BIOSYNTHESIS AND FUNCTION OF THE CORONAVIRUS SPIKE PROTEIN

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INTRODUCTION

One of the most interesting aspects of coronavirus replication is their intracellular assembly. Budding is localized in the ER-pre Golgi region (8, 26). Both coronavirus glycoproteins are synthesized in the RER on membrane bound ribosomes (16). The integral membrane protein (M) accumulates in the perinuclear region and is believed to determine the site of budding. The spike protein (S) mediates binding of virions to the host cell receptor, possesses a fusogenic activity and is the major target for virus neutralizing antibodies (22). The primary nucleotide sequence and the predicted amino acid sequence of a number of spike protein genes revealed features characteristic of type I membrane proteins (22).

The S protein of all coronaviruses studied so far is cotranslationally glycosylated. The transport and maturation of S has been studied in coronavirus infected cells (22, 24). Due to the fact that virus budding takes place intracellularly, S protein can be transported in association with cellular or viral membranes. These two transport processes cannot be studied separately. In the present study we have used vaccinia virus recombinants expressing the S gene of three different coronaviruses to study the intracellular transport and maturation of the S protein in the absence of virus budding.

During its synthesis and transport in coronavirus infected cells the S protein is folded and assembled into the structure which finally constitutes the viral spike. From experimental data (1) combined with theoretical analyses (4) a model was deduced picturing the spike as a dimer of the S protein. In order to study this aspect of the maturation of S we have begun to analyze its oligomerization process.

MATERIALS AND METHODS

Cloning of full-length spike protein genes; construction of recombinant vaccinia viruses. The S genes of FIPV strain 79-1146, MHV-A59 and IBV M41 have been cloned and sequenced in our laboratory. The coding sequence of the FIPV S gene was recloned from cDNA clone B1 (5) as described (3). The coding region of the S gene of MHV was reconstructed from two overlapping cDNA clones, B24 and 1F11, (13). The S gene coding

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sequence of IBV was isolated from cDNA clone 39 (18). The VSV G coding region was isolated from plasmid pSVGL11, a derivative of pSVGL (19). Isolated gene fragments were recloned in transfer vectors pGS20 (15), or pSC11 (2). Recombinant vaccinia viruses were constructed using established procedures described previously (2, 15).

Cell culture and protein labelling. FIPV infection was carried out on NLFK cells at a multiplicity of infection (m.o.i.) of 10 $TCID_{50}$ per cell. Subconfluent monolayers of NLFK or HeLa cells were infected with different vaccinia virus recombinants at a m.o.i. of 10 PFU per cell. Protein labelling was carried out at the times indicated in the legends in methionine free medium supplemented with ^{35}S-methionine at a concentration of 0.1mCi/ml. In some cases a pulse labelling period was followed by a chase with medium containing 4 mM methionine and 10% fetal calf serum. Cells were lysed in 20mM Tris-HCl pH7.5, 1mM EDTA, 100mM NaCl (TESV), containing 1% Triton X-100 and 2mM phenyl-methyl-sulphonyl fluoride (PMSF).

Radio immunoprecipitation and endo H analysis. Immunoprecipitations were carried out overnight at 4°C in TESV, 0.4% Triton X-100, at a 600-fold antiserum dilution for lysates of recombinant vaccinia virus infected cells and at a 100-fold dilution for lysates of FIPV and mock infected cells. After the addition of KCl to 0.5M, immunecomplexes were bound to Staph A (Pansorbin Cells, Calbiochem) for 2.5h at 4°C and pelleted by centrifugation. Pellets were washed three times in TESV, 0.1% Triton X-100. The final pellet was suspended in 50mM Tris-HCl pH 6.8, 0.25% SDS and heated for 2 min at 95°C to dissolve immune complexes. Immunoprecipitated proteins were treated with endo H (Boerhinger Mannheim) by adding 15µl 150mM Na-Citrate pH 5.3 and 1mU endo H to 10µl supernatant of dissolved immunecomplexes and overnight incubation at 37°C or mock treated by incubation without enzyme and analyzed in 10% SDS polyacrylamide gels.

Oligomerization assay. ^{35}S-labelled MHV-A59 was prepared from the medium of infected Sac$^-$ cells (25cm^2) incubated from 6-9h p.i. in cysteine-free MEM containing 2% foetal calf serum (FCS) to which was added 150µCi ^{35}S-cysteine at 6h and at 7.5h p.i. Virus was collected by precipitation with 8% PEG in the presence of 2.3% NaCl and dissolved in MNT (20mM MES, 30mM Tris-HCl, 100mM NaCl, 1.25mM EDTA, 1mM EGTA, pH 5.8) containing 1% Triton X-100. The cells were lysed in the same buffer, cleared by centrifugation and the supernatant was used for gradient analysis. For the pulse-chase experiment MHV-A59 infected Sac$^-$ cells (75cm^2) were trypsinized and labelled in suspension in 200µl of the above labelling medium to which now was added 400µCi ^{35}S-cysteine. After the 3 min pulse one 30µl sample of the cells was lysed immediately in 220µl ice-cold MNT containing 1% Triton X-100. Five similar samples were suspended in preincubated chase medium (DMEM containing 5% FCS and 2mM cysteine), incubated for various times, harvested and lysed in the same buffer.

For sedimentation analysis samples were loaded onto 5-20% (w/w) continuous sucrose gradients in MNT buffer containing 0.1% Triton X-100 and centrifuged in a SW50.1 rotor (Beckman Instruments) for 10h at 45,000rpm and 4°C. Fractions were collected from the bottom. Viral proteins were precipitated using 1µl rabbit polyclonal antiserum against MHV-A59 and 15µl Staph A and prepared for SDS-PAGE. To analyze possible complexes pelleted through the gradient, material at the bottom of the tube was dissolved and immuneprecipitated as well. All samples were heated for 5 min at 95°C prior to PAGE. As sedimentation markers the following proteins were used: thyroglobulin, β-glucuronidase, catalase, lactate dehydrogenase and BSA, which have molecular weights of 669, 280, 232, 140, and 67kD. Their positions in the gradients were determined by Coomassie blue staining of gels run with material from each fraction.

RESULTS

Expression of recombinant S proteins. Recombinant vaccinia viruses encoding S proteins of FIPV, MHV and IBV under control of the vaccinia 7.5kD promoter were constructed as described in materials and methods and designated vFS, vMS and vIS, respectively. Recombinant S proteins were labelled with ^{35}S-methionine and compared to their respective coronavirus counterparts after immunoprecipitation and SDS-PAGE analysis. Figure 1 shows the results for the FIPV spike protein. Endoglycosidase H (Endo H) was used to deglycosylate glycoproteins. Both the untreated as well as the deglycosylated recombinant and FIPV spike proteins comigrated in SDS-PAGE (FIG. 1). Similar results were obtained for IBV and MHV spike proteins (data not shown).

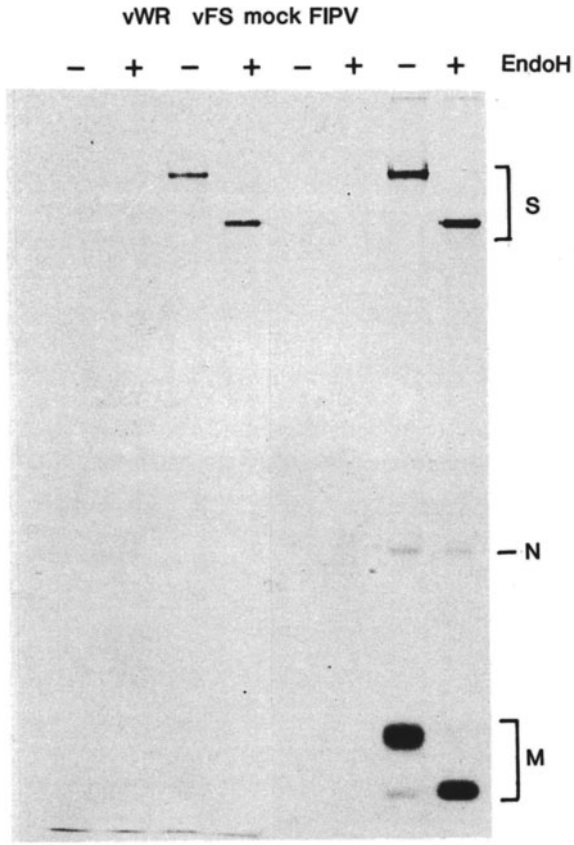

FIG. 1. Radio immunoprecipitation (RIP) and SDS-PAGE analysis of recombinant FIPV S protein. Recombinant (vFS) and wild type (vWR) vaccinia virus infected cells were labelled for 30 min at 16h p.i.. FIPV and mock infected cells were labelled at 8h p.i.. RIP was carried out. FIPV structural proteins (S, N and M) are indicated. Immunoprecipitates were split in two samples; one half was mock treated and the other was treated with endo H (indicated with - and +, respectively).

Biological activity of recombinant S proteins. Cell surface expression was demonstrated by immunofluorescence staining of unfixed cells (manuscript submitted). S protein expressed on the surface of infected cells is thought to induce cell-cell fusion (24). This phenomenon was evident from the appearance of large syncytia in vFS infected NLFK cells and vMS infected Sac$^-$ cells (FIG. 2). No syncytia formation was observed in NLFK or Sac$^-$ cells infected with wild type vaccinia virus or with vIS. Induction of cell fusion by recombinant vFS was restricted to cells of feline origin, whereas vMS induced fusion was observed only in murine cells. These results suggest a relation between susceptibility of cells for a certain coronavirus and the ability of the respective S protein to induce fusion.

Intracellular transport of recombinant S proteins. During the transfer from the RER to the trans-Golgi compartment, the oligosaccharide side chains of N-glycosylated glycoproteins are processed and modified. The co-translationally added high mannose side chains which are endo H sensitive become endo H resistant in the medial-Golgi compartment (9). Therefore, the rate of acquisition of endo H resistance can be used to study intracellular transport kinetics. To analyze the rate of acquisition of resistance to digestion by endo H we performed pulse chase experiments, followed by RIP and endo H digestions, with vFS, vMS and vIS infected NLFK cells. In addition a recombinant vaccinia virus, designated vVG, expressing the VSV G

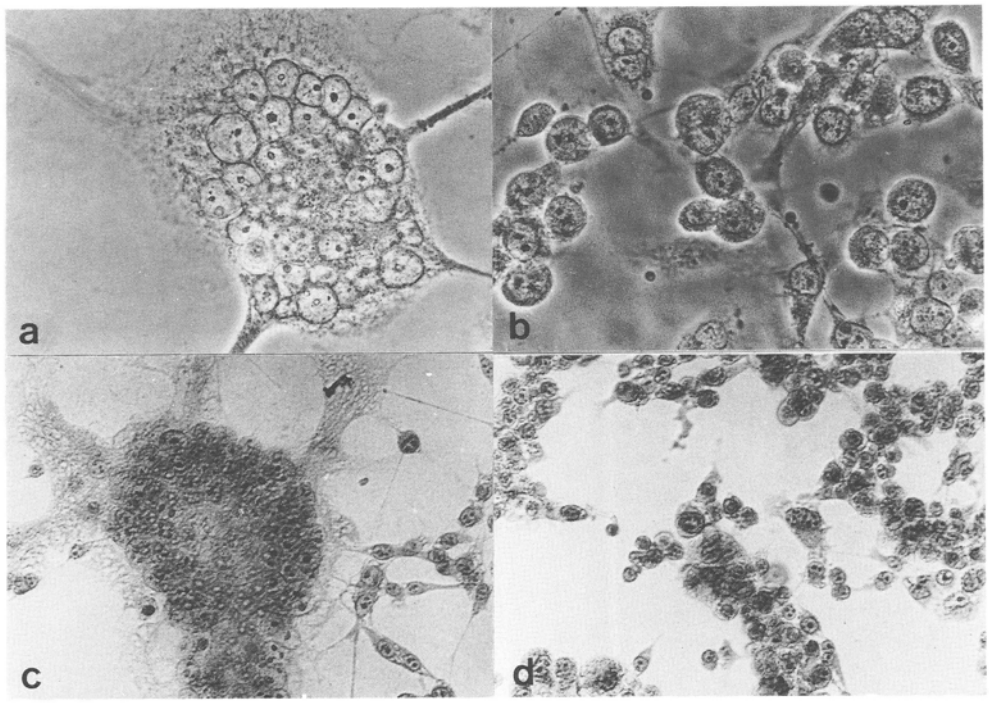

FIG. 2.Biological activity of recombinant S proteins. NLFK cells were infected with vFS (a) or vWR (b). Infected NLFK cells were photographed using dark field microscopy, x400. Sac$^-$ cells were infected with vMS (c) or vWR (d). Infected Sac$^-$ cells were stained for nuclei using hematoxylin and photographed under light microscopy, x100.

