MHV-A59 Gene 1 Proteins are Associated with Two Distinct Membrane Populations

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1. INTRODUCTION

All of the stages of coronavirus replication that have been investigated have been shown to occur on or within intracellular membranes. We and others have shown that mouse hepatitis virus (MHV) RNA synthesis occurs in association with intracellular membranes (Bi *et al.*, 1998; Denison *et al.*, 1999; Dennis and Brian, 1982; Sethna and Brian, 1997; Shi *et al.*, 1999; van der Meer *et al.*, 1999). Several proteins processed from the gene 1 (replicase gene) polyprotein have been shown by immunofluorescence and electron microscopic approaches to be associated with intracellular membranes. Specifically, membranes containing markers for late endosomes have been shown to be sites of localization of newly synthesized viral RNA as well as at least one of the mature gene 1 proteins (van der Meer *et al.*, 1999). However, different patterns of gene 1 protein localization and interaction have been reported in the settings of distinct cell types, experimental approaches and virus strains. Most recently, it has been shown by confocal microscopic analysis of MHV-A59 infected cells that multiple replicase gene proteins as well as structural proteins may not completely colocalize but rather are organized in closely associated or "interdigitated" membranes, suggesting that proteins involved in MHV RNA synthesis may be localized to more than one membrane population (Bost *et al.*, 2000).

In this report we have used biochemical fractionation of MHV-A59 infected cells to determine if gene 1 proteins, MHV structural proteins M

The Nidoviruses (Coronaviruses and Arteriviruses).
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and N, and viral RNA localize to one or more membrane populations. In addition, we have defined nature of the membranes to which the viral proteins localize using both enzymatic and marker protein analyses. We have shown that gene 1 proteins localize to at least two biochemically distinct membrane populations, only one of which is the location of newly synthesized viral RNA.

2. METHODS AND MATERIALS

2.1 Virus Infection and Radiolabeling

DBT cells were infected with MHV-A59 as previously described (Sims *et al.*, 2000). Cells were propagated on 150 cm^2 flasks and were infected for 6 hr, with actinomycin D added at 2.5h post infection. For radiolabeling and lysis, cells were removed from the flasks by trypsinization and 1 x 10^8 cells were suspended in 2ml of DMEM lacking methionine and cysteine but containing 2% FCS and isotope for labeling protein [^{35}S met/cys] or RNA [^3H uridine]. All labeling was performed for 1-2 hrs between 6 and 8 h p.i.

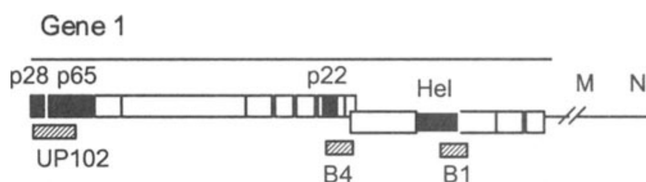

Figure 1. Organization of the MHV genome. The location of the domains within gene 1 encoding p28, p65, p22 and Hel are shown as black boxes within the gene 1 coding region. The hatched boxes show the polypeptides used to generate the rabbit polyclonal sera used in this study directed against the proteins shown in black. Antibodies against M and N are described in the text.

2.2 Cell Fractionation, Antibodies and Immunoprecipitation

Following infection and radiolabeling, cells were lysed using a ball bearing homogenizer in a sucrose-Tris buffer in the absence of detergent (Sims *et al.*, 2000). Differential centrifugation of the lysed cells was performed to obtain pellets and cytosol at 1000 x g (P1 or nuclear pellet), 2,300 x g (P2.3), 100,000 rpm (P100), and the residual cytosol (S100). The

membranes in the P100 pellet were fractionated on a 10 to 30% Iodixanol (Optiprep-Nycomed) gradient. Both crude differential fractionation pellets, S100, and gradient fractions were analyzed for the presence of MHV proteins by immunoprecipitation in TTK buffer containing 1% Triton X100. Antibodies used in these experiments were rabbit polyclonal sera directed against p28 and p65 (UP102) (Denison *et al.*, 1995), p22 (B4) (Lu *et al.*, 1998), Hel (B1) (Denison *et al.*, 1999), and mouse monoclonal antibodies directed against M (J.1.3) and N (J.3.3) obtained from John Fleming (Fig. 1).

2.3 Assays for Cellular Proteins

Iodixanol gradient fractions were assessed for endosomes/lysosomes (aLAMP-1 antibodies) Golgi membranes (galactosyltransferase activity), endoplasmic reticulum (NADPH-cytochrome C reductase activity), as previously described (Sims *et al.*, 2000).

3. RESULTS

3.1 Association of MHV Replicase Proteins with Membranes

Antibodies directed gene 1 proteins and the structural proteins M and N were used to immunoprecipitate proteins both from crude differential fractionation pellets and gradient fractions. When the crude pellets and cytosol were analyzed, the gene 1 proteins p28, p65, p22, and Hel, as well as the structural M protein were detected almost exclusively to the high speed P100 pellet. N was most abundant in P100, but was also readily detected in the nuclear pellet, low speed P2.3 pellet and the S100 cytosol. The P100 pellet is post mitochondrial, and has been shown to contain small membranes of endosomes, lysosomes, ER, and Golgi. Thus these results were consistent with previous studies showing localization of M to Golgi and of gene 1 proteins to endosomes, and possible to ER. Since all of the replicase proteins studied localized to P100, this pellet was selected for further fractionation on the iodixanol gradient, as well as for studies of RNA localization

3.2 MHV Proteins Segregate to Distinct Membranes

The P100 pellets were fractionated on the iodixanol gradient and separated into 10 fractions before immunoprecipitation. A summary of the

results is shown in Fig. 2. A clear pattern emerged with the proteins localizing toward the two extremes of the gradient. P28 and helicase were detected only in fractions 7 through 10 (most dense), with peak of detection in fractions 8 and 9. N was also most abundant in fractions 8 and 9, although it had a broader distribution on the gradient, being readily detectable in fractions 5 through 9. The pattern with p22 and p65 was remarkably distinct, with peaks in fractions 2 and 3 and detectability in fractions 1 through 5 (least dense).

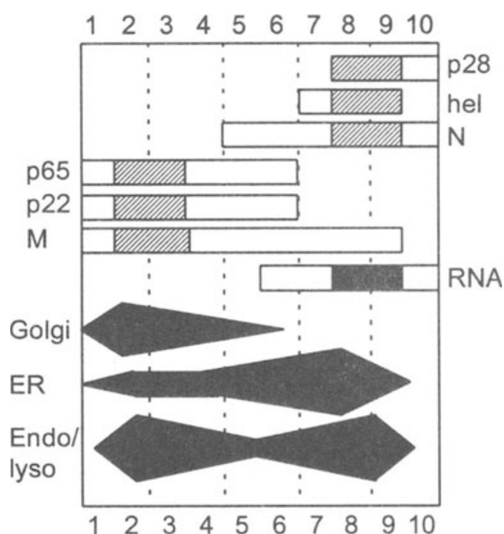

Figure 2. Fractionation of gene 1 proteins, structural proteins, viral RNA and cellular marker proteins on an iodixanol gradient. Fraction numbers are above and below the figure. White rectangles show the extent of detectability on the gradient. Hatched boxes show areas of maximal detection. Black box shows area of maximal RNA detection. Black polygons show relative detection of markers for Golgi, ER and endo/lysosomes

3.3 Viral RNA Localizes to Membranes with p28, Hel, and N

MHV specific, actinomycin D resistant viral RNA synthesized between 6 and 7 h p.i. was predominantly detected in the P100 crude fraction, as determined by total TCA precipitable [^{3}H]uridine incorporation. When the P100 pellet was fractionated, viral RNA was detected only in fractions 6 through 10, with a very distinct peak in fractions 8 and 9.

3.4 Identification of Membrane Proteins in Gradient Fractions

The enzymatic marker for Golgi (Gal-T) was concentrated in fraction 2 and 3, although as expected, some activity above baseline was detected in almost all fractions. This was consistent with localization of M to the same fractions. The ER marker (NADPH cyt C Red) showed a detectable level across the gradient but a distinct peak in fractions 6 through 8. Finally, the protein marker for endosomes/lysosomes (LAMP 1) showed a bimodal pattern, with clear peaks in fractions 1 through 3 and 8 through 10, respectively. Thus the marker for endo/lysosomes was present in both membrane populations containing gene 1 proteins, and the ER marker overlapped only with those containing p28, N, hel and viral RNA.

4. DISCUSSION

The results of this study demonstrate that gene 1 and structural proteins may segregate to distinct membrane populations during infection. If the proteins analyzed in this study are representative of the remainder of gene 1 proteins, then two different membrane-associated complexes will be the sites of gene 1 protein localization and function. This outcome was suggested in our previous confocal microscopic study that demonstrated that several gene 1 proteins did not completely colocalize but rather colocalized to different extents and demonstrated areas of close approximation and interdigitation at the resolution of light (Bost *et al.*, 2000). The present study confirms and extends those results by showing that several membrane-associated gene 1 proteins localize to distinct membranes that are readily separable using the least stringent approaches to cell lysis and fractionation. In fact, given that even cell marker proteins show some distribution across the entire gradient under these conditions, it was remarkable that the gene 1 protein/membrane populations showed no overlap on the gradient. This result suggests that the interaction of the membrane/protein populations may serve different functions, or may interact to mediate one function, specifically viral RNA synthesis, in a manner that is easily altered and may change over time. At the least, it appears that the interaction of these populations observed by both light and electron microscopy is not mediated by covalent or strong hydrophobic forces that are difficult to disrupt.

It was also interesting that all viral RNA synthesized during this same period of time (6 through 8h p.i.) was detected only in the fractions containing p28, helicase, and the peak of N. The presence of hel in these fractions suggests that this membrane population might be the site for RNA

synthesis. However, the localization of the RdRp (polymerase) has not been determined using these approaches. Thus it is possible that replication may occur in the interface of the closely approximated membrane/protein complexes, and that newly synthesized RNA may then target to the membranes along with hel and N.

Finally, the cell marker experiments along with previous data support the conclusion that the membranes containing p22 and p65 are derived from late endosomes. The membranes containing p28, hel, N and RNA either contain a mixed population of ER and lysosomal membranes or possibly ER alone. The latter possibility is suggested by the fact that proteins such as hel do not colocalize with lysosomes as determined by markers for acidic compartments (data not shown). Together these data suggest a model in which the interface of different membrane/protein complexes forms a "macro" complex where protein expression, protein processing, and RNA synthesis occurs.

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