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Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes

Harry Vennema[1], Gert-Jan Godeke, John W.A.Rossen, Wim F.Voorhout[2], Marian C.Horzinek, Dirk-Jan E.Opstelten[3] and Peter J.M.Rottier

Department of Infectious Diseases and Immunology, Division of Virology, Yalelaan 1, 3584 CL Utrecht, PO Box 80.165, 3508 TD Utrecht and [2]Department of Functional Morphology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[3]Present address: Center for Biotechnology, Karolinska Institute, Novum, 141 57 Huddinge, Sweden

[1]Corresponding author

Budding of enveloped viruses has been shown to be driven by interactions between a nucleocapsid and a proteolipid membrane. By contrast, we here describe the assembly of viral envelopes independent of a nucleocapsid. Membrane particles containing coronaviral envelope proteins were assembled in and released from animal cells co-expressing these proteins' genes from transfected plasmids. Of the three viral membrane proteins only two were required for particle formation, the membrane glycoprotein (M) and the small envelope protein (E). The spike (S) protein was dispensable but was incorporated when present. Importantly, the nucleocapsid protein (N) was neither required nor taken into the particles when present. The E protein, recently recognized to be a structural protein, was shown to be an integral membrane protein. The envelope vesicles were found by immunogold labelling and electron microscopy to form a homogeneous population of spherical particles indistinguishable from authentic coronavirions in size (~100 nm in diameter) and shape. They were less dense than virions and sedimented slightly slower than virions in sucrose velocity gradients. The nucleocapsid-independent formation of apparently bona fide viral envelopes represents a novel mode of virus assembly.

Keywords: budding/coronavirus/envelope protein/virus assembly/virus-like particle

Introduction

Basically, viruses are mobile genetic elements equipped for swift spread from cell to cell; enveloped viruses employ a membrane to facilitate their intercellular transport. Budding at cellular membranes allows the virus to acquire its envelope and leave the cell without injury. The viral envelope protects the genome in the extracellular environment and provides a convenient device to deliver it into the target cell's cytoplasm by membrane fusion. Like intracellular transport vesicles, which also use processes of budding and fusion, the viral envelope contains proteins that mediate binding to, and fusion with, the acceptor membrane. Envelope proteins are also involved in the formation of the particle and in maintaining its integrity.

The process of viral envelope formation is not well understood. Available evidence points to a crucial role played by the viral nucleocapsid (NC). In retroviruses, it is all that is required, as the gag core particle can direct its envelopment without the need for envelope proteins (Delchambre *et al.*, 1989; Gheysen *et al.*, 1989; Karacostas *et al.*, 1989). Capsids of togaviruses are not sufficient to drive their own budding. Here, the viral membrane proteins are indispensable, and virion assembly involves direct interactions between them and the NC (Suomalainen *et al.*, 1992; Hobman *et al.*, 1994; Lopez *et al.*, 1994; Qiu *et al.*, 1994; Zhao *et al.*, 1994). Probably, the icosahedral capsid acts as a scaffold responsible for the curvature of the envelope. Viral membrane proteins are in some cases secreted in the absence of core components. Expression of the hepatitis B virus surface antigen, for instance, leads to the assembly and release of subviral particles (Patzer *et al.*, 1986; Simon *et al.*, 1988; Bruss and Ganem, 1991), which, however, are not simply 'empty' envelopes as they are morphologically distinct from hepatitis B virions (Dubois *et al.*, 1980; Laub *et al.*, 1983; Patzer *et al.*, 1986). Subviral lipoprotein particles were also observed after synthesis of flavivirus envelope proteins (Mason *et al.*, 1991; Konishi *et al.*, 1992; Yamshchikov and Compans, 1993; Allison *et al.*, 1995).

Coronaviruses are among the largest enveloped RNA viruses, measuring 80–120 nm in diameter. Their membrane, which surrounds the 30 kb genome packaged in a helical nucleocapsid, contains two major glycoproteins. The type I spike (S) glycoprotein makes up the surface projections typical for the viruses. The M glycoprotein is most abundant, largely embedded within the membrane bilayer and spans the membrane three times. The small envelope protein (E) is a third, minor structural component in infectious bronchitis virus (IBV), transmissible gastroenteritis virus (TGEV) and mouse hepatitis virus (MHV) particles (Liu and Inglis, 1991; Godet *et al.*, 1992; Yu *et al.*, 1994); it has only recently been identified and is not extensively characterized. Some coronaviruses contain an additional membrane protein with haemagglutinin/esterase activity (HE; reviewed by Spaan *et al.*, 1988).

Coronaviruses are assembled intracellularly at membranes of the intermediate compartment, between the endoplasmic reticulum (ER) and the Golgi complex (Tooze *et al.*, 1984, 1988; Klumperman *et al.*, 1994; Krijnse Locker *et al.*, 1994). The helical nucleocapsids which are synthesized in the cytoplasm align at these membranes (Dubois-Dalcq *et al.*, 1982) and probably interact with the cytoplasmic domains of the viral membrane proteins. They are incorporated into viral particles by budding. The immature virions are exported from the cell along the

exocytic pathway while undergoing various post-assembly maturation events including proteolytic and oligosaccharide processing.

Little is known about the molecular interactions that drive coronaviral budding and control the specificity of the process. Direct interactions have been demonstrated between the M and the S protein: when co-expressed from their cloned genes they associate into large heteromeric complexes (Opstelten *et al.*, 1995). Surprisingly, these complexes do not localize to the intermediate (i.e. budding) compartment but rather accumulate in the Golgi complex, the site where the coronaviral M protein generally localizes when synthesized alone (Krijnse Locker *et al.*, 1992; Klumperman *et al.*, 1994). The S protein, which on its own is transported to the plasma membrane, is thus retained in the Golgi complex through its interaction with M. Interestingly, M also forms complexes in the absence of S. Independently synthesized M protein accumulates in the Golgi apparatus in homomultimeric, detergent-insoluble structures, presumably as part of its retention mechanism (Krijnse Locker *et al.*, 1995).