protein was included in this study. Figure 3 shows the results of a 30 min pulse labelling and a 60 min chase period. It can be seen that none of the three coronavirus S proteins acquired any detectable resistance to endo H during the 1h chase period. In the lanes of the vMS lysates two background bands can be seen besides the S protein band. The upper band appears to be a glycoprotein. At the end of the pulse labelling period this protein was already partially resistant to endo H and completely so after the 1h chase period.

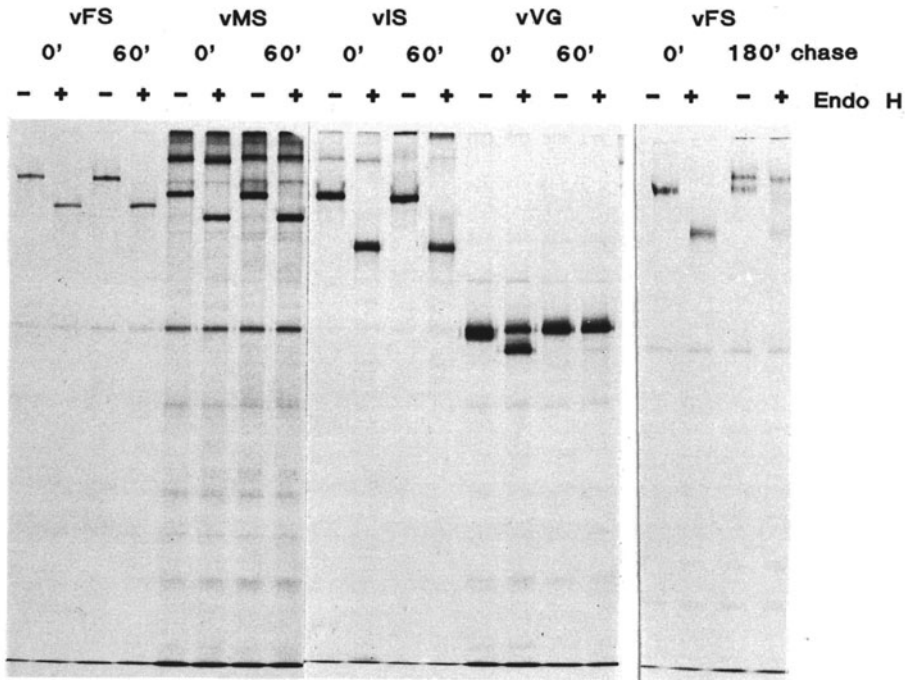

FIG. 3. Endo H analysis of three different recombinant coronavirus S proteins and of recombinant VSV G protein. Infected NLFK cells were pulse-labelled at 12h p.i for 30 min and lysed immediately or after a chase of 1h or 3h (vFS). Recombinant vaccinia viruses are indicated above each panel of 4 lanes.

In contrast to the S proteins, the VSV G-protein has become partially resistant to digestion by endo H during the 30 min pulse labelling period. After the 60 min chase period all of the VSV G-protein was resistant to endo H. During longer chase periods a higher molecular weight form of the recombinant FIPV S protein appeared which was resistant to digestion by endo H, albeit not completely. After a 3h chase period about half of the recombinant S protein was still sensitive to digestion by endo H (FIG. 3). The half time of acquisition of endo H resistance of the recombinant S proteins of MHV and IBV was also estimated to be approximately 3h (data not shown).

Biosynthesis of the S protein in FIPV infected cells. To study the biosynthesis of S in FIPV infected cells we analyzed the structural proteins in cell lysates and of virions released into the extracellular medium. Endo H analysis of intracellular and virion protein showed that the virion S protein was endo H resistant. The amount of intracellular S protein decreased during chases due to release into the medium. Intracellular S protein was endo H sensitive even after prolonged incubation (manuscript submitted).

