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Folding of the mouse hepatitis virus spike protein and its association with the membrane protein

D.-J. E. Opstelten, P. de Groote, M. C. Horzinek, and **P. J. M. Rottier**

Institute of Virology, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, The Netherlands

Summary. Coronaviruses are assembled by budding into pre-Golgi membranes. Using different approaches we have demonstrated that the spike (S) protein and the membrane (M) protein of mouse hepatitis virus (MHV) associate to form large complexes. Newly synthesized M was found in these complexes almost immediately after its synthesis, whereas the S protein started to appear in heterocomplexes after 10–20 min. This is consistent with the slow rate of folding of S and with the observation that folding of S preceeds its association with M. While the folding of S involves the formation of multiple disulfide bonds, folding of M is disulfide-independent. This contrast was reflected by the differential sensitivity of the two proteins to reduction with dithiothreitol (DTT). Addition of DTT to the culture medium of MHV-infected cells drastically impaired the folding of S, but not of M. Consequently, the S protein was unable to interact with M. Under these conditions, S stayed in the ER while M was transported efficiently beyond the site of budding to the Golgi complex. We conclude that the association of S with M is an essential step in the formation of the viral envelope and in the accumulation of both proteins at the site of virus assembly.

Introduction

Budding through cellular membranes is the last step in the assembly of enveloped viruses. The assembly process is driven by specific interactions between the nucleocapsid and the viral envelope proteins [12]. Depending on the virus, budding takes place at the plasma membrane or at intracellular membranes. The site of budding appears to be determined by the envelope proteins because virus assembly occurs where these proteins accumulate. Accordingly, viruses that assemble at the plasma membrane have envelope proteins that are rapidly transported to the

cell surface after synthesis. In contrast, membrane proteins from intracellularly budding viruses are retained in the budding compartment [4, 8].

We study the assembly of the coronavirus mouse hepatitis virus (MHV). MHV particles are composed of three structural proteins. The nucleocapsid (N) protein is complexed with the genome, thereby forming the helical nucleocapsid. The spike (S) glycoprotein constitutes the large peplomers and functions in cell attachment and fusion during virus entry. The membrane (M) protein is a small glycoprotein, which is largely embedded in the lipid bilayer. MHV matures by budding into intracellular smooth membranes located between the endoplasmic reticulum (ER) and the Golgi complex [13], and it has been concluded that the M protein determines the site of budding. The correlation between this site and the intracellular accumulation of M protein strongly argues for such a role [13]. In addition, tunicamycin treatment of MHV-infected cells resulted in the secretion of spikeless virions suggesting that only the M protein is required for budding [5, 9]. When expressed independently, however, the M protein is transported beyond the site of budding to the trans side of the Golgi complex [7, 11]. The same holds true for the S protein which, when not incorporated into virions in infected cells or when expressed independently, is transported to the plasma membrane (Vennema and Rottier, unpubl. res.). Clearly, neither envelope proteins localize to the budding compartment by themselves, implying that in MHV-infected cells they have to interact in order to be retained and to co-accumulate at the site of budding. Such an interaction is probably specific because cellular membrane proteins are virtually absent in virions.

Complex formation of the viral envelope proteins

Until now no experimental data in support of the proposed interaction between the two coronaviral envelope proteins have been reported. We reasoned that any complexes between S and M might simply have escaped detection due to the analytical conditions used, e.g. by disruption of the complexes during solubilization of the infected cells. Therefore, we studied the effects of different detergents with the aim of finding conditions that might preserve the interaction between the two proteins. A large panel of buffer compositions was tested and the conclusion was reached that the nature of the detergent(s) used for cell lysis and during further analysis indeed had profound effects and that M/S complexes do exist. Optimal preservation of the complexes was achieved when we used a combination of the non-ionic detergent Nonidet-P40 (NP-40) and the ionic detergent sodium deoxycholate (DOC), both at

