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Biosynthesis, Structure, and Biological Activities of Envelope Protein gp65 of Murine Coronavirus

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We have previously shown that gp65 (E3) is a virion structural protein which varies widely in quantity among different strains of mouse hepatitis virus (MHV). In this study, the biosynthetic pathway and possible biological activities of this protein were examined. The glycosylation of gp65 in virus-infected cells was inhibited by tunicamycin but not by monensin, suggesting that it contains an *N*-glycosidic linkage. Glycosylation is cotranslational and appears to be complete before the glycoprotein reaches the Golgi complex. Pulse–chase experiments showed that this protein decreased in size after 30 min of chase, suggesting that the carbohydrate chains of gp65 undergo trimming during its transport across the Golgi. This interpretation is supported by the endoglycosidase treatment of gp65, which showed that the peptide backbone of gp65 did not decrease in size after pulse–chase periods. This maturation pathway is distinct from that of the E1 or E2 glycoproteins. Partial endoglycosidase treatment indicated that gp65 contains 9 to 10 carbohydrate side chains; thus, almost all of the potential glycosylation sites of gp65 were glycosylated. *In vitro* translation studies coupled with protease digestion suggest that gp65 is an integral membrane protein. The presence of gp65 in the virion is correlated with the presence of an acetylesterase activity. No hemagglutinin activity was detected.

INTRODUCTION

Mouse hepatitis virus (MHV), a member of the Coronaviridae family, is an enveloped virus with a positive-sensed RNA genome of more than 6×10^6 molecular weight (Lai and Stohlman, 1978; Wege *et al.*, 1978). The virion particle is composed of three structural proteins: two glycosylated envelope proteins, gp180/gp90 (E2) and gp23 (E1), and a phosphorylated nucleocapsid protein pp60 (N). The E2 protein forms the spikes on the surface of virion, is responsible for viral interaction with target cells and fusion activity, and also elicits neutralizing antibodies (Sturman and Holmes, 1983). Monoclonal antibodies against E2 alter the pathogenicity of MHV (Fleming *et al.*, 1986; Dalziel *et al.*, 1986). E1 is a transmembrane matrix protein (Sturman *et al.*, 1980; Rottier *et al.*, 1984, 1986), the function of which is not yet clear. Some monoclonal antibodies against E1 can also alter the pathogenicity of the virus (Fleming *et al.*, 1989). The N protein is a phosphorylated nucleocapsid protein (Stohlman and Lai, 1979), which interacts with virion RNA (Sturman *et al.*, 1980; Baric *et al.*, 1988). Some strains of MHV, e.g., JHM or MHV-S, contain an additional glycoprotein gp65 (Lai and Stohlman, 1981; Siddell, 1982; Makino *et al.*, 1983). Since bromelain treatment of JHM virion removed gp65, it has been suggested that this protein is also an envelope protein, part of which is exposed on the viral surface of this virus (Makino *et al.*, 1983).

The structural proteins of MHV are translated from virus-specific monocistronic mRNAs in the virus-infected cells (Rottier *et al.*, 1981; Leibowitz *et al.*, 1982). These mRNAs have a 3′-coterminal nested-set structure (Lai *et al.*, 1981; Leibowitz *et al.*, 1981). Only the 5′-terminal unique region of each mRNA is used for translation (Leibowitz *et al.*, 1982; Siddell, 1983). The E2 protein is translated from mRNA 3, and glycosylated at asparagine residues via *N*-glycosidic bonds (Holmes *et al.*, 1981; Rottier *et al.*, 1981). It is first synthesized as a p110 precursor, and glycosylated to gp150 and then to gp180 (Siddell *et al.*, 1981; Rottier *et al.*, 1981). It is subsequently cleaved into two gp90 subunits (Sturman *et al.*, 1985). This cleavage is required for viral infectivity and is carried out by cellular proteases (Storz *et al.*, 1981; Yoshikura and Tajima, 1981; Sturman *et al.*, 1985). In contrast, E1 protein is an O-linked glycoprotein, which is probably glycosylated in the Golgi complex post-translationally (Holmes *et al.*, 1981; Niemann and Klenk, 1981; Niemann *et al.*, 1984; Rottier and Rose, 1987). The glycosylation of the E1 protein is resistant to tunicamycin but is sensitive to monensin, which acts at the Golgi complex level (Niemann *et al.*, 1982). In contrast to what is known about E1 and E2, the biosynthetic pathway of gp65 in MHV-infected cells is not yet clear. Recently we have shown that gp65 is translated from a new mRNA designated

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mRNA 2b, which is synthesized by only a few isolates of JHM strains of MHV (Makino and Lai, 1989; Shieh *et al.*, 1989). This mRNA corresponds to an open reading frame (ORF) 2b which encodes gp65 (Shieh *et al.*, 1989). The unique aspect of this protein is that its expression is variable among different MHV strains and is regulated by the sequences of leader RNA at the 5′-end of genomic RNA and the intergenic region preceding the ORF 2b. For instance, JHM(3) virus, which contains three UCUAA repeats at the 3′-end of leader RNA, makes only a small quantity of gp65 (Makino and Lai, 1989; Shieh *et al.*, 1989). In contrast, JHM(2) virus with two UCUAA repeats makes an abundant quantity of the protein (Shieh *et al.*, 1989). Although the genomic RNA of the A59 strain of MHV contains ORF 2b and two UCUAA repeats, it lacks both correct transcriptional and correct translational start signals for this gene, resulting in the absence of gp65 in A59 virus (Luytjes *et al.*, 1988; Shieh *et al.*, 1989).

The gp65 protein has also been detected in another coronavirus, bovine coronavirus (BCV) (King and Brian, 1982). This protein, termed E3, contains both an esterase activity, which is similar to the receptor-destroying activity of influenza C virus (Vlasak *et al.*, 1988a), and a hemagglutinin activity (King *et al.*, 1985). Significantly, the predicted sequence of gp65 of MHV is homologous to the hemagglutinin protein of influenza C virus (Luytjes *et al.*, 1988), although hemagglutinin activity has not been demonstrated for MHV (Talbot 1989). The presence of this protein in certain strains of coronaviruses raises the question whether the added biological activity of this protein contributes to the biological or pathogenic properties of the viruses. To answer such a question, we characterized the biochemical properties of gp65 of MHV. In this report, we determined its structure, biosynthetic pathway, and esterase activity.

MATERIALS AND METHODS

Virus and cells

The plaque-cloned JHM(2), which contains two UCUAA repeats at the 3′-end of leader sequence (Makino and Lai, 1989), was used for most of the experiments described in this study. Two other MHV isolates, JHM(3), which contains three UCUAA repeats (Makino and Lai, 1989), and A59 strains were also used. Viruses were propagated on DBT cell, a mouse astrocytoma cell line (Hirano *et al.*, 1974), according to the methods described previously (Makino *et al.*, 1984).

Preparation of rabbit antiserum specific for JHM(2) virion proteins

JHM(2) virus was purified from approximately 1 liter of media from infected DBT cells according to the published method (Makino *et al.*, 1983). Briefly, virus particles were purified by discontinuous and continuous sucrose gradients, pelleted by centrifugation in a Beckman SW41 rotor at 40,000 rpm for 2 hr, and then suspended in TE buffer (0.01 *M* Tris–HCl, pH 7.4 and 1 m*M* EDTA) containing 0.1% SDS. Purity of virus was examined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with Coomassie blue. The purified virus was injected into rabbits intramuscularly three times with 2-week intervals. The first injection was made with complete Freund's adjuvant, whereas second and third ones were with incomplete adjuvant. After checking the titer of antiviral antibody by ELISA, rabbits were bled. Serum was complement-inactivated at 56° for 30 min, adsorbed with uninfected DBT cells, and filtered before use.

^{35}S-labeling of intracellular proteins and preparation of cell extracts

Confluent monolayers of DBT cells (1.5×10^6 cells) were infected with virus at a multiplicity of infection of 2. After 1 hr adsorption at 37°, virus inoculum was replaced with minimum essential media (MEM) containing 5% dialyzed fetal calf serum (FCS). At 8.5 hr postinfection, medium was changed to methionine-free MEM containing 2% dialyzed FCS. After 30-min starvation for methionine, ^{35}S-translabel (1193 Ci/mmol; ICN Biochemicals) was added to a final concentration of 50 μCi/ml. Cells were pulse-labeled for either 20 or 5 min and chased for different periods of time. Cells were then placed on ice, washed three times with cold phosphate-buffered saline (PBS), and lysed with 400 μl of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing 1 m*M* phenyl methyl sulfonyl fluoride (PMSF). Cell lysate was immediately passed through a 19-gauge syringe needle five times and centrifuged at 12,000 *g* for 10 min at 4°. The supernatant fluid was stored at −70°.

Immunoprecipitation and SDS–PAGE

Each cytoplasmic lysate was incubated with rabbit hyperimmune serum prepared against purified JHM(2) at 4° for 2 hr. Protein A (Pansorbin, Calbiochem) (10% suspension in 0.05% NP-40) was then added to the reaction mixture, which was further incubated at 4° for 2 hr. Precipitates were washed with lysis buffer several times. The final pellet was suspended in 2× sample buffer (0.06 *M* Tris–HCl, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, and 0.1% bromphenol blue), heated at 95° for 3 min, and centrifuged at 12,000 *g* for 5 min. The supernatants were electrophoresed on 7.5 to 15% gradient polyacrylamide gel containing 0.1% SDS.

Tunicamycin and monensin treatments

At 3 hr postinfection, tunicamycin (Sigma) was added to media to a final concentration of 0.3–1 μg/ml. At 8 hr p.i., cells were pulse-labeled in the presence of tunicamycin with ^{35}S-translabel for 20 min and processed for immunoprecipitation as described above. For monensin treatment, monensin (Sigma) was added to the media to a final concentration of 10^{-7} to 10^{-5} *M* at 3 hr p.i. At 8 hr p.i., cells were pulse-labeled with ^{35}S-translabel for 20 min and then chased with cold methionine for 2 hr before immunoprecipitation.

Endoglycosidase H treatment

After immunoprecipitation of infected cell lysates, the immune complexes bound to Pansorbin were eluted by heating in 0.5% SDS and 50 m*M* Tris–HCl, pH 7.5, at 95° for 4 min (Kakach *et al.*, 1989) and centrifuged. The supernatant was mixed with an equal volume of 1 *M* sodium citrate buffer (pH 7.0) containing 1 m*M* PMSF and 1 to 25 mU of endoglycosidase H (Boehringer-Mannheim) and incubated at 37° for different periods of time ranging from 1 min to 16 hr. After incubation, the reaction mixture was mixed with an equal volume of 2× sample buffer and heated at 95° for 4 min before SDS–PAGE analysis.

In vitro transcription and translation

Recombinant plasmid pT7(2B) (Shieh *et al.*, 1989) was linearized by digestion with *Eco*RI or *Bam*HI and transcribed *in vitro* with T7 RNA polymerase as previously described (Soe *et al.*, 1987; Tabor and Richardson, 1985). The resulting RNA was translated in mRNA-dependent rabbit reticulocyte lysate (Promega Biotech) in the presence of [^{35}S]methionine. Reactions were carried out in a final volume of 25 μl under conditions recommended by the manufacturer. Where specified, 2 μl of canine pancreatic microsomal membranes (Promega Biotech) were added to the reaction mixtures. Translation products were analyzed by electrophoresis on 10% polyacrylamide gels containing 1% SDS (Laemmli, 1970).

Protease digestion

Translation mixtures were diluted 1:2 with 50 m*M* Tris–HCl buffer, pH 7.4, containing 100 m*M* NaCl and 3 mg/ml Pronase (Sigma), and incubated at 0° for 30 min. Proteolysis was terminated by treatment with a protease-inhibitor cocktail containing 10 m*M* PMSF, 2.5 m*M* EDTA, 1.4 mg/ml Pepstatin A, and 3 mg/ml Aprotinin. Samples were analyzed by SDS–PAGE.

Acetylesterase assays

Media collected at 14 hr p.i. from the virus-infected cells were precipitated by ammonium sulfate (Makino *et al.*, 1984). Virus precipitates were suspended in PBS, and incubated with 1 m*M* *p*-nitrophenylacetate (Sigma) at room temperature. Hydrolysis of the substrate was monitored at 400 nm at 2-min intervals as previously described (Vlasak *et al.*, 1988a), using a chart recorder. Media from mock-infected cells were also precipitated with ammonium sulfate as a control. The background counts of this control were subtracted from those of virus samples. An aliquot of virus precipitate was used for plaque assay to determine virus titers of each virus preparation. The $O.D._{400}$ values of each virus were normalized with respect to the virus titer used.

Hemagglutination assay

Fresh red blood cells from mice (BALB/C and C57BL) were washed and resuspended in PBS to a final concentration of 0.5%, and then aliquoted (50 μl into each well) into V-shaped microtiter plates. Equal volumes of a series of twofold dilutions of the different virus preparations were added to each well. Plates were incubated at 4° overnight before results were read.

RESULTS

Detection of intracellular virus-specific gp65

To study the structure, biosynthesis, and possible biological activities of gp65, we first determined the intracellular form of gp65 in cells infected with different strains of MHV, including A59 and two strains of JHM, JHM(2), and JHM(3), which differ in the number of UCUAA repeats in the leader sequence (Makino and Lai, 1989; Shieh *et al.*, 1989). Cells were pulse-labeled with ^{35}S-translabel for 20 min, immunoprecipitated with antibodies made against JHM(2) virion which contains a large quantity of gp65 (Shieh *et al.*, 1989), and then analyzed by SDS–PAGE. As can be seen from Fig. 1, JHM(2) synthesizes a prominent species of 65-kDa protein in addition to N, E1, and E2 proteins. This virus has been shown to synthesize a large amount of mRNA 2b in infected cells (Makino and Lai, 1989; Shieh *et al.*, 1989). In contrast, JHM(3), which synthesizes a very small amount of mRNA 2b, synthesized a comparatively smaller quantity of gp65. This is in agreement with the previous finding that mRNA 2b is the mRNA for gp65 (Makino and Lai, 1989; Shieh *et al.*, 1989). Surprisingly, A59, which does not synthesize mRNA 2b (Makino and Lai, 1989) and does not contain ORF 2b with an initiator AUG (Luytjes *et al.*, 1988), also makes a small amount of gp65. Since this protein was precipi-

