Identification of a Golgi Complex-Targeting Signal in the Cytoplasmic Tail of the Severe Acute Respiratory Syndrome Coronavirus Envelope Protein[▽]

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The 2003 global outbreak of progressive respiratory failure was caused by a newly emerged virus, severe acute respiratory syndrome coronavirus (SARS-CoV). In contrast to many well-studied enveloped viruses that assemble and bud at the plasma membrane, coronaviruses assemble by budding into the lumen of the endoplasmic reticulum-Golgi intermediate compartment and are released from the cell by exocytosis. For this to occur, the viral envelope proteins must be efficiently targeted to the Golgi region of the secretory pathway. Although the envelope protein (E) makes up only a small percentage of the viral envelope, it plays an important, as-yet-undefined role in virus production. To dissect the targeting of the SARS-CoV E protein to the Golgi region, we exogenously expressed the protein and various mutants from cDNA and determined their localization using immunofluorescence microscopy and biochemical assays. We show that the cytoplasmic tail of the SARS-CoV E protein is sufficient to redirect a plasma membrane protein to the Golgi region. Through site-directed mutagenesis, we demonstrate that a predicted beta-hairpin structural motif in the tail is sufficient for Golgi complex localization of a reporter protein. This motif is conserved in E proteins of beta and gamma coronaviruses (formerly referred to as group 2 and 3 coronaviruses), where it also functions as a Golgi complex-targeting signal. Dissecting the mechanism of targeting of the SARS-CoV E protein will lead to a better understanding of its role in the assembly and release of virions.

Coronaviruses (CoVs), named for their crownlike appearance under the electron microscope, are pathogens that infect vertebrates and cause a range of diseases, such as respiratory infections, gastroenteritis, encephalitis, and hepatitis (48). They are enveloped viruses that contain a positive-sense RNA genome of ~30 kb. In contrast to many well-studied enveloped viruses that assemble and bud at the plