

**COMPLEX FORMATION BETWEEN THE SPIKE  
PROTEIN AND THE MEMBRANE PROTEIN DURING  
MOUSE HEPATITIS VIRUS ASSEMBLY**

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**SUMMARY**

Using different approaches we have demonstrated the formation of a complex between the S protein and the M protein in the process of mouse hepatitis virus (MHV) assembly. Preservation of the M/S heterocomplexes was critically dependent on the solubilization conditions. Pulse-chase labeling of MHV-infected cells followed by a co-immunoprecipitation assay revealed that newly synthesized S and M engage in complex formation with different kinetics, the S protein reacting much slower. Sedimentation experiments showed the M/S heteromultimer complexes to be very large. A model for the role of the complex formation in MHV assembly is presented.

**INTRODUCTION**

Coronaviruses mature by budding into intracytoplasmic pre-Golgi membranes (1). In the case of mouse hepatitis virus (MHV) electronmicroscopic observations have shown helical nucleocapsids to attach to regions of these membranes (2). Assuming that the viral envelope proteins localize the budding one would expect these proteins to accumulate here. However, when expressed independently both the M and the S proteins appear to localize differently: M accumulates in the Golgi apparatus (3, 4) while S is transported to the plasma membrane (H.Vennema and P.Rottier, unpublished observations). This suggests that in infected cells an interaction between the two viral membrane proteins may be required to direct them to and retain them at the site of budding. This interaction is probably very specific since virions contain only trace amounts of host proteins. Using different

approaches we describe here the detection of complexes between the S protein and the M protein and their initial characterization.

## **MATERIALS AND METHODS**

### **Virus, cells and antisera**

MHV strain A59 (MHV-A59) was propagated in Sac(-) cells as described by Spaan et al. (5). Sac(-) and 17Cl1 cells were maintained in Dulbecco's minimal essential medium containing 5% fetal calf serum, penicillin, and streptomycin (DMEM/5%FCS). The production of the polyclonal antiserum to MHV-A59 has been described (6). The monoclonal antibodies J7.6 (anti-S; 7) and J1.3 (anti-M; 8) were kindly provided by Dr. J. Fleming.

### **Infection and metabolic labeling**

Subconfluent monolayers of Sac(-) or 17Cl1 cells in 35 mm-dishes were inoculated for 60 min at a multiplicity of infection of 10-50. From 5.5 hr post inoculation cells were incubated for 30 min in MEM (GIBCO) without methionine and then pulse-labeled with 20-200  $\mu$ Ci ExpressS<sup>35</sup>S-label (Dupont). Cells were washed once with PBS and either lysed directly or chased for various times in DMEM/5% FCS supplemented with 2 mM L-methionine. The cells were lysed in 600  $\mu$ l detergent solution (50 mM Tris-Cl, [pH 8.0], 62.5 mM EDTA, 0.5% sodiumdeoxycholate, 0.5% Nonidet P-40 [NP40]) containing 2mM phenylmethylsulfonyl fluoride and 40  $\mu$ g/ml aprotinin (Sigma). In the experiment of Fig. 1 detergent solutions were varied as indicated. The composition of the MNT detergent solution has been described before (9). For the analysis of complexes from virions (Fig. 3A) detergent solution was added from a 5 times concentrated stock solution directly to the cleared culture medium.

### **Sucrose gradient centrifugation**

Samples of solubilized <sup>35</sup>S-labeled cells or virions were analyzed in sucrose gradients (15-30% [wt/wt] sucrose in detergent solution). The gradients were centrifuged for 30 min at 50,000 rpm in an SW50.1 rotor (Beckman Instruments Inc., Palo Alto, CA). They were fractionated from the bottom into 15 fractions of approx. 330  $\mu$ l. Any material pelleted to the bottom of the tube was dissolved in 333  $\mu$ l detergent solution.