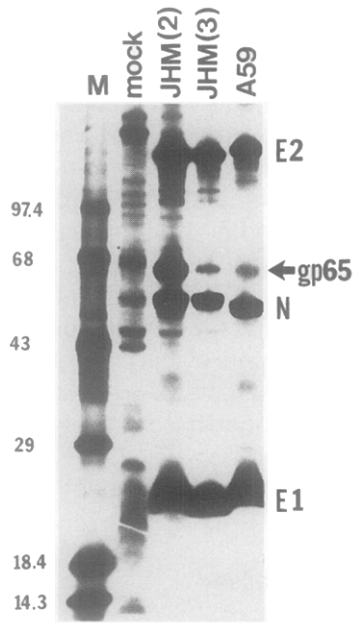

FIG. 1. Intracellular viral proteins of JHM(2), JHM(3), and A59. Virus-infected cells were radiolabeled with ^{35}S-translabel at 9 hr p.i. for 20 min. Cell lysates were immunoprecipitated with anti-JHM(2) rabbit serum and analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. Gels were overexposed to reveal gp65 in A59-infected cells. M, ^{14}C-protein molecular weight markers.

tated with JHM(2)-gp65-monospecific antibodies (data not shown), it is probably related to the JHM-specific gp65. The significance of this protein in A59 will be discussed later.

The relative amount of gp65 in infected cells is considerably higher than that in virion (Shieh *et al.*, 1989), suggesting that the incorporation of gp65 into virion is poor.

Biosynthesis of gp65 in the presence of tunicamycin and monensin

To understand the biosynthetic pathway of gp65, we studied the synthesis of viral glycoproteins in JHM(2) virus-infected cells in the presence of different inhibitors of glycosylation. Tunicamycin has been shown to inhibit the synthesis of *N*-glycosidic bond-linked glycoproteins (Takatsuki *et al.*, 1975). As shown in Fig. 2, tunicamycin inhibited the synthesis of gp65. Instead, a protein of 45 kDa was detected. This result is consistent with the previous *in vitro* translation studies which indicated that the peptide portion of this glycoprotein is of 45 kDa (Shieh *et al.*, 1989). Furthermore, it demonstrates that gp65 is an N-linked glycoprotein. In agreement with the published data (Niemann and Klenk, 1981), the glycosylated E2 was not detectable in the presence of tunicamycin. Instead, a p110 precursor protein was detected. In contrast, the size of gp23(E1) was not affected by the presence of tunicamycin, since it is an O-linked glycoprotein (Niemann and Klenk, 1981; Holmes *et al.*, 1981; Rottier and Rose, 1987). It is interesting to note that the 45-kDa protein detected in the presence of tunicamycin appears to consist of two bands, in agreement with the previous *in vitro* translation studies of mRNA 2b (Shieh *et al.*, 1989). The significance of this finding is unknown at the present time.

Another inhibitor of glycoprotein transport, monensin, affected the synthesis of the three viral glycoproteins differently. As shown in Fig. 3, monensin reduced the size of E1. This result is in agreement with the published finding that E1 glycosylation occurs in Golgi complex (Niemann *et al.*, 1982; Rottier and Rose, 1987). In contrast, E2 maturation occurred even in the presence of monensin. However, the size of the mature E2(gp90) was slightly smaller than that of E2 synthesized in the absence of monensin. The two protein bands corresponding to E2 (90K) probably represent different glycosylation states of the protein. This result indicates that the final carbohydrate residues on E2 glycoproteins are added in the Golgi apparatus and that incomplete glycosylation does not interfere with

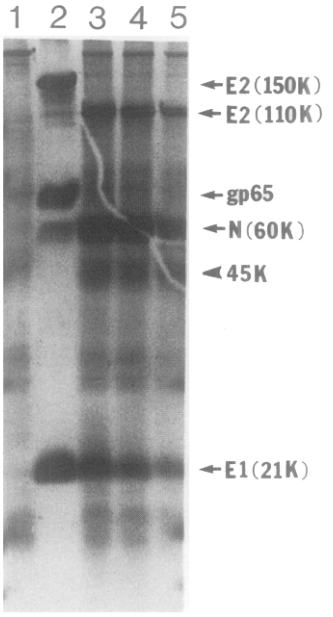

FIG. 2. Effects of tunicamycin treatment on the synthesis of intracellular viral proteins. JHM(2)-infected DBT cells were radiolabeled with ^{35}S-translabel at 9 hr p.i. for 20 min in the presence of different concentrations of tunicamycin. Cell lysates were immunoprecipitated with anti-JHM(2) rabbit serum. Lane 1, mock-infected cells; lane 2, JHM(2)-infected cells without tunicamycin; lane 3, JHM(2)-infected cells with tunicamycin 0.3 μg/ml; lane 4, JHM(2)-infected cells with tunicamycin 0.6 μg/ml; lane 5, JHM(2)-infected cells with tunicamycin 1 μg/ml.

