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Disulfide Bonds in Folding and Transport of Mouse Hepatitis Coronavirus Glycoproteins

DIRK-JAN E. OPSTELTEN, PIETER DE GROOTE, MARIAN C. HORZINEK, HARRY VENNEMA, AND PETER J. M. ROTTIER*

Institute of Virology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

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We have analyzed the effects of reducing conditions on the folding of the spike (S) protein and on the intracellular transport of the membrane (M) protein of the mouse hepatitis coronavirus. These proteins differ in their potential to form disulfide bonds in the lumen of the endoplasmic reticulum (ER). Intrachain disulfide bonds are formed in the S protein but not in M, which was demonstrated in a pulse-chase experiment by analyzing the viral proteins under nonreducing conditions. To reduce disulfide bonds in vivo, we added dithiothreitol (DTT) to the culture medium of mouse hepatitis coronavirus-infected cells following a procedure recently described by Braakman et al. (I. Braakman, J. Helenius, and A. Helenius, EMBO J. 11:1717–1722, 1992). Short exposure to DTT resulted in the complete reduction of newly synthesized S protein and affected its conformation as judged by the change in mobility in nonreducing gels and by the loss of recognition by a conformation-specific monoclonal antibody. Using this antibody in an immunofluorescence assay, we monitored the reducing effect of DTT in situ. DTT was found to initially affect only the S protein present in the ER; also, after longer treatment, the remaining signal also gradually disappeared. In contrast, folding and transport of the M protein were not inhibited by DTT. Under reducing conditions, M was transported efficiently to the *trans* side of the Golgi complex, indicating that cellular processes such as ER-to-Golgi transport, O-glycosylation, and Golgi retention were unaffected. In the presence of DTT, the M protein even moved at an increased rate to the Golgi complex, which is probably because of its failure to interact with unfolded S protein. The effects of in vivo reduction were reversible. When DTT was removed from pulse-labeled cells, the S protein folded posttranslationally and aberrantly; during its oxidation, most of S now transiently aggregated into large disulfide-linked complexes from which subsequently folded S molecules dissociated.

Membrane and secretory proteins synthesized in the rough endoplasmic reticulum (ER) fold during and after translation to become functional and transportable. The primary amino acid sequence essentially dictates the ultimate conformation of a protein, and the co- and posttranslational formation of disulfide bonds often plays a decisive role in the generation of the final structures. Chaperones usually assist in the process of protein folding (14, 17, 26). The disulfide bonds are formed by oxidation in the lumen of the ER; their formation and possible rearrangements are catalyzed by the enzyme protein disulfide isomerase (12, 13). In some secretory and membrane proteins, folding and disulfide bond formation begin on the nascent chain and proceed vectorially from the N terminus towards the C terminus (3, 5, 27).

Recently, Braakman et al. (4) reported a method of interfering with the formation of disulfide bonds in vivo. Upon addition of dithiothreitol (DTT) to the culture medium, the oxidizing state in the ER was found to be drastically affected as judged by the inhibition of disulfide bond formation in newly synthesized influenza virus hemagglutinin (HA) and the reduction of already oxidized HA present within the ER. The effects of DTT were reversible: when conditions were restored, HA was rapidly oxidized and correctly folded, indicating that, for the folding of HA, disulfide bond formation does not need to occur cotranslationally. Consequently, DTT treatment can be used to study the protein folding process separately from the vectorial processes of translation and translocation.

We have studied the role of disulfide bond formation in the folding and transport of the spike (S) and membrane (M) proteins of the coronavirus mouse hepatitis virus (MHV). Both are membrane glycoproteins which differ in many respects. The S protein constitutes the large peplomers and is involved in binding of virions to and fusion with the host cell membrane during virus entry (33). It is a type I membrane protein that is synthesized as a precursor (gp150) in the rough ER and cotranslationally N-glycosylated. In the case of MHV strain A59 (MHV-A59), the luminally exposed domain of the precursor contains 42 cysteine residues (23) which are available for disulfide linkages. We showed that S oligomerizes slowly with a half-time of 40 to 60 min (40). It becomes incorporated into virions during budding at pre-Golgi membranes (37, 38), and a small fraction is transported to the plasma membrane.

The M protein is a triple-spanning membrane protein (2, 32) which uses an uncleaved internal signal sequence to assemble in the rough ER membrane (29). When expressed independently, the M protein localizes to the *trans*-Golgi cisterna and *trans*-Golgi network (TGN) (19, 31); it contains only O-linked oligosaccharide side chains (15, 30) consisting of Ser/Thr-GalNAc(-SA)-Gal-SA (19, 25, 38). The first sugar (GalNAc) is added posttranslationally in the budding compartment (38). The addition of both Gal and SA occurs in the Golgi complex while two other, as-yet-unidentified, terminal modifications take place in the TGN (19). Interestingly, the amino acid sequence of the MHV-A59 M protein (2) does not allow for any disulfide bond formation in the ER lumen; the four cysteine residues present in the molecule reside in the second transmembrane domain and in the region facing the cytoplasm (32).

In view of these different characteristics of S and M, we have

* Corresponding author.

used MHV to analyze simultaneously the fate of a membrane protein that requires extensive disulfide bond formation to acquire and stabilize its structure and that of one in which such covalent interactions are absent.

MATERIALS AND METHODS

Virus, cells, and antisera. Sac(−) cells were maintained in Dulbecco's minimal essential medium containing 5% fetal calf serum, penicillin, and streptomycin (DMEM–5% FCS). OST7-1 cells, a kind gift of B. Moss (11), were maintained in DMEM–10% FCS supplemented with 400 μg of G-418 (Geneticin, GIBCO) per ml. MHV-A59 was propagated in Sac(−) cells as described previously (34). The preparation of the recombinant vaccinia virus expressing the MHV-A59 M protein has been described previously (20). The production of the rabbit peptide antiserum to the M protein and the rabbit polyclonal antiserum to MHV-A59 has been described previously (references 20 and 30, respectively). The monoclonal antibody (MAb) J7.6 and the rabbit antipeptide serum 5415, both specific for the S protein, were kindly provided by J. Fleming and M. Buchmeier, respectively.

Infection, metabolic labeling, and in vivo reduction. (i) MHV infection. Subconfluent monolayers of Sac(−) cells in 16- or 35-mm dishes were washed with phosphate-buffered saline (PBS) containing 50 μg of DEAE-dextran per ml (PBS-DEAE) and inoculated with MHV-A59 for 60 min at a multiplicity of infection of 10 to 50 in PBS-DEAE–1% FCS at 37°C.

(ii) Infection with recombinant vaccinia virus expressing MHV-A59 M and transfection of OST7-1 cells. Subconfluent monolayers of OST7-1 cells in 35-mm dishes were inoculated with vaccinia virus expressing MHV-A59 M at a multiplicity of infection of 10 in PBS for 1 h at 37°C. After inoculation, the cells were transfected with the vector PTUM-M, a construct of PTUG31 (39) in which a *Bam*HI fragment containing the MHV M gene (20) was cloned. For this purpose, the cells were washed twice with DMEM, and a mixture of 800 μl of DMEM with 2 to 4 μg of plasmid DNA and 10 μl of lipofectin reagent (Bethesda Research Laboratories, Life Technologies, Inc.) was added. After 3 h, the transfection medium was replaced by 2 ml of DMEM–10% FCS.

(iii) Labeling. At 5.5 h after inoculation, the cells were starved for 30 min in MEM (GIBCO) without methionine. When indicated, brefeldin A (Boehringer Mannheim) was added to a concentration of 6 μg/ml. Cells were then pulse-labeled with 20 to 200 μCi of Expre^{35}S label (NEN-Dupont) for the times indicated and then washed once with PBS and either put on ice and lysed immediately or chased for various times in DMEM–5% FCS supplemented with 2 mM L-methionine (chase medium). The cells were lysed on ice in TES (20 mM Tris [pH 7.4], 1 mM EDTA, 100 mM NaCl)–1% Triton X-100 (lysis buffer) containing 2 mM phenylmethylsulfonyl fluoride and 40 μg of aprotinin per ml (Sigma). The lysates were spun for 3 min at 12,000 × *g* at 4°C to pellet nuclei and cell debris. When disulfide bond formation was analyzed, cells were washed twice for 1 min with ice-cold PBS containing 20 mM *N*-ethylmaleimide (Sigma) just before treatment with lysis buffer containing 20 mM *N*-ethylmaleimide.

For in vivo reduction, the culture medium was replaced at the time points indicated by medium containing 5 mM DTT (Boehringer Mannheim). For reoxidation, the cells were washed twice with PBS and further incubated with chase medium.

Immunoprecipitation and gel electrophoresis. Viral proteins were immunoprecipitated with the polyclonal MHV-A59 antiserum (2 μl), the M peptide antiserum (2 μl), or the monoclonal anti-S serum J7.6 (10 μl). Serum was added to aliquots of cell lysates diluted with lysis buffer to a final volume of 600 μl. After overnight incubation at 4°C, immune complexes were collected with 15 μl of a 10% (wt/vol) suspension of formalin-fixed *Staphylococcus aureus* cells (Bethesda Research Laboratories, Life Technologies, Inc.). After a 30-min incubation at 4°C, they were washed three times with lysis buffer and finally suspended in 25 μl of 62.5 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–10% glycerol (sample buffer). Where indicated, sample buffer was supplemented with 20 mM DTT to reduce disulfide bonds prior to gel electrophoresis. The proteins were analyzed in SDS–7.5 to 15% polyacrylamide gels (PAGs). Since M aggregates when heated in sample buffer (35), only those samples in which S was assayed were heated for 5 min at 95°C before loading on the gel. Reduced and nonreduced samples, when analyzed in one gel, were separated by at least three lanes to avoid unintentional reduction due to diffusion of DTT.

Indirect immunofluorescence. Sac(−) cells, grown on 12-mm coverslips, were infected with MHV-A59 at a multiplicity of infection of 10 to 50. At the time points indicated, 5 mM DTT or 0.1 mg of cycloheximide per ml was added to the culture medium. The cells were fixed with 3% paraformaldehyde for 20 to 30 min and washed three times with PBS containing 10 mM glycine (PBS-glycine). Cells were permeabilized with PBS–1% Triton X-100 for 5 min and then washed three times with PBS-glycine. They were then treated for 30 min with antisera diluted in PBS-glycine–5% FCS: MAb J7.6, 1:20 dilution; S peptide antiserum 5415, 1:150 dilution; and M peptide antiserum, 1:150 dilution. Antibodies were washed away, and the cells were stained for 30 min with affinity-purified rhodamine-conjugated goat anti-rabbit immunoglobulin G (IgG) and fluorescein-conjugated goat anti-mouse IgG (Protos Immunoresearch, San Francisco, Calif.), both diluted 1:150 in PBS-glycine. All incubations were done at room temperature. Finally, the coverslips were washed extensively and mounted in Mowiol 4-88 (Hoechst, Frankfurt, Germany) containing 25 mg of 1,4-diazabicyclo-[2.2.2]octane (Sigma) per ml. Fluorescence was viewed with an Olympus BHS-F microscope.

RESULTS

Disulfide bond formation and folding of the spike protein. We have used SDS-gel electrophoresis under nonreducing conditions to analyze disulfide bond formation in the MHV membrane proteins. This method takes advantage of the increase in mobility as the protein acquires more compact conformations stabilized by the formation of disulfide bonds, which reflects the progress in folding towards the native state. We refer to this process as disulfide-dependent folding. With 42 cysteine residues in its luminal domain (23), the MHV-A59 S protein precursor was expected to become heavily disulfide bridged. This was verified by analysis of the protein from MHV-A59-infected Sac(−) cells during a 5-min pulse-labeling with [^{35}S]methionine and after chases for various periods. Before lysis, the cells were washed twice with ice-cold PBS containing the alkylating agent *N*-ethylmaleimide to block free sulfhydryl groups, thereby preventing further disulfide bond formation (8). The viral proteins were precipitated with a polyclonal anti-MHV serum which recognizes immature as well as mature forms of S. The precipitates were split into two equal portions, to one of which DTT was added to 20 mM. As shown in Fig. 1A, the nonreduced forms of S had a higher electrophoretic mobility than did the reduced form. Immedi-

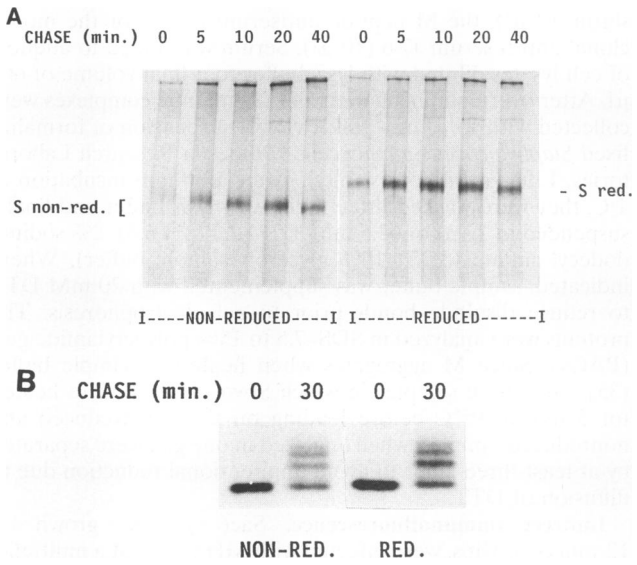

FIG. 1. (A) Oxidation of the MHV S protein. MHV-infected Sac(−) cells were pulse-labeled for 5 min and chased for the time periods indicated. The S protein was immunoprecipitated with a polyclonal anti-MHV serum. Immunoprecipitates were each split into two equal portions, one of which was reduced with 20 mM DTT before analysis in an SDS–7.5% PAG. (B) Analysis of the MHV M protein under reducing and nonreducing conditions. MHV-infected Sac(−) cells were pulse-labeled for 5 min and immediately lysed or chased for 30 min. The M protein was immunoprecipitated with the polyclonal anti-MHV serum, and equal fractions were analyzed under reducing and nonreducing conditions in an SDS–15% PAG.

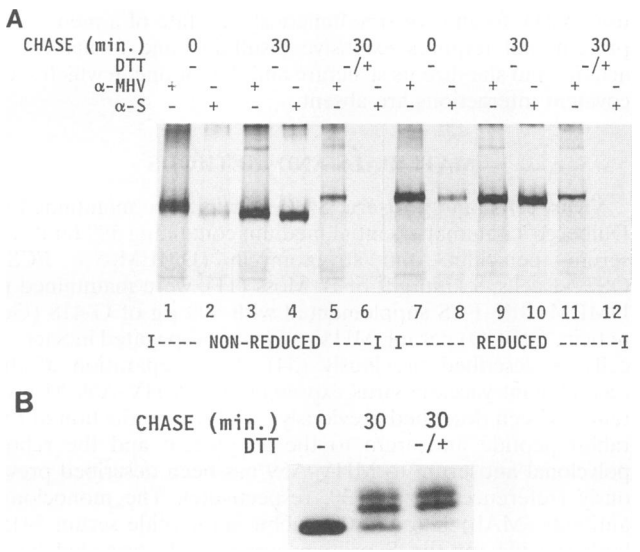

FIG. 2. Posttranslational effects of in vivo reduction with DTT on the MHV S and M proteins. MHV-infected Sac(−) cells were pulse-labeled for 10 min and immediately lysed or chased for 30 min in the presence (−/+) and in the absence (−) of 5 mM DTT, as indicated. (A) MHV S protein was immunoprecipitated with the polyclonal anti-MHV serum or MAb J7.6 anti-S and analyzed under reducing and nonreducing conditions in an SDS–7.5% PAG. (B) MHV M protein was immunoprecipitated with the polyclonal anti-MHV serum and analyzed in an SDS–15% PAG.

