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Protein Interactions during Coronavirus Assembly

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Coronaviruses assemble and obtain their envelope at membranes of the intermediate compartment between the endoplasmic reticulum and Golgi complex. Like other enveloped viruses, coronavirus assembly is presumably dependent on protein localization and protein-protein as well as protein-RNA interactions. We have used the bovine coronavirus (BCV) as a model to study interactions between the viral proteins in virus-infected cells that are important for coronavirus assembly. BCV is a prototype for the coronaviruses that express an additional major structural protein, the hemagglutinin esterase (HE), in addition to the spike(S) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein. Complexes consisting of the M, S, and HE proteins were detected in virus-infected cells by coimmunoprecipitations. Kinetic analyses demonstrated that S protein and HE each quickly formed a complex with M protein after synthesis, whereas heterocomplexes consisting of all three proteins formed more slowly. The kinetics of HE biosynthesis revealed that the half-life of oligomerization was ~30 min, which correlated with the appearance of complexes consisting of M, HE, and S proteins, suggesting that oligomerization and/or conformational changes may be important for the S-M-HE protein complexes to form. Only HE dimers were found associated with the heterocomplexes consisting of all three proteins. S-M-HE protein complexes were detected prior to processing of the oligosaccharide chains on HE, indicating that these protein complexes formed in a premedial Golgi compartment before trimming of sugar chains. Transient coexpressions and double-labeling immunofluorescence demonstrated that HE and S proteins colocalized with M protein. This was further supported by coimmunoprecipitation of specific HE-M and S-M protein complexes from transfected cells, indicating that these proteins can form complexes in the absence of other viral proteins.

Coronaviruses constitute a large family of enveloped, positive-stranded RNA viruses that replicate in the cytoplasm. Mature virions consist of two or three major structural glycoproteins anchored in an envelope that surrounds a helical nucleocapsid. One recent study raised questions about the actual structure of the nucleocapsid, suggesting that transmissible gastroenteritis coronavirus contains a spherical core consisting of membrane (M) glycoprotein and nucleocapsid (N) protein (29); however, further detailed studies will be required to determine if the description of the coronavirus helical nucleocapsid (25) requires modification. As we currently understand it, the nucleocapsid consists of at least the approximately 31-kb genome and N protein. The spike (S) and M proteins are major glycoproteins present in all coronaviruses. A third major structural component, hemagglutinin esterase (HE), is present in some, but not all, coronaviruses. In addition to the major structural proteins, a few molecules of the small membrane (E) protein are also present in the virion (9, 24, 36).

Coronaviruses assemble and bud at membranes of the intermediate compartment (IC), located between the endoplasmic reticulum (ER) and Golgi complex (19, 20, 33, 34). Like other enveloped viruses, the nucleocapsid is presumed to interact with the glycoproteins at the membranes where virions bud (7, 10, 28, 32). Protein localization and some specificity must exist at the level of protein-protein interactions, as well as protein-nucleic acid interactions, to ensure proper assembly of virions. Although the M glycoprotein is localized beyond the IC when expressed independently, M protein is the only viral gene product that is retained in its transport at intracellular membranes when expressed in either virus-infected cells or alone in transfected cells (19, 20, 21). Complexes consisting of the M and S proteins are found in mouse hepatitis virus (MHV)-infected cells (26). Recent coexpression studies have shown that expression of M and E proteins alone is sufficient for virus-like particles to form (1, 17, 35).

To further our understanding of the assembly of coronaviruses, we sought to identify protein-protein interactions by using the bovine coronavirus (BCV) as the prototype for the coronavirus strains that express HE. BCV consistently expresses HE as a major structural component of the virion, in addition to the S, M, and N proteins (18). HE is thought to play a role in the pathogenesis of those coronaviruses that express the protein (for review, see reference 2). In this investigation, we focused on HE since its association with other virion components and the mechanism of its incorporation into virions were not previously demonstrated. HE, like S protein, is a typical type I glycoprotein with a single membrane-spanning anchor and a short cytoplasmic tail (16). The protein exists in the mature virion as a disulfide-linked homodimer that is glycosylated with N-linked sugars (6, 12). Extracellular virions contain HE that is partially resistant to endoglycosidase H (endo H) (12). The S protein, bearing N-linked oligosaccharides, is thought to be a trimer, even though this has not been definitively shown for BCV (for review, see reference 3). The M protein spans the membrane three times and contains O-linked oligosaccharides (for review, see reference 31).