For the study of coronavirus assembly a system is required in which the roles of and the interactions between the assembly partners can be analysed in molecular detail. Since an infectious cDNA clone is presently not feasible due to the large size of the coronaviral genome we have exploited the co-expression approach. We show here that viral envelope proteins can self-assemble into membrane-bound structures that are morphologically indistinguishable from coronavirions.

Results

Co-expression of selected MHV-A59 genes

Expression of viral envelope protein genes has been shown in some cases to result in the production of subviral lipoprotein particles. We never observed such particles in the culture medium when expressing the coronavirus MHV-A59 M or S genes either individually or together (Opstelten *et al.*, 1995). Minor amounts of the S1 cleavage product of S were detected but this was merely due to dissociation from its membrane-anchored partner S2 at the cell surface (see below). In the experiments to be described below we included the E protein gene and this proved to be essential for the formation of coronavirus-like particles.

Particle assembly and secretion were initially assayed by measuring the release of structural proteins into the culture medium of cells expressing MHV protein genes by the recombinant vaccinia virus bacteriophage T7 RNA polymerase system. In the experiment of Figure 1 the cells and media were harvested separately and processed for immunoprecipitation with a polyclonal anti-MHV rabbit serum mixed with anti-S monoclonal antibodies, followed by SDS–PAGE analysis. We found that neither the M protein nor the 180 kDa S protein (S/gp180) was released into the medium when their genes were expressed individually (lanes 2 and 4). However, co-expression of the M and E gene resulted in release of the M protein into the medium (lane 6). Release of the uncleaved mature S/gp180 form of S and of the M protein occurred when the three envelope protein genes, S, M and E, were co-expressed (lane 8). This did not occur when either the M

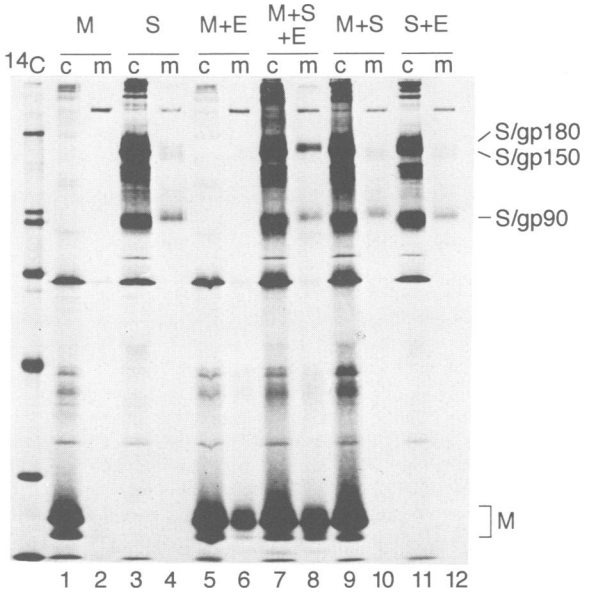

Fig. 1. Co-expression of MHV envelope protein genes. Recombinant vaccinia virus vTF7-3-infected OST7-1 cells were transfected with one or a combination of plasmids each containing one of the MHV genes M, E or S downstream a T7 promoter. Cells were labelled for 1 h with [^{35}S]methionine followed by a 2 h chase. Both the cells (c) and the culture medium (m) were prepared and used for immunoprecipitation and the precipitates were analysed by SDS–PAGE. The MHV genes used are indicated above each set. The positions of the different forms of the S protein and of the M protein, which occurs as a set of differently *O*-glycosylated species, are indicated on the right. Separation of intracellular S/gp180 and S/gp150 was only visible on a short exposure of the gel. The long exposure is shown to demonstrate the absence of S/gp150 in the medium. Lane marked ^{14}C contains marker proteins of 200, 100, 92.5, 69, 46, 30 and 14 kDa.

gene or the E gene were omitted (lanes 10 and 12). Appearance of the proteins in the medium did not result from cell lysis since the 150 kDa immature S protein precursor (S/gp150), which was still abundant in the cell lysate after 2 h of chase (lanes 3, 7, 9 and 11), was not found in the medium. Thus, co-expression of selected MHV genes leads to specific release of MHV envelope proteins. All medium samples from cells expressing the S gene contained the 90 kDa S1 cleavage product. This was therefore not a suitable marker for particle assembly and release. Rather, the M protein and the 180 kDa S protein species were used as such, since these were not released from cells transfected with non-productive combinations of genes. In later experiments we used the non-productive combination of the M and S gene as our negative control.

Subsequently, we also included the gene encoding the viral nucleocapsid protein (N) in our transfections. Co-expression of N with the three envelope protein genes did not affect the release of the 180 kDa S protein nor of the M protein into the culture medium (data not shown). Some N protein was found in the medium but this was also the case and to a similar extent when its gene was expressed alone. Therefore, the N protein was not released specifically and release of envelope proteins was independent of nucleocapsid.

We also tested the generality of our findings by repeating the above experiments with the analogous genes of another coronavirus, feline infectious peritonitis virus (FIPV).

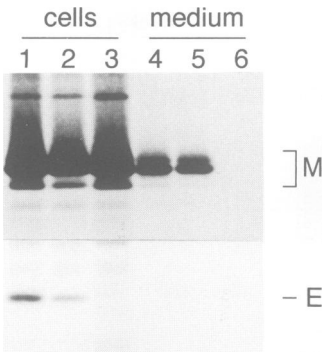

Fig. 2. Co-expression of the M gene with three different gene 5 constructs. Lanes 1 and 4, pTM5ab, containing the intact ORFs 5a + 5b. Lanes 2 and 5, pTM5b, having only ORF 5b. Lanes 3 and 6, pTM5a, containing only ORF 5a. Cells were labelled with [^{35}S]methionine and chased, followed by immunoprecipitation of the viral proteins from the cell lysates and the culture media. Precipitates were analysed by electrophoresis in a 15% polyacrylamide gel. The upper part shows the different M protein bands. The lower part of the figure which displays the section of the gel containing the E protein, is shown after prolonged exposure.