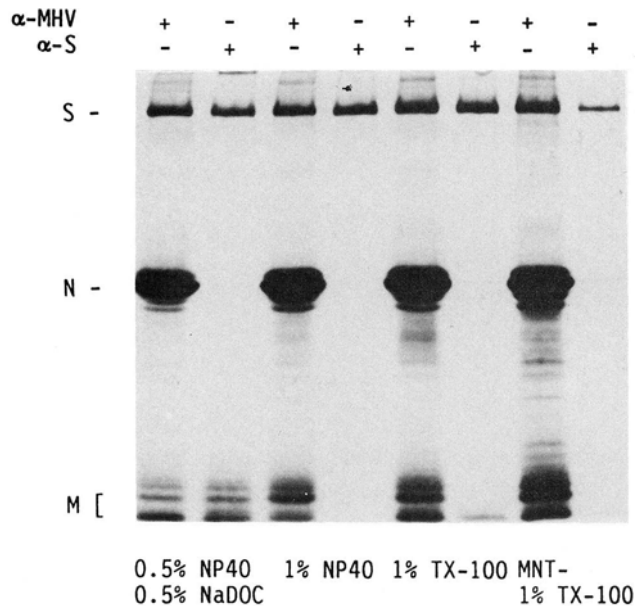
### **Immunoprecipitation**

Samples of cell lysates or gradient fractions were brought to 600  $\mu$ l with detergent solution and antibodies were added (2  $\mu$ l anti-MHV; 10  $\mu$ l anti-S; 2  $\mu$ l anti-M). The samples were incubated at 4°C for at least 3 hr and immune complexes were then bound to Staph. A (Pansorbin Cells, Calbiochem) for at least 30 min at 4°C. Staph. A was pelleted by centrifugation and washed three times with detergent solution. The final pellets were resuspended in Laemmli sample buffer containing 20 mM dithiothreitol and the samples heated for 2 min at 95°C. They were analyzed in 10 or 15% SDS poly-acrylamide gels.

## RESULTS

### Detection of M/S complexes

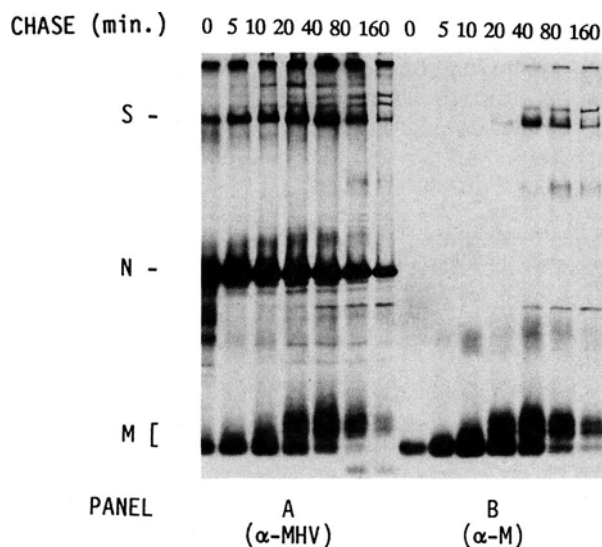
Sofar, specific complexes between the S and M protein of MHV-A59 have not been clearly observed. We reasoned that such complexes may have escaped detection due to the analytical conditions used. Assuming that these conditions were not suitable to preserve the intermolecular interactions in the complexes we studied the effects of different detergents and buffers used to solubilize the membrane proteins from infected cells. Fig. 1 shows some results. Parallel cultures of MHV-A59 infected cells were labeled for 60 min with  $^{35}\text{S}$ -methionine and lysed with a panel of detergent solutions containing ionic as well as nonionic detergents. Each lysate was split in two parts and the viral proteins were immunoprecipitated with a polyclonal anti-virion serum or with a monospecific anti-S serum. It should be noted that the same buffer/detergent conditions were maintained throughout the analytical procedure. Clearly, under all conditions shown in Fig.1 the anti-S serum exclusively precipitated the S protein except in one case. When a combination of NP40 and NaDOC was used, significant co-precipitation of M with S was observed. Thus, in infected cells a major fraction of M is present in a physical complex with S and these complexes are well preserved when suitable lysis conditions are used. No co-immunoprecipitation of the nucleocapsid protein was observed. It is interesting to note that the detergent solution MNT/1% Triton X-100 which we have been using to detect viral spike oligomers (9) apparently disrupted the interactions between S and M.



**Figure 1.** MHV-infected Sac(-) cells were labeled for 1 hr and solubilized with a set of different detergent solutions as indicated. Each lysate was split in two parts and the viral proteins were precipitated with the polyclonal anti-MHV serum and with the monospecific anti-S serum. Structural proteins are indicated (S, N, and M).