In the present study, we have identified protein complexes that are present in virus-infected cells and have begun studying the interactions between the viral glycoproteins that are required for assembly of virions. The data indicate that the M

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protein plays a key role in assembly of coronaviruses by its ability to form a complex with the S and HE glycoproteins.

MATERIALS AND METHODS

Viruses and cells lines. HCT-8, a human adenocarcinoma ileocecal cell line was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Baby hamster kidney (BHK-21) cells were grown in 10% FCS-supplemented Glasgow minimal essential medium. Both cell lines were obtained from the American Type Culture Collection. Plaque-purified bovine enteric coronavirus (BCV) Mebus strain was grown and the titer on HCT-8 cells was determined as previously described (11). The vTF7-3 recombinant vaccinia virus expressing T7 RNA polymerase (8) was grown on HeLa cells, and the titer on CV-1 cells was determined.

Virus infection. Subconfluent monolayers of HCT cells were infected with BCV in serum-free DMEM at a multiplicity of infection of 5 to 10. After adsorption for 1 h at 37°C, virus was removed, and cells were cultured in DMEM that contained 5% FCS. Transient expressions were carried out essentially as previously described with some modifications (14). BHK-21 cells were infected with vTF7-3 at a multiplicity of infection of 10 for 1 h. Subconfluent cells were then transfected by Lipofectin (Gibco BRL) with a total of 3 μg of plasmid DNA according to the manufacturer's recommendations.

Expression vectors. Plasmids containing the HE gene (pHET7) and the S gene (pTZ18R), both under the control of the T7 promoter, were previously described (16, 27). The BCV M gene was subcloned by PCR from a previously described clone, MA7 (22). PCR primers homologous to sequences flanking the gene also included restriction sites for subcloning into pGem-3Zf(+) (Promega). The sequence was confirmed by sequencing after PCR. The influenza virus HA gene (pGem3HA) was obtained from Debi Nayak (University of California at Los Angeles).

Radiolabelling, immunoprecipitation, and gel electrophoresis. At 15 h postinfection with BCV or 10 h posttransfection, cells were starved for 30 min in methionine-deficient medium. The medium was replaced for 15 min with 200 μCi of [^{35}S]methionine-cysteine mix per ml (EXPRE^{35}S^{35}S protein labeling mix; Dupont NEN). The cells were then washed and chased with medium containing an excess of methionine and cysteine. The [^{35}S]methionine-cysteine mix was supplemented with additional [^{35}S]cysteine (translation grade; Dupont NEN) in some experiments during this study to increase the chances of detecting the E protein. At the indicated time points, BCV-infected cells were washed with cold phosphate-buffered saline and lysed on ice in NP40-DOC buffer (50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA, 0.5% Nonidet P-40 [NP40], 0.5% sodium deoxycholate [DOC]). Transfected BHK cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% sodium dodecyl sulfate [SDS]). RIPA buffer was used to decrease the nonspecific background due to vaccinia virus proteins. All buffers included 1 mM phenylmethylsulfonyl fluoride. Iodoacetamide was included at a concentration of 25 mM in experiments in which HE dimerization was analyzed. Nuclei and cell debris were removed by centrifugation at 4°C for 10 min at 13,000 × *g*. Lysates were precleared with protein A–Sepharose CL-4B (Pharmacia BioTech) prior to being immunoprecipitated with specific anti-BCV protein antibodies. Protein A-Sepharose-bound immune complexes were washed four times in RIPA buffer and once in RIPA buffer containing no detergent and eluted in Laemmli sample buffer. Proteins were resolved on 5 to 20% gradient gels by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography with Amplify (Amersham).

Antibodies. Monoclonal antibodies against the BCV S, HE, and M proteins were kindly provided by L. Babiuk (VIDO). Monoclonal antibodies HB10-4, JB5-6, and HF8-8 against the S protein; 56-40, HC10-5, and BD9-8C against HE; and E1-1 and CC7-3 against the M protein have all been described previously (5, 6). The monoclonal antibodies to each specific protein were used as a pool. Rabbit polyclonal antibodies made against the M and HE proteins from purified BCV virions have been previously described (11). A rabbit polyclonal antibody that recognizes the BCV N protein was made against histidine-tagged N protein (3a). Rabbit antibodies against the WSN strain of influenza virus and a monoclonal antibody against influenza virus HA were obtained from Debi Nayak (University of California at Los Angeles).

endo H digestion. BCV-infected HCT cells were pulse-chased, and immunoprecipitated with anti-HE or anti-M protein antibodies as described above. endo H (Boehringer Mannheim) digestions were carried out as previously described with modifications (13). Protein complexes were eluted from protein A-Sepharose in 50 mM sodium citrate (pH 5.5) containing 0.02% SDS. Samples were digested with 10 mU of endo H for 16 to 18 h at 37°C. Mock control digestions were carried out in parallel. Reactions were stopped by adding 4× Laemmli sample buffer. Samples were boiled for 3 min prior to SDS-PAGE.

