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COEXPRESSION AND ASSOCIATION OF THE SPIKE PROTEIN AND THE MEMBRANE PROTEIN OF MOUSE HEPATITIS VIRUS

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ABSTRACT

The M and S envelope glycoproteins of mouse hepatitis virus associate in the process of virus assembly. We have studied the intrinsic properties of M/S heterocomplexes by coexpressing M and S in the absence of other coronaviral proteins. The formation of M/S complexes under these conditions indicates that M and S can interact independently of other coronaviral factors. Pulse-chase analysis revealed that M and S associate in a pre-Golgi compartment. M/S complexes are efficiently transported beyond the coronavirus budding compartment to the Golgi complex. The failure to detect complexes at the surface of coexpressing cells demonstrated that they are retained intracellularly. Thus, coexpression of the envelope glycoproteins drastically affects the intracellular transport of the S protein: instead of being transported to the cell surface, S is retained intracellularly by its association with M.

INTRODUCTION

The spike (S) protein and the membrane (M) protein of the mouse hepatitis virus strain A59 (MHV-A59) are involved in intermolecular interactions in the process of virus assembly. Using specific detergent conditions heterocomplexes consisting of M and S can be extracted from solubilized virions and from MHV-infected cells[1]. The interaction between M and S is likely to be essential for the incorporation of the S protein into virus particles since the latter is thought to be dispensable for virus assembly. Tunicamycin treatment of MHV-infected cells results in the secretion of spikeless virions[2,3] suggesting that the S protein

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is not required for the budding process. A specific interaction between the M and S proteins, therefore, could effect the incorporation of the latter into virus particles.

Coronaviruses are assembled by budding of the nucleocapsid into pre-Golgi membranes[4,5,6]. According to a prevailing hypothesis the envelope proteins of membrane viruses determine the site of budding[7,8]. In the case of coronaviruses, however, neither of the envelope glycoproteins contain targeting signals that specify their accumulation in the budding compartment. When expressed independently, the M protein localizes to the Golgi complex[6,9,10] whereas the S protein is transported to the cell surface (Vennema et al., in prep.). M/S complexes, however, might have acquired a signal that prevents their transport beyond the budding compartment. To investigate the effect of the association between M and S on their intracellular transport we have coexpressed the envelope glycoprotein genes in the absence of other coronaviral proteins.

MATERIALS AND METHODS

Cells, Virus and Antisera

OST7-1 cells, a kind gift of Dr. B. Moss, were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, penicillin, and streptomycin (DMEM-10%FCS) supplemented with 400 μg/ml G-418 (Geneticin, GIBCO). The recombinant vaccinia virus vTF7-3 expressing the T7 RNA polymerase[11] was also obtained from Dr. B. Moss. The production of the rabbit polyclonal antiserum to MHV-A59 has been described previously[2]. The monoclonal antibodies (MAbs) J7.6 and J1.3 against S and M, respectively, were kindly provided by Dr. J. Fleming.

Infection, Transfection and Metabolic Labeling

Infection and transfection of OST7-1 cells: Subconfluent monolayers of OST7-1 cells in 35-mm dishes were washed with DMEM and inoculated with vTF7-3 at a multiplicity of infection (m.o.i.) of approx. 10 in DMEM for 45 min at 37°C. After inoculation the cells were washed with DMEM and transfected with the vector PTUM-M[12] and/or PTUM-S (Vennema et al., in prep.) which contain a cDNA copy of the MHV M and S protein, respectively, under the control of the T7 promotor. For this purpose, a mixture of 200 μl DMEM with plasmid DNA and 10 μl lipofectin reagent (Bethesda Research Laboratories, Life Technologies, Inc.) was added to the cells. After a 10-min incubation at room temperature 800 μl DMEM was added and cells were incubated at 37°C.