## Kinetics of co-immunoprecipitation

To further confirm the existence of the M/S complexes and to determine the kinetics with which they are formed we performed a pulse-chase labeling of MHV-infected cells. The cells were labeled for 5 min and chased for various time periods. Using the detergent solution containing 0.5% NP40 and 0.5% NaDOC the cells were lysed and the viral proteins were precipitated with the polyclonal anti-MHV serum and with a monospecific anti-M serum. As Fig. 2 demonstrates the anti-M serum exclusively precipitated the M protein from the pulse-labeled sample. However, after 10-20 min of chase a detectable amount of the spike precursor gp150 started to become co-precipitated. Using the amount of labeled S protein precipitated with the polyclonal serum as a reference, the fraction of S that co-pre-



**Figure 2.** MHV-infected Sac(-) cells were pulse labeled for 5 min and chased for various time periods as indicated. Viral proteins in one half of each cell lysate was precipitated with anti-MHV serum (panel A) and in the other half with the anti-M serum (panel B).

cipitated with M gradually increased during longer chase times. This result indicates that S becomes involved in complexes with M post-translationally. Note that the S/gp180 and S/gp90 forms of the spike protein appearing during the chase were co-precipitated by the anti-M serum, too. In a separate pulse-chase experiment we examined the complex formation vice versa by precipitating with the monospecific anti-S serum. The results of this experiment indicated that the amount of co-immunoprecipitated M reached its maximum already after about 10-20 min of chase (data not shown). We conclude that the envelope proteins engage in heterocomplex formation with different kinetics M appearing in complexes earlier after its synthesis than S.

## Sedimentation of M/S complexes

Our second approach to study the complex formation between S and M was sedimentation analysis of the viral proteins using the same NP40/DOC detergent conditions both for solubilization and in the sucrose gradients. Therefore, MHV-infected 17Cl1 cells were labeled for 10 min and chased for 60 min and 180 min. To examine complexes derived from virions, the culture medium from the cells chased for 180 min was treated with the detergents and analyzed in 15-30% sucrose gradient. Fig. 3A shows that under the conditions described the major fraction of M and S co-sedimented to fractions 7-9. Given the composition of the gradient and the relatively short run time this position in the gradient implies that the complexes must be very large. The nucleocapsid protein appeared to sediment much slower confirming the conclusion from the co-immunoprecipitation assay that N was not engaged in M/S complexes. A fraction of S and M stayed at the top of the gradient. The nature of these molecules is unclear; they may have been derived from disrupted cells or from dissociation of the complexes.

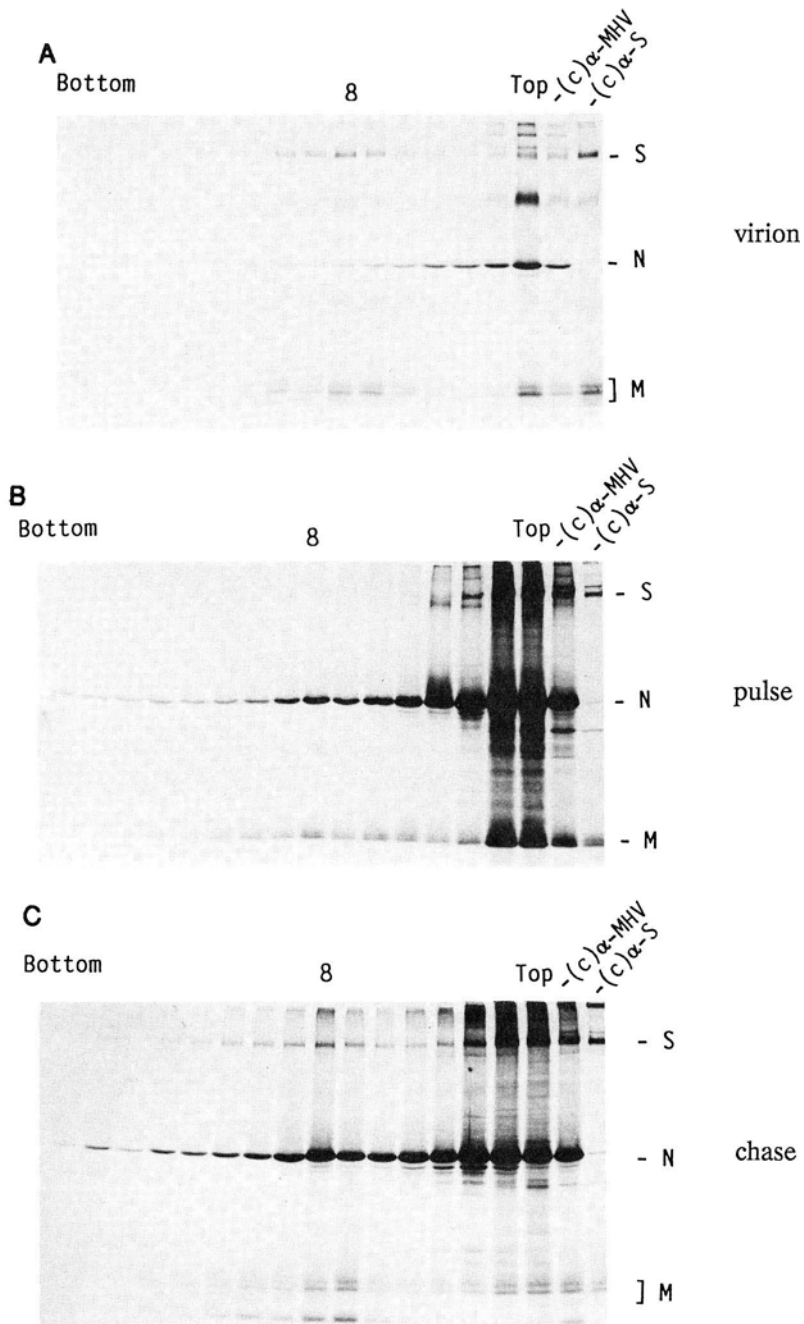
In parallel with the virion material the gradient analyses of the labeled cell samples were carried out. Most viral protein labeled during the 10 min pulse was recovered from the top of the gradient (Fig. 3B). A significant fraction of M, however, sedimented heterogeneously from the top to deep positions in the gradient and was apparently present in complexes. Although no labeled S protein was detected in these fractions the M protein was shown in a separate experiment to be precipitated by the anti-S monoclonal antibody (data not shown). After the 60 min chase a significant fraction of the labeled S protein also appeared in the M/S complexes of heterogeneous sizes (Fig. 3C). At this time point most of the M protein was present in these complexes. Comparison of the different profiles in Fig. 3 shows that the intracellular complexes are more heterogeneous than those derived from virions. Part of the nucleocapsid protein from the cell samples co-sedimented with the M/S complexes. We do not believe that this N protein was physically associated with the M/S complexes as the protein did not co-immunoprecipitate with these complexes.