Indirect immunofluorescence. BHK-21 cells were grown on Lab-Tek slides (Nunc), infected and transfected as described above. Indirect immunofluorescence was performed between 9 and 10 h posttransfection essentially as described previously (14). To detect the M and HE proteins, fixed cells were incubated with rabbit anti-HE (11) and/or a mix of anti-M protein monoclonal antibodies, E1.1 and CC7.3 (6). Rhodamine-conjugated antirabbit (Boehringer Mannheim) and fluorescein isothiocyanate-conjugated antimouse (Fisher) secondary antibodies were used. Following extensive washings, coverslips were mounted with Fluoromont G (Fisher) and examined with an Olympus Vanox epifluorescence microscope. The ×40 objective was used for photography.

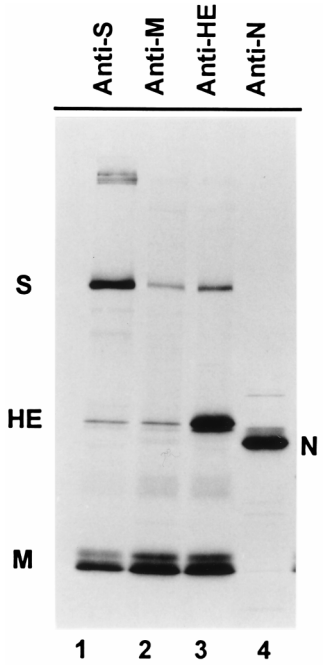

FIG. 1. Protein complexes in BCV-infected cells. HCT cells infected with BCV were labeled for 15 min with [^{35}S]methionine-cysteine and chased for 60 min. Cells were lysed and immunoprecipitated with specific anti-BCV protein antibodies. Proteins were resolved on a 5 to 20% gradient gel by SDS-PAGE followed by fluorography.

RESULTS

Detection of viral protein complexes in BCV-infected cells. As a first step toward understanding the protein interactions required for virus assembly, we sought to determine if protein complexes could be detected in BCV-infected cells. A human adenocarcinoma ileocecal cell line, HCT-8, was infected with the BCV Mebus strain, metabolically labeled, and immunoprecipitated with antibodies specific for each of the major BCV structural proteins. Specific complexes composed of S, HE, and M proteins were detected only in BCV-infected cell lysates (Fig. 1, lanes 1 to 3 [see negative controls in Fig. 2 and 3]). Each antibody coimmunoprecipitated the other two glycoproteins in addition to the protein against which the antibody was directed. Essentially no N protein was coimmunoprecipitated with the antibodies to the S or HE proteins (Fig. 1, lanes 1 and 3), whereas a very small amount of N protein was detected with the antibodies to M protein (Fig. 1, lane 2). No S protein or HE was coimmunoprecipitated with the antibody to N protein (Fig. 1, lane 4), indicating that stable complexes consisting of only the glycoproteins are present in infected cells. The same complexes were also detected in purified virions (data not shown). In some experiments, supplemental [^{35}S]cysteine was included in the labeling mix in an attempt to determine if the E protein could be detected in these complexes; however, the protein was never convincingly detected by coimmunoprecipitation (data not shown). Detection of E protein by direct immunoprecipitation is difficult, since good antigenic reagents against E protein are not currently available.

Kinetics of association between the viral proteins. Pulse-chase experiments were performed with BCV-infected cells to assess the rate of complex formation. Both uninfected and

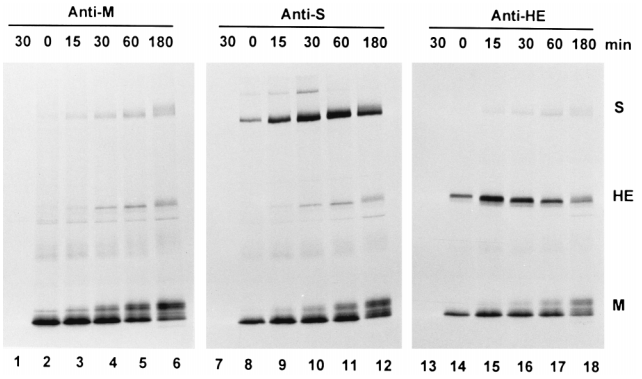

FIG. 2. Kinetics of association between BCV glycoproteins. BCV-infected HCT cells were pulse-labeled for 15 min with [^{35}S]methionine-cysteine and chased in medium containing an excess of methionine and cysteine. At the indicated time points, cells were lysed and immunoprecipitated with anti-M, anti-S, and anti-HE antibodies. Mock-infected cell lysates were used as controls for each immunoprecipitation (lanes 1, 7, and 13).