The rate of acquisition of resistance to endo H digestion was determined using combined lysates, which were prepared by adding concentrated lysis buffer and protease inhibitor to a culture dish without removing the medium. FIPV infected NLFK cells were pulse labelled for 20 min at 9h p.i. and chased for different periods. The results of this experiment are shown in Figure 4. Approximately half of the labelled spike protein has become resistant to digestion by endo H after a chase period of 1h. After 2h the amount of endo H sensitive material has further decreased. A small amount of endo H sensitive material remains after a 4h chase period.

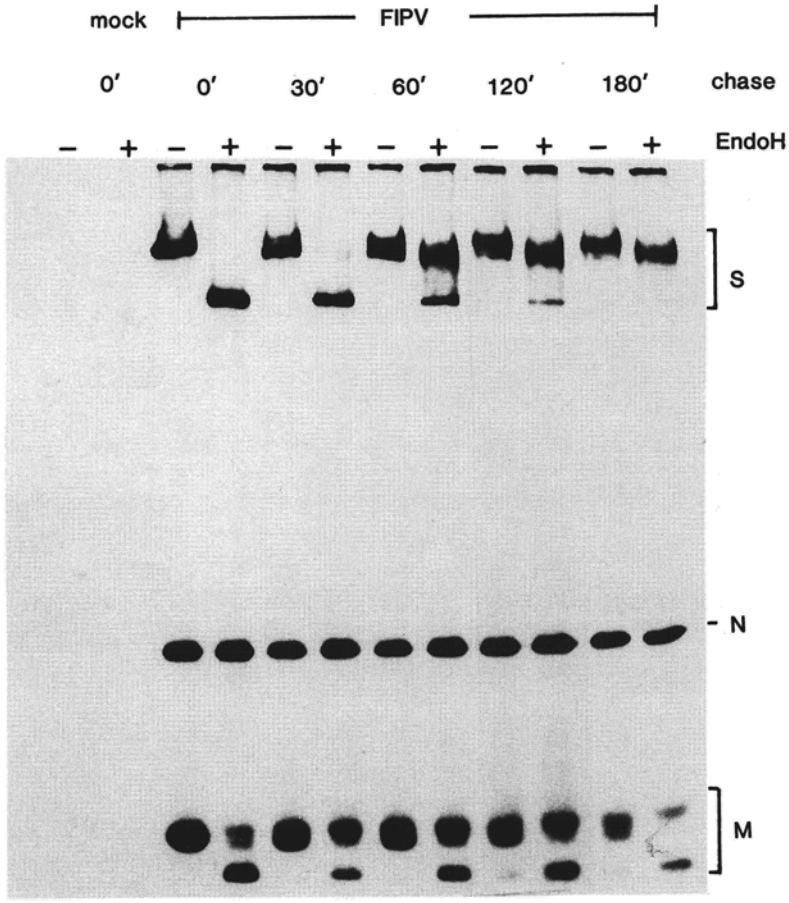

FIG. 4. Kinetics of endo H resistance acquisition of the S protein in FIPV infected NLFK cells. Metabolic labelling was carried out at 9h p.i. for 30 min. Lysates were prepared immediately or after the indicated chase times. Intracellular and virion proteins were analyzed together in combined lysates. More than half of the labelled S protein is endo H resistant after 1h.

Oligomeric structure and kinetics of oligomerization of the S protein in MHV infected cells. The analysis of the oligomeric state of the S protein in cells as well as in virions was performed in sucrose density gradients. Under the conditions described, the S protein of purified MHV-A59, dissolved in 1% Triton X-100 sedimented deep into the gradient peaking in fractions 2 and 3 (Fig. 5). It occurred there both in its 180kD uncleaved form and as the 90kD cleavage products. The same species were also found at the same position in gradients loaded with a lysate of MHV-A59 infected cells after a 3h labelling period. In addition, the S protein precursor gp150 was found in this gradient peaking at two positions namely around fractions 3 and 8 (Fig. 5). In order to determine the complexity of these S species their sedimentation rates were compared with those of a number of marker proteins. These analyses were perfomed both in the SW50.1 rotor and using the SW40 (data not shown). From this it followed that gp150 occurs as a momomer and as a dimer (peaks around fraction 8 and 3, respectively) and that the gp180 and gp90 species are present as the homodimer $(gp180)_2$ and the cleaved form thereof, the heterotetramer $(90A/90B)_2$.