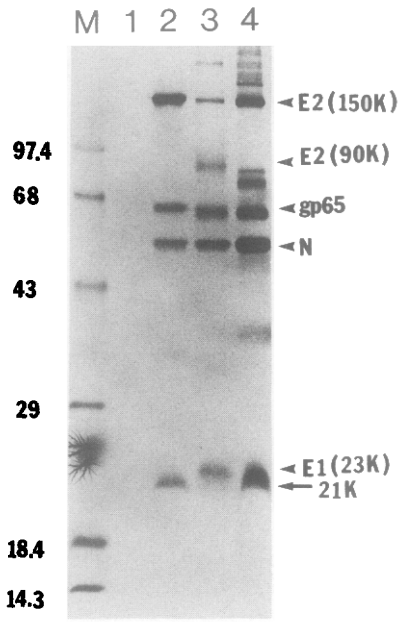

FIG. 3. Effects of monensin treatment on the synthesis of intracellular viral proteins. JHM(2)-infected cell lysate was prepared as described under Materials and Methods and immunoprecipitated with anti-JHM(2) rabbit serum. M, ^{14}C-protein molecular weight markers; lane 1, mock-infected; lane 2, 20-min [^{35}S] pulse-labeling at 9 hr p.i.; lane 3, 2-hr chase after 20 min of pulse-labeling; lane 4, the same condition as lane 3, but in the presence of monensin (10^{-5} *M*).

the cleavage of gp180 into gp90. In contrast, the size of gp65 was not affected by the presence of monensin (Fig. 3, lanes 3 and 4), suggesting that the glycosylation of gp65 was completed before the protein was transported into Golgi complex.

Careful examination of Fig. 3 also revealed that, after 2 hr of chase with cold methionine, gp65 slightly decreased in size (Fig. 3, lanes 2 and 3), suggesting that further processing of gp65 takes place after its glycosylation (see also Fig. 4). Monensin did not affect this processing (Fig. 3, lane 4).

Pulse–chase studies of gp65 biosynthesis

To further study the biosynthetic pathway of gp65, additional pulse–chase experiments were carried out. Figure 4 shows that a fully glycosylated gp65 could be detected after 5 min of pulse-labeling. No precursor 45-kDa protein could be detected, even after pulse-labeling for as short as 2 min (data not shown). This result suggests that glycosylation of gp65 may be cotranslational. After 30 min of chase, a slight decrease of gp65 size was noticeable (Fig. 4, lane 4). The size reduction of gp65 reached a maximum after 1 hr of chase (lane 5). These kinetics of gp65 processing suggest that a trimming of carbohydrate or peptide of gp65 occurred in the Golgi complex (Johnson and Spear, 1983). In contrast, E1 was not glycosylated during the 5-min pulse-labeling period. The glycosylation of E1 did not begin to occur until after 30 min of chase period. The fully glycosylated E1 could not be detected until after 2 hr of chase. This result is consistent with the monensin studies which suggest that the glycosylation of E1 occurs in the Golgi complex. The glycosylation of E2 occurred rapidly during the 5-min pulse-labeling period, resulting in the detection of gp150. The cleaved E2 (gp90) could not be observed until after 1 hr of chase period. Thus, the final glycosylation of E2 probably also occurred in the Golgi complex.

Removal of carbohydrates by endoglycosidase treatments

To determine whether the decrease in the size of gp65 during chase period is because of trimming of carbohydrate or cleavage of peptide backbone, we treated the intracellular viral glycoproteins with either endoglycosidase H or endoglycosidase F to remove carbohydrate side chains. As shown in Fig. 5, the endo H treatment reduced gp65 to a size of 45 kDa, indicating that most of the carbohydrate side chains have been removed. The sizes of endo H-treated gp65 were identical between samples chased for 10 and 60 min, even though the untreated gp65 at these two time