infected cells were metabolically labeled for 15 min, chased, lysed, and immunoprecipitated with antibodies against HE, M, or S protein. Complexes consisting of the HE and M proteins, as well as those consisting of the S and M proteins, were detected immediately after the pulse (Fig. 2, lanes 2, 8, and 14). The data indicated that the complexes formed immediately or shortly after translation. Only small amounts of S and HE proteins were coimmunoprecipitated with the anti-M antibodies immediately after the pulse (Fig. 2, lane 2). The amounts of S and HE proteins increased during the chase (Fig. 2, lanes 3 to 6). This is similar to what was reported for M-S protein complexes detected in MHV-infected cells (26). Complexes consisting of the HE, M, and S proteins were detected during the chase between 15 and 30 min (Fig. 2, lanes 10 and 16).

The M protein detected in complexes immediately after the pulse appeared to be the unglycosylated form which acquired sugar side chains during the chase (Fig. 2, compare lanes 8 and 14 with lanes 12 and 18). The MHV M protein has been shown to be unglycosylated in the ER, and becomes fully O glycosylated in the Golgi complex (20, 34). Therefore, the results indicated that M protein associated with the HE and S proteins in a pre-Golgi compartment, most likely the ER. If BCV M protein becomes initially glycosylated in the IC, as previously reported for the MHV M protein (20, 34), the data suggest that both the S and HE proteins complex with the M protein at a point prior to IC localization.

Requirement of HE dimerization for its association with M and S glycoproteins. HE has been shown to rapidly form disulfide-linked dimers after synthesis (6, 12). To determine whether monomeric and dimeric HEs were equally competent to enter into complexes, infected cells were pulse-labeled for 15 min and chased for the times indicated. Cell lysates were immunoprecipitated with antibodies against HE or S protein. Immunoprecipitated complexes were analyzed under both nonreducing and reducing conditions. Both forms of HE were readily detected with antibodies against HE under nonreducing conditions (Fig. 3, upper panel, lanes 1 to 5). The kinetics of HE dimerization correlated well with previously reported data (6, 12). Essentially all of HE had dimerized by 60 min (Fig. 3, upper panel, lane 4). Antibodies against the S protein coimmunoprecipitated the M protein after the 15-min pulse; however, complexes consisting of the HE, M, and S proteins were detected only after the 15- to

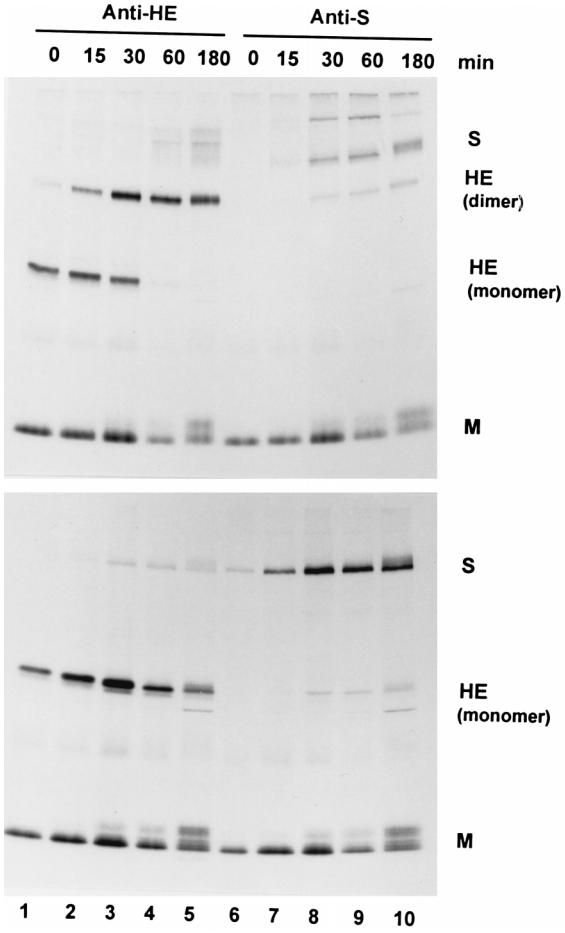

FIG. 3. HE dimerization correlates with its appearance in HE-M-S protein complexes. BCV-infected HCT cells were pulse-labeled with [^{35}S]methionine-cysteine and chased for the times indicated. Half of each lysate was immunoprecipitated with anti-HE (lanes 1 to 5) or anti-S (lanes 6 to 10) protein antibodies. Immunoprecipitates were divided into two parts and eluted in sample buffer without (upper panel) or with (lower panel) β-mercaptoethanol and analyzed by SDS-PAGE.