ately after the pulse, the nonreduced S protein appeared heterogeneous, which is presumably because of the occurrence of different conformations. The mobility of S gradually increased during the chase, reflecting the transition to increasingly more compactly folded conformations as a result of formation and/or redistribution of disulfide bonds. Completely oxidized S molecules started to appear 10 to 20 min after translation. The mobility of the reduced form of S did not change during the first 40 min. Faster-migrating forms resulting from trimming of the oligosaccharide side chains appeared after longer chases (not shown). The increase in intensity of S during the first 10-min chase period is probably caused by the completion of nascent S chains which were not terminated at the end of the pulse. In addition, the polyclonal serum seems to have a lower affinity for the less folded forms of S. The material on the top of the gel presumably represents, at least in part, M protein, which is known to aggregate during heating in electrophoresis sample buffer (35).

MHV membrane protein does not contain disulfide bonds. To confirm that the cysteines present in the MHV-A59 M protein are not involved in disulfide bonding as predicted, we compared the mobility of newly synthesized M with that of fully matured M under reducing and nonreducing conditions. Again, MHV-infected cells were labeled for 5 min, and a chase of 30 min followed. The M protein was immunoprecipitated by the polyclonal anti-MHV serum and the precipitates were run in an SDS–15% polyacrylamide gel to provide optimal resolution of the different forms of the protein. As shown in Fig. 1B, no difference in mobility between the reduced and nonreduced forms of pulse-labeled, unglycosylated M could be detected. The same was found for the various O-glycosylated forms synthesized posttranslationally after the protein has left the rough ER. Given the high sensitivity of the electrophoretic system, this result is consistent with the predicted absence of inter- or intramolecular disulfide bridges.

Effects of in vivo reduction on S and M. To determine the effects of in vivo reduction on the folding of S, we applied DTT to MHV-infected cells. In the experiment whose results are shown in Fig. 2A, parallel cultures of MHV-infected cells were pulse-labeled for 10 min and the cells were either lysed directly or subsequently chased for 30 min in the absence or presence of 5 mM DTT. The lysates were split: from one part, the spike protein was precipitated by the polyclonal anti-MHV serum, and from the other part, it was precipitated by the MAb J7.6 (41). The analyses under nonreducing conditions revealed the S protein precursor as a diffuse band (lane 1). This band disappeared during the chase in the absence of DTT, becoming a more discrete band of material that migrated faster (lane 3). In contrast, when DTT was added during the chase the S protein comigrated with the fully reduced form obtained after in vitro reduction (lane 5). This indicates that the partially oxidized S protein synthesized during the pulse-labeling had become reduced posttranslationally by the DTT treatment. Apparently, this had affected its conformation drastically since the reduced form migrated much more slowly than the (partially) oxidized forms. The reduced S molecules were still recognized by the polyclonal serum, albeit less efficiently than were the oxidized forms. The reduction of S was accompanied by a loss of recognition by MAb J7.6. After the pulse, only small amounts of S were precipitated by the MAb (lane 2) while the antibody precipitated S almost quantitatively after the protein had been chased into the faster-migrating form (lane 4). This indicates that the epitope recognized by MAb J7.6 is formed during the oxidation and folding of S. The absence of S in lane 6 confirms this conclusion.