Labeling: 2 hr after inoculation, cells were transferred to 32°C. Starting at 4.5 hr after inoculation, the cells were starved for 30 min in MEM (GIBCO) without methionine. Cells were pulse-labeled with 100-200 μCi ^{35}S-in vitro labeling mix (Amersham) for the times indicated, then washed once with DMEM-10%FCS supplemented with 10 mM HEPES, 2 mM L-methionine, and 2 mM L-cysteine (chase medium) and chased for various times in chase medium. The cells were lysed on ice in 50 mM Tris (pH 8.0), 62.5 mM EDTA, 0.5% Nonidet P-40, 0.5% Na-deoxycholate (detergent solution) containing 2mM phenylmethylsulfonyl fluoride (PMSF). The lysates were spun for 3 min at 12,000 x *g* at 4°C to pellet nuclei and cell debris.

Immunoprecipitation and Gel Electrophoresis

Viral proteins were immunoprecipitated with the polyclonal MHV-A59 antiserum (10μl), the MAb J1.3αM (10μl), or with the MAb J7.6αS (20 μl). Serum was added to

aliquots of cell lysates which had been diluted with detergent solution to a final volume of 600 µl. After overnight incubation at 4°C immune complexes were collected using 50 µl of a 10% (w/v) 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 detergent solution and finally suspended in 25 µl 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 20 mM DTT (sample buffer). The proteins were analysed in 10% SDS-polyacrylamide gels. The samples were heated for 2 min at 95°C before loading on the gel.

Surface Immunoprecipitation

Transfected cells were labeled with ^{35}S-in vitro labeling mix from 5-5.5 hr p.i. and subsequently chased for 3 hr in chase medium. The cells were put on ice and washed with PBS/5%FCS and incubated for 2 hr in 800 µl PBS/5%FCS containing the MAb J1.3αM (15µl) and/or MAb J7.6αS (30µl). Thereafter, cells were extensively washed with PBS/5%FCS and lysed with detergent solution containing 2 mM PMSF. The lysates were spun for 3 min at 12,000 x *g* and 4°C and 50 µl of a 10% (w/v) suspension of formalin-fixed *Staphylococcus aureus* cells was added to collect the immune complexes. After a 30 min incubation at 4°C the cells were pelleted by centrifugation. The supernatant was subjected to a second round of immunoprecipitation using the same antibodies. The bacterial cells were washed three times with detergent solution and finally suspended in sample buffer.

RESULTS AND DISCUSSION

Association of the Coexpressed M and S Proteins

The interaction between the M and S proteins of MHV-A59 can be detected under specific analytical conditions. When a combination of ionic and nonionic detergents is used for the solubilization of virions or infected cells M/S heterocomplexes can be immunoprecipitated using monospecific antisera against M or S[1,13]. To study whether M and S associate independently of other viral proteins we have coexpressed their respective genes in the absence of other coronaviral proteins using the vaccinia virus infection/transfection expression system[11].

Cells coexpressing M and S were labeled for 30 min with ^{35}S-methionine and chased for 90 min and subsequently lysed using a buffer containing 0.5% NP40 and 0.5% NaDOC. Equal fractions of the cell lysate were used for immunoprecipitation using a polyclonal anti-MHV serum or a monoclonal antibody (MAb) against S. As shown in Fig. 1, the material precipitated using the anti-MHV serum represents the total amount of labeled viral proteins. It mainly consists of the spike precursor S/gp150 and differentially glycosylated forms of M. The two other bands presumably represent vaccinia virus proteins which were precipitated nonspecifically. The S protein as well as a significant fraction of the M protein were also precipitated using the MAbαS. In addition, a fraction of labeled S was coprecipitated with the M specific antibodies. This indicates that M and S were associated under these conditions. Apparently, the formation of M/S complexes does not require other coronaviral proteins. To exclude the possibility that M and S had formed complexes after lysis we expressed the proteins independently in separate cell cultures and performed the immunoprecipitations using a mixture of the cell lysates (data not shown). Under these conditions no complexes between M and S were found indicating that M/S complexes derived from coexpressing cells were specific.