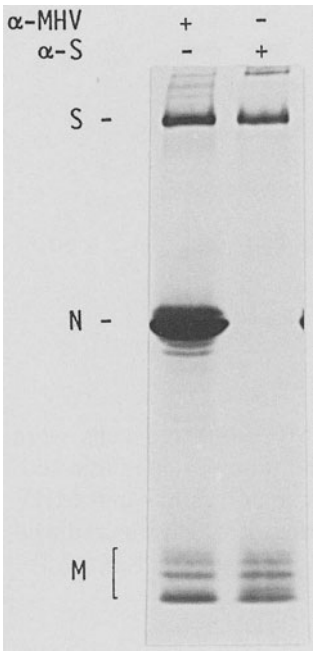

Fig. 1. Co-immunoprecipitation of the MHV M and S protein. MHV-infected cells were labeled with ^{35}S-methionine for 1 h and lysed in a buffer containing 0.5% Nonidet-P40 and 0.5% Na-deoxycholate. Viral proteins were precipitated from half of the lysate with a polyclonal anti-MHV serum (α-MHV); for the other half a monoclonal antibody to S was used (α-S). MHV structural proteins are indicated (*S*, *N*, *M*)

0.5%. We have characterized the specificity and nature of the interaction in several ways.

(i) We detected heterocomplexes of S and M by co-precipitation of the S protein with monospecific antibodies to the M protein, and vice versa. Similar amounts of M protein can be precipitated with a monoclonal antibody to S as with a polyclonal anti-virion serum (Fig. 1). Our experiment also illustrates the specificity of the interaction because only the viral envelope proteins were co-precipitated by the monoclonal antibodies: scarcely any nucleocapsid protein or cellular protein was observed in the immunoprecipitates.

(ii) Pulse-chase analysis demonstrated that the complexes are formed post-translationally. Interestingly, we found that M and S engage in complex formation with different kinetics. Our data indicate that M associates with S very soon after its synthesis while newly synthesized S protein starts to appear in complexes only after a lag-time of 10–20 min. This implies that immediately after synthesis M molecules associate with S molecules synthesized some time previous.

(iii) Sucrose gradient analysis under the detergent conditions described above demonstrated that S and M occur in huge multimeric complexes. These have been observed after detergent treatment of virions as well as in lysates of infected cells, being more heterogeneous in the latter.

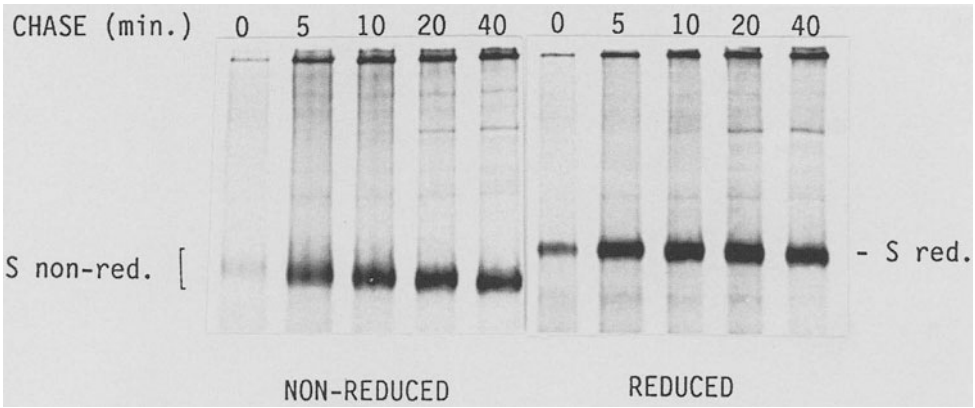

Fig. 2. Disulfide bond formation in the MHV S protein. MHV-infected cells were pulse-labeled for 5 min with ^{35}S-methionine and chased for the time periods indicated. Viral proteins were precipitated from the cell lysates with a polyclonal anti-MHV serum. The immunoprecipitates were split into two portions one of which was reduced with 20 mM DTT. The samples were heated for 5 min at 95°C and analyzed in a 7.5% SDS-polyacrylamide gel

On the basis of these results we hypothesize that S and M congregate at the site of budding to form a matrix into which viral nucleocapsids can bud.

Folding of the spike protein

The typical surface projections of coronavirions are formed solely by the S protein. In previous work we studied their biogenesis by analyzing the oligomerization process [15]. It was found that S forms oligomers with a half-time of 40–60 min, rather slow as compared to most other viral spike oligomers [6]. Since the conditions used in our earlier experiments did not preserve the M/S interactions we were not able to link the oligomerization of the S protein to the complex formation. It is now obvious that both processes take place slowly. This suggested to us that the folding of the S protein is the rate-limiting step.