Again, viral membrane protein release was fully dependent on the M and E gene products (data not shown).

The E protein is essential for secretion of M

The E gene expression construct pTM5ab used above contains the unique region of MHV-A59 mRNA 5 behind a T7 promoter. In this region the ORFs 5a and 5b are present, the latter encodes the E protein which is translated by internal initiation (Thiel and Siddel, 1994). The mRNA transcribed from our construct may allow translation of both ORFs, as has previously been shown for a similar construct by Budzilowicz and Weiss (1987). To confirm that the observed effect was due to the ORF 5b expression product alone we prepared two additional constructs. In construct pTM5b, ORF 5a was eliminated and ORF 5b was positioned immediately behind a T7 promoter. In construct pTM5a, a frameshift mutation was introduced into pTM5ab to render ORF 5b inactive. Both constructs and pTM5ab were co-transfected with the M gene expressing plasmid pTUMM, and viral proteins were assayed as before. Analysis of the cell lysates (Figure 2) showed a protein of the expected size (9.7 kDa) in the lanes of pTM5ab and pTM5b, not of pTM5a. The protein was detected only after prolonged exposure of the gel. Since the rabbit antiserum used had been raised against highly purified virus, its ability to immunoprecipitate the E protein, though only to a low extent, indicates that E is a structural protein of MHV. However, we could not rule out the possibility that the overproduced protein was precipitated non-specifically or co-precipitated with the M protein. No potential 5a protein band was seen after immunoprecipitation. In the functional assay, elimination of ORF 5a, as in construct pTM5b, had no noticeable effect: the M protein was released into the medium as efficiently as with the complete construct. Knocking out the expression of ORF 5b as in pTM5a, however, completely abolished the release of M; its secretion from cells is therefore fully dependent on the E protein alone. Secretion of E was not detected in these experiments.

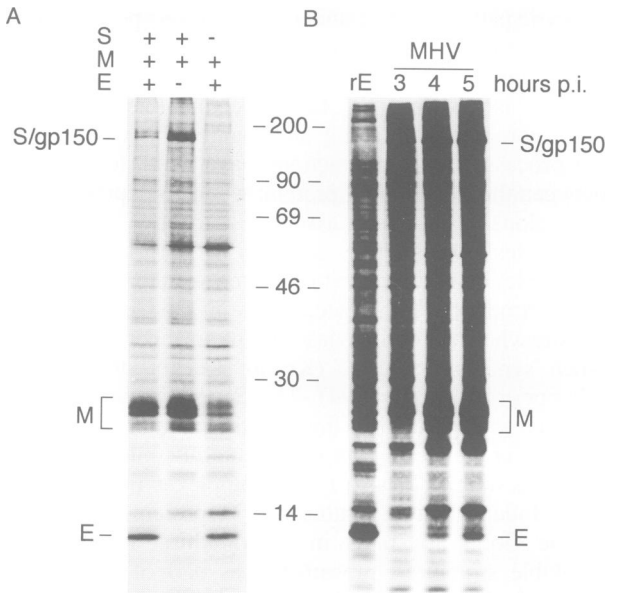

Fig. 3. Characterization of the MHV E protein. Cells were labelled for 1 h with [^{35}S]methionine. Membranes were isolated by ultracentrifugation of cell homogenates after sodium carbonate treatment at pH 11 and analysed directly in a 15% polyacrylamide gel. (**A**) Cells were infected with vTF7-3 and transfected with vectors expressing the genes indicated by a + sign. A band of ~10 kDa is seen only in the samples of cells transfected with the E gene vector. (**B**) Cells infected with MHV were labelled starting at the times indicated above the lanes. The E protein appears concomitant with the other structural proteins. A sample from recombinant E gene expressing cells was co-electrophoresed for reference (lane marked rE).

The MHV E protein is an integral membrane protein

In view of its apparent importance for MHV assembly the E protein was further characterized. Its primary sequence predicted a large NH_2-terminal hydrophobic domain that might anchor the protein in the membrane. To demonstrate that it is an integral membrane protein we used an alkaline extraction method. Dounce homogenates were prepared of cells expressing either the M, S and E genes, the M and S genes, or the M and E genes. After removal of the nuclei by pelleting, the homogenates were treated with 100 mM Na_2CO_3 at pH 11 for 10 min on ice. This treatment disrupts membrane vesicles, releases their protein content, and strips off peripheral membrane proteins, leaving integral membrane proteins unaffected (Fujiki *et al.*, 1982). The membrane pellet after ultracentrifugation was analysed by SDS–PAGE and found to contain all three proteins (Figure 3A). The E protein was readily identified by its absence from the sample of cells transfected with the M and S gene only. No coronaviral proteins were detected in the supernatant fractions. Subsequently, MHV-infected cells were fractionated similarly after three consecutive 1-h periods of ^{35}S-labelling, in order to follow the appearance of E (Figure 3B). The protein was identified by co-electrophoresis of the recombinant expression product prepared in the same way. Between 3 and 4 h post-infection most viral proteins are still at low levels of synthesis which increase during the subsequent hours when the E protein starts to appear together with M and S. The experiments show that the MHV E protein is an

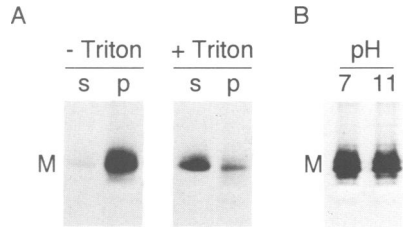

Fig. 4. Analysis of secreted coronaviral structures. [^{35}S]methionine-labelled material released from cells expressing the MHV E and M protein genes was analysed by ultracentrifugation after various treatments. (**A**) To one of a set of two equal samples of culture medium Triton X-100 was added to a concentration of 1%. The samples were ultracentrifuged and the supernatants (s) and pellets (p) were analysed after immunoprecipitation. (**B**) Two other samples of culture medium were diluted 2-fold with carbonate buffer adjusted to pH 11 or to pH 7 as indicated, and ultracentrifuged. The resulting pellets were analysed directly in a 15% polyacrylamide gel. Only the sections of the gels containing the M proteins are shown.

integral membrane protein. In MHV-infected cells it is one of the specific proteins that are increasingly produced in the course of infection.

