H and F proteins to interact early during their biosynthesis within the endoplasmic reticulum. Independently, the RNA genome and the N, P, and L proteins associate into the RNP in the cytosol (step 3). Interactions with the M protein enable the M-RNP complex to associate with the rafts (step 4). The interaction of the M lattice with the cytoplasmic tails of the F proteins allows merging of the M-RNP complex with the membrane envelope H and F glycoproteins within the raft platform (step 5). These steps do not necessarily occur sequentially and may be synchronized. Furthermore, this model is likely to be oversimplified; for example, it is not unlikely that the assembly process involves at one stage the coalescence of several membrane rafts (see text).

these two components of the virus envelope to interact with each other. Indeed, the H and F proteins can be coimmunoprecipitated (18), and several observations argue for their association into a functional complex allowing the fusion of the virus envelope with the target cell membrane. (i) Mutant H and F proteins should be appropriately paired (5). (ii) The fusion helper function of H is ablated by a single amino acid substitution at residue 98 (16). (iii) Some anti-H monoclonal antibodies inhibit the fusion step without inhibiting attachment of the H protein to the CD46 cellular receptor (11, 16).

The M protein can interact with the cytoplasmic tail of the F protein. Indeed, it is specifically recruited into the recombinant virus MG/FV, where the H and F genes have been replaced by a gene encoding VSV-G fused to the cytoplasmic tail of MV-F, whereas the M protein was excluded from the MGV particles (28). The association of the M protein with the rafts is, however, not enhanced when M is coexpressed with the F protein. This suggests that a stable interaction of the M protein with the F cytoplasmic tail requires its association with the MV RNP and enhanced localization into the rafts. Likewise, the inability of the M-RNP complex to drag the H glycoprotein into the rafts indicates that the putative interaction of M with the cytoplasmic tail of H is too weak or absent. While this report was under review, Naim et al. showed that in MV-infected polarized human epithelial cells, F and H glycoproteins are intrinsically targeted to the basolateral site and that M protein reverts the transport of F and H glycoproteins from the basolateral site to the apical cell surface, where MV budding occurs (20). Since the later targeting is often associated with the interaction of sorting signals in the *trans*-Golgi network with membrane rafts (26), they postulated that the membrane rafts could be involved in the apical sorting of MV nucleocapsid and glycoproteins. If this is true, one prediction derived from their and our findings would be that the M protein expressed in the absence of the RNP should not be appropriately targeted to the apical site and/or able to redirect the F protein to the apical site.

Taken together, our data suggest that the membrane rafts can act as platforms for the assembly of MV prior to virus budding. We propose the following assembly model for MV (Fig. 4). The F glycoprotein is spontaneously targeted to the rafts (step 1), and its ability to associate with its envelope partner, H, allows the localization of H-F complexes within the rafts (step 2). Which of these two steps comes first will depend on the ability of H and F proteins to interact early during their biosynthesis within the endoplasmic reticulum. Independently, the RNA genome and the N, P, and L proteins associate to form the RNP in the cytosol (step 3); this allows scaffolding of the M lattice and targeting of the M-RNP complex to the rafts owing to the discrete intrinsic propensity of the M protein to associate with membrane rafts (step 4). Through the interaction of the M lattice with the cytoplasmic tail of the F protein, the M-RNP complex merges with the membrane envelope H and F glycoproteins within the raft platform (step 5). The envelope-RNP complex would now be ready for budding into mature MV particles. These steps do not necessarily occur sequentially and may be synchronized. Furthermore, this model is likely to be oversimplified. The assembly process could involve at one stage the coalescence of several membrane rafts, owing to their small size (<70 nm in diameter) determined on the plasma membrane of viable cells (22, 30). The latest step of virus formation into close vesicles is also not drawn because analysis of mature virus particles has shown that they are made partly of nonraft membranes (19), indicating that any budding trough membrane rafts would be associated with capture of adjacent nonraft membranes. This model is now being tested to establish whether MV buds exclusively through membrane rafts or also through a raft-independent pathway.

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