## DISCUSSION

We here describe the detection of complexes between the spike protein and the membrane protein of MHV-A59 in infected cells. Specific detergent conditions appeared to be required to preserve the intermolecular interactions in the complexes. Pulse-chase labeling of MHV-infected cells followed by co-immunoprecipitation and sedimentation analyses revealed that M after its synthesis readily engages in complex formation with S. In contrast, S appears to associate with M with much slower kinetics. The data imply that M interacts with S molecules that were already synthesized. Apparently, S needs more time to become interaction-competent. Preliminary experiments show that this behaviour correlates well with the slow folding characteristics of the protein.

We do not know yet where in the cell the M/S complexes are formed. An important observation, however, is that already after a 10 min pulse labeling a significant fraction of unglycosylated M protein was found in a complex with S. This suggests that the complexes are formed before or at the site of budding.

Our sedimentation analyses demonstrated that the M/S complexes isolated from infected cells are rather heterogeneous, in contrast to the complexes obtained from



**Figure 3.** MHV-infected 17Cl1 cells were labeled for 10 min and chased for 60 min and 180 min. Detergent treated culture medium from the 180 min chase (A) and lysates of 10 min pulse labeled cells (B) or the 60 min chase (C) were run for 30 min at 50,000 rpm into a 15-30 % (wt/wt) sucrose gradient. Viral proteins in the fractions from bottom to top were precipitated with anti-MHV serum. Viral proteins in the control lanes (c) were precipitated with anti-MHV and with anti-S serum from aliquots taken from the samples before loading them onto the gradient.

virions. A significant fraction of the intracellular complexes sedimented slower than those derived from virions, while also faster sedimenting complexes were observed. The homogeneous nature of the virion M/S complexes suggests that the structures we are observing are real and do not represent procedural artefacts. Though no size determinations have been done yet it is clear that the complexes are very large.

Collectively, the data lead us to hypothesize that S and M by virtue of their interaction congregate at the site of viral budding thereby forming a matrix into which the nucleocapsids can bud. Implicit to this hypothesis is the idea that the formation of the complexes between S and M causes the proteins to be retained at the budding site instead of being transported beyond this site when expressed independently. Finally, since coronaviruses like most other viruses are very selective in incorporating only virus-specific proteins, we postulate that the interaction between S and M is very specific. Accordingly, our results so far demonstrate that the viral M/S complexes are not contaminated with non-viral proteins.

## ACKNOWLEDGEMENTS

We thank Harry Vennema for helpful discussions and Pieter de Groot for technical assistance. We are grateful to Dr. J. Fleming for providing the monoclonal antibodies J7.6 and J1.3.

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