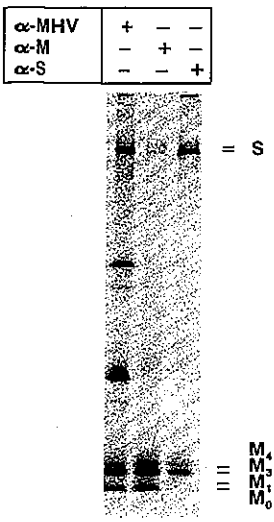

Figure 1. Association of the coexpressed MHV M and S proteins. Cells coexpressing M and S were labeled for 30 min and chased for 90 min. The cell lysate was split into three equal fractions and the viral proteins were precipitated using a polyclonal anti-MHV serum, a MAbαM, or a MAbαS, respectively.

Formation and Intracellular Transport of M/S Complexes

To determine the kinetics of complex formation and to analyze the intracellular transport of M/S complexes we performed a pulse-chase labeling. Cells coexpressing M and S were pulse labeled for 15 min and chased for various time periods. The cell lysates were split into three equal portions from which the viral proteins were immunoprecipitated using the polyclonal anti-MHV serum, a MAbαM, and a MAbαS, respectively.

Again, the material precipitated using the polyclonal anti-MHV serum represents the total amount of labeled viral proteins (Fig. 2). After the pulse, predominantly precursor forms of M (M_0) and S (S/gp150) can be detected. The M protein becomes glycosylated posttranslationally when it is transported from the ER to the Golgi complex giving rise to the formation of different glycosylated forms (M_1-M_3-M_4). The addition of the first sugar, GalNAc, takes place in the intermediate compartment - which is identical to the budding compartment[5,14] - and M_3 and M_4 appear after the protein has reached the Golgi complex[10]. A fraction of S/gp150 was converted into S/gp180 during the chase as a result of modifications of its N-linked oligosaccharides. Cleaved forms of the S protein were hardly detected in this experiment.

Using the MAbαS we analyzed which fraction of the labeled M molecules was engaged in M/S complexes. After the pulse, only small amounts of labeled M protein were coprecipitated by the S specific antibodies. The amount of M that was coprecipitated with S increased during the chase and reached a maximum around 60 min of chase. The coprecipitation of the unglycosylated form of M (M_0) seen after short chase periods indicates that M/S complexes are formed in a pre-Golgi compartment. After longer chase periods, this form was efficiently processed into M_3 and M_4 demonstrating that the complexes were transported to the Golgi complex. Surprisingly, no accumulation of M_1 is observed indicating that M/S complexes are not retained in the budding compartment by themselves.

When M/S complexes were precipitated using the MAbαM we found that coprecipitation of labeled S started to appear after 15 min of chase. Apparently, newly synthesized M and S become engaged in heterocomplexes with different kinetics. The S protein needs more time to become association competent which is probably the result of its slow folding[12,13,15]. Like the M protein, the S protein present in M/S complexes underwent