The released envelope proteins are membrane associated

Detergent treatment of the structures released from the co-transfected cells gave us a first indication that the proteins were contained in membranes. Culture medium from cells co-expressing the M and E genes was analysed by ultracentrifugation directly or after treatment with the detergent Triton X-100. The pellets were solubilized and processed for RIPA in parallel with their respective supernatants. In the untreated controls, the M protein was present in the pellet and virtually absent in the supernatant (Figure 4). After treatment of the culture medium with Triton X-100, however, the M protein remained largely in the supernatant; only a small fraction was pelleted.

The alkaline extraction method was then applied to the culture medium. Medium samples were incubated for 10 min on ice in 100 mM Na_2CO_3 at pH 11 or, as a control, at pH 7. After centrifugation the pellets were solubilized in sample buffer and analysed directly by SDS–PAGE. The high pH treatment did not affect the M protein's appearance in the pellet (Figure 4), indicating that it is an integral part of membranes. These data support the notion that the envelope proteins secreted from the cells are constituents of membranous structures, presumably in the form of particles.

Density of particles in sucrose gradients

The particle density was analysed by flotation in sucrose gradients. To this end we dissolved crystalline sucrose in the culture supernatant to a final concentration of 60% (w/w). Subsequently, 1.5 ml of this solution was overlayed with 0.9 ml each of 50, 40, 30 and 20% (w/w) sucrose solutions. The gradients were centrifuged and five fractions of 1 ml were collected. This fractionation scheme allowed us to collect each interphase in one fraction. Fractions were diluted 2-fold to allow pelleting of particles by ultracentrifugation. The selected vector combinations for transfection were M+S+E, M+E and M+S, the latter serving as a negative control. As a positive control we used culture medium of MHV-infected cells treated in the same manner. The MHV particles were found at the highest density, in fraction 3 which contains the 40/50% interphase (Figure 5A). A polypeptide of ~10 kDa, presumably representing the E protein (indicated with an arrowhead), co-purified with MHV virions. The particles derived from M+S+E-expressing cells always floated to a slightly higher position in these gradients and appeared in fractions 3 and 4 corresponding with the 40/50% and 30/40% interphases, respectively. Here, a weak band of the E protein could be discerned in fraction 4 after overexposing the gel. Particles devoid of the S protein produced by M+E-expressing cells, on the other hand, floated consistently to lower density than particles containing S; they were found mainly in fraction 4, which contains the 30/40% interphase (Figure 5B). No viral proteins were seen floating when the S and M genes were co-expressed. In fraction 5 of the sample of MHV-infected cells (Figure 5A) there appears to be only M protein present. These particles might be very similar to the particles produced by M+E-expressing cells.

The data allow a comparison of the yields of viral particles released from transfected and MHV-infected cells. Taking the radioactivity in the M protein as a measure we estimated that the amounts of particles produced by cells expressing M+S+E and M+E were ~20% and ~10% that of virions secreted from infected cells, respectively. The yields of particles varied between 5% and 50% relative to MHV among different experiments, probably due to variations in transfection efficiency. Frequently, more particles were released when the S protein was co-expressed than in its absence. Apparently, S protein-induced fusion of neighbouring cells containing incomplete sets of genes increased productivity by increasing the availability of individual components to the assembly system.

Analysis of particles by electron microscopy

In order to visualize the particles that were apparently assembled when coronaviral envelope proteins were co-synthesized in cells, preparations of extracellular material purified by pelleting through a 30% (w/w) sucrose cushion were analysed by electron microscopy (EM). Immunogold labelling was employed for particle identification using the polyclonal anti-MHV serum and a protein-A–gold conjugate. Labelled particles were observed in material from cells expressing the M+E or M+S+E genes (Figure 6). The particles were spherical and had the same appearance as MHV particles analysed in parallel. They also had the same dimensions. While the mean diameter of MHV virions was 108.9 ± 13.4 nm (n = 30), that of the M+S+E particles was 102.6 ± 15.2 nm (n = 30) and that of M+E particles 102.6 ± 9.8 nm (n = 30). Material derived from cells expressing the M+S genes showed no particles. Occasional gold labelling was seen here, probably due to the presence of some S1 material released into the medium (data not shown). No labelling of particles occurred with control sera.

Sedimentation analysis of particles

MHV and the virus-like particles (VLPs) were compared by velocity centrifugation in 5–30% (w/w) sucrose gradients. Fractions were collected and analysed as described above. Consistently, MHV particles sedimented slightly