In the same experiment, we analyzed the M protein in a

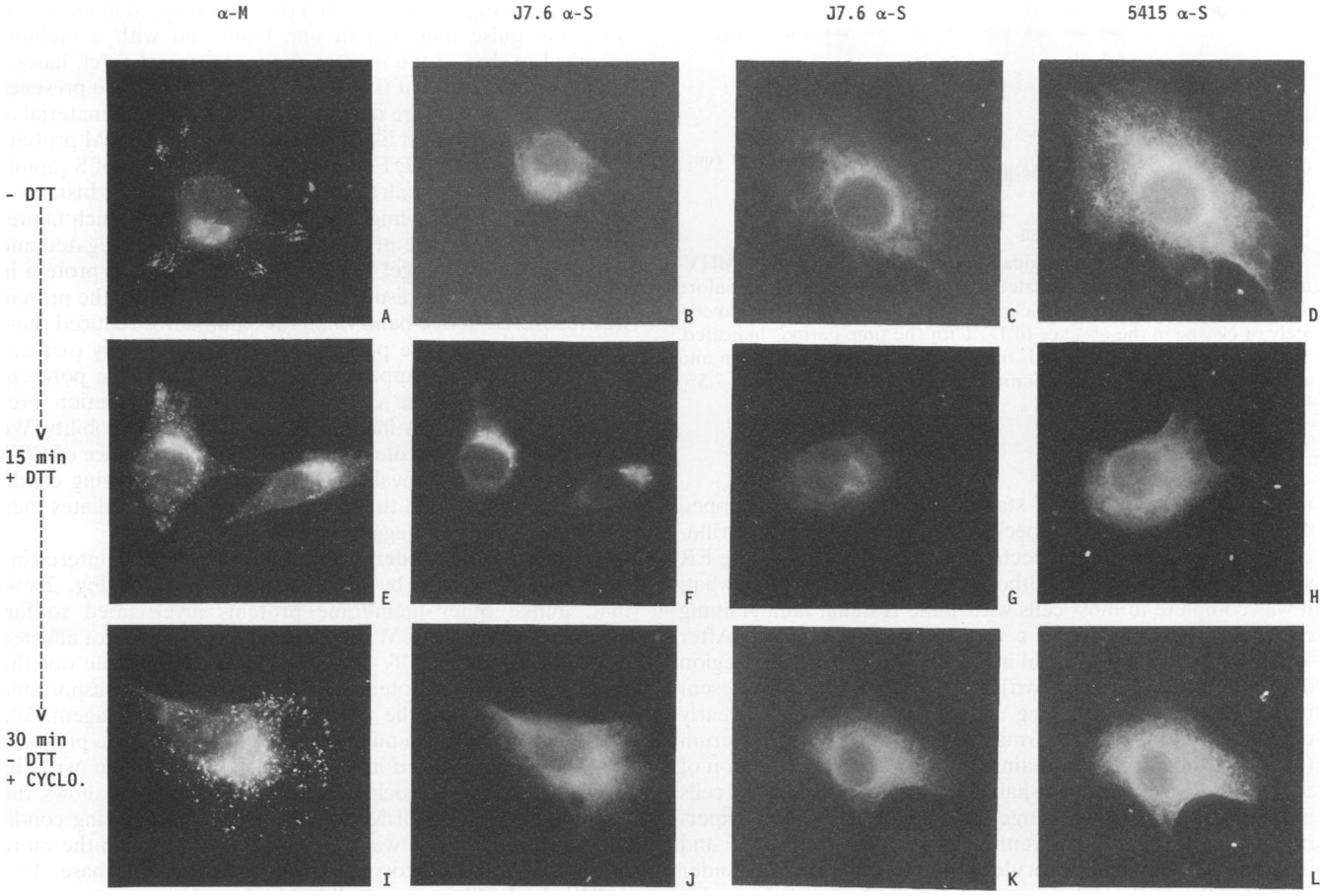

FIG. 3. In situ localization of the effects of DTT. Sac(−) cells grown on six coverslips in one 60-mm culture dish were infected with MHV-A59. At 6 h postinoculation, one pair of coverslips was removed for fixation and 5 mM DTT was added to the remaining cells. After a 15-min treatment with DTT, a second pair of coverslips was removed for fixation and DTT was washed out of the remaining cells. The latter were subsequently incubated for 30 min in the absence of DTT but in the presence of 0.1 mg of cycloheximide per ml and finally fixed. One coverslip of each pair was used to localize the M and S proteins by indirect double immunofluorescence. M was labeled with the peptide anti-M serum (A, E, and I), and S was labeled with MAb J7.6 anti-S (B, F, and J). The second coverslip of each pair was used to localize the folded S molecules within the total pool of S protein present in the cells. MAb J7.6 anti-S was used to label folded S molecules (C, G, and K) while the peptide anti-S serum 5415 was used to visualize all forms of S (D, H, and L). The binding of peptide anti-M antibodies and the peptide anti-S serum 5415 was performed with rhodamine-conjugated anti-rabbit IgG (left and right columns). In the middle columns, a fluorescein-conjugated anti-mouse IgG second antibody was used for the staining of MAb J7.6 anti-S in the same cells.

separate gel (Fig. 2B). After the pulse, the unglycosylated form was observed. This form normally becomes modified by O-linked sugars as the protein is transported to and through the Golgi complex, giving rise to the typical set of glycosylated forms. Surprisingly, the glycosylated forms of M appeared both in the absence and in the presence of DTT. This suggests that neither transport nor glycosylation of M is affected by DTT, as will be further substantiated below.

In vivo reduction initially affects the S protein in the ER. To monitor the state of folding of S, we exploited the observation that MAb J7.6 recognizes a conformational epitope. By biochemical experiments (also Fig. 2A), we established that the epitope for the antibody appears during oxidation and folding of the S precursor gp150 and remains in the mature form, gp180, as well as in its cleavage products, S1 and S2 (data not shown). By using this antibody in combination with a polyclonal M-specific serum, we were able to perform immunofluorescence double staining for S and M to analyze the effects of in vivo reduction in MHV-infected cells. In addition, to simultaneously monitor the distribution of folded versus unfolded S we performed double staining with MAb J7.6 and with another anti-S serum. This latter had been raised in rabbits against a synthetic peptide corresponding to a part of the COOH terminus of S and was found to immunoprecipitate all forms of S, including the reduced ones (data not shown). The experiment described below was done with cells grown in one culture dish containing six coverslips to ascertain that all cells were treated identically.

In untreated cells, the S protein was observed predominantly in a typical ER-like reticular pattern throughout the cytoplasm with additional intense fluorescence in a distinct perinuclear region (Fig. 3B). This latter region appeared to be the major site of the M protein (Fig. 3A) and is presumably the site of virus budding. From the analysis of a parallel coverslip, it was clear that the staining patterns of S obtained with MAb J7.6 (Fig. 3C) and with the anti-S peptide serum (Fig. 3D) were indistinguishable under these control conditions.

