The Hydrophobic Domain of Infectious Bronchitis Virus E Protein Alters the Host Secretory Pathway and Is Important for Release of Infectious Virus

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The coronavirus (CoV) E protein plays an important role in virus assembly. The E protein is made in excess during infection and has been shown to have ion channel activity in planar lipid bilayers. However, a role in infection for the unincorporated E or its ion channel activity has not been described. To further investigate the function of the infectious bronchitis virus (IBV) E protein, we developed a recombinant version of IBV in which the E protein was replaced by a mutant containing a heterologous hydrophobic domain. The mutant virus, IBV-EG3, was defective in release of infectious virus particles. Further characterization of IBV-EG3 revealed that damaged particles appeared to accumulate intracellularly. The phenotype of IBV-EG3 suggested that the hydrophobic domain of IBV E may be important for the forward trafficking of cargo, so we determined whether IBV E facilitated the delivery of cargo to the plasma membrane. Surprisingly, we found that IBV E, but not EG3, dramatically reduced the delivery of cargo to the plasma membrane by impeding movement through the Golgi complex. Furthermore, we observed that overexpression of IBV E, but not EG3, induced the disassembly of the Golgi complex. Finally, we determined that the delivery of IBV S to the plasma membrane was reduced in cells infected with wild-type-IBV compared to those infected with IBV-EG3. Our results indicated that the hydrophobic domain of IBV E alters the host secretory pathway to the apparent advantage of the virus.

Coronaviruses (CoVs) pose a significant threat to human health. In addition to causing ~20% of common cold cases, CoVs can cause deadly illness in humans, as exemplified during the outbreak of severe acute respiratory syndrome (SARS) in 2003. Since the emergence of SARS-CoV, two other CoVs that cause respiratory disease in humans have been identified (24), emphasizing that continued study of CoV biology has important implications for human health.

CoVs are enveloped, positive-strand RNA viruses. Unlike many enveloped viruses that bud at the plasma membrane, CoVs assemble and bud intracellularly into the lumen of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (7). After budding, CoVs use the host secretory pathway to exit the cell. Virions are much larger than normal protein cargo, and it is unknown whether the virus alters the secretory pathway to meet the demands of trafficking its virions. CoVs encode four major structural proteins that facilitate host entry and virus assembly. The membrane protein (M) has three transmembrane domains and forms the scaffolding for virion assembly, the nucleocapsid protein (N) and packages the RNA genome, the spike protein (S) is the fusion protein that facilitates virus entry, and the envelope protein (E) plays a role in virus budding. The CoV E protein is a small (~75- to 109-amino-acid) structural protein that contains a single hydrophobic domain (HD). CoV E was first implicated in virion budding, where it was shown that coexpression of E along with M drives virus-like particle (VLP) formation (31).

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Interestingly, only a fraction of the CoV E produced during infection is incorporated into the virion envelope. This suggests that additional functions for the pool of unincorporated E protein may exist.

Recombinant CoVs lacking the E gene show decreased production of virus, indicating that CoV E is necessary for efficient virus production (6, 9, 21). The E proteins from different CoVs are variable in both length and amino acid composition. However, it has been shown that the E proteins from different CoVs can rescue a mutant of the mouse hepatitis virus (MHV) lacking the E gene, suggesting that the E proteins from different CoVs perform a similar function during viral infection (8). Additionally, studies have demonstrated that some CoV E proteins have ion channel activity in planar lipid bilayers (35, 36), leading to speculation that CoV E may oligomerize and act as an ion channel during infection. Consistent with this idea, computational modeling of the putative HDs of several different CoV E proteins predicts that a pentamer is a stable arrangement (28). This model is supported by a recent nuclear magnetic resonance (NMR) structure of the SARS-CoV E HD (22).

Further evidence for the ion channel activity of CoV E was obtained using the Na+/H+ exchanger inhibitor hexamethylenamine amiloride (HMA), which blocks the ion channel activity of human CoV 229E E and MHV E in planar lipid bilayers. When cells infected with either virus were treated with HMA, their growth was inhibited, but HMA had no effect on the replication of a mutant MHV lacking the E gene. (35). Alanine-scanning mutagenesis of the HD of MHV E showed that disrupting the pitch of the putative transmembrane helix resulted in a virus with lower titer as well as a defect in release of infectious virus (37). Finally, whole-cell patch clamp data suggest that SARS-CoV E has ion channel activity in transfected
cells; however, it was unclear whether the expressed E protein in these studies was present at the plasma membrane (22). These results point to an important role for the HD of CoV E in the virus life cycle and suggest that it may act as an ion channel during viral infection. In this study we showed that the HD of the infectious bronchitis virus (IBV) E protein is important for the release of infectious virus and alters the host secretory pathway to the apparent advantage of the virus.