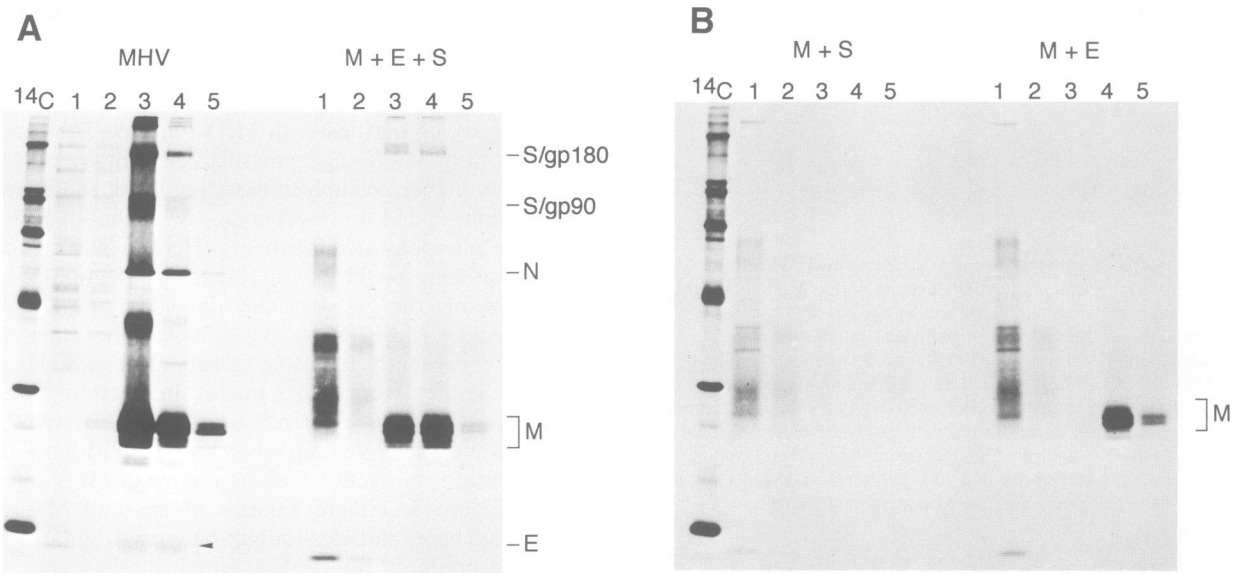

Fig. 5. Flotation gradient analysis of particles and MHV. Cells infected with MHV and cells expressing combinations of MHV envelope protein genes were labelled with [^{35}S]methionine and chased for 2 h. Medium samples were adjusted to 60% sucrose and overlaid with a sucrose gradient. After centrifugation, five fractions were collected from which the particles were analysed in a 15% polyacrylamide gel. Vector combinations used are indicated above the panels and the positions of the viral proteins are given at the right. Fractions 1 and 5 are bottom and top fraction, respectively.

ahead of M+S+E VLPs which in turn sedimented slightly faster than M+E particles (Figure 7). The sedimentation profiles were similar with respect to peak width for all types of particles, indicating that the particles were of comparable homogeneity. Sedimentation analysis of VLPs produced by cells expressing M+E+S+N did not reveal N protein co-purifying with the particles containing S and M, indicating that it was not physically associated with the envelopes.

Discussion

Co-expression of coronaviral envelope protein genes in cells leads to the assembly and secretion of particles morphologically indistinguishable from authentic coronavirions. Only the M and E proteins are required to drive their formation; the S protein and the N protein (and, by consequence, the nucleocapsid) are dispensable. Earlier we have shown that the MHV M protein alone can associate into large homomeric complexes, presumably by lateral interactions between M molecules in the plane of the membrane (Krijnse Locker *et al.*, 1995). Extensive EM work with M proteins from various coronaviruses never gave any indication that the protein produced on its own would form particles or induce curvature in the intracellular membranes in which it appears (Machamer *et al.*, 1990; Krijnse Locker *et al.*, 1992; Klumperman *et al.*, 1994). This implies that the E protein is pivotal in inducing this curvature and that coronaviruses acquire their particular shape and dimension by the concerted action of just these two membrane proteins.

Through our observations we have identified a novel budding principle for enveloped viruses: nucleocapsid-independent assembly of bona fide virion envelopes. In the two other well-studied models the nucleocapsid plays a central role (Figure 8). In type D retroviruses the gag core particle is the sole requirement: It is able to bud in the absence of envelope proteins (see Hunter, 1994). Specific interactions between the nucleocapsid and viral membrane proteins are the driving force in the alphavirus model (Garoff *et al.*, 1994; Strauss and Strauss, 1994). Here, enveloped particles are formed neither by the nucleocapsid alone nor by the membrane proteins alone (Suomalainen *et al.*, 1992). However, nucleocapsid-independent formation of enveloped particles has been demonstrated before. Subviral particles were, for instance, produced by the synthesis of the hepatitis B or Japanese encephalitis virus envelope proteins (Simon *et al.*, 1988; Bruss and Ganem, 1991; Konishi *et al.*, 1992). Heterogeneous enveloped particles were also released from cells producing the VSV G protein (Rolls *et al.*, 1994). Apparently, these were generated by vesiculation at the plasma membrane rather than by a specific assembly process. We can only speculate why coronaviruses have the capacity to assemble uniform envelopes from their membrane proteins: the helical nature of the (probably random-coil) nucleocapsid and the lack of a classical matrix protein may necessitate an intrinsic morphogenetic potential of the envelope proteins.

While the M protein has long been recognized as being essential for coronavirus assembly (for a review, see Rottier, 1995), the crucial role of the E protein came as a surprise. Only recently has E been identified as a virion protein, having been overlooked due to its small size and low abundance. It was estimated to occur in IBV and TGEV at a rate of about 100 and 20 molecules per particle, respectively (Liu and Inglis, 1991; Godet *et al.*, 1992). For MHV our preliminary estimates were some 5–10 molecules per virion using M as a reference, with 726 molecules per virion as calculated for human coronavirus (HCV) OC43 (Hogue and Brian, 1986). The stoichiometry of M and E in VLPs appeared similar although the low particle yields did not allow accurate measurements. This low abundance suggests that the E protein has no genuine structural function in the virion envelope in that it occupies frequent, regular positions within the lattice built by

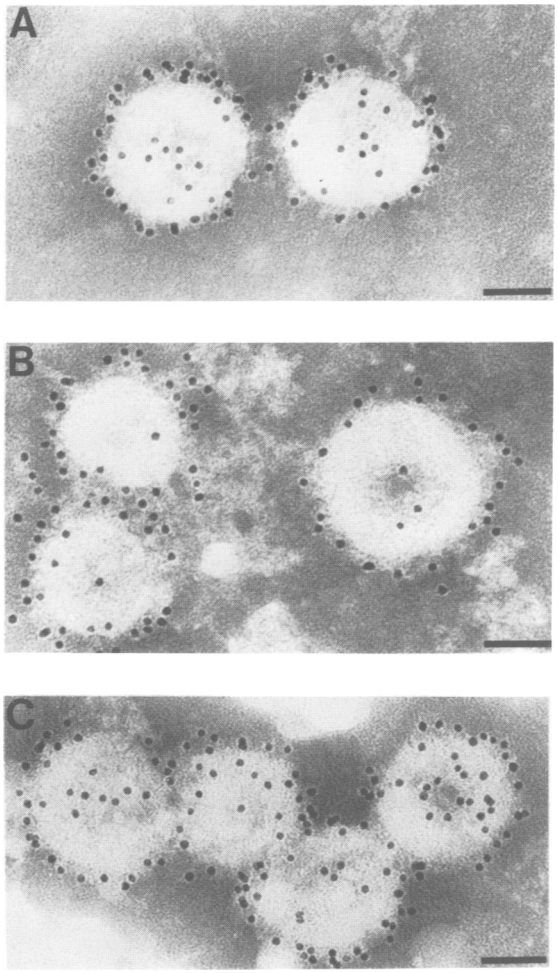

Fig. 6. Electron microscopy of particles and MHV. Virus released from MHV-A59-infected cells (**A**) and particles secreted from cells expressing the M, E and S genes (**B**) or M and E (**C**) were purified by pelleting through a 30% (w/w) sucrose cushion. Particles were viewed by EM after immunogold labelling. Bar markers represent 50 mm.

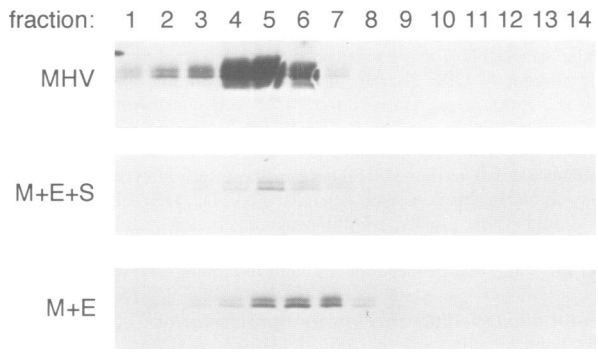

Fig. 7. Sedimentation analysis of VLPs and MHV. Cells infected with MHV and cells expressing combinations of MHV envelope protein genes were labelled for 4 h with [^{35}S]methionine. Viral particles released into the culture media were sedimented into a 5–30% sucrose gradient by centrifugation. Fractions were collected from the bottom and particles were pelleted from each fraction for direct analysis in 15% polyacrylamide gel, of which the section containing the M protein is shown to locate the position of the particles. Fractions 1 and 14 are bottom and top fraction, respectively.

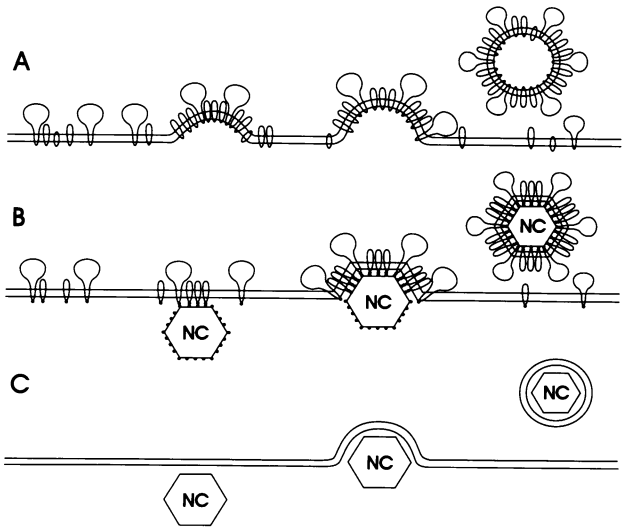

Fig. 8. Models of budding of enveloped viruses. In model (**A**), budding is nucleocapsid-independent and driven only by lateral interactions between viral membrane proteins. Nucleocapsid-dependent assembly is depicted in models (**B**) and (**C**). In the former, exemplified by the alphaviruses, the specific interactions between nucleocapsid and viral membrane proteins are the driving force for virus assembly. Budding driven by direct interactions of the nucleocapsid with the host cell membrane, without the necessary involvement of viral membrane proteins, as has been demonstrated for type D retroviruses, is shown in model (C). NC, nucleocapsid.

M molecules. Rather, it may have a morphogenetic function by taking infrequent but strategic positions within the lattice to generate the required membrane curvature. Alternatively, its role might merely be 'catalytic', serving for instance to close the 'neck' of the viral particle as it pinches off from the membrane in the terminal phase of budding.

Too little is known about the E protein to understand its function. The polypeptide of 80–110 residues is hydrophobic, particularly in its NH_2-terminal domain, though a classical cleaved signal sequence is not predicted. Consistently, it was shown by immunofluorescence to be associated with the cell surface and with internal membranes (Abraham *et al.*, 1990; Smith *et al.*, 1990; Godet *et al.*, 1992; Yu *et al.*, 1994); we demonstrate here that in MHV the E protein is membrane-anchored. Its membrane structure and topology can only be guessed. Antibodies to the C-terminal domain resulted in cell surface fluorescence (Godet *et al.*, 1992) and neutralized infectivity in the presence of complement (Yu *et al.*, 1994); these observations suggest that the C-terminal region is translocated into the lumen of the ER during biogenesis. The MHV-A59 E protein is acylated (Yu *et al.*, 1994), probably at sites within the conserved cysteine cluster close to the middle of the molecule.

Small, often acylated proteins occur in the membranes of many enveloped RNA viruses. Examples are the influenza virus M2 and the alphavirus 6K proteins. Like E, these are synthesized in ample amounts in infected cells but are incorporated into virions in only minor amounts. The 6K protein was suggested to facilitate the budding stage (Liljeström *et al.*, 1991; Loewy *et al.*, 1995). The E protein has been speculated to function as an ion channel-like influenza virus M2 (Liu and Inglis, 1991), but this activity

has not yet been demonstrated. Of the several small proteins induced by lentiviruses the HIV-1 vpu protein bears resemblance to E in that it enhances virion maturation and release; however, vpu has not been detected in extracellular virions and is not essential for virus replication (see Subramanian and Cohen, 1994). An intriguing protein is $p6^{Gag}$, a peptide derived from the C-terminus of the Gag precursor of primate lentiviruses. It has been implicated in a late step in budding (Göttlinger *et al.*, 1991; Huang *et al.*, 1995). The equivalent of this latter function was recently also identified in an unrelated retrovirus, the avian Rous sarcoma virus which lacks a similar cleavage product but has instead an assembly domain located between the matrix and capsid sequences (Parent *et al.*, 1995). Defects in this domain lead to the accumulation of Gag proteins at the plasma membrane due to the impairment of viral particle release (Wills *et al.*, 1994).

An intriguing feature of coronaviruses is their flexibility in accommodating various numbers of different envelope proteins. As we show here, VLPs can be assembled without S, the spike protein which mediates binding of virions to and fusion with the host cell membrane. This observation is consistent with earlier findings showing that spikeless, hence non-infectious, virions are secreted when infected cells are treated with tunicamycin (Holmes *et al.*, 1981; Rottier *et al.*, 1981a). Inhibition of N-glycosylation induces the S protein to aggregate in the ER (our unpublished data) thereby blocking its incorporation into viral particles. Tunicamycin similarly affects HE: when HCV OC43 was grown in the presence of the inhibitor, virions were produced which now lacked both S and HE (Mounir and Talbot, 1992). Another illustration of the flexibility of the coronavirus envelope was the incorporation of HE protein into a genetically HE-deficient strain, as shown by Liao *et al.* (1995). Expression of the MHV JHM(2) HE gene in MHV-A59-infected cells, yielded A59 particles containing the heterologous protein.

We have recently shown that incorporation of S into virions is mediated by its specific interaction with M (Opstelten *et al.*, 1994, 1995). S associates with M into large, detergent-insoluble complexes when they are co-expressed from their cloned genes and in infected cells. Since the M protein alone can assemble into higher-order complexes (Krijnse Locker *et al.*, 1995), we speculate that specific positions are available in such an M protein matrix where S protein trimers can be accommodated. Whether the HE protein can occupy similar positions or is incorporated into virions through a different mechanism remains to be seen. The specificity of the interactions between the viral envelope proteins may function not only to include kin components into viral particles, it probably also serves to exclude host membrane proteins from them.

The picture of coronaviral envelope formation thus emerging is one in which membrane proteins (viral and cellular) are sampled for fit into the M protein lattice. The specificity of the molecular interactions acts as a quality control system to warrant the formation of defined two-dimensional assemblies containing the full complement of viral membrane proteins and excluding cellular ones. The involvement of the E protein in these structures is decisive in inducing the membrane curvature and causing budding. As the nucleocapsid is not required, it needs to entertain specific interactions with the envelope proteins in order to become sequestered into particles. As a prediction of this view, nucleocapsid-less particles might also be produced during coronavirus infection. Such particles have indeed been described for IBV. They had a typical coronavirus morphology and a lower density than virions (Macnaughton and Davies, 1980).

Materials and methods

Cells and viruses

The A59 strain of mouse hepatitis virus (MHV-A59) was used throughout. Recombinant vaccinia virus vTF7-3 encoding the T7 RNA polymerase (Fuerst *et al.*, 1986) was obtained from Dr B.Moss. OST7-1 cells (Elroy-Stein and Moss 1990; obtained from Dr B.Moss) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 IU penicillin/ml, 100 μg streptomycin/ml and 500 μg G418/ml (all from Gibco Laboratories, Life Technologies Ltd, Paisley, UK).

Infection and transfection

Subconfluent monolayers of OST7-1 cells in 10-cm^2 tissue culture dishes were infected with MHV-A59 in phosphate-buffered saline containing 50 mg DEAE–dextran/l (PBS–DEAE), or with recombinant vaccinia virus in DMEM, for 1 h at 37°C at a multiplicity of infection of 20. At 1 h after vaccinia virus infection cells were transfected with 5 μg DNA of each selected construct and 10 μl Lipofectin© (Gibco Laboratories) in 0.5 ml DMEM for 10 min at room temperature. Incubation was continued at 37°C for 10 min after which 0.5 ml DMEM was added. Cells were transferred to 32°C at 2 h after infection with the vaccinia virus to allow efficient folding of the S protein (H.Vennema, H.Blaak, G.-J.Godeke, M.C.Horzinek and P.J.M.Rottier, manuscript in preparation).

Expression vectors

Expression constructs used for transfection were pTUMS, with a *Bam*HI-fragment containing the MHV S gene (Vennema *et al.*, 1990) and pTUMM, with a *Xho*I-fragment containing the MHV M gene (Rottier and Rose, 1987), both cloned in pTUG3 (Vennema *et al.*, 1991), which provides the bacteriophage T7 transcription regulatory sequences. The MHV N gene was cloned as a *Sty*I–*Pst*I fragment from pRG68 (Bredenbeek *et al.*, 1987) downstream of the T7 promoter in pBluescript SK^- (Stratagene) to yield pBMN. Construct pTM5ab contains the MHV gene 5 ORFs 5a and 5b, the latter encoding the E protein. It was prepared by ligating a T7 promoter/MHV leader fragment, similar to a synthetic fragment described previously (van der Most *et al.*, 1991), to the insert of clone pRG68, which contains a nearly full-length copy of mRNA 5, including most of the leader sequence. The tandemly duplicated leader sequences were eliminated by limited T4 DNA polymerase digestion in the absence of nucleotides, followed by re-annealing of complementary single-stranded ends and transformation. Correct repair in the bacterial host was confirmed by sequence analysis. The sequences downstream of ORF 5b were deleted by restriction enzyme digestion and re-ligation. A derivative of pTM5ab was constructed by digestion with the restriction enzyme *Bst*EII, which has a single recognition site in ORF 5b. The overhanging ends were filled in with Klenow DNA polymerase and re-ligated, resulting in the insertion of five nucleotides in ORF 5b. In this construct, designated pTM5a, ORF 5b is inactivated by a frameshift. Finally, pTM5b was constructed by cloning a *Taq*I–*Eag*I restriction fragment from pRG68 containing ORF 5b into *Acc*I- and *Eag*I-digested pBluescript SK^- and re-cloning into pTUG3.