We studied the folding of S by following the formation of disulfide bonds. These play an important role in the folding and stability of secretory and membrane proteins and are usually crucial for the generation of functional structures. We used the approach that has recently been described for the hemagglutinin protein (HA) of influenza virus [2]. As illustrated in Fig. 2, different folding intermediates of the S protein could be visualized in non-reducing gels on the basis of their differences in electrophoretic mobility. The large mobility difference between the fully reduced form and the unreduced folding intermediates demonstrates that the formation of the disulfide bonds in the S molecules has a pro-

nounced effect on the protein's conformation. Disulfide bond formation apparently starts co-translationally; even after very short pulse labelings the S intermediates always migrated faster than the fully reduced species. In contrast to influenza virus HA no distinct intermediates were detected. Instead, the S protein appeared to undergo many conformational changes that did not resolve. This probably reflects the high number of cysteines present in the luminal domain of the S protein, giving rise to a wide spectrum of forms as a result of the formation or redistribution of disulfide bonds.

After synthesis the S protein undergoes its major folding transitions during the first 10–20 min, as judged from electrophoretic analyses. The more compact, faster migrating conformations occur after some 20–30 min. This time-course corresponds well with the lag-time, after which newly synthesized S protein starts to appear in complexes with the M protein. It also suggests that the S molecule must have reached a certain conformational maturity before it can engage in complex formation. To verify this point we analyzed the S protein present in M/S complexes in non-reducing gels. Indeed, only the faster migrating forms of S were detected in the heterocomplexes, which indicates that the molecule acquires its competence to associate with M as a result of folding.

Manipulation of disulfide bond formation in the spike protein

By adding dithiothreitol (DTT) to the cell culture medium the oxidizing state in the lumen of the ER can be drastically affected. As Braakman et al. [3] have demonstrated, this treatment prevents disulfide bond formation in newly synthesized HA and even leads to the reduction of oxidized HA present in the ER. We wondered whether in vivo reduction of the MHV S protein would also affect its folding and what implications this would have for its association with M.

When 5 mM DTT was added to the culture medium of MHV-infected cells we observed reduction of partially as well as fully oxidized S protein. The effect was monitored in non-reducing gels; the oxidized forms were converted to the slower migrating reduced form. Interestingly, this reduction was accompanied by changes in epitopes, as judged from the loss of recognition by several S-specific monoclonal antibodies. We used some of these antibodies to localize the S protein in MHV-infected cells by indirect immunofluorescence. In untreated cells the antibodies predominantly stained the ER, with additional intense fluorescence in a distinct perinuclear region. Double immunofluorescence identified the latter region as the major site of M protein in MHV-infected cells, presumably the viral budding compartment. A short exposure (<20 min)