30-min chase (Fig. 3, lower panel, lanes 3 and 8). Only dimeric HE was detected in these complexes (Fig. 3, upper panel, lanes 8 to 10).

Subcellular compartment of initial association between viral glycoproteins. The processing pattern of the M glycoprotein suggested that M protein initially interacted with HE and S proteins at a pre-Golgi site (Fig. 2). To help support this conclusion, the status of N-linked glycosylation on HE was monitored for sensitivity to endo H digestion. Infected cells were pulse-labeled. At each time point during the chase, cell lysates were divided into equal aliquots and immunoprecipitated with anti-HE or anti-M protein antibodies. Following immunoprecipitation, samples were eluted, divided into equal parts, and either mock treated or incubated with endo H. Initially HE was totally endo H sensitive (Fig. 4A and B, lanes 8 to 10), but slowly it acquired partial endo H resistance after appearing in a complex with the M and S proteins (Fig. 4A and B, lanes 11 and 12), indicating that the protein complexes formed prior to the Golgi complex. This was not surprising, since viral assembly takes place in the IC.

Complex formation in the absence of other BCV proteins. To determine whether the glycoprotein complexes detected in

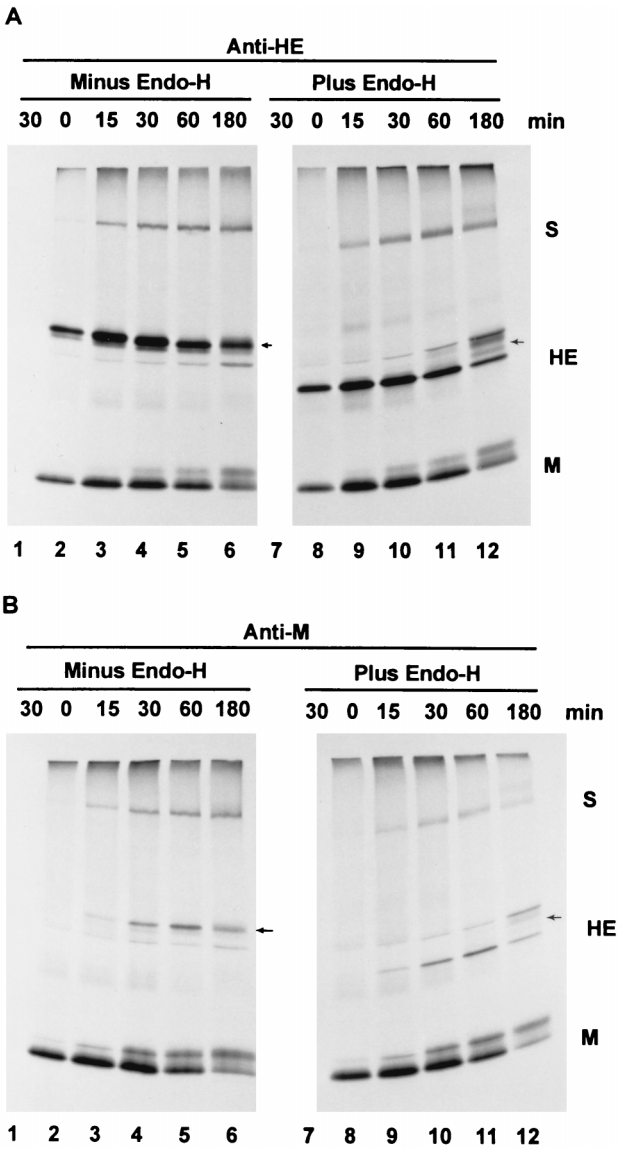

FIG. 4. HE-M-S protein complexes form in a pre-Golgi compartment. BCV-infected HCT cells were metabolically pulse-labeled with [^{35}S]methionine-cysteine and chased as indicated. Lysates from each time point were immunoprecipitated with anti-HE (A) or anti-M (panel B) protein antibodies. After washing and elution, immunoprecipitates were divided into equal parts and mock digested (lanes 1 to 6) or digested (lanes 7 to 12) overnight with endo H prior to being analyzed on 5 to 20% gradient gels by SDS-PAGE. Uninfected controls are shown in lanes 1 and 7 (A and B). The arrows indicate the positions of endo H-partially resistant forms of HE (A and B [compare lanes 5 and 6 with lanes 11 and 12]).

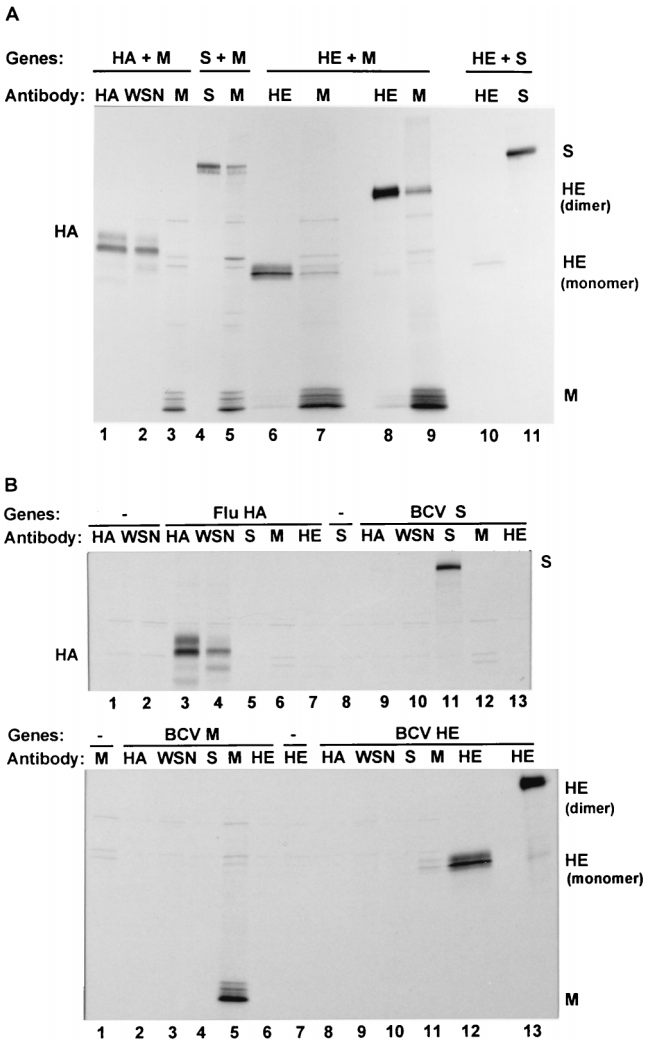

FIG. 5. Coexpression of viral proteins in transfected BHK-21 cells. (A) Semiconfluent BHK-21 cells were infected with vTF7-3 expressing T7 RNA polymerase and transfected with plasmids containing the genes coding for the indicated proteins under control of the T7 promoter. At 9 to 10 h posttransfection, cells were labeled with [^{35}S]methionine-cysteine for 1 h, chased for 1 h, and lysed in RIPA buffer. Immunoprecipitations were carried out with antibodies against the indicated BCV proteins. A monoclonal antibody (HA) and a rabbit polyclonal antibody against the WSN strain of influenza virus (WSN) were used to detect the influenza virus HA. Eluted proteins were resolved by SDS-PAGE on a 5 to 20% gradient gel. HE-M protein complexes were analyzed under both nonreducing conditions (lanes 8 and 9) and reducing (lanes 6 and 7) conditions. (B) Lysates from untransfected cells and cells transfected individually with each gene were used to establish the specificity of the antibodies. HE was analyzed under reducing (lane 12) and nonreducing (lane 13) conditions.

virus-infected cells were able to form in the absence of other viral proteins, BCV glycoproteins were coexpressed with the vaccinia virus T7 expression system. Complexes consisting of HE and M proteins were detected in cells coexpressing only these proteins (Fig. 5A, lanes 6 to 9). As expected, M and S protein complexes were also detected when only M and S proteins were coexpressed (Fig. 5A, lanes 4 to 5). The M-S protein complexes were detected only with the anti-M protein antibodies (Fig. 5A, lane 5), but were not immunoprecipitated with the pool of S protein monoclonal antibodies (Fig. 5A, lane 4). The pool of S protein monoclonal antibodies clearly recognized S protein in M-S protein complexes in virus-infected cells (Fig. 1 to 3). In the context of infected cells, but not in transfected cells, S protein in S-M protein complexes may undergo conformational changes which allow the protein to be recognized by the mix of anti-S protein antibodies used in this study. These changes could result from association of M-S protein complexes with other viral components or from the extent of transport and glycoprotein processing of the protein complexes in coronavirus-infected cells.

The results with a number of controls supported the conclusion that the observed protein complexes were not just artifacts of our experimental conditions. First, the specificity for all antibodies was established by determining that they did not cross-react with the other viral proteins (Fig. 5B). Second,

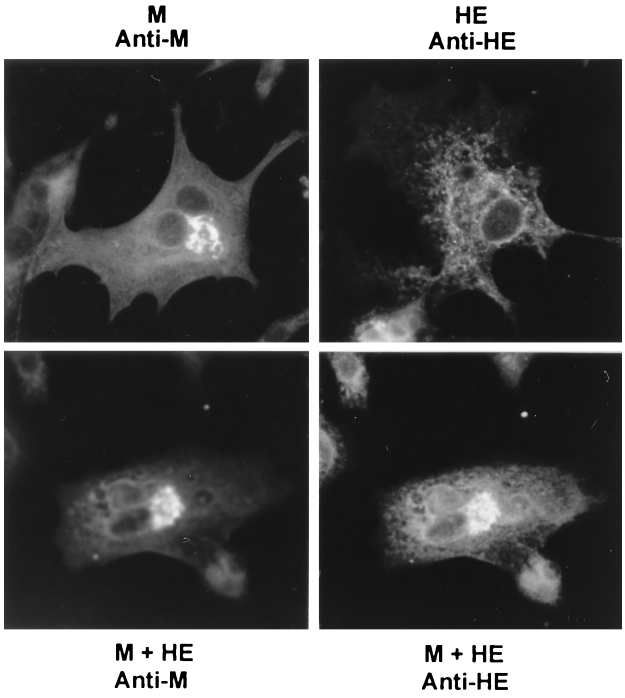

FIG. 6. Localization of HE and M glycoproteins by indirect immunofluorescence of transfected BHK-21 cells. Following infection with vTF7-3, plasmids encoding the HE and M proteins were transfected either individually (upper panels) or together (lower panels). At 9 h posttransfection, cells were fixed with methanol-acetone and stained singly with rabbit anti-HE antibody (upper right) or a mix of mouse anti-M monoclonal antibodies (upper left). Cells expressing both plasmids were double stained with both anti-HE and anti-M antibodies (lower panels). Secondary antibodies were rhodamine-conjugated antirabbit immunoglobulin G (upper and lower right) or fluorescein-conjugated antimouse immunoglobulin G (upper and lower left).

no complexes were detected when the proteins were expressed individually and cell lysates were mixed prior to immunoprecipitation (data not shown). Third, HE and S glycoproteins did not associate when coexpressed in the same cells (Fig. 5A, lanes 10 and 11), further supporting the idea that M protein plays a key role in ensuring that the other glycoproteins are assembled into the virion. Finally, the specificity of the complexes was assessed by coexpressing influenza virus HA with the BCV M protein. Complexes between HA and M protein were not detected (Fig. 5A, lanes 1 to 3).

Colocalization of HE and M glycoproteins. To further substantiate the detected interactions between M protein and the other two glycoproteins, transfected cells were analyzed by indirect immunofluorescence. In cells expressing only M protein, the protein was localized predominantly on one side of the cell nucleus, a characteristic of Golgi localization (Fig. 6, upper left panel). In cells expressing HE alone, the staining was reticular, consistent with the trafficking of a protein along the exocytic pathway to the cell surface (Fig. 6, upper right panel). However, in cells coexpressing the HE and M proteins, part of the expressed HE (Fig. 6, lower right panel) colocalized with M protein (Fig. 6, lower left panel). This clearly indicated that M protein is capable of retaining a fraction of the HE molecules at intracellular membranes. Similar data were obtained when the S and M proteins were coexpressed (data not shown).

DISCUSSION

In this study, we have demonstrated for the first time that glycoprotein complexes are present in BCV-infected cells. The complexes were identified by coimmunoprecipitations with antibodies specific for each of the viral proteins. These complexes formed rapidly after protein synthesis, most likely in the ER or in the IC where coronavirus assembly and budding take place. We assume that these complexes are prerequisites for virion assembly. The M protein apparently plays a key role as a central organizer in assembly of these complexes and in ensuring that S and HE proteins are assembled into virions, since both proteins initially associated with M protein. In addition, both proteins clearly interacted with M protein and were retained at intracellular membranes when coexpressed with M protein, indicating that no other viral protein is required for these complexes to form. The data further contribute to the well-recognized importance of M protein in coronavirus assembly (for review, see reference 31).