Metabolic labelling and immunoprecipitation

Metabolic labelling was performed in methionine-free MEM without FCS with 50 μCi/ml ^{35}S *in vitro* cell labelling mix (Amersham) for 1 h. Pulse labelling was preceded by a starvation period of 30 min in methionine-free MEM. During chase periods the cells were incubated in DMEM containing 10% FCS and four times the normal amount of methionine. Cells were lysed in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl (TES) containing 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride. Lysates were kept on ice for 15 min and cleared by centrifugation for 15 min at 10 000 *g* and 4°C. Medium samples were cleared likewise by centrifugation at room temperature and subsequently used for radioimmunoprecipitation (RIP) or gradient

analysis. For RIP, the medium was brought to 1× TES and 1% Triton X-100 by adding a concentrated stock solution, followed by a further dilution with 2 volumes TES containing 0.1% Triton X-100. To 200 µl cell lysate, 1 ml detergent solution containing 50 mM Tris–HCl, pH 8, 62.5 mM EDTA, 0.4% sodium deoxycholate, 1% Nonidet P-40 was added and sodium dodecyl sulfate (SDS) to 0.25%. Antisera were added at a 100-fold dilution. For immunoprecipitation of MHV proteins hyperimmunized rabbit serum 134 (Rottier *et al.*, 1981b) was used, mixed with monoclonal antibodies A.1.3, A.3.10 and A.1.4 (Gilmore *et al.*, 1987), and J.7.6 (Fleming *et al.*, 1983). The latter were added to more efficiently precipitate the S protein. RIP was carried out overnight at 4°C. The immune complexes were bound to Immunoprecipitin (Gibco Laboratories) for 1 h at 4°C and subsequently pelleted by centrifugation. Pellets were washed three times in 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40 (RIPA buffer) for the cell lysates and in TES–0.1% Triton X-100 for the medium samples. The final pellets were suspended in sample buffer and heated at 95°C for 3 min before analysis by SDS–PAGE according to Laemmli (1970).

Alkaline extraction of cellular membranes

Cells were lysed in a 10-fold diluted TES buffer by 20 strokes in a tight-fitting Dounce homogenizer on ice. Nuclei were pelleted by centrifugation at 1000 *g* for 10 min. The supernatant was diluted 2-fold by adding an equal volume of 200 mM Na_2CO_3 adjusted to pH 11, and incubated for 10 min on ice. Membranes were pelleted in a Beckman TLA100 rotor (Beckman Instruments, Inc., Fullerton, CA, USA) at 65 000 r.p.m. for 30 min at 4°C. The pellet was dissolved in sample buffer and analysed by SDS–PAGE.

Flotation analysis

Samples of cell culture medium were made up to 60% sucrose (w/w) with crystalline sucrose and 1.5 ml was transferred into a centrifuge tube and overlayed with 0.9 ml each of 50, 40, 30 and 20% (w/w) sucrose solutions in TES. The gradients were centrifuged for 72 h at 50 000 r.p.m. in a Beckman SW50 rotor and fractionated into five fractions, each of 1 ml. This fractionation scheme allowed each interphase to be collected in one fraction. Fraction 1 was 60% sucrose, fraction 2 contained the 50–60% interphase, fraction 3 the 40–50% interphase, fraction 4 the 30–40% interphase, and fraction 5 the 20–30% interphase. Fractions were diluted 2-fold with TES to allow pelleting of particles by ultracentrifugation for 30 min at 4°C in a Beckman TLA100.2 rotor at 65 000 r.p.m. Pellets were dissolved in sample buffer and analysed by SDS–PAGE.

Electron microscopy

Culture media of cells were collected at 8 h post-infection and spun for 10 min at 10 000 *g* to pellet cell debris. The cleared medium was then layered on a sucrose gradient consisting of 1 ml 30% (w/w) sucrose, 1 ml 20% (w/w) sucrose/1% glutaraldehyde and 1 ml 10% (w/w) sucrose in TES, pH 6.5. The gradients were centrifuged for 3 h at 30 000 r.p.m. in a SW50.1 rotor at 4°C. After the run the pellet was suspended in TES, pH 6.5. A formvar–carbon-coated copper grid was placed on top of 5 µl of the suspension for 30 min. Subsequently, the grids were rinsed three times with PBS containing 50 mM glycine (PBG) and treated for 30 min with 5% bovine serum albumin (BSA, fraction 5; Sigma) and 1% acetylated BSA (BSA-C; Aurion) in PBG. Samples were incubated for 1 h with the rabbit polyclonal antiserum 134 to MHV-A59 diluted 200-fold in PBG containing 0.5% BSA and 0.1% BSA-C (PBG^+). After three washes with PBG^+, samples were incubated with protein A–5 nm gold diluted to an OD_{520} of 0.1 in PBG^+ (Slot and Geuze, 1985) for 30 min, rinsed three times with PBG^+ and twice with water. Finally, samples were negatively stained by placing the grids for 30 s in 2% potassium phosphotungstate solution, pH 6.8. The grids were viewed and photographed in a Philips CM10 electron microscope at 100 kV.

Velocity gradient sedimentation

Cells infected with MHV and cells expressing combinations of MHV envelope protein genes were labelled for 4 h with 133 µCi/ml ^{35}S *in vitro* cell labelling mix (Amersham). Culture media were collected and cleared by centrifugation at 4°C for 15 min at 10 000 *g*. Particles present in medium samples were sedimented into gradients of 5–30% (w/w) sucrose in TES, pH 6.5, by centrifugation for 1 h at 40 000 r.p.m. in a Beckman SW41 rotor at 4°C. Fractions of 0.5 ml were collected from the bottom of the tubes. Particles were pelleted from each fraction by ultracentrifugation for 30 min at 4°C in a Beckman TLA100.2 rotor at 65 000 r.p.m. and analysed directly by SDS–PAGE.

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