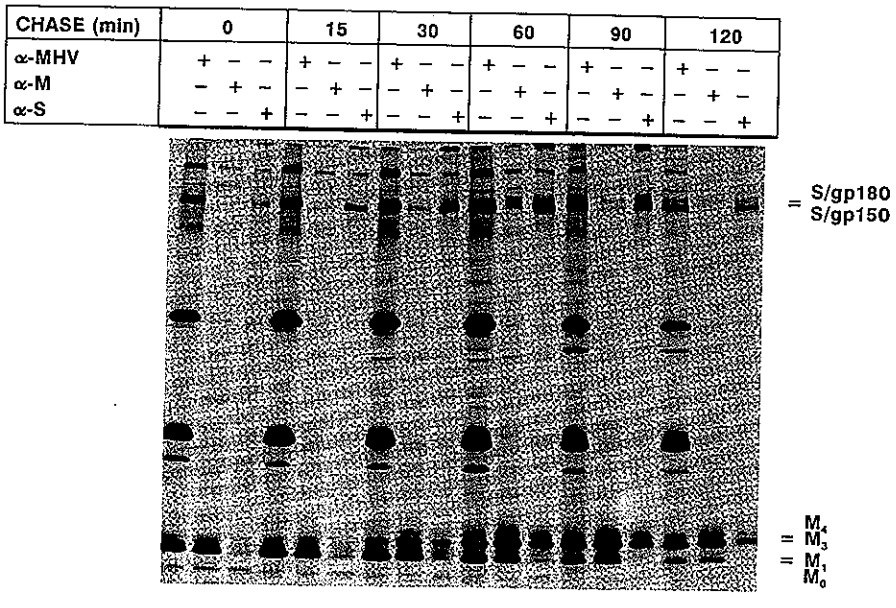

Figure 2. Formation and intracellular transport of M/S complexes. Cells coexpressing M and S were labeled for 10 min and chased for the indicated periods. Each cell lysate was split into three equal fractions which were used for immunoprecipitations with the polyclonal anti-MHV serum, the MAbαM, or the MAbαS, respectively.

processing during longer chase periods: its precursor form S/gp150 was converted into S/gp180 which indicates that it was transported from the ER to the Golgi complex. We conclude from this experiment that M and S associate in a pre-Golgi compartment and that they are transported as a complex beyond the site of virus budding to the Golgi region. This implies that additional factors, e.g. the nucleocapsid or the recently identified small membrane protein[16], determine pre-Golgi budding of MHV.

Intracellular Accumulation of S by Its Interaction with M

Knowing that M/S complexes are transported beyond the budding compartment and that the independently expressed M and S proteins are transported to different cellular locations, i.e. Golgi complex and plasma membrane, respectively, we were interested in the destination of M/S complexes. The previous experiment (Fig. 2) shows that M/S complexes reach the Golgi complex but the observation that only a very small fraction of S was cleaved into S/gp90 suggests that the complexes do not reach the cell surface. To investigate the cell surface expression of M/S complexes we have performed cell surface immunoprecipitations. Cells expressing S alone or together with M were labeled for 30 min and chased for 3 hr to allow ample time for the proteins to reach their final destinations. As described in the Methods section, we then first precipitated the viral proteins that were expressed at the plasma membrane and in a second round of immunoprecipitation we collected the proteins which were kept within the cells.

When expressed independently, a large fraction of the cleaved form of S as well as some uncleaved S/gp180 were found at the cell surface (Fig. 3). As expected, and at the same time illustrating the validity of the approach, the remaining precursor S/gp150 was only detected intracellularly. When coexpressed with M, however, hardly any S protein was

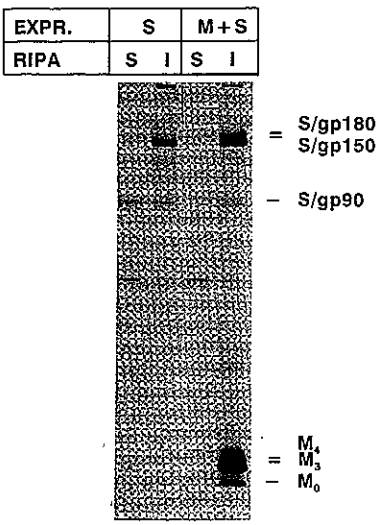

Figure 3. Intracellular accumulation of M/S complexes. Cells expressing S alone or together with M were labeled for 30 min and chased for 3 hr. Viral proteins expressed at the cell surface (S) were collected in a first round of immunoprecipitation and the remaining fraction of intracellular proteins (I) were immunoprecipitated in a second round.

detected at the cell surface. Instead, we now observed an intracellular accumulation of S/gp180 and S/gp90. Although the M protein had reached the Golgi complex as judged by the appearance of M_3 and M_4 it could not be detected at the cell surface. We conclude that coexpression of M and S does not affect the localization of M. Rather, it specifically results in the intracellular retention of the S protein due to its interaction with M.

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