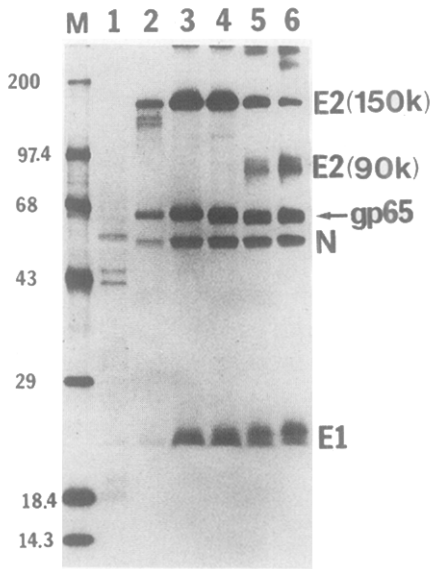

FIG. 4. Pulse-chase labeling of JHM(2)-infected DBT cells. At 9 hr p.i., JHM(2)-infected DBT cells were pulse-labeled in medium containing 150 μCi/ml of ^{35}S-translabel for 5 min and chased with cold methionine for various periods of time. Cell extracts were immunoprecipitated with anti-JHM(2) rabbit serum. M, ^{14}C-protein molecular weight markers; lane 1, mock infection; lane 2, 5-min labeling without chase; lane 3, 10-min chase after labeling; lane 4, 30-min chase; lane 5, 1-hr chase; lane 6, 2-hr chase.

points differed in size. This result suggests that the decrease in the size of gp65 during the chase period is because of trimming of carbohydrate side chains. It is also noted that two proteins of approximately 45 kDa were detected after endo H treatment. Prolonged treatment (16 hr) and higher endo H concentration (25 mU) did not eliminate either of these two proteins (data not shown). Thus, these two proteins were not the result of incomplete endo H treatment. Similar results were obtained after treatments with endoglycosidase F (data not shown). The significance of these two proteins will be discussed later.

It has previously been determined that gp65 of JHM contains 10 potential *N*-glycosylation sites (Shieh *et al.*, 1989). To determine the actual number of carbohydrate side chains in this protein, we treated gp65 with endoglycosidase H under very mild conditions to achieve partial and stepwise removal of carbohydrate side chains. As shown in Fig. 6, at least 9 or 10 distinct protein species could be detected, ranging from the complete gp65 to the final digestion products. These products most likely represent the intermediates of endo H-digestion products. This result suggests that most, if not all, of the potential *N*-glycosylation sites are glycosylated.

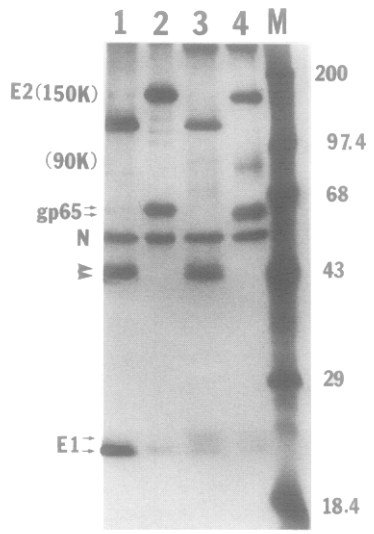

FIG. 5. Endoglycosidase H treatment of intracellular viral glycoproteins. JHM(2)-infected cells were pulse-labeled with ^{35}S-translabel for 5 min and chased for 10 min or 1 hr. Lysates were immunoprecipitated, dissolved in 50 m*M* Tris–HCl (pH 7.4) containing 0.5% SDS, and boiled for 4 min before endoglycosidase H treatment (3 mU, at 37° for 16 hr). Lane 1, 5-min pulse, 10-min chase, endoglycosidase H (3 mU); lane 2, 5-min pulse, 10-min chase, no endoglycosidase H treatment; lane 3, 5-min pulse, 1-hr chase, endoglycosidase H (3 mU); lane 4, 5-min pulse, 1-hr chase, no endoglycosidase H treatment; M, ^{14}C-protein molecular weight markers.

Topology of gp65 in the membrane

Since gp65 contains hydrophobic domains at both N- and C-termini (Shieh *et al.*, 1989), it is very likely an integral membrane protein. To determine the likely topology of gp65 in the membrane, we performed *in vitro* translation of the ORF 2b RNA transcribed from a pT7 plasmid construct. Translation was performed in the presence of canine pancreatic microsomal membranes, and translation products were digested with Pronase to remove the portion of proteins not translocated into the lumen of microsomal membranes. As shown in Fig. 7, *in vitro* translation of this RNA yielded a primary translation product of 45 kDa, which was glycosylated to a protein of 65 kDa in the presence of microsomal membranes. The latter protein was converted to a protein of 35 kDa after Pronase digestion. This result suggests that gp65 is indeed a membrane protein with roughly a 35-kDa portion protruding into the lumen of microsomal membrane. Translation of a truncated RNA representing the 5′-end 768 nucleotides of ORF 2b resulted in a primary translation product of 28 kDa which corresponds to the N-terminal portion of gp65. In the presence of microsomal membrane, this protein was converted to a protein of 33 kDa. Thus, the N-terminal portion alone of gp65 has the capacity to be translocated across the membrane.