It was recently shown that complexes consisting of M and S proteins are present in MHV-infected cells (26). The data presented here extend and provide evidence that similar complexes are also present in cells infected with another coronavirus, supporting the idea that these are conserved assembly complexes in coronavirus-infected cells. In addition, these are the first data demonstrating that HE forms complexes with M protein and that begin to define how a second major structural protein is assembled into the coronavirus virion. Mechanistically, as far as can be defined from our data, HE appears to form complexes and to be incorporated into virions in the same manner as S protein. The data clearly indicate that the coronavirus assembly process can accommodate a third membrane glycoprotein. The inclusion of this additional glycoprotein is not unique to those viruses such as BCV and the hemagglutinating encephalomyelitis virus that naturally express HE. A previous study demonstrated that HE can be incorporated into an MHV that does not synthesize HE (23).

The synthesis and processing of the BCV glycoproteins have been previously analyzed (6, 12). Our earlier data suggested that the glycoproteins are processed on virions as they are transported through the exocytic pathway. The data presented here are in agreement with this idea, since we observed that protein complexes formed quickly, prior to acquisition of any complex sugars on S protein or HE.

Coronaviruses differ from other enveloped, single-stranded RNA viruses that have a helical nucleocapsid. Unlike the orthomyxoviruses, paramyxoviruses, and rhabdoviruses, coronaviruses do not contain a classical matrix protein that underlies the membrane. The classical matrix protein of these viruses interacts with the viral envelope proteins and the helical nucleocapsid cores to initiate virus assembly. The coronavirus M protein is an integral membrane protein that appears to be a matrix-like protein. The long cytoplasmic tail of the coronavirus M protein may provide a function analogous to that of the matrix protein of other viruses. In addition, the coronavirus M protein interacts with the S and HE proteins like the matrix proteins of these other viruses. Through its interactions with the S and HE proteins, M protein also plays an additional role in retaining the other glycoproteins at internal membranes for virion assembly. Both S and HE proteins are transported to the cell surface in virus-infected cells and when expressed alone (16, 27). The interactions between M protein and the other two glycoproteins appear to be specific, since complexes between the influenza virus HA and M proteins were not detected when these proteins were coexpressed. The mechanism by which S and HE proteins interact with M protein is currently not

known and will require detailed mapping of the interacting protein domains.

The data presented here indicate that only dimers of HE were associated with the complexes consisting of HE, M, and S proteins. Since the appearance of HE in the HE-M-S protein complexes correlated with the kinetics of HE dimerization, proper oligomerization is most likely a requirement for incorporation of HE into complexes consisting of all three proteins. It is likely a requirement for HE to complex with M protein as well, even though we were not able to definitively determine that only dimers are coimmunoprecipitated with M protein in this study. In most cases, protein oligomerization takes place in the ER and is a prerequisite for transport out of the ER (15, 30). Therefore, complexes consisting of all three proteins must occur following this step. Even though in the present study, we were unable to follow trimerization of S protein, the analysis of complexes under nonreducing conditions suggests that higher oligomeric forms of S protein appeared in the HE-M-S protein complexes coincident with HE dimers. Others have suggested that specific positions are available within large M protein complexes that can accommodate S trimers (35). If this is the case, the complexes can accommodate other oligomeric structures as well.

Complexes consisting of all three major structural glycoproteins were identified in BCV-infected cells. These complexes were identified during the chase following the initial detection of M-S and M-HE protein complexes. No direct interactions were detected between S protein and HE when these proteins were expressed alone. In this respect, the BCV HE and S glycoproteins are like those of influenza virus. HE and S protein form homo-oligomers and do not complex directly with each other, as is the case with the influenza virus HA (4) and neuraminidase (13).

The data presented here, along with data from a previous study (26), are consistent with a budding model in which M-S and M-HE protein complexes initially form. These complexes then come together to form larger assembly-competent complexes consisting of all three proteins. The membrane-bound complexes most likely provide a site at which nucleocapsids are captured for budding. These complexes presumably form such that cellular proteins are excluded from this environment. The complexes we describe here and those that others have described recently (26) must govern, at least in part, virion assembly.

Since recent studies showed that the E protein is required for formation of mouse hepatitis coronavirus-like-particles (1, 17, 35), E protein may play a key role in the organization of the larger complexes within the membrane. It was previously suggested that E protein may have a morphogenetic role in assembly by positioning within the lattice of membrane proteins to generate the required membrane curvature for budding (35). We have been unsuccessful in detecting complexes consisting of M, S, and HE proteins when all three proteins were coexpressed (data not shown), whereas M-S and M-HE protein complexes did form (data presented here). This suggests that formation of HE-M-S protein complexes may require some other factor.

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