When MHV-infected cells were treated for 15 min with 5

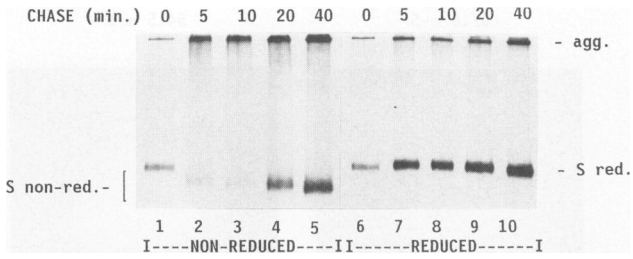

FIG. 4. Posttranslational oxidation of the MHV S protein. MHV-infected Sac(−) cells were treated with 5 mM DTT for 5 min before and during a 15-min pulse-labeling. Cells were either lysed immediately or chased in the absence of DTT for the time periods indicated. S was immunoprecipitated with the polyclonal anti-MHV serum and analyzed under reducing and nonreducing conditions in an SDS–7.5% PAG.

mM DTT, the intensity of staining of S in the ER dropped dramatically while the S-specific fluorescence in the perinuclear region remained unaffected (Fig. 3F). The loss of the ER signal was clear in all cells although its extent varied somewhat; it was complete in most cells with some residual faint staining remaining in some of the cells as shown in Fig. 3G. After longer treatments, the signal in the distinct perinuclear region also declined (data not shown). The S protein was still present in the ER under reducing conditions, which was clearly demonstrated by the conformation-independent anti-S serum (Fig. 3H); the intensity of staining as well as the distribution of the protein was indistinguishable from that in untreated cells and did not change after longer DTT treatment. These experiments illustrate the differential distribution of folded and incompletely folded S molecules in MHV-infected cells under reducing conditions. They suggest that folding of S is readily reversible under the conditions prevailing in the ER but that the conformation of the protein is not easily affected by in vivo reduction once it has left this compartment.

In contrast to the S protein, no such effects of DTT were observed for the M protein: neither the intensity nor the location of the fluorescence staining was significantly affected by the treatment (Fig. 3E). Only longer exposures to DTT led to an overall reduction of the signal, which in addition became more concentrated in a distinct, Golgi-like region of the cell (data not shown).

To investigate whether the effects of DTT were reversible, DTT was washed out of the cells after a 15-min treatment and the incubation was continued for 30 min in the presence of cycloheximide to inhibit protein synthesis. As shown in Fig. 3J and K, this procedure efficiently restored the typical ER staining of S by the conformation-specific MAb. The different fluorescence patterns obtained with the MAb and with the conformation-independent anti-S serum seen after the DTT treatment were no longer observed (Fig. 3L). Apparently, the oxidizing state of the ER had recovered after removal of DTT, allowing the unfolded S protein to refold. As expected, these procedures did not noticeably affect staining of the M protein.

Oxidation of S after removal of DTT. To monitor the posttranslational oxidation of S biochemically, we pulse-labeled S under reducing conditions, after which we washed out the DTT and the label and incubated the cells for various time periods in chase medium. To make sure that no disulfide bonds were formed during the pulse, the DTT treatment was started 5 min before labeling. The viral proteins were immunoprecipitated with the polyclonal anti-MHV serum and analyzed by gel electrophoresis under reducing and nonreducing conditions. As shown in Fig. 4, the labeled S protein analyzed immediately after the pulse migrated in one band and with a mobility identical to that of the in vitro-reduced form of S (cf. lanes 1 and 6), confirming that the protein synthesized in the presence of DTT did not acquire disulfide bonds. Again, the material at the top of the gel most likely represents aggregated M protein. After the removal of DTT, the fully reduced form of S rapidly disappeared and had already vanished after 5 min. Instead, a diffuse band of faster-migrating forms which was much fainter appeared; most of the protein apparently had aggregated and stayed on top of the gel in large complexes. The S protein in these aggregates was disulfide cross-linked since all the protein was recovered in one band when the sample was reduced (lane 7). After longer chase periods, apparently normally oxidized molecules gradually appeared (lanes 2 to 5). These polypeptides acquired a more compactly folded conformation over time as judged by their increasing electrophoretic mobility. We conclude that S molecules synthesized in the presence of DTT transiently fall into covalent complexes once oxidizing conditions are restored and that normal folding intermediates then dissociate from these aggregates.

Transport of M under reducing conditions. An interesting conclusion suggested by the results presented in Fig. 2 was that, unlike other membrane proteins investigated so far, transport of the MHV M protein from the ER was not affected by reduction with DTT. However, we could not rule out the possibility that the protein had already become transportable during the pulse in the absence of the reducing agent. We therefore performed a pulse-chase experiment in the presence of DTT and analyzed the transport of M, again with its O-glycosylation as a biochemical marker. As Fig. 5A shows, the unglycosylated form of M synthesized under reducing conditions during the pulse was efficiently converted into the more slowly migrating, glycosylated forms during the chase. Evidently, the DTT treatment did not affect the proper assembly of the triple-spanning membrane protein, because it adopted the right orientation to become glycosylated. In addition, since O-glycosylation takes place while M is being transported through the intermediate compartment to Golgi and TGN, the protein was still transportable in the presence of DTT. One might argue that this glycosylation was unrelated to transport and was just caused artifactually by the reducing conditions. DTT does indeed have some side effects illustrated by, e.g., its inhibiting influence on protein synthesis, especially during longer treatments (data not shown). To verify that the O-glycosylation observed in the presence of DTT was dependent on transport of the protein, we analyzed the addition of oligosaccharides in MHV-infected cells treated with brefeldin A. This drug causes a redistribution of all Golgi compartments up to the *trans* cisterna back to the ER and inhibits transport to the TGN (18, 21). We have used this drug earlier to demonstrate that the most mature forms of M, designated M4 and M5, are made in the TGN (19). As shown in Fig. 5B, under reducing conditions the formation of these species was fully blocked by the addition of brefeldin A. The glycosylation of the M protein that we observed in the presence of DTT must thus have been the result of ER-to-Golgi transport.