The order and time-course of appearance of the various S structures in MHV-A59 infected cells were determined by gradient analysis of samples from a pulse-chase experiment. As illustrated in Fig. 6, S is synthesized as its precursor gp150 in the form of a monomer that dimerizes slowly. Then $(gp150)_2$ is converted to the $(gp180)_2$ form which subsequently undergoes cleavage giving rise to the $(90A/90B)_2$ structure.

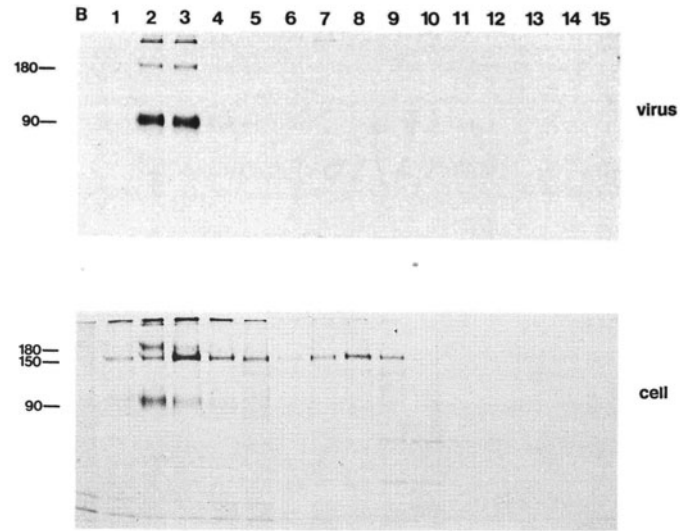

FIG. 5. Sedimentation analysis of MHV-A59 S protein from purified virus and from infected cells. Viral and cellular material was prepared by a 3h labelling with ^{35}S-cysteine and analyzed after sucrose gradient centrifugation. Numbers on top refer to the fractions obtained, 1 and 15 representing bottom and top of the gradient, respectively. In lane B the material was analyzed that pelleted through the gradient to the bottom of the tube. Arrows indicate the positions of the markers β-glucuronidase (280kD) and catalase (232kD) run in a parallel gradient.

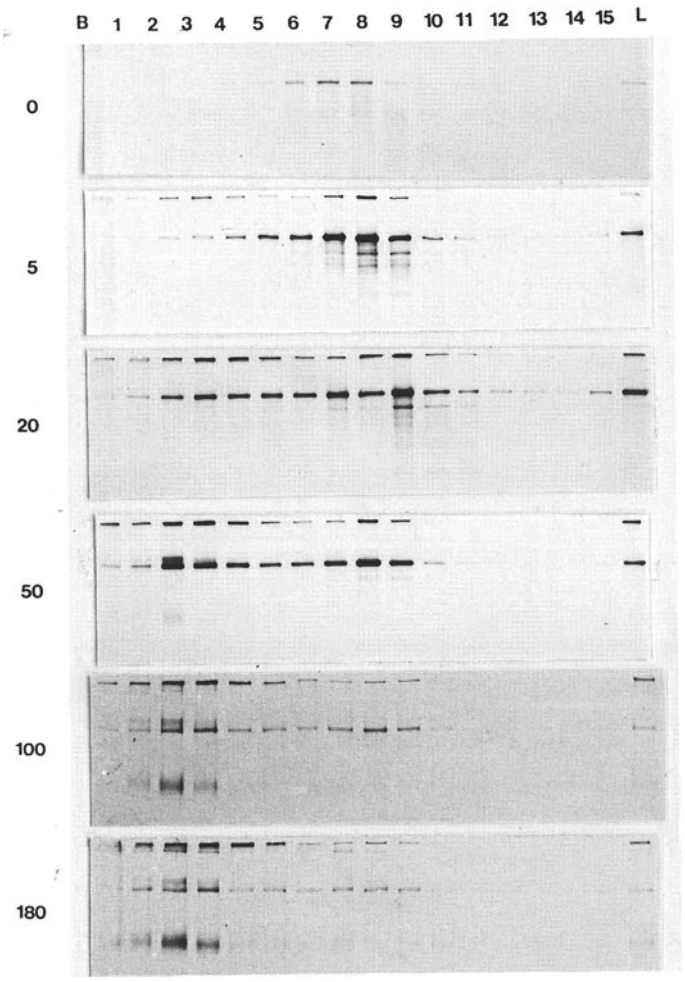

FIG. 6. Oligomerization and maturation of the S protein. The fate of S synthesized as its precursor gp150 during a 3 min pulse labelling was followed by analysis of cells chased for 5, 20, 50, 100. and 180 min. In the lanes marked L an unfractionated sample of each lysate was analyzed after immuneprecipitation. The precursor is seen to dimerize, to be subsequently converted to $(gp180)_2$ and to finally be cleaved.

DISCUSSION

In this paper we showed that several coronavirus S proteins expressed from cloned cDNA are transported slowly to the medial-Golgi where endo H resistance is acquired. Comparison of the transport rates of the recombinant S protein to the S protein in FIPV infected cells showed that the retention of the S protein only occurs in the absence of budding. This specific retention can be interpreted as transient accumulation between RER and medial-Golgi, possibly to allow efficient incorporation in budding

virions. A second possible interpretation of this retention may be that the S protein localizes the budding event itself. The fact that the M protein of MHV accumulates in the perinuclear region in infected cells has led to the assumption that the M protein determines the site of budding (12). This view was supported by the observation that the M protein accumulates in the Golgi apparatus in cDNA expressing cells (14, 20). However, the rate of O-glycosylation and terminal sialylation of the MHV-A59 M protein in transfected COS cells (20) was the same as in MHV-A59 infected Sac^- cells. This means that the M protein is not specifically retained in the region where budding takes place, since sialylation occurs in trans-Golgi.

It has been shown that in the presence of tunicamycine spikeless non-infectious virus particles are assembled (12, 17, 21, 23). On the basis of these data it was assumed that the S protein is not necessary for virus budding. As was already pointed out, the unglycosylated S protein is probably degraded and this may occur after incorporation in virions (21, 23).

Control experiments with VSV G-protein expressed in the same way as the coronavirus S proteins showed that the observed phenomenon of slow intracellular transport is coronavirus specific and not due to the expression system we used.

In contrast to the M protein S is transported to the plasma membrane. This intrinsic property of the S protein may be responsible for transport of virus particles. Cell surface expression of recombinant S proteins was demonstrated among others by the induction of cell-to-cell fusion. The cell fusion data suggest a relationship between cell susceptibility for a coronavirus and the ability of the respective S protein to induce cell-to-cell fusion. Recombinant vaccinia viruses now offer the possibility to test factors influencing cell fusion, independent of coronavirus replication.

Our studies of the complexity of S in viral particles confirmed the models put forward earlier (1, 4). They demonstrated that the peplomers are indeed composed of 2 S molecules which in cases like MHV-A59 and IBV are cleaved. The biogenesis of the spikes involves the dimerization of the primary translation product of the S-mRNA. This is a slow process when compared to the trimerization of G and HA from VSV (6) and influenza virus (10), respectively, but occurs faster than trimerization of the Rous sarcoma virus env glycoprotein (11). Exactly where dimerization of gp150 occurs is still unclear. It is likely, however, to take place before its passage through the trans-Golgi compartment since it preceeds the appearance of gp180, the charactistic product resulting from the processing of the oligosaccharides in the Golgi apparatus. Assuming that the S protein is incorporated into the virion at the time of budding, the gp150-dimer constitutes the initial spike structure. Interestingly, no monomer forms of gp180 were detected in the gradients. This indicates that dimerization is a very efficient process or that it is a prerequisite for transport through the Golgi system. The latter has been shown for some viral glycoproteins destined for the plasma membrane (6, 7, 10). It may also indicate that dimerization is a precondition as well for incorporation into viral particles.

Cleavage of viral membrane glycoproteins is known to occur with many viruses. It generally constitutes one of the last steps in the maturation of the proteins and is required to activate viral infectivity. Though cleavage is not essential for all coronaviruses, the same principles seem to hold true for MHV-A59 (26). Our gradient analyses show that the first 90kD cleavage products are detectable as soon as gp180-dimers have appeared. This implies that cleavage of S occurs either while still in the trans-Golgi compartment or shortly after its exit to the cell surface.

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