Esterase and hemagglutinin activities of gp65

The gp65 (E3) of BCV has been shown to contain both hemagglutinin (King *et al.*, 1985; Parker *et al.*, 1989) and esterase activities (Vlasak *et al.*, 1988a). We therefore determined whether gp65 of MHV also contains similar activities. We purified JHM(2), JHM(3), and A59, which contain different amounts of gp65 in the virion, and tested the presence of esterase activities, using *p*-nitrophenylacetate as a substrate. Figure 8 shows that, although both JHM strains could hydrolyze *p*-nitrophenylacetate, JHM(2) has a considerably higher activity. Since the only difference among these three viruses in virion structural proteins is the amount of gp65 (Fig. 1) (Shieh *et al.*, 1989), this result is consistent with gp65 being responsible for the esterase activity. A59 has a very low but detectable esterase activity, consistent with the presence of a trace amount of gp65 in the virion.

We have also tested the possible presence of a hemagglutinin activity of these three strains of MHV, using red blood cells from several different animal species, including mouse, sheep, chicken, bovine, guinea pig, rat, and human. While BCV was demonstrated to hemagglutinate all of these red blood cells, none of the MHV strains tested agglutinated these cells (data not shown). Thus, MHV gp65 does not possess hemagglutinin activities detectable under these conditions.

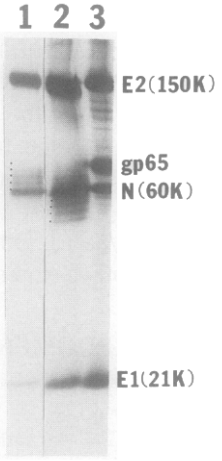

FIG. 6. Partial endoglycosidase H treatment of intracellular viral glycoproteins. Infected cell lysate labeled with ^{35}S-translabel for 5 min and chased for 10 min as shown in lane 1 of Fig. 5 was treated with endoglycosidase H (0.5 mU) for 4 (lane 1) and 5 min (lane 2), respectively. Lane 3 is the untreated sample.

DISCUSSION

gp65 (E3) is an optional structural protein among coronaviruses. It has been detected in BCV (King *et al.*, 1985), human coronavirus OC43 (Hogue and Brian, 1986), and turkey coronavirus (Dea and Tijssen, 1988) and is absent in avian infectious bronchitis virus (IBV) (Cavanagh, 1981). The best characterized gp65 is the one in BCV. It is a membrane glycoprotein, which forms short spikes on virion surface (King *et al.*, 1985). It contains hemagglutinin (King *et al.*, 1985; Parker *et al.*, 1989) and esterase activities (Vlasak *et al.*, 1988a). Some strains of MHV also contain gp65 (Lai and Stohlman, 1981; Siddell, 1982; Makino *et al.*, 1983). Even within a particular strain, e.g., JHM, the amount of gp65 appeared to be variable with different virus stocks. Because of this variability and because no known viral genes or mRNAs were capable of encoding this protein, the identity of this glycoprotein in MHV has long been in question. Recently, our laboratory has identified a genetic region in MHV genome capable of encoding gp65 (Shieh *et al.*, 1989). Furthermore, we have determined the molecular basis of differential transcription of this gene in different MHV strains (Makino and Lai, 1989; Shieh *et al.*, 1989). Thus, this glycoprotein is an optional protein among MHVs. However, our current study showed that even A59 strain, which does not have a complete gene 2b (Luytjes *et al.*, 1988), synthesizes a small amount of gp65 (Shieh *et al.*, 1989). Therefore, this protein may possibly be indispensable for MHV.

The data presented in this report provide information on the biosynthetic pathway of gp65 and also additional details on the biosynthesis of E1 and E2. It is interesting that the three glycoproteins of MHV have different biosynthetic pathways. E1 is glycosylated via an *O*-glycosidic bond (Holmes *et al.*, 1981; Niemann and Klenk, 1981) and is thus resistant to tunicamycin (Fig. 2). Our studies also suggest that its glycosylation occurs entirely within the Golgi complex. This conclusion is supported by the findings in this report that the glycosylation of E1 was inhibited by monensin, which interferes with protein transport in Golgi complex, and that glycosylation of E1 took nearly 2 hr to complete (Fig. 4), which is the length of time required for protein transport across Golgi. In contrast, E2 is glycosylated via an *N*-glycosidic bond, which occurs probably cotranslationally (Niemann *et al.*, 1982). Our study also showed that the final step of glycosylation of E2 occurs within Golgi complex, since monensin treatment reduced the size of E2 (gp90), which was not detected until after 1 hr of chase period (Fig. 4). The data in this study suggest that gp65 is also glycosylated cotranslationally via an *N*-glycosidic bond. However, the glycosylation of gp65 is completed before the protein reaches *cis*-Golgi. This conclusion is supported by the findings that monensin did not affect the size of gp65 and that a full-length gp65 was detectable after only 5 min of labeling (Fig. 4). Interestingly, the pulse–chase