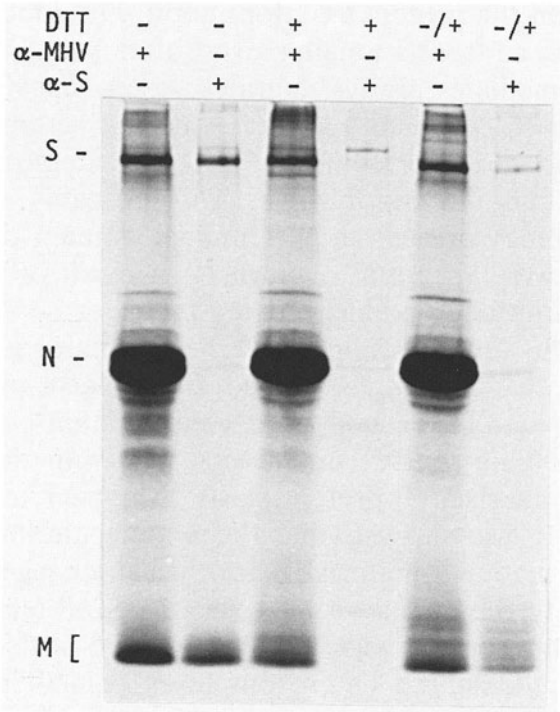

Fig. 3. Effects of in vivo reduction. MHV-infected cells were labeled for 10 min in the absence or presence of 5 mM DTT. In the latter case the cells were treated with 5 mM DTT for 5 min before labeling. As a control, MHV-infected cells were labeled for 10 min in the absence of DTT and chased for 10 min in the presence of 5 mM DTT. The cell lysates were split and the viral proteins were precipitated with the polyclonal anti-MHV serum and with the monoclonal anti-S serum. MHV structural proteins are indicated (*S*, *N*, *M*)

of the cells to a reducing milieu resulted in almost complete absence of ER staining by the conformation-specific anti-S monoclonal antibodies. However, the fraction of S protein that co-localized with the M protein was still recognized by the antibody. Thus, in vivo reduction with DTT affects the conformation of S present within the ER while the protein outside the ER appears to be much more resistant to DTT.

Do M/S complexes still form during in vivo reduction? As mentioned above, only the oxidized S protein occurs in complexes with M under normal conditions. Thus, under reducing conditions one would not expect the formation of M/S complexes to take place. To confirm this assumption we labeled MHV-infected cells under reducing conditions and analyzed the complex formation by co-immunoprecipitation. While under normal conditions much of M can be co-precipitated with S already after a 10-min pulse labeling, no co-precipitation was observed in the presence of DTT (Fig. 3). This result indicated either that the complexes

were not formed or that existing complexes were no longer recognized by the anti-S monoclonal antibody. To rule out the latter possibility we prelabeled MHV-infected cells for 10 min in the absence of DTT and then chased for 10 min in its presence. As shown in Fig. 3, a significant fraction of the M/S complexes formed during the pulse were still recognized by the conformation-specific antibody after DTT treatment. We therefore conclude that M/S complexes are no longer formed under reducing conditions. Apparently, the folding of S is a prerequisite for its association with M.

Differential effects of in vivo reduction on transport of the coronaviral envelope proteins

The endoplasmic reticulum controls the exit of proteins to the Golgi complex. Only properly folded molecules are allowed to leave, misfolded proteins are generally retained in the ER. This "quality control" still functions during in vivo reduction, as was shown for the influenza HA protein, the reduced form of which is unable to leave the ER [3]. As a general consequence of this finding, those proteins that require disulfide bond formation for their proper folding will accumulate in the ER under reducing conditions. Accordingly, we found that the reduced MHV S protein stably stayed in the ER and was re-oxidized upon DTT removal.

The M protein of MHV does not form luminal disulfide bonds, as can be deduced from its structure; no cysteines are present in the luminally exposed part of the protein [1, 10]. This enabled us to study whether transport of such proteins to the Golgi complex still occurs during DTT treatment. Taking advantage of its well-characterized O-glycosylation pattern [7, 14], the M protein allows us to follow its intracellular transport biochemically. Pulse-chase analysis under reducing conditions (Fig. 4) showed that M was efficiently transported out of the ER and reached the trans side of the Golgi complex; the slower migrating forms appearing during the chase are indicative of modifications occurring in this part of the Golgi complex. Thus, transport of M is independent of disulfide bond formation. Moreover, the behavior of M under reducing conditions shows that cellular processes such as glycosylation and ER-to-Golgi transport are not disturbed.

Another interesting observation from these experiments was that the M protein was transported to the Golgi complex faster under reducing than under normal conditions (Fig. 4; compare M in lanes 3 and 7). In contrast to its glycosylation in the presence of DTT, after a 30 min chase in the absence of DTT a large fraction of M had still not been modified by Golgi enzymes, suggesting that it was retained somewhere before the Golgi complex. As concluded from the effects of DTT on the M protein