MATERIALS AND METHODS

Cell culture. HeLa and Vero cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen/Gibco, Grand Island, NY) with 10% or 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), respectively, and 0.1 mg/ml Normocin (InvivoGen, San Diego, CA) at 37°C under 5% CO2.

Viruses and infection. The recombinant IBV Beaudette virus used as the wild type and the generation of the IBV-EG3 mutant have been previously described (15, 40). After three rounds of plaque purification, 12 plagues were expanded and the E gene was sequenced by reverse transcription-PCR (RT-PCR). Two mutant clones carrying the mutation (clone 8 and clone 10) were chosen for further study. After four passages, the mutation was stable, and no other mutations were present in the region of the genome encoding the structural proteins. Vero cells were inoculated with virus diluted into serum-free DMEM, and virus was adsorbed for 1 h with rocking. The inoculum was removed, and the cells were rinsed with phosphate-buffered saline (PBS) and placed in DMEM with 5% FBS. For the one-step growth curve, triplicate wells of Vero cells were infected with wild-type IBV (wt-IBV), IBV-EG3, or IBV-EG10 at a multiplicity of infection (MOI) of 1. The supernatants and cells were collected separately every 4 h for 24 h. The supernatants were clarified and frozen at –80°C. The cells were washed with PBS, covered with 0.5 ml of DMEM with 5% FBS, and subjected to three freeze-thaw cycles. The virus titer was determined by plaque assay on Vero cells. Each sample was plated in triplicate, and the plaques were stained by overlay of 0.8% agarose in DMEM with 0.015% neutral red.

Plasmids. cDNAs encoding IBV E, EG3, and IBV M have been described previously (3, 5). The pcAGGS expression vectors for each were constructed by excising the coding sequence from pBlueScript (Stratagene, La Jolla, CA) and subcloning into pCAGGS-MCS (20) with SacI and EcoRI restriction sites. The cDNA for a soluble version of the membrane protein dipheriae pertussis (Invivogen/Molecular Probes, Eugene, OR). The Alexa Fluor 594-conjugated transferrin was from Invitrogen/Molecular Probes. The Texas Red-conjugated donkey anti-rat and fluorescein-conjugated donkey anti-rat IgGs were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Electron microscopy. For analysis of infected cells by transmission electron microscopy (TEM), Vero cells were infected with IBV-wt or IBV-EG3 at an MOI of 0.1. At 14 h postinfection (p.i.), cells were fixed and embedded essentially as described previously (2). Briefly, cells were fixed in 1% glutaraldehyde and 0.1 M sodium cacodylate buffer, treated with 1% OsO4 in 0.1 M sodium cacodylate with 1% potassium ferricyanide, dehydrated, and embedded in Epon. For quantitation, carriers were defined as membrane-bound structures containing three or more virions. Abrupt material was defined as structures other than virions present within the limiting membranes of carriers. For IBV-wt-infected cells, 16 cells and 54 carriers were counted, and for IBV-EG3-infected cells, 10 cells and 83 carriers were counted. Thin sections were viewed at 80 kV. Images were collected on a Hitachi 7000 microscope using an AMT charge-coupled device (CCD) camera.

Comunmunoprecipitation. Vero cells were infected with IBV-wt or IBV-EG3 at an MOI of 0.1. At 20 h p.i. cells were starved in cysteine-methionine-free DMEM for 15 min, labeled with 150 μCi of ExperEss™[35S]methionine-cysteine (Perkin-Elmer, Waltham, MA) in cysteine-methionine-free DMEM for 90 min and chased for 30 min in normal growth medium. Labeled cells were washed with PBS-BSA, cross-linked with 1 mM dithiobis(succinimidyl propionate) (DSP) for 10 min, and quenched in 40 mM glycine in PBS. After cross-linking, cells were lysed in detergent solution (62.5 mM EDTA, 1% NP-40, 0.4% deoxycholic acid, 50 mM Tris-HCl [pH 8]) with protease inhibitor cocktail (Sigma). Samples were clarified, and SDS was added to 0.2%. All samples were precleared with Staphylococcus aureus Pansorbin cells (Calbiochem, San Diego, CA). IBV E was immunoprecipitated with the rabbit anti-IBV E antibody. Immune complexes were collected with 20 μl of Staphylococcus aureus Pansorbin cells and washed two times in radiol immunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.4], 0.1% SDS, 1% deoxycholic acid, 1% NP-40, 150 mM NaCl). The cross-linking was reversed prior to gel electrophoresis with 5% β-mercaptoethanol. Samples were separated by 15% SDS-PAGE, and labeled proteins were visualized by using a Molecular Imager FX phosphorImager (Bio-Rad) using Quantity One software.

Transient assay. In all trafficking assays, HeLa cells were used at 18 to 22 h posttransfection. Cells were starved in cysteine-methionine-free DMEM for 15 min, labeled with 50 to 100 μCi of ExperEss™[35S]methionine-cysteine (Perkin-Elmer, Waltham, MA) per dish in cysteine-methionine-free DMEM for 20 min, and chased for various times in normal growth medium. Labeled cells were washed with PBS and lysed in detergent solution with protease inhibitor cocktail. Samples were clarified, and SDS was added to 0.2%. All samples were precleared with Staphylococcus aureus Pansorbin cells. After incubation with the appropriate antibody, immune complexes were collected with 20 μl of Staphylococcus aureus Pansorbin cells and washed two times in RIPA buffer. For the VSV G surface immunoprecipitation assay, HeLa cells expressing VSV G along with IBV M, IBV E, or EG3 were pulse-labeled as described above. At each time point, labeled dishes were washed two times with cold PBS-BSA (1% BSA and 0.02% NaN3 in PBS) and then incubated on ice with rabbit anti-VSV diluted into 400 μl PBS-BSA for 4 h. Cells were lysed in detergent solution, and immune complexes were isolated as described above. This pool of protein was counted as “surface.” The internal pool of VSV G was determined by immunoprecipitating the samples with rabbit anti-VSV. Immune complexes were eluted in loading sample buffer and separated by 10% SDS-PAGE. For the solDPPIV secretion assay, HeLa cells coexpressing solDPPIV along with IBV E, IBV M, or EG3 were pulse-labeled as described above. The supernatants were taken from each sample and clarified, and 5% detergent solution was added to 1×, and SDS was added to 0.2%. solDPPIV was immunoprecipitated from the cell and supernatant fractions with mouse anti-C-Myc antibody. For the VSV G endo-N-acetyl-glucosaminidase H (endo H) assay, HeLa cells coexpressing VSV G along with IBV E, IBV M, or EG3 were pulse-labeled and collected as described above. VSV G was immunoprecipitated using rabbit anti-VSV antibody. Immune complexes were eluted in 1× SDS (pH 6.8) at 100°C and digested in 75 mM Na-citrate (pH 5.5) with 0.2 μl endo H (100 units) (New England Biolabs, Beverly, MA) at 37°C overnight. For Tac-S endo H assays, HeLa cells coexpressing Tac-S along with IBV E, IBV M, or EG3 were pulse-labeled and collected as described above. Tac-S was immunoprecipitated using rabbit anti-SARS-CoV S antibody (17). Immune complexes were eluted and digested with endo H. Concentrated sample buffer (200 mM Tris-HCl [pH 6.8], 8% SDS, 60% glycerol, 0.2% bromophenol blue) was added to each sample, and the proteins were run on 10% SDS-PAGE. Labeled proteins were visualized by using a Molecular Imager FX phosphorImager (Bio-Rad) using Quantity One software.

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Imaged FX phosphorimager (Bio-Rad) and quantified using Quantity One software.

[^H]palmitate incorporation. Vero cells were infected with either IBV-wt or IBV-EG3 at an MOI of 0.1. At 12.5 h.p.i., cells were labeled with either 25 µCi of [^35S]methionine-cysteine ([^35S]-Promix; Amersham) or 500 µCi of [^3H]palmitate for 3 h as previously described (3). Cells were lysed in detergent solution and clarified. We assessed the rabbit anti-IBV E antibody as described above. Samples were subjected to SDS-PAGE, and gels were impregnated with 2,5-diphenyloxazole (PPO) and processed by fluorography at –80°C.