In pulse-chase studies such as those whose results are shown in Fig. 2B and 5A, we repeatedly observed that the M protein became glycosylated faster in the presence of DTT than in its absence. Since we had reasons to believe that this might relate to the interaction of M with S (see Discussion) rather than being caused by the DTT per se, we analyzed the effect of DTT on the rate of M glycosylation in cells expressing the protein from a recombinant vaccinia virus. For this purpose, we made use of OST7-1 cells (11) which constitutively express T7

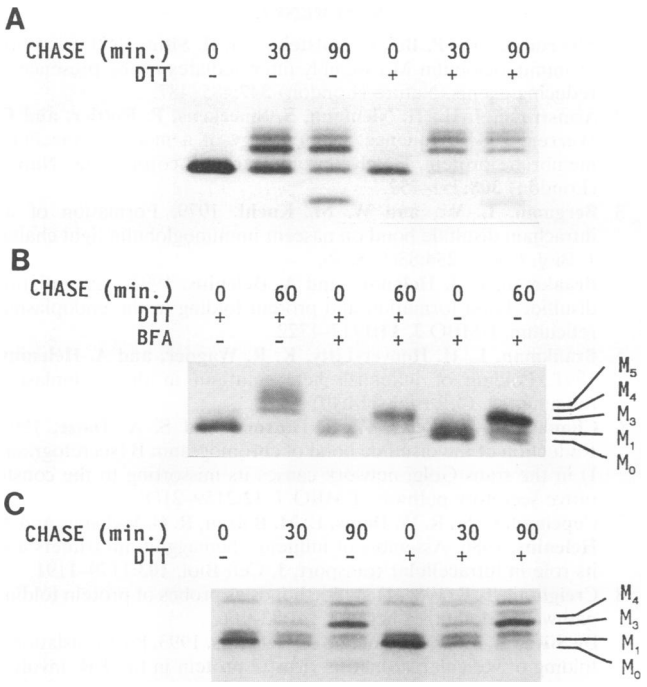

FIG. 5. (A) Effects of DTT on newly synthesized M protein. Parallel cultures of MHV-infected Sac(−) cells were pulse-labeled for 10 min and chased for the time periods indicated. Both the labeling and the chase were done in the absence and presence of 5 mM DTT as indicated. The DTT treatment was started 5 min before the start of the labeling. M was immunoprecipitated with the polyclonal anti-MHV serum and analyzed in an SDS–15% PAG. The lower-molecular-weight protein appearing during the 90-min chase periods is an M degradation product. (B) Intracellular transport of M under reducing conditions. MHV-infected Sac(−) cells were pulse-labeled for 10 min and directly lysed or chased for 60 min. In some cultures, 5 mM DTT was present during the labeling and the chase as indicated, the treatment again being started 5 min before the labeling. To some cultures, brefeldin A (BFA) was added to 6 μg/ml 30 min before the labeling, and the drug was kept present during the labeling and the chase. M was further analyzed as in panel A. (C) Effects of DTT on independently expressed M. In this experiment, the M protein was expressed by a recombinant vaccinia virus in OST7-1 cells. The effects of in vivo reduction on the M protein in the absence of other coronaviral proteins were analyzed in an experiment identical to that described for panel A. The uppermost band present in all lanes is a background band unrelated to the MHV M protein.

polymerase. The results of a pulse-chase experiment are shown in Fig. 5C. M was found to be glycosylated at the same rate irrespective of the presence of DTT. Thus, DTT does not seem to interfere with transport of M directly. As this experiment suggests, glycosylation of the vaccinia virus-expressed M is slower than in MHV infection (data not shown). The reason for this is not clear.

DISCUSSION

The correct folding of membrane proteins in the ER is drastically impaired by in vivo reduction, as has been shown for the influenza virus HA protein (4), for the H1 subunit of the asialoglycoprotein receptor (22), and for the vesicular stomatitis virus (VSV) G protein (9). These examples all bear upon proteins which form intramolecular disulfide bonds. We have taken advantage of the characteristic differences between the membrane proteins S and M of MHV to simultaneously analyze the folding and transport of one membrane protein that contains multiple intramolecular disulfide bonds and of another that does not. Our data confirm and extend the conclusions concerning disulfide-dependent folding drawn from other studies (4, 6, 9, 22, 36) which show that key cellular processes, both within and outside the ER, are unaffected by the reducing conditions introduced into the cell by DTT. Notably, while folding and transport of the MHV S protein were largely impaired, DTT treatment did not affect the disulfide-independent folding of the M protein, nor did it prevent its transport from the ER to the Golgi complex, interfere with its O-glycosylation, or affect its Golgi retention.

The MHV S protein assembles into homo-oligomeric structures with a half-time of 40 to 60 min (40), which is slow compared with the influenza virus HA and VSV G (7, 10, 28). We think that this is mainly because of its low rate of folding. Our pulse-chase analyses demonstrate that the major conformational events involving disulfide bond formation take about 20 min. Folding of the S protein could be followed by electrophoresis under nonreducing conditions as well as by the appearance of a conformational epitope. Since we never detected completely reduced S molecules, even after very short labeling periods, it is likely that disulfide bond formation already starts in the nascent chains, as has been shown for some other proteins (3, 5, 24, 27). It continues posttranslationally, during which the protein passes through a continuous spectrum of folding intermediates. No discrete intermediate forms were resolved in contrast to the influenza virus HA protein (5). This is probably explained by the high number (a total of 42) of cysteine residues present in the S molecule, which allows for numerous alternative disulfide linkages before the native state is reached.

Perturbation of the redox state in the cell by addition of DTT has a selective effect on the different maturation forms of S. Just-completed S molecules as well as nascent polypeptides are reduced and unfolded instantaneously as could be monitored by the immediate loss in the recognition of the pulse-labeled protein by conformation-specific antibodies. However, the S molecules become gradually more resistant to reduction and eventually can be unfolded only by prolonged treatment with the reducing agent. These observations are consistent with the immunofluorescence data which show that upon reduction the protein immediately unfolds when still in the ER; once outside, the definite epitopes appear to be more stable. This has also been reported for the influenza virus HA and the H1 subunit of the asialoglycoprotein receptor (4, 22). In addition, Tatu et al. (36) recently demonstrated that the VSV G protein and the influenza virus HA become DTT resistant already before reaching the Golgi complex. The conversion to DTT resistance of HA was found to coincide with (or may even precede) its trimerization (36). In the case of the S protein, it is still unknown where and when this conversion takes place. Interestingly, the disulfide bond in the secretory protein secretogranin I is susceptible to DTT even in the TGN (6). This reduction diverts the protein from the regulated to the constitutive pathway.

Artificially, the S protein can be driven into disulfide-linked complexes. When it is allowed to fold posttranslationally after DTT washout, it becomes transiently trapped in aggregates formed by interchain disulfides. Correctly folded molecules emerge from these complexes as judged from the regain of specified epitopes. This indicates that the aberrantly formed interchain disulfides are replaced by correct bonds in the rescue of aggregated molecules. A similar process has been observed during the posttranslational oxidation of the VSV G protein (9) but not during that of the influenza virus HA

protein (4). Apparently, some proteins tend to aggregate under these conditions whereas others do not. In all cases, however, even proteins with a large number of intrachain disulfide bonds (such as the S protein) can pass the aggregated state and fold successfully.

The major finding of our study is that the biogenesis and transport of the coronaviral M protein are insensitive to reduction. The M protein does not form any inter- or intramolecular disulfide bonds in the lumen of the ER. Several observations provide evidence that the protein is correctly assembled under reducing conditions. Firstly, it becomes normally glycosylated, which shows that the amino-terminal domain is exposed luminally. Secondly, since O-glycosylation takes place in post-ER compartments the protein is apparently transportable and thus able to pass the quality control in the ER (16). Thirdly, the protein has the right conformation to be retained in the Golgi complex. Golgi retention is sensitive to structural changes in the protein, as we have learned from mutational studies (unpublished observations).

As is clear from our work and that of others (4, 6, 9, 22, 36) in vivo reduction with DTT does not induce a general state of alarm in the cell. Most processes seem to continue normally. This even holds true for processes occurring in the ER, where reduction appears to selectively affect the folding of only those proteins whose native structure is dependent on the formation of disulfides. Other proteins such as the secretory protein, secretogranin II (6), and the M protein are handled undisturbed. Moreover, aberrantly folded proteins are prevented from exiting the ER while at the same time transport-competent proteins are able to leave the ER. Assuming that the latter proteins are actively controlled by the ER quality control system, these observations suggest that the system is quite insensitive to short-term treatment with DTT. The intracellular transport of glycoproteins along the secretory pathway seems not to be affected by in vivo reduction. This has been previously demonstrated for the secretory proteins IgM (1) and secretogranins I and II (6) as well as for the trimerized forms of influenza virus HA (36).

We found that in MHV-infected cells the M protein was transported to the Golgi complex faster in the presence of DTT than in its absence, as judged by the increased rate at which the protein acquired its Golgi modifications. No such difference was evident when the protein was expressed independently. This discrepancy probably relates to the interactions between the coronaviral membrane proteins in the process of virus assembly. As we have recently observed, the M protein associates with the S protein almost immediately after its synthesis, forming large complexes which presumably accumulate at the site of budding (unpublished results). Since budding occurs in pre-Golgi membranes, this association is aimed, among others, at retaining the M protein and preventing it from escaping to the Golgi apparatus. Interaction between S and M is critically dependent on the folding of the former. Under reducing conditions, the S protein is unfit to associate with M, which, as a consequence, is no longer retained and is transported swiftly to the Golgi complex.

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