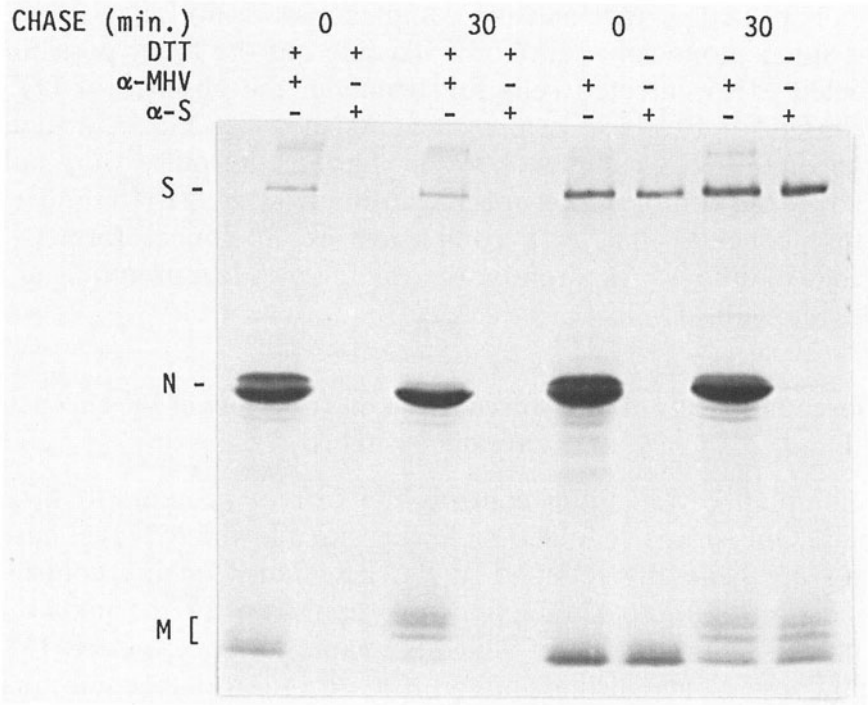

Fig. 4. Effects of in vivo reduction on transport of the MHV-M protein. Parallel cultures of MHV-infected cells were labeled for 10 min and chased for 30 min in the presence or absence of 5 mM DTT. In the former case the cells had been treated with 5 mM DTT for 5 min before labeling. The cell lysates were split and the viral proteins were precipitated with polyclonal anti-MHV serum and with monoclonal anti-S serum. MHV structural proteins are indicated (*S*, *N*, *M*)

expressed by a recombinant vaccinia virus, the reducing agent itself did not affect the transport kinetics. M was transported to the Golgi complex at the same rate in the presence and absence of DTT. Thus under conditions of in vivo reduction of the S protein, M is no longer retained in a pre-Golgi compartment. These data strongly suggest that complex formation between S and M plays an important role in the retention of both proteins at the site of virus budding.

Envelope protein interaction and viral budding

The data obtained so far raise several questions. One is where the two envelope proteins associate. The presence of unglycosylated M protein in complexes with S strongly suggests that the proteins interact in the ER. In this instance the proteins may either be transported to the budding compartment as small oligomers of a discrete composition or in the form of larger aggregates. It cannot yet be excluded, however, that the two proteins are transported individually to the budding com-

partment before they associate. Clearly, complex formation can occur in the ER as budding takes place in this compartment late in infection, probably as the result of the abundant co-accumulation of the envelope proteins.

Like other RNA viruses that assemble intracellularly, coronaviruses lack a matrix protein; the nucleocapsid must interact directly with one or both envelope proteins. There are no indications that the RNA genome is involved in the budding process, and protein-protein interactions probably are the driving force. Two models can be envisioned. The envelope proteins may form large rafts, which are composed solely of M and S, in the plane of the budding compartment's membrane. In this model the nucleocapsids would interact with the envelope proteins present in these preformed patches. Alternatively, the nucleocapsid may be the organizing factor, and the nucleocapsid protein would selectively recruit the envelope proteins for formation of the viral membrane. Our findings support the first model in which S and M form large complexes by lateral interactions, the specificity of which would exclude non-viral proteins. Because we did not observe co-immunoprecipitation of the nucleocapsid protein in our assays, the interaction between the viral membrane proteins is apparently capsid-independent. An obvious way to obtain more conclusive information on these and other issues related to coronaviral budding is by co-expressing the structural genes in the absence of other viral components. Such experiments are currently in progress.

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Authors' address: Dr. P. J. M. Rottier, Institute of Virology, Yalelaan 1, 3584 CL Utrecht, The Netherlands.