Indirect-immunofluorescence microscopy. HeLa cells plated on glass coverslips were processed for immunofluorescence at 18 to 20 h after transfection. For staining p230, GM130, and giantin, cells were washed with PBS and fixed in 3% paraformaldehyde. The fixative was quenched in PBS containing 10 mM glycine (PBS-Gly), and the cells were permeabilized in 0.5% Triton X-100 (TX-100) for 3 min. For staining calnexin and LAMP1, cells were fixed in 3% paraformaldehyde and permeabilized in 0.05% saponin for 10 min. Saponin was included in all antibody dilutions for the latter samples. For staining tubulin, cells were fixed and permeabilized in methanol for 20 min at –20°C. Coverslips were incubated in primary antibody with 1% BSA for 18 min at room temperature and washed twice with PBS-Gly. Primary antibodies were used at the following dilutions: rabbit anti-IBV E, 1:800; mouse anti-p230, 1:200; mouse anti-GM130, 1:800; mouse anti-LAMP1, 1:200; rat anti-IBV E, 1:500; rabbit anti-giantin, 1:800; rabbit anti-disRed, 1:500; and rabbit anti-calnexin, 1:100. After being washed two times with PBS-Gly, cells were incubated in secondary antibody with 1% BSA for 18 min. Secondary antibodies were used at the following dilutions: Alexa Fluor 488-conjugated anti-rabbit IgG, 1:1,000; Alexa Fluor 568-conjugated anti-mouse IgG, 1:1,100; Texas Red-conjugated anti-rat IgG, 1:500; and fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG, 1:200. Coverslips were washed two times with PBS-Gly and mounted on microscope slides in glycerol containing 0.1 M N-propylgallate. All images and enlargements were adjusted independently.

The surface staining for IBV S was carried out as follows. Vero cells were infected with IBV-wt or IBV-EG3 at an MOI of 0.1. At 9 to 10 h.p.i., cells were incubated with mouse anti-IBV S antibody (1:4) for 12 min at 37°C. Cells were then fixed with 3% paraformaldehyde and permeabilized with 0.5% TX-100 for 3 min. Rabbit anti-IBV S antibody (1:800) was used to stain for total IBV S. The surface staining for IBV S was carried out as described above. Vero cells were infected with IBV-wt or IBV-EG3 at an MOI of 0.1. At 9 to 10 h.p.i., cells were incubated with mouse anti-IBV S antibody (1:4) for 12 min at 37°C. Cells were then fixed with 3% paraformaldehyde and permeabilized with 0.5% TX-100 for 3 min. Rabbit anti-IBV S antibody (1:800) was used to stain for total IBV S. The surface staining for IBV S was carried out as described above.

RESULTS

The HD of IBV E is important for efficient viral replication. To better understand the role of the HD of IBV E, we created a chimeric version of IBV E in which the HD (amino acids 12 to 32) was replaced with the HD of the vesicular stomatitis virus glycoprotein (VSV G) (amino acids 463 to 482) (Fig. 1A). The HD of VSV G was chosen because it is the same length as the HD of IBV E and does not contain any known localization or oligomerization signals. The HD of VSV G was chosen because it is the same length as the HD of IBV E and does not contain any known localization or oligomerization signals. We then made a recombinant version of IBV wherein EG3 was inserted in place of E in the virus genome. The recombinant virus (IBV-EG3) was plaque puriﬁed, and several clones were sequenced by RT-PCR to conﬁrm the mutation. Selected clones carrying EG3 in place of E were grown and passed in Vero cells. After expansion and passage of several clones, RT-PCR sequencing showed that the mutation was stable and no additional mutations were present within the structural proteins.

One-step growth curves were performed for IBV-wt and IBV-EG3. Since the whole genome of the recombinant virus was not sequenced, we used two different clones of IBV-EG3 to control for any clonal variation. At each time point, the released and cell-associated viruses were collected separately. Infectivity was determined by triplicate plaque assay, and the total infectivity (the sum of the released and cell-associated titers) was plotted. Error bars indicate the standard deviation for each time point. (C) Percentage of infectious virus in the supernatant at 12, 16, 20, and 24 h p.i. Error bars indicate the standard deviation for each time point.

FIG. 1. Defect in growth and release of IBV-EG3 infectious particles. (A) The HD of IBV E was precisely replaced with the HD of VSV G. (B) Vero cells were infected with IBV-wt or two clones of IBV-EG3 (8 and 10) at an MOI of 1, and a one-step growth curve was performed. At each time point, the released and cell-associated viruses were collected separately. Infectivity was determined by triplicate plaque assay, and the total infectivity (the sum of the released and cell-associated titers) was plotted. Error bars indicate the standard deviation for each time point. (C) Percentage of infectious virus in the supernatant at 12, 16, 20, and 24 h p.i. Error bars indicate the standard deviation for each time point.
These results indicate that IBV-EG\textsuperscript{3} is defective in the production of infectious virus (reduced 10-fold) and has an even larger defect in the release of infectious virus (reduced \textsuperscript{44}fold).

**Damaged IBV-EG\textsuperscript{3} virions accumulate intracellularly.** Preliminary characterization of IBV-EG\textsuperscript{3} demonstrated that most particles purified from infected cells lacked surface spikes and that the majority of IBV S was cleaved near the virion envelope (15). Additionally, the total protein content of purified virions indicated that IBV-EG\textsuperscript{3} particles were released at 2- to 3-fold-lower levels than IBV-wt (data not shown), even though released infectious particles were reduced \textsuperscript{44}fold.

To address whether IBV-EG\textsuperscript{3} virions were accumulating intracellularly, we performed transmission electron microscopy (TEM) on Vero cells infected with either IBV-wt or IBV-EG\textsuperscript{3}. In cells infected with IBV-wt, virions were present in pleomorphic structures packed with virions (Fig. 2A, upper panels, arrows). However, in IBV-EG\textsuperscript{3}-infected cells, virions accumulated in vacuole-shaped carriers along with aberrant structures that may be degraded material (Fig. 2B, lower panels, arrowheads). We next quantified how often virion carriers contained aberrant material, and we found that IBV-EG\textsuperscript{3} virions were much more likely to have aberrant material than IBV-wt. It is possible that IBV-EG\textsuperscript{3} virions are trafficked incorrectly and end up in degradative compartments, resulting in the release of damaged virions.

**The HD of IBV E is not necessary for interaction with IBV M or palmitoylation of E.** We have previously shown that E and EG\textsuperscript{3} can both drive VLP formation when coexpressed with IBV M, suggesting that both proteins are competent for virus assembly (4). Here we tested how well M interacts with EG\textsuperscript{3} in the context of an infection. We radiolabeled infected cells with \textsuperscript{35}S methionine and immunoprecipitated IBV E after cross-linking with dithiobis(succinimidyl propionate) (DSP) to preserve the interaction after detergent solubilization. The results showed that both E and EG\textsuperscript{3} coimmunoprecipitated M (E/M signal ratio of \textasciitilde 1.2 for both), suggesting that altering the HD of IBV E does not interfere with E-M interaction (Fig. 3A). This is consistent with our previous observation that bud-
ded IBV-EG3 virions were present in Golgi stacks as determined by TEM (15).

It has been shown that palmitoylation of MHV E is important for virus production (1, 13). Therefore, we determined whether replacing the HD of IBV E affected its palmitoylation. We radiolabeled infected cells with either [35S]methionine-cysteine or [3H]palmitate and compared the amount of palmitate incorporated to total protein using fluorography. The results showed that E and EG3 were palmitoylated to similar extents (Fig. 3B). Thus, the heterologous HD of IBV-EG3 does not disrupt the interaction with IBV M or affect protein palmitoylation.

