Revised: JVI01237-15

A Coronavirus E Protein is Present in Two Distinct Pools with Different Effects on Assembly and the Secretory Pathway

Running Title: Two Pools of Coronavirus IBV E

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Word Count Abstract: 233
Word Count Text: 6,876
Key words: Coronavirus, E protein, Golgi, Oligomers, Viroporin

ABSTRACT

Coronaviruses (CoVs) assemble by budding into the lumen of the early Golgi prior to exocytosis. The small CoV envelope (E) protein plays roles in assembly, virion release, and pathogenesis. CoV E has a single hydrophobic domain (HD), is targeted to Golgi membranes, and has cation channel activity *in vitro*. However, the precise functions of the CoV E protein during infection are still enigmatic. Structural data for the severe acute respiratory syndrome (SARS)-CoV E protein suggests that it assembles into a homo-pentamer. Specific residues in the HD regulate the ion-conducting pore formed by SARS-CoV E in artificial bilayers and the pathogenicity of the virus during infection. The E protein from the avian infectious bronchitis virus (IBV) has dramatic effects on the secretory system, which requires residues in the HD. Here, we use the known structural data from SARS-CoV E to infer residues important for ion channel activity and oligomerization of IBV E. We present biochemical data for the formation of two distinct oligomeric pools of IBV E in transfected and infected cells, and residues required for their formation. A high-order oligomer of IBV E is required for the production of virus-like particles (VLPs), implicating this form of the protein in virion assembly. Additionally, disruption of the secretory pathway by IBV E correlates with a form that is likely monomeric, suggesting that the effects on the secretory pathway are independent of E ion channel activity.

IMPORTANCE

CoVs are important human pathogens with significant zoonotic potential as demonstrated by the emergence of SARS-CoV and Middle East respiratory syndrome (MERS)-CoV. Progress has been made toward identifying potential vaccine candidates in mouse models of CoV infection, including use of attenuated viruses that lack the CoV E protein or express E mutants. However, no approved vaccines and anti-viral therapeutics exist. We previously reported that the hydrophobic domain of the IBV E protein, a putative viroporin, causes disruption of the mammalian secretory pathway when exogenously expressed in cells. Understanding the mechanism of this disruption could lead to the identification of novel anti-viral therapeutics. Here, we present biochemical evidence for two distinct oligomeric forms of IBV E, one essential for assembly and the other with a role in disruption of the secretory pathway. Discovery of two forms of CoV E protein will provide additional targets for anti-viral therapeutics.

INTRODUCTION

Coronaviruses (CoVs) are enveloped viruses with positive-sense, single-stranded RNA genomes that infect avian and mammalian species. These viruses cause about 20% of common colds in humans. However, CoVs have presented a more serious threat to human health in recent years. The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 and the Middle East respiratory syndrome coronavirus (MERS- CoV) in 2012 demonstrate the zoonotic potential of this family of viruses (1). There has been some success in the development of mouse models of SARS and MERS infection, and candidate vaccines where the E protein is deleted or

mutated have been described (2–7). However, there is still much that is unclear regarding the role of the CoV E protein in infection.

CoVs acquire their membrane envelope by budding into the ER-Golgi intermediate compartment (ERGIC), a characteristic that sets CoVs apart from other well studied enveloped viruses (8). The infectious virions that bud into the ERGIC lumen must then navigate the host secretory pathway to be released from the cell. CoVs have three major structural proteins that are constituents of the virion envelope (1). The CoV S protein is the attachment and fusion protein. The CoV M protein coordinates the process of virion assembly and is the most abundant protein in the virion envelope. Lastly, the CoV E protein contains a single hydrophobic domain and is a minor component of the virion envelope. Only a small portion of the E protein expressed during infection is incorporated into the virion envelope; the majority of E remains localized to Golgi membranes (9–12). The E protein has been shown to be required for the robust production of virus, since recombinant CoVs lacking the E protein grow to a significantly reduced titer or are propagation-incompetent (13–15).

Three roles for CoV E protein have been proposed. A role for CoV E in assembly has been suggested based on the observation that CoV E along with the M protein can drive the production of VLPs; the interaction occurs via sequences in the cytoplasmic tails (16, 17). A role in release of infectious virus that requires the hydrophobic domain (HD) of the E protein has been reported (18, 19). Lastly, residues in the HD of SARS-CoV E have been shown to promote viral fitness and pathogenesis in a mouse adapted model of infection (20).

91 Early reports on several CoVs including IBV, SARS-CoV, mouse hepatitis virus
92 (MHV) and the human CoV 229E demonstrated that CoV E proteins form cation-
93 selective ion channels in planar lipid bilayers, suggesting that CoV E functions as a
94 viroporin (21, 22). Structural data support the idea that CoV E can oligomerize and form
95 a channel. Bacterially expressed or synthetic peptides corresponding to the SARS-CoV
96 E protein HD form pentameric α-helical bundles in planar lipid bilayers (23–26). Solution
97 NMR analysis of a synthetic peptide of SARS-CoV E protein in micelles also revealed a
98 homo-pentameric structure (27). Recent studies have suggested that the MERS-CoV E
99 protein also forms pentameric ion channels in lipid bilayers (28). Furthermore, SARS-
100 CoV E forms a proteolipidic pore in which negatively charged lipids in bilayers enhance
101 ion conductance and cation selectivity (29, 30).
102 SARS-CoV E residues N15 and V25, both in the HD, are necessary for ion channel
103 activity in lipid bilayers (24, 29). N15 and V25 promote viral fitness and pathogenesis in
104 a mouse adapted SARS-CoV model of infection, presumably through the necessity of
105 these residues for ion channel activity (20). Despite the plethora of *in vitro* evidence
106 supporting the role of CoV E as an ion channel and the role of E as a pathogenic
107 determinant, the precise function of E as an ion channel in infected cells and animals is
108 unknown. The best evidence for the ion channel activity of the CoV E protein during
109 infection comes from experiments demonstrating that the drug hexamethylene
110 amiloride, a known channel inhibitor, reduces the titer of MHV grown in cultured cells,
111 but not of a mutant of MHV with the entire E protein deleted (22).
112 We previously reported that overexpression of IBV E induces disassembly of the
113 Golgi as well as reduced trafficking of cargo molecules through the Golgi (19). Alanine

mutagenesis of the HD of IBV E revealed that a single residue, T16, was required for the Golgi disassembly and membrane trafficking disruption (31). Given that IBV E T16 is in the equivalent position as N15 in the SARS-CoV E, we predicted the ability of IBV E to disrupt the secretory pathway is dependent on its ion channel activity. Further, We hypothesize that the HD, and T16 specifically, is required for modification of intracellular compartments to allow assembly and release of infectious virions.