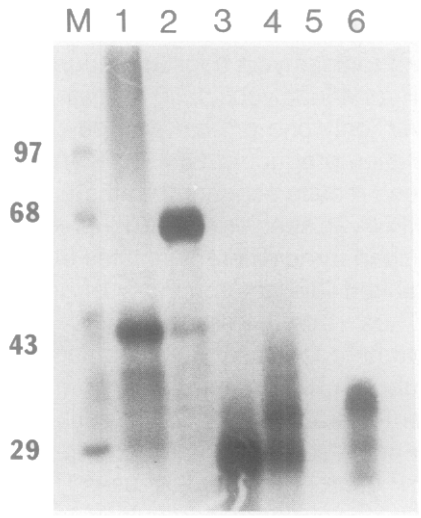

FIG. 7. *In vitro* translation of the ORF 2b RNAs. Plasmid pT7(2b) was linearized with either *Eco*RI, generating a full-length transcript by T7 RNA polymerase, or *Bam*HI, generating a truncated 0.78-kb RNA. These RNAs were translated in rabbit reticulocyte lysates in the absence or presence of canine microsomal membrane. Aliquotes of the samples were treated with Pronase before applying to gels. M, ^{14}C-protein molecular markers; lane 1, *Eco*RI-generated transcript, without membrane; lane 2, *Eco*RI-generated transcript, with membrane; lane 3, *Bam*HI-generated transcript, without membrane; lane 4, *Bam*HI-generated transcript with membrane; lane 5, *Eco*RI-generated transcript, without membrane, Pronase-treated; lane 6, *Eco*RI-generated transcript, with membrane, Pronase-treated.

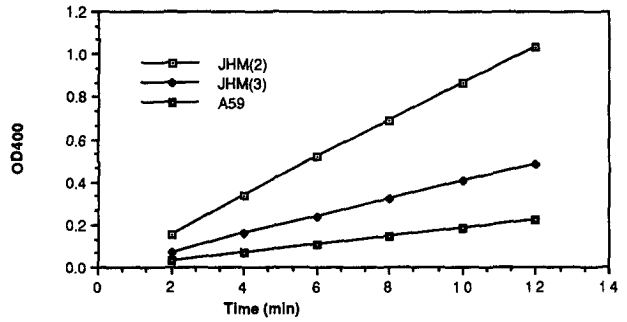

FIG. 8. Esterase activity assay of different viruses performed as hydrolysis of *p*-nitrophenylacetate. The hydrolysis of substrate was monitored at 400 nm at 2-min intervals. The background counts of the mock-infected control media were subtrated from those of virus samples. The experiment was duplicated and average was taken.

experiment showed that gp65 underwent a trimming of carbohydrate chains during its transport across Golgi and that the trimming was not inhibited by monensin. This carbohydrate trimming is not observed with either E1 or E2. Thus, the maturation processes of the three glycoproteins of MHV appear to be different from each other.

The detection of probably two unglycosylated precursor proteins of gp65 is of considerable interest. These two protein species were seen in the *in vitro* translation products of purified mRNA 2b (Shieh *et al.*, 1989), in the precursor proteins accumulated in the presence of tunicamycin (Fig. 2), and in the endoglycosidase H- or endoglycosidase F-treated protein (Fig. 5). However, only one protein was detected in the *in vitro* translation product of ORF 2b RNA (Shieh *et al.*, 1989). These results suggest that there might be two different mRNA 2b species in JHM(2)-infected cells. We have examined three different isolates of JHM(2), all of which exhibited this heterogeneity of gp65 (data not shown). Thus, this heterogeneity is probably not caused by heterogeneity of virus population, but rather is the result of mRNA heterogeneity, which could be derived from alternative initiation or possible RNA editing. The latter mechanism has been suggested from studies of P gene of paramyxoviruses (Thomas *et al.*, 1988). This possibility is particularly tempting since A59, which does not have a functional ORF 2b because of the lack of an initiator AUG (Luytjes *et al.*, 1988) and does not have a consensus transcriptional initiation sequence (Shieh *et al.*, 1989), also synthesizes a small amount of gp65 (Fig. 1). The origin of gp65 in A59 strain and of gp65 heterogeneity in JHM strain is currently under study in our laboratory.

Similar to BCV, the gp65 of JHM strain also contains an esterase activity. However, no hemagglutinin activity was detected, in contrast to gp65 of BCV. This functional dichotomy during the evolution of viruses is interesting. Probably because of the hemagglutinin activity of gp65, BCV has been found to share the same receptor as influenza C virus (Vlasak *et al.*, 1988b). The esterase activity of BCV is also similar to the receptor-destroying enzyme of influenza C virus (Vlasak *et al.*, 1988a). The presence of gp65 in MHV may alter the tissue tropism, antigenicity, and pathogenicity of virus. Such a possibility is currently being studied.

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