IBV E, but not EG3, affects delivery of cargo to the plasma membrane. Our data demonstrate that the HD of IBV E is important for the efficient release of infectious virus but does not appear to be critical for particle assembly. We hypothesized that the HD of IBV E facilitates the forward trafficking of virions through the host secretory pathway and that rapid movement through the secretory pathway helps virions avoid cellular degradation pathways. To this end, we tested whether IBV E enhances the movement of protein cargo toward the plasma membrane. We coexpressed IBV E or EG3 along with the model cargo glycoprotein VSV G in HeLa cells. As a control, we coexpressed VSV G with IBV M, a membrane protein that is also targeted to the Golgi region. Transfected cells were pulse-labeled with [35S]methionine-cysteine, and the delivery of VSV G to the plasma membrane was determined by surface immunoprecipitation at various times postchase. To our surprise, the overexpression of IBV E reduced delivery of VSV G to the plasma membrane, while overexpression of EG3 had no effect (Fig. 4A). This result was the opposite of what we expected; instead of facilitating the movement of VSV G to the plasma membrane, IBV E inhibited the trafficking of VSV G. To further characterize how IBV E affected protein trafficking, we examined other cargo molecules. Since VSV G is a membrane-bound protein and virions are soluble cargo, we next assayed the effect of overexpressing IBV E and EG3 on the secretion of a soluble version of the membrane protease dipetidyl peptidase IV (solDPPIV). solDPPIV is a secreted version of DPPIV that was created by inserting a signal sequence cleavage site at the end of the transmembrane domain (32). Cells coexpressing solDPPIV along with either IBV E or EG3 were pulse-labeled with [35S]methionine-cysteine, the supernatants were collected at various times postchase, and the amount of solDPPIV secreted was determined by immunoprecipitation followed by SDS-PAGE and phosphorimaging. We
observed that overexpression of IBV E severely inhibited the secretion of solDPPIV, while overexpression of EG3 had no effect (Fig. 4B). Taken together, these results show that IBV E inhibits the delivery of protein cargo to the cell surface.

**IBV E delays movement of cargo through the Golgi complex.** To determine if the decrease in protein trafficking caused by IBV E was occurring at the Golgi complex or at a post-Golgi step, we analyzed the processing of N-linked oligosaccharides on cargo proteins. Measuring the acquisition of resistance to endoglycosidase H (endo H), which occurs in the medial Golgi compartment, is a common method for determining the rate of glycoprotein movement through the Golgi complex. Cells co-expressing VSV G along with IBV E, IBV M, or EG3 were pulse-labeled and chased for various times. After collection of the cell lysates at each time point, VSV G was immunoprecipitated and digested with endo H. The percentage of VSV G that was resistant to endo H at each time point was determined after SDS-PAGE and phosphorimaging. The results showed that IBV E, but not EG3 or IBV M, dramatically reduced the amount of processed VSV G (Fig. 5A). This suggests that IBV E impedes trafficking at or prior to the medial Golgi compartment. We next assayed the effect of IBV E on a different cargo protein, Tac-S. This protein contains the ectodomain of interleukin-2 and the endodomain of SARS-CoV S, which contains a weak dibasic ER retrieval signal (17). IBV E also-delayed the movement of Tac-S through the medial Golgi compartment (Fig. 5B). The results of these experiments show that IBV E, but not EG3, decreases the anterograde movement of several cargo proteins through the Golgi complex. This result is consistent with the observation that IBV E reduces the delivery of cargo proteins to the cell surface.

**Overexpression of IBV E causes the Golgi complex to disassemble.** Given the trafficking defect observed in cells expressing IBV E, we determined whether the overexpression of IBV E had any effect on the morphology of secretory organelles. Using indirect-immunofluorescence microscopy of HeLa cells overexpressing either IBV E or EG3, we examined the staining pattern for the cis-Golgi protein GM130. GM130
staining was dispersed in cells overexpressing IBV E but not in cells overexpressing EG3 (Fig. 6A). However, lysosomes (LAMP1), early endosomes (transferrin receptor), and the ER (calnexin) were all unaffected by the overexpression of IBV E (data not shown). We also examined the distributions of actin and tubulin to determine if IBV E induced any rearrangement of the cytoskeleton, which could disrupt the Golgi structure. However, both actin filaments and microtubules appeared to be normal in cells overexpressing IBV E (data not shown).

We next tested several different Golgi markers to see if their localization was affected by IBV E. Both the trans-Golgi protein p230 and the membrane-bound cis/medial Golgi protein giantin were dispersed in cells overexpressing IBV E (Fig. 6B). In addition, the localization of the ERGIC marker ERGIC-53 was also disrupted (data not shown). This suggests that the overexpression of IBV E alters the structure of the entire Golgi complex. Closer examination of the staining patterns for GM130, p230, and giantin showed that IBV E only partially colocalized with each marker (Fig. 6). Additionally, we observed that both p230 and GM130 only partially colocalized with giantin in cells with disrupted Golgi complexes (Fig. 7). Taken together, the results suggest that the Golgi complex is

FIG. 6. IBV E disrupts the morphology of the Golgi complex. (A) HeLa cells overexpressing IBV E or EG3 were double labeled with anti-IBV E and anti-GM130 antibodies. The same field is shown for each pair of samples. (B) HeLa cells overexpressing IBV E were double labeled with rat anti-IBV E and rabbit antigiantin (cis/medial Golgi), or rabbit anti-IBV E and mouse anti-p230 (trans-Golgi). Boxed areas in each field are enlarged at the right; arrows indicate areas of overlap, and arrowheads indicate areas where only one marker is present. (C) Color merges of the enlargements shown in panels A and B. IBV E is shown in green, and the respective marker is shown in red.
not simply disassembling into ministacks, where the Golgi resident proteins should mostly colocalize (29). Instead, more extensive disassembly must be occurring, which could also explain the reduction in cargo trafficking.

To compare the level of overexpression of IBV E in transfected cells to that during infection, we used immunoblotting and normalized the signal to the number of E-expressing cells determined by indirect-immunofluorescence microscopy. We found that the level of E expressed per cell at 18 h posttransfection was similar to the level present at 16 h p.i. at an MOI of 0.2 (data not shown). Thus, transfected cells express an amount of IBV E per cell similar to that expressed by infected cells at a late time postinfection.

The HD of IBV E reduces accumulation of IBV S on the surface of infected cells. Because IBV E reduces protein traffic through the Golgi complex in transfected cells, we assayed whether IBV E could also affect trafficking in infected cells. We measured the accumulation of IBV S on the surface of cells infected with IBV-wt or IBV-EG3 using a surface immunofluorescence assay. We labeled the surface of cells infected with IBV-wt or IBV-EG3 with mouse anti-IBV S antibodies. We then fixed and permeabilized the cells and labeled the total amount of IBV S by coinjection with a rabbit anti-IBV S antibody. When we compared the signal from the surface to the total for each sample, we found that cells infected with IBV-EG3 had a greater proportion of surface IBV S (Fig. 8A), although the total signal for IBV S was similar. This result is consistent with our observation of reduced cargo trafficking. Since more IBV S was at the surface of cells infected with IBV-EG3, we reasoned that cells infected with IBV-EG3 should also produce larger syncytia. We examined the size of syncytia in cells infected with either IBV-wt or IBV-EG3 by phase microscopy and found that syncytia were larger and more prevalent in IBV-EG3-infected cells (Fig. 8B). We quantified the difference by counting the number of nuclei per syncytia in IBV-wt- or IBV-EG3-infected cells at 11.5 h p.i. Over 80% of IBV-wt syncytia had three to five nuclei, while over 80% of IBV-EG3 syncytia contained more than five nuclei. Thus, IBV E, but not EG3, decreases the accumulation of IBV S on the surface of infected cells and reduces the size of syncytia in IBV-wt-infected cells.

**DISCUSSION**

We have shown that the HD of IBV E plays an important role in the release of infectious IBV particles from Vero cells. A recombinant virus with IBV E containing a heterologous HD (EG3) was competent for virus assembly but showed a defect in the release of infectious particles. The finding that the HD was not required for assembly is consistent with our earlier observation that the cytoplasmic tail of IBV E is sufficient for interaction with IBV M (4). Further characterization of the mutant virus showed that it accumulated intracellularly in vacuole-like structures along with aberrant material. We hypothesized that the mutant virions were accumulating intracellularly and becoming damaged and were subsequently released as noninfectious particles. Thus, we initially thought that the HD of IBV E might alter the secretory pathway to promote anterograde trafficking. However, in overexpression experiments IBV E, but not EG3, caused a dramatic reduction in protein trafficking to the plasma membrane by impeding cargo trafficking through the Golgi complex. We also observed that overexpression of IBV E disrupted Golgi morphology but did not affect the ER or endosomal compartments. Finally, we observed that cells infected with IBV-EG3 had increased surface levels of IBV S, leading to larger syncytia.
Previously our lab reported that expression of IBV E using a recombinant vaccinia virus in BHK-21 cells did not disrupt Golgi structure (5). In the current study we expressed IBV E from a plasmid using lipid-based transient transfection of HeLa cells. One possible reason for the difference we observed in the previous study could be the different cell types used. We tested this by overexpressing IBV E via lipid-based plasmid transfection in several different cell lines, including BKH-21. In all cases, IBV E disrupted the Golgi morphology (data not shown). We next tested whether the lipid-based transfection method was responsible for the effects that we observed. We used a CaPO4-based transfection method as well as nucleofection and observed the same effects on the Golgi complex as we saw using lipid-based methods (data not shown). Thus, the discrepancy in the data is likely due to the method used to express IBV E. Vaccinia virus expression produces a large amount of protein very rapidly, whereas transient transfection tends to produce a more modest amount of protein over a longer period of time.

Our lab previously reported that IBV does not replicate efficiently when IBV S accumulates on the surface of cells early in infection (39). The ability of IBV E to reduce protein trafficking is likely beneficial to the virus because it prevents the accumulation of IBV S on the surface of infected cells, thereby reducing syncytium size and number. Large syncytia may die prematurely, which prevents robust virus replication. Increased syncytium size may also make virion trafficking more challenging due to the intracellular rearrangements caused by cell-cell fusion. Furthermore, reducing protein trafficking during host infection may have other positive effects for the virus, such as reducing the amount of antigen on the cell surface or preventing antigen display to the immune system by the major histocompatibility complex I.

The importance of the E protein in the release of infectious particles has been observed for other CoVs. Mutations introduced into the HD of MHV E via alanine scanning produced mutant viruses which, among other defects, were compromised in release of infectious virus (37). Studies investigating the role of transmissible gastroenteritis coronavirus (TGEV) E showed that when E protein was deleted from the virus, virions accumulated intracellularly, and infectious virus could not be recovered unless E was provided in trans (21). These results, combined with our data showing that IBV E alters the secre-
tory pathway, suggest that the CoV E protein supports the release of infectious particles.

The apparent disassembly of the Golgi complex in response to expression of IBV E raises some interesting questions. Previously it has been reported that MHV infection drives the rearrangement of the Golgi complex in a two-step process, where initially the Golgi complex is dispersed from its juxtanuclear position by an unknown mechanism, followed by the condensation of the Golgi complex in the centers of syncytia, seemingly driven by cell-cell fusion (10). More recent work by Ulasli et al. has greatly expanded our understanding of the membrane rearrangements caused by coronaviruses. These authors describe the formation of large virion-containing vacuoles from ERGIC/Golgi membranes concurrent with the scattering of the Golgi complex (30). These results suggest that the rearrangement of the Golgi complex may be important for forming virion carriers. It is interesting to speculate on why the virus would need to alter the secretory pathway in order to properly traffic its virions. Virions are much larger than normal cargo and may traverse the secretory pathway using a different route, or they may require different machinery than conventional cargo. The morphological changes in the Golgi complex that we observed in the presence of IBV E may help to create an environment that promotes virion trafficking.

The mechanism by which IBV E modifies the secretory pathway is not understood. One possibility is that IBV E oligomerizes and forms an ion channel at the Golgi complex. Other viruses encode small hydrophobic proteins that oligomerize and form ion channels, with the best characterized being influenza virus M2 (23). The proton channel activity of influenza virus M2 plays an important role during the entry of influenza virus by acidifying the lumen of the virion following endocytosis and forms ion channels at the Golgi complex. Other ways are not understood. One possibility is that IBV E oligomers from ERGIC/Golgi membranes concurrent with the scattering of the Golgi complex (30). These results suggest that the rearrangement of the Golgi complex may be important for forming virion carriers. It is interesting to speculate on why the virus would need to alter the secretory pathway in order to properly traffic its virions. Virions are much larger than normal cargo and may traverse the secretory pathway using a different route, or they may require different machinery than conventional cargo. The morphological changes in the Golgi complex that we observed in the presence of IBV E may help to create an environment that promotes virion trafficking.

While the CoV E proteins are small, they appear to have multiple functions. Previous characterization of IBV E showed that the C-terminal tail of the protein contains targeting information and facilitates interaction with IBV M (3, 4). Here, we took advantage of a mutant version of IBV E that was competent for assembly but defective in release of infectious particles. We showed that the HD of IBV E alters the cellular secretory pathway. This indicates that multiple domains of IBV E are important for its proper function, and this is possibly true for all CoV E proteins. Future studies will examine which residues within the HD of IBV E are critical for its effect on the secretory pathway. Additionally, it will be important to determine how IBV alters the secretory pathway through direct ion channel activity, protein-protein interaction, or some other mechanism.

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