Herein, we investigated how the IBV E protein and two HD mutants behave in cells. We present evidence for two distinct pools of IBV E in transfected and infected cells. HD mutants suggest that the Golgi phenotypes observed with exogenous expression are independent of IBV E ion channel activity, leading to a model that IBV E functions as (I) a monomer, potentially interacting with a cellular protein(s) to alter the host secretory machinery, and (II) as a high molecular weight homo-oligomer with a function in virion assembly.

MATERIALS AND METHODS

Cell culture. HeLa and Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen/Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 0.1mg/ml Normacin (InvivoGen, San Diego, CA) at 37˚C under 5% CO_2.

Plasmids. The pBluescript and pCAGGS IBV E, pCAGGS IBV E-T16A, pCAGGS IBV M, pCAGGS IBV N and pCAGGS VSV G plasmids have been previously described (19, 31). The pCAGGS IBV E-A26F plasmid was constructed using Quikchange

(Stratagene) site-directed mutagenesis of the pBluescript IBV E expression plasmid. The IBV E-A26F coding sequence was then subcloned into the pCAGGS-MCS using EcoRI and SacI restriction sites.

Transient transfection. X-tremeGENE 9 DNA Transfection Reagent (Roche, Indianapolis, IN) was used to transiently transfect cells according to the manufacturers protocol. Unless otherwise noted, subconfluent HeLa cells in 35 mm dishes were transfected with the following amounts of plasmid diluted into Opti-MEM (Invitrogen/Gibco) with a 1:3 ratio of X-tremeGENE 9: 1.0 µg pCAGGS IBV E, 1.0 µg pCAGGS IBV E-T16A, 1.0 µg pCAGGS IBV E-A26F, 1.0 µg pCAGGS VSV G for sucrose gradient analysis, 0.5 µg pCAGGS VSV G for endo H trafficking assay (see below). The cells were used in experiments at 16 to 22 h after transfection, unless otherwise noted.

Antibodies. The rabbit polyclonal and rat polyclonal antibodies recognizing the C-terminus of IBV E, have been described previously (32). The rabbit anti-IBV M and anti-IBV N antibodies have also been described (31). The rabbit polyclonal and mouse monoclonal antibodies recognizing VSV or VSV G, respectively, have been previously described (33, 34).The mouse anti-GM130 antibody was from BD Biosciences (San Diego, CA). The horseradish peroxidase-conjugated donkey anti-rabbit and the horseradish peroxidase-conjugated sheep anti-mouse antibodies were from GE Healthcare (Piscataway, NJ). The horseradish peroxidase-conjugated donkey anti-rat antibody was from Jackson ImmunoResearch Laboratories (Bar Harbor, Maine). The Alexa-Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-mouse IgG were from Invitrogen/Molecular Probes (Eugene, OR).

Sucrose gradient analysis. HeLa cells transiently expressing wild type or mutant IBV E or VSV G were washed with PBS at 16 h post transfection. The cells were lysed for 10 min on ice with 100µl of DDM lysis buffer (20 mM HEPES-KOH [pH 7.4], 100 mM NaCl, 20 mM Imidazole, 1 mM $CaCl_2$) containing 4.2% *n*-Dodecyl-*β*-D-maltoside (DDM), protease inhibitor cocktail (Sigma), and 10% glycerol in all cases except for the comparison of VSV G and IBV E in Figure 1A; qualitative analysis suggested that IBV E gradient peaks were sharper in the presence of glycerol, thus glycerol was included in the lysis buffer and gradients for all subsequent experiments. The lysates were centrifuged at 20,000×g for 10 min at 4˚C and the supernatants were loaded onto 5 ml, 5-20% linear sucrose gradients consisting of DDM lysis buffer with 0.42% DDM, over a 300 µl 60% sucrose cushion. The gradients were spun at 192,000×g for 24 h at 4˚C in a Beckman SW55Ti ultracentrifuge rotor. Fifteen fractions per gradient were collected using a Buchler Instruments Auto Densi-Flow II C. The fractions were then analyzed by either western blot or by phosphoimaging after immunoprecipitation, described below. Lysates were treated with 1% SDS prior to sucrose gradient analysis when specified.

Western blot analysis. 4× concentrated sample buffer (200 mM Tris-HCl [pH 6.8], 8% SDS, 60% glycerol, 0.2% bromophenol blue) was added to 15% of each fraction collected. The samples were heated at 100˚C for 3-5 min in the presence of 2-5% BME unless otherwise noted, and run on a 15% SDS-PAGE gel. Gels were transferred to polyvinylidene fluoride (PVDF) Immobilon membranes (Millipore). The membranes were blocked 1 h at room temperature in 5% milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20). Proteins were detected using rabbit or rat anti-IBV E (1:10,000) in 2.5% milk in TBST overnight at 4˚C. After washing in TBST membranes were then

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incubated in secondary horseradish peroxidase-conjugated donkey anti-rabbit or anti-rat (1:10,000) for 1 h at room temperature. After washing, the membranes were incubated with HyGlo Quick Spray chemiluminescent detection reagent (Denville Scientific Inc.). Images were collected using a Versa Doc model 5000 (Bio-Rad) and analyzed with Quantity One software.

Sucrose gradient analysis of IBV E from infected cells. The Beaudette strain of recombinant IBV used in this study has been previously described (Youn et al., 2005; Machamer and Youn, 2006). Vero cells were inoculated with virus diluted in serum-free DMEM, and virus was adsorbed for 1h with rocking. The inoculum was removed and the cells were rinsed with DMEM containing 5% FBS, and then incubated at 37˚C in DMEM containing 5% FBS. For sucrose gradient analysis of IBV E during infection, Vero cells infected at an MOI of 1.0 were rinsed with PBS and lysed in DDM lysis buffer and protease inhibitor cocktail (Sigma) at 8 h post infection. For sucrose gradient analysis of secreted IBV virions Vero cells were inoculated at an MOI of 1.0 for 1 h at 37˚C and treated as above. At 24 h post-infection the clarified cell supernatant was placed on a 4 ml 20-50% sucrose step gradient in TNE buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA). The step gradients were spun at 198,000×g for 90 min in an SW41Ti Beckman ultracentrifuge rotor at 4˚C. The interface containing virions was collected, diluted in TNE, and virions were pelleted with a 50 min spin as above. The virions were resuspended in DDM lysis buffer containing protease inhibitor cocktail. Sucrose gradient analysis and western blot analysis with the rabbit anti-IBV E antibody were performed as described above.

205 *Crosslinking and anti-IBV E Dynabead immunoprecipitation.* HeLa cells transiently
206 expressing IBV E, IBV E-T16A, or IBV E-A26F were used at 18 h post transfection.
207 Lysis of transfected cells, or purification of virions, and sucrose gradient analysis was
208 performed as described above. Fifteen fractions were collected for each gradient and
209 fractions representing the low molecular weight pool (4,5, and 6) and high molecular
210 weight pool (7, 8, and 9) were combined. The crosslinker Dithiobis(succinimidyl
211 propionate) (DSP, Pierce, Rockford, IL) was added to the combined fractions to 1 mM
212 final. The samples were incubated for 1 h at 4˚C with rotation. The crosslinked samples
213 were then added to 1 mg of washed rabbit anti-IBV E IgG conjugated Dynabeads (Life
214 Technologies AS, Oslo, Norway). The beads were prepared by conjugating 20 µg of
215 protein G Sepharose-purified rabbit anti-IBV E IgG per mg of Dynabeads as specified
216 by the manufacturer's instructions. The samples were incubated for 1 h at 4˚C with
217 rotation. Dynabeads were then washed in 1 ml of DDM lysis buffer. The Dynabeads
218 were placed on a magnet for 1 min, the supernatant was removed and 100 µl of 1×
219 sample buffer was added to each sample, then split in half. One half was treated with
220 5% BME to reverse the crosslinks and the other half was left unreduced. The samples
221 were heated at 100˚C for 5 min. The crosslinked samples were then evaluated on a
222 15% SDS-PAGE gel along with various controls and analyzed by western blot as
223 described above with the rat anti-IBV E polyclonal antibody.
224 *Pulse-chase analysis of IBV E on sucrose gradients.* HeLa cells expressing IBV E
225 were analyzed at 16 h post-transfection. Cells were starved in cysteine-methionine-free
226 DMEM for 15 min, labeled with 250 µCi of EasyTag Express Protein Labeling Mix ^{35}S-
227 cysteine-methionine (PerkinElmer, Boston, MA) per dish in cysteine-methionine-free

DMEM for 15 min, and chased for various times in normal growth media. Cells were washed with PBS and lysed in 100 µl of DDM lysis buffer containing protease inhibitor cocktail (Sigma). The samples were clarified and subjected to sucrose gradient analysis as described above. Two-fold concentrated detergent solution (125 mM EDTA, 2% NP-40, 0.8% deoxycholic acid, 100 mM Tris-HCl [pH 8.0]) and SDS were added to the sucrose gradient fractions to 1× and 0.2% final, respectively. IBV E was immunoprecipitated-using 2 µl of the rabbit anti-IBV E antibody per fraction with incubation at 4˚C for 2h. Immune complexes were collected using 20 µl of washed *Staphylococcus aureus* Pansorbin cells and washed three times with radioimmunprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.4], 0.1% SDS, 1% deoxycholic acid, 1% NP-40, 150 mM NaCl). Concentrated sample buffer was added to each sample, the immunocomplexes were eluted at 100˚C for 3 min in the presence of 2% BME and run on 15% SDS-PAGE. Labeled IBV E was visualized using a Molecular Imager FX phosphoimager (Bio-Rad) and quantified using Quantitiy One software.

VSV G endo-β-N-acetyl-glucosaminidase H (endo H) assay. HeLa cells coexpressing VSV G and IBV E, IBV E-T16A, or IBV E-A26F were used at 20 h post transfection. Cells were starved in cysteine-methionine-free DMEM for 15 min, labeled with 60 µCi of EasyTag Express Protein Labeling Mix ^{35}S-cysteine-methionine per dish in cysteine-methionine-free DMEM for 10 min, and chased for various times in normal growth media. Cells were washed with PBS and lysed in 500 µl of 1x detergent solution with protease inhibitor cocktail. The samples were clarified and SDS was added to 0.2%. The samples were immunoprecipitated with rabbit anti-VSV for 2 h at 4˚C. Immune complexes were collected with 20 µl of washed *S. aureus* Pansorbin cells and

washed three times with RIPA buffer. The immune complexes were eluted in 50 mM Tris-HCL [pH 6.8] containing 1% SDS at 100˚C for 5 min. The *S. aureus* cells were spun out, and the supernatant was diluted with an equal amount of 150 mM Na-citrate (pH 5.5) with endo H (50 units per sample) (New England Biolabs , Beverly, MA) at 37˚C overnight. Concentrated sample buffer was added, the samples were boiled for 5 min at 100˚C in the presence of 3.75% BME and run on 10% SDS-PAGE. Labeled VSV G was visualized using a Molecular Imager FX phosphoimager (Bio-Rad) and quantified using Quantitiy One software.

Indirect-immunofluorescence microscopy.

Golgi Disassembly. HeLa cells plated on glass coverslips were processed for immunofluorescence at 16 h post transfection. Cells were washed with PBS and fixed in 3% paraformaldehyde in PBS for 10 min at 22˚C. The fixative was quenched in PBS containing 10 mM glycine (PBS-Gly), and the cells were permeabilized in 0.5% Triton X-100 for 3 min. The coverslips were washed twice with PBS-Gly and incubated in primary antibody with 1% BSA for 20 min at room temperature. Rabbit anti-IBV E was used at 1:1,000 and mouse anti-GM130 was used at 1:300. The cells were then washed twice with PBS-Gly and incubated for 20 min in secondary antibody with 1% BSA. Alexa-Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-mouse IgG were used at 1:1,000. The coverslips were washed twice in PBS-Gly and incubated with Hoescht 33285 [0.1 µg/ml] to stain DNA, rinsed twice in PBS-Gly and mounted on slides in glycerol containing 0.1M *N*-propylgallate. Images were captured using an Axioscop microscope (Zeiss) equipped for epifluorescence with an ORCA-03G charge-coupled-device camera (Hamamatsu, Japan). Data analysis was performed using iVision

software (Bio Vision Technologies) and Microsoft Excel. Golgi complex disassembly was addressed in cells expressing IBV E, IBV E-T16A, or IBV E-A26F. GM130 staining of the Golgi was outlined. The outlined area for the Golgi was measured and Golgi complexes with an area larger than 1.5 standard deviations from the average area of untransfected cells were considered disassembled. For each IBV E protein the percent of cells with a disassembled Golgi was determined by dividing number of cells scored as disrupted by the total number of cells measured (n ≥100 cells).

Virus-like particle (VLP) assay. Subconfluent HeLa cells in 60 mm dishes were transfected with the following plasmids in indicated combinations diluted into Opti-MEM (Invitrogen/Gibco) with a 1:3 ratio of X-tremeGENE 9: 0.1 µg pCAGGS IBV E, 0.1 µg pCAGGS IBV E-T16A, 0.1 µg pCAGGS IBV E-A26F, 2.0 µg pCAGGS IBV M, and 1.5 µg pCAGGS IBV N. Samples were prepared at 48 h post transfection. The medium was clarified at 4500 ×g for 20 min. The supernatant was loaded on a 20% sucrose cushion and centrifuged at 234,000 ×g in a TLA-110 rotor for 60 min. To simplify quantification of heterogeneously glycosylated IBV M, samples were deglycosylated prior to SDS-PAGE. The pellet containing VLPs was resuspended in 2X glycoprotein denaturation buffer (New England Biolabs). The cell fraction was pelleted in PBS at 4,000 ×g for 2 min. The cell pellets were resuspended in detergent solution containing protease inhibitor cocktail. Glycoprotein denaturation buffer was added to 2X final. Both the VLP and cell fractions were heated to 100˚C for 1 min. All samples were digested with PNGase F (New England Biolabs) according to the manufacturers protocol. 4× concentrated sample buffer was added to the samples and they were heated to 100˚C for 3 min. 10% of the cell fractions and 100% of the VLP fractions were separated on 15% SDS-PAGE

gels. The proteins were western blotted as described above. Proteins were detected using rabbit anti-IBV E (1:10, 000), rabbit anti-IBV M (1:5,000), and rabbit anti-IBV N (1:10, 000) primary antibodies and horseradish peroxidase conjugated donkey anti-rabbit IgG (1:10,000) secondary antibody.

RESULTS

IBV E is enriched in two pools by sucrose gradient analysis. Structural data for SARS-CoV E suggests that its ion channel activity in artificial membranes can be attributed to homo-pentamer formation (23–27). To address the possibility that IBV E ion channel activity was responsible for secretory pathway disruption, we asked if IBV E forms homo-oligomers during transient expression in cells. Native lysates from HeLa cells expressing IBV E were prepared in lysis buffer containing *n*-Dodecyl-*β*-D-maltoside (DDM), and separated on a 5-20% sucrose gradient also containing DDM. Gradient fractions were collected and analyzed by western blot. On a parallel control gradient the vesicular stomatitis virus glycoprotein (VSV G), a 68 kDa membrane protein known to be 4 Svedburg units (4S) in its monomeric form on a neutral 5-20% sucrose gradient, was analyzed as a reference for molecular size under our assay conditions (35). IBV E was enriched in two peaks, while VSV G was enriched in one (Fig 1A). We will refer to the two IBV E peaks as the low molecular weight (LMW) and high molecular weight (HMW) pools, respectively. VSV G ran slightly further into the gradient than the LMW pool of IBV E, while the HMW pool of IBV E ran slightly further than VSV G. This led us

to predict that the HMW pool of IBV E was in the range of 65-75 kDa, potentially representing a higher order oligomer of the protein (e.g. homo-pentamer).

In order to characterize the two pools of IBV E further we tested the effect of SDS on the lysate prior to being run on a sucrose gradient (Fig. 1B). When the lysate containing IBV E was treated with SDS, IBV E was enriched in a single peak, which should reflect IBV E in its monomeric state (~12 kDa). In the absence of SDS, IBV E was again enriched in two peaks. The LMW peak formed in the absence of SDS ran further into the gradient than IBV E in the presence of SDS and we concluded that this LMW peak represented a pool of IBV E that was larger than a predicted monomer. It should be noted that estimation of molecular weight of membrane proteins by sucrose gradient analysis in the presence of detergent is difficult, as various factors including molecular size and shape, as well as the amount of bound detergent all contribute to the migration of the protein in the gradient (36, 37). These results suggest that IBV E is present in two pools in transfected cells, possibly representing different homo-oligomeric states, or differential association of IBV E with unknown cellular protein(s).

Kinetics of formation of the LMW and HMW pools of IBV E. In an effort to better characterize the LMW and HMW pools of IBV E, we followed the newly synthesized pool of IBV E protein by metabolic labeling. HeLa cells transiently expressing IBV E were pulse-labeled for 15 min with ^{35}S methionine-cysteine and chased for 0 or 60 min. The cells were lysed and run over sucrose gradients as described above. IBV E was immunoprecipitated from each fraction and analyzed by SDS-PAGE. Directly after the pulse, IBV E was predominantly found in the LMW pool, with a smaller proportion in the HMW pool. The fraction of IBV E in the HMW pool slightly increased following a 60 min

chase, but the LMW pool did not substantially decrease over time (Fig 2A). To elaborate on this finding, we performed a time course of IBV E expression from 12 to 20 h post-transfection (Fig. 2B). At 12 h more IBV E was in the LMW pool relative to the HMW pool; however at 16 and 20 h post transfection the percent total E in the LMW and HMW pool approached equal enrichment. Taken together, these data suggest that the LMW pool of IBV E forms more rapidly than the HMW pool, and that it is unlikely to be a precursor of the HMW pool. An alternative possibility is that the LMW pool of IBV E reaches a steady state early during expression, and slowly transitions into the HMW pool. Analysis of IBV E mutants (see below) makes this explanation less likely, however.

The LMW and HMW pools contain IBV E in different oligomeric states. To determine the oligomeric states of IBV E in the two pools, we crosslinked the LMW and HMW gradient fractions using the reversible crosslinker DSP. IBV E was immunoprecipitated and eluates were treated with or without reducing agent to reverse the crosslinks and analyzed by western blot. In the absence of reducing agent, the LMW pool, which correlates with a species of IBV E slightly larger than monomer on the sucrose gradient, consisted primarily of monomeric IBV E, with a small amount of a second, larger species (Fig. 3). The HMW pool consisted of a series of six evenly spaced bands: one representing IBV E monomer, another that appears to be the second band observed in the LMW pool, as well as four other bands (Fig. 3). The addition of reducing agent collapsed the crosslinked species to the 12 kDa monomer. Although we cannot rule out crosslinking of cellular proteins to IBV E, the evenly spaced

crosslinked bands suggest the IBV E species represent homo-oligomers ranging from homo-dimer to homo-hexamer.

The IBV E protein in infected cells is also present in two pools by sucrose gradient analysis. To determine if the oligomeric states of IBV E in transfected cells were relevant during IBV infection, a native lysate from IBV-infected Vero cells was evaluated by sucrose gradient analysis. Vero cells were infected at a MOI of 1.0 for 8 h and the cell lysate was analyzed as described above (Fig. 4A, black line). Indeed, IBV E was enriched in the same two pools as observed during exogenous expression. Although the percent of total E in the LMW pool in infected cells (~10%) was less than that in the same pool in transfected cells (~50%), this was consistent over multiple experiments, suggesting the LMW form is relevant in infection. Clearly, the majority of IBV E in infected cells is in the predicted homo-oligomeric form. One possibility is that the formation of the LMW pool of IBV E could be tightly regulated during infection but not transfection. It should also be noted that more IBV E was present in fraction 15 at the bottom of the gradient in infected cells compared to transfected cells.

The majority of virion associated IBV E is in the HMW pool and forms homo-oligomers. We next addressed the forms of IBV E in the virion envelope. Vero cells were infected for 24 h at an MOI of 1.0 and virions were purified from the supernatant (see Materials and Methods). The virions were solubilized with DDM and run over a 5-20% sucrose gradient (Fig. 4A, grey line). The sucrose gradient profile of IBV E in secreted virions was very similar to IBV E from infected cells, with the majority of the E protein in the HMW pool, relative to the LMW pool. We again observed some IBV E in fraction 15. Next, we used crosslinking and immunoprecipitation of the HMW fraction

387 from secreted virions to show the IBV E in this pool consisted of higher order oligomers
388 (Fig. 4B), with a banding pattern identical to the HMW pool in transfected cells.
389 Together our data from infected Vero cells and from secreted virions suggest that the
390 majority of IBV E in both is in a higher order homo-oligomeric state.

391 **IBV E HD mutants have different effects on the cellular secretory pathway.** We
392 previously reported that the wild type (WT) IBV E protein alters the cellular secretory
393 pathway during transient expression in HeLa cells, which requires residue T16 in the
394 HD (19, 31). Given that the LMW and HMW pools of IBV E are both present during IBV
395 infection, we wanted to understand the role of IBV E oligomerization in the context of
396 secretory pathway disruption. N15 in SARS-CoV E (at the equivalent position to IBV E
397 T16) along with V25, is necessary for ion channel activity in lipid bilayers (26, 31).
398 Whether IBV E-T16A loses the capacity to disrupt the secretory pathway due to loss of
399 ion channel activity, improper oligomerization, or through another mechanism is not
400 known. SARS-CoV E V25 is located at the predicted monomer-monomer interface of
401 the SARS-CoV E homo-pentamer. It has been suggested that the V25 residue in SARS-
402 CoV E may be important for the formation of a stable homo-oligomer given its position
403 in the predicted structure (20). The residue at the equivalent position to V25 in SARS-
404 CoV E in IBV E is A26. We mutated IBV E A26 to phenylalanine to determine the effect
405 on the cellular secretory pathway. HeLa cells expressing WT IBV E or the mutants T16A
406 or A26F were evaluated for Golgi disassembly by indirect immunofluoresence
407 microscopy (Fig. 5A,B). As previously reported, IBV E caused disassembly of the Golgi
408 as determined by the dispersal of the *cis*-Golgi marker GM130, while the T16A mutant

409 did not. The IBV E-A26F protein did elicit Golgi disassembly, despite the predicted role
410 of A26 in oligomer formation.

411 We then tested effects of the IBV E-A26F mutant on the trafficking of the model
412 cargo protein VSV G by measuring oligosaccharide processing rates. Cells expressing
413 VSV G along with IBV E or IBV E mutants were pulse-labeled, chased for various times,
414 and immunoprecipitated VSV G was subjected to glycosidase digestion (Fig. 5C). As
415 previously reported, WT IBV E dramatically reduced the rate of acquisition of
416 endoglycosidase H resistance of VSV G, while IBV E-T16A did not. However, IBV E-
417 A26F, in accordance with the disassembly of the Golgi, reduced the trafficking of VSV G
418 similar to WT IBV E. These data suggest that two HD mutants of IBV E predicted to
419 interfere with ion channel activity have different effects on the cellular secretory
420 pathway.

421 **HD mutants of IBV E are differentially enriched in LMW and HMW pools.** We
422 next examined the oligomerization profiles of the two HD mutants of IBV E. At steady
423 state the IBV E-T16A protein was nearly exclusively in the HMW pool, while the IBV E-
424 A26F protein was largely present in the LMW pool (Fig. 6A). Interestingly, we observed
425 an increase in the amount of IBV E-T16A in fraction 15 at the bottom of the sucrose
426 gradient compared to the WT protein and the A26F mutant, an observation we also
427 made for IBV E expressed during infection. Preliminary data using steeper sucrose
428 gradients suggested that IBV E T16A in fraction 15 exists in a distinct peak and is not a
429 heterogeneous aggregate (our unpublished data). Experiments to characterize this form
430 of IBV E are in progress.

Next, we observed a window of IBV E expression for the two HD mutants by ^{35}S-cysteine-methionine metabolic labeling as described above for WT IBV E. The pulse-chase analysis revealed that the majority of IBV E-T16A was present in the HMW pool even at 0 min of chase, and the percent total at the bottom of the gradient in fraction 15 increased with chase (Fig. 6B). On the other hand, IBV E-A26F was predominantly in the LMW pool, remained in the LMW pool over time, and did not accumulate in fraction 15 (Fig. 6C).

We performed crosslinking followed by immunoprecipitation of the pooled LMW and HMW fractions for each mutant. IBV E-T16A formed the predicted HMW homo-oligomer robustly, while the IBV E-A26F did not (Fig 6D), consistent with the migration of these proteins on sucrose gradients. These results indicate that the T16 residue, predicted to be required for ion channel activity and secretory pathway phenotypes, is not required for the HMW oligomerization of IBV E, but is required for stable formation of the LMW pool. The A26F mutation, which was predicted to affect ion channel activity and oligomerization of the E protein, did markedly reduce the predicted oligomerization in the HMW pool. These results suggest that the effects of IBV E on the cellular secretory pathway correlate with the LMW pool, and thus are likely to occur by a mechanism distinct from the proposed ion channel activity of this protein.

Virus-like particle (VLP) production requires the HMW pool. We previously reported that cells expressing IBV E and IBV E-T16A produce VLPs to a similar extent when co-expressed with IBV M and IBV N, suggesting that T16 in IBV E is required for secretory pathway disruption, but not for virion assembly (31). We evaluated VLP production from HeLa cells expressing IBV E-A26F compared to WT IBV E and IBV E-

T16A (Fig 7). Cell lysates and supernatants were collected, and VLPs were concentrated from the supernatants by centrifugation through a 20% sucrose cushion. VLP release was determined by comparing the percentage of expressed IBV M released into the supernatant, as quantified by western blot. As expected, VLPs were released into the supernatant of cells expressing IBV E, M and N. Surprisingly, cells expressing IBV E-T16A along with IBV M and N released ~4-fold more IBV M into the supernatant than the WT protein. We believe that the difference from our previous study (31) can be attributed at least in part to differences in the transfection protocol. Intriguingly, cells expressing IBV E-A26F were unable to produce VLPs. These results suggest that the LMW pool of IBV E present during expression of IBV E-A26F does not support VLP production. On the other hand, the higher-order oligomer, present in the HMW pool supports the robust production of VLPs.

DISCUSSION

Two distinct pools of IBV E. Our sucrose gradient analysis of IBV E indicate that the protein exists in two populations during both transient expression and infection. Pulse-chase and steady-state analysis led us to conclude that the LMW and HMW pools of IBV E represent distinct populations of the protein, and that the LMW pool is unlikely to be a precursor of the HMW pool. Rather, the LMW pool of IBV E appears to represent a persistent population of the protein, potentially possessing its own function.

476 We determined that the LMW pool of IBV E is slightly larger than the predicted
477 monomer present on the gradient in the presence of SDS, and that the HMW pool of
478 IBV E is slightly larger than the 68 kDa VSV G protein. Though we cannot calculate the
479 precise size of the HMW form of IBV E by sucrose gradient analysis, it is similar to the
480 predicted size of an IBV E homo-pentamer. Crosslinking and subsequent
481 immunoprecipitation of the gradient fractions containing the two pools of IBV E
482 suggested that the LMW pool contains a monomer or dimer, whereas the HMW
483 represents a homo-oligomer up to a hexamer. It is thus possible that IBV E may be
484 different from both SARS-CoV E and MERS-CoV E, which have both been shown to
485 form homo-pentamers in artificial membranes (23–28). However, our experiments do
486 not rule out the possibility that some bands could contain IBV E crosslinked to host cell
487 proteins in the case of transfection, and host or other viral proteins in the case of
488 infection. Preliminary mass spectroscopy data suggest that immunoprecipitates of IBV E
489 from the LMW pool from transfected cell lysates contain several host proteins, whereas
490 immunoprecipitates of IBV E from the HMW pool do not (our unpublished data). These
491 preliminary data suggest a small host protein interacting with IBV E could explain the
492 migration of the LMW pool further into the gradient than the E monomer. Additionally,
493 the sole presence of IBV E in the immunoprecipitates of the HMW pool would lend
494 support to the interpretation that IBV E forms a homo-hexamer. However, if an
495 interacting host protein is present it is possible that IBV E does indeed form a homo-
496 pentamer in the HMW pool. We are currently pursuing these preliminary results. Our
497 data suggesting that mutation of IBV E A26 inhibits homo-oligomer formation would lend
498 support to a model for homo-pentamer formation given that it is the analogous residue

499 to SARS-CoV E V25. Additional evidence for a model of homo-pentamer formation by
500 IBV E has been reported by Parthasarthy et al, in which they demonstrate that IBV E
501 purified from bacteria can form an oligomer consistent with homo-pentamer by
502 perfluorooctanoic acid-PAGE and analytical ultracentrifugation (38).

503 **IBV E mutants support distinct functions for the two pools.** IBV E has been
504 proposed to play roles in both virion assembly and in the release of infectious virions
505 from the host cell (8). However, the mechanism(s) by which the protein executes these
506 roles remains unknown. Based on the persistence of the two pools of IBV E in pulse-
507 chase experiments, we hypothesize that the LMW and HMW pools may represent
508 populations of IBV E with distinct functions. The sucrose gradient profiles of the IBV E-
509 T16A and A26F mutants support this idea and revealed several interesting points. First,
510 the T16A mutant, predicted to be deficient in ion channel activity, formed very little of
511 the LMW pool. Second, the sucrose gradient profile for the IBV E-A26F mutant, which
512 we predicted would prevent homo-oligomerization, behaved as expected, with formation
513 of the HMW pool severely inhibited. Crosslinking and immunoprecipitation of the IBV E-
514 T16A and A26F LMW and HMW fractions corroborated our interpretation of the sucrose
515 gradient profiles. Additionally, analysis of the IBV E mutants by pulse-chase labeling
516 followed by sucrose gradient analysis showed that IBV E-T16A formed very little of the
517 LMW pool even at 0 min of chase. Alternatively, the IBV E-A26F protein remained in the
518 LMW pool from 0 to 60 min. of chase. The pulse-chase analyses of the HD mutants
519 correlate with the findings for the WT protein, suggesting distinct populations of IBV E.
520 In addition, we conclude that A26 is required for robust formation of the higher-order
521 homo-oligomers observed in the HMW pool of IBV E, and T16 is required for the

formation of the LMW pool. The fact that the sucrose gradient profiles for the T16A mutant during transfection and WT E during infection are similar has led us to hypothesize that formation of the LMW pool of IBV E during infection may be a regulated event, which would suggest that T16 is not only required for putative ion-channel activity, but could also take part in this regulatory event during infection, possibly though its interaction with a host cell protein(s). We predict that this regulated interaction is crucial for modification of the host cell environment leading to proper navigation of the host secretory pathway by undamaged, infectious virions.

Golgi phenotypes in transfected cells correlate with the LMW pool. We previously reported that IBV E has significant effects on the host cell secretory pathway when exogenously expressed, including the disassembly of the Golgi as well as a reduction in the rate of protein trafficking through the secretory pathway. T16 in the HD is required for these phenotypes (31). In this study we show that IBV E-T16A migrates predominantly in the HMW pool in sucrose gradients, compared to relatively equal distribution of the WT protein in the LMW and HMW pools. By contrast, the A26F mutation shifted the majority of IBV E into the LMW pool. Since expression of A26F disrupted the Golgi complex while T16A did not, we suggest that the secretory pathway phenotypes are induced by the fraction of IBV E in the LMW pool. Thus, secretory pathway disruption is unlikely to be associated with the ion channel activity of the E protein, which we assume requires higher-order oligomerization as found in the HMW pool.

Assembly of VLPs correlates with the HMW pool. VLP analysis together with the sucrose gradient profiles and oligomerization data from our crosslinking studies suggest

the HMW pool of IBV E has a role in the production of VLPs, while the LMW pool does not. The lack of VLP production by the IBV E-A26F mutant suggests that higher-order oligomerization is required for VLP production; these data thus implicate the HMW pool of IBV E in the process of virion assembly. The fact that IBV E-T16A released 4-fold more IBV M into the supernatant of expressing cells in our VLP assay is intriguing. Perhaps VLPs are released at a faster rate when T16 is mutated, as a consequence of losing the LMW pool and secretory pathway disruption. If so, virions with the T16A mutation would also travel more quickly through the secretory pathway, leading to damage from host proteases and reduced infectivity. Future studies will address this possibility, as well as if the higher-order oligomer of IBV E found in the HMW pool is required for induction of membrane curvature, membrane scission during budding, or efficient exocytosis of virions.

Precedent for mutliple functions of the CoV E protein. SARS-CoV E interacts with the tight junction protein PALS1, and was suggested to play a role in pathogenesis by disassembling tight junctions in lung epithelium, although this interaction has not been characterized further (39). More recently it has been suggested that SARS CoV E interacts with syntenin, resulting in the activation of p38 MAPK signaling during infection (40). Together with the fact that the ion channel activity of SARS CoV E has been shown to be a pathogenic determinant in a mouse infection model (20), these examples set a strong precedence for our hypothesis suggesting multiple functions for IBV E. Other viroporins have been shown to have interactions with host and other viral proteins (41). The HIV-1 Vpu1 viroporin has been shown to interact with and induce the degradation of CD4, tetherin, and the tetraspanin protein CD81 (42–44). The influenza

A M2 viroporin has roles in assembly, virus release and entry, and the HCV p7 viroporin is required for assembly and release of infectious virus (41, 45–50). While the ion channel activity of various viroporins has been implicated in certain roles it is likely that some functions are ion channel-independent, similar to our findings here.

In summary, this study has established that IBV E exists in two populations when expressed in mammalian cells and that it forms oligomers consistent with a homo-pentamer or homo-hexamer. Importantly, the secretory pathway phenotypes induced by the WT IBV E protein in transfected cells are likely to be independent of virus ion channel activity. We suggest that T16 in the HD of IBV E is not only required for the secretory pathway phenotypes, but is required for the formation of the LMW pool of IBV E, which correlates with the secretory pathway phenotypes. This could occur through a regulated interaction with a host cell protein. Additionally, we demonstrate that the HMW pool of IBV E present during IBV E-T16A expression is capable of robust VLP production, thus suggesting a role for a higher-order oligomer of IBV E in the process of assembly. Understanding the dynamics of oligomerization, secretory pathway disruption, ion channel activity and E protein interactors during infection will help resolve some of the interesting questions posed by this study.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant R01 GM42522 and the Biochemistry, Cellular and Molecular Biology Graduate Program (T32 GM007445).

We thank Travis Ruch, Catherine Gilbert, David Zuckerman, and Jason Berk for helpful discussions and critical reading of this manuscript. We would also like to thank Travis Ruch for the development of some of the tools used in this study.

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FIGURE LEGENDS

Figure 1. IBV E forms two pools in transfected cells. (A) HeLa cells expressing IBV E (black line) or VSV G (gray line) were lysed and run on a 5-20% sucrose gradient as described in Materials and Methods. Gradient fractions were collected and analyzed for the presence of IBV E or VSV G by western blot. Plots indicate the percent of total

754 protein in each fraction. (B) HeLa cells expressing IBV E were lysed and treated with
755 SDS (black line) or not (gray line), prior to being run on a 5-20% sucrose gradient
756 containing glycerol. Gradient fractions were collected and analyzed for the presence of
757 IBV E by western blot. Qualitative observations suggest that sucrose gradients
758 containing glycerol resulted in sharper gradient peaks; glycerol was thus included in all
759 subsequent gradients. Error bars represent +/- SEM for two and five independent
760 experiments for the +SDS and –SDS conditions, respectively.
761
762 **Figure 2. The LMW pool of IBV E persists over time, and the HMW pool**
763 **increases with time.** (A) HeLa cells expressing IBV E were pulse-labeled with [^{35}S]
764 cysteine-methionine for 15 min and lysed at 0 (black line) or 60 min (gray line) of chase.
765 The cell lysates were run on 5-20% sucrose gradients. Fractions were collected and
766 immunoprecipitated with anti-IBV E antibody, analyzed by SDS-PAGE and visualized by
767 phosphormaging. (B) HeLa cells expressing IBV E were lysed at 12 (black line), 16
768 (dark gray line) or 20 h (gray line) post-transfection. Cell lysates were separated on 5-
769 20% sucrose gradients, and fractions were analyzed by western blot.
770
771 **Figure 3. The HMW pool contains species of IBV E consistent with higher-**
772 **order homo-oligomers.** Pooled sucrose gradient fractions representing the LMW or
773 HMW forms of IBV E from HeLa cells expressing IBV E were crosslinked with a
774 reducible crosslinker (DSP), immunoprecipitated with anti-IBV E antibody and analyzed
775 by western blotting. One-half of each immunoprecipitate was treated with BME to
776 reverse the crosslinked species. Input lanes represent ~2.0% of the pre-

immunoprecipitated sample to demonstrate the relative amount of IBV E in the LMW and HMW pools. The dots on the non-reduced panel indicate crosslinked species. The bead lane indicates antibody-conjugated beads that were analyzed in parallel as a negative control. This blot is representative of four independent experiments with similar results.

Figure 4. IBV E is present in two pools in infected cells and virions with the majority in the HMW pool. (A) Vero cells infected with IBV were lysed at 8 hpi and run on a 5-20% sucrose gradient (black line), or virions were purified from infected cell supernatant at 24 hpi, resuspended in lysis buffer containing DDM, and run on a 5-20% sucrose gradient (gray line). Fractions were collected and analyzed by western blot. Error bars represent +/- SEM for two independent experiments for each condition. (B) Pooled sucrose gradient fractions representing the LMW and HMW forms of IBV E from purified virions were crosslinked with DSP, immunoprecipitated with anti-IBV E antibody and analyzed by western blot. One-half of each immunoprecipitate was reduced with BME to reverse the crosslinked species. Input lanes represent ~2.0% of the pre-immunoprecipitated sample to demonstrate the relative amount of IBV E in the LMW and HMW pools. The bead lane indicates antibody-conjugated beads that were analyzed in parallel as a negative control. Dots on the non-reduced panel indicate crosslinked species. This blot is representive of two independent experiments that produced similar results.

Figure 5. Predicted HD ion channel mutants of IBV E have different effects on the cellular secretory pathway. (A) HeLa cells expressing IBV E, IBV E-T16A or IBV E-A26F were analyzed by indirect immunofluorescence microscopy at 16 h post-transfection. Cells were labeled with rabbit anti-IBV E and mouse anti-GM130, a cis-Golgi marker. Secondary antibodies were Alexa-Fluor 488-conjugated anti-rabbit IgG and Alexa-Fluor 568-conjugated anti-mouse IgG. The DNA was stained with Hoescht 33285. White arrows indicate disrupted Golgi complexes. (B) Quantification of the percent of cells with Golgi disruption (See Materials and Methods). N≥ 100 cells for each condition. (C) HeLa cells co-expressing VSV G and IBV E, IBV E-T16A, IBV E-A26F or IBV M (as a control) were pulse-labeled with [^{35}S] cysteine-methionine for 10 min, and VSV G was immunoprecipitated at the indicated times of chase and digested with Endo H. The graph represents the percent of Endo H-resistant VSV G in cells co-expressing each IBV E construct.

Figure 6. HD mutants of IBV E are differentially enriched in the LMW and HMW pools. (A) HeLa cells expressing IBV E-T16A (black line) or IBV E-A26F (gray line) were lysed and run on a 5-20% sucrose gradient and fractions were analyzed by western blot. Error bars represent +/- SEM for three independent experiments for each HD mutant. The relative position of the LMW and HMW pools for wild-type IBV E are indicated in bold, boxed lettering and were determined in parallel. HeLa cells expressing IBV E-T16A (B) or IBV E-A26F (C) were pulse-labeled for 15 min with [^{35}S] cysteine-methionine and lysed at 0 (black line) or 60 min (gray line) chase. The cell lysates were run on 5-20% sucrose gradients and fractions were immunoprecipitated and analyzed

by SDS-PAGE and phosphorimaging. (D) Pooled sucrose gradient fractions representing the LMW and HMW pools of IBV E-T16A or IBV E-A26F from transfected cells were crosslinked with DSP, immunoprecipitated with anti-IBV E antibody and analyzed by western blot. One-half of each immunoprecipitate was reduced with BME to reverse the crosslinked species. Input lanes represent ~2.0% of the pre-immunoprecipitated sample to demonstrate the relative amount of each IBV E HD mutant in the LMW and HMW pools. Dots on the non-reduced panel indicate crosslinked species. The bead lane indicates antibody-conjugated beads that were analyzed in parallel as a negative control. This blot is representative of two independent experiments with similar results. The blot images or phosphorimages are illustrated under the graphs for A, B, and C, with the 0 min timepoint on top and the 60 min timepoint on bottom for B and C.

Figure 7. IBV E-T16A supports VLP production while IBV E-A26F does not. (A) A representative immunoblot showing the amount of IBV N, M, and E co-expressed in 10% of HeLa cell fractions and 100% of VLP fractions. (B) Quantification of the amount of IBV M released with no E, WT E, E-T16A, or E-A26F. The amount of M released with WT E was set to 1. Error bars represent +/- SEM for three independent experiments. The asterisk denotes a significant change in VLP level compared to WT E by Student's t-test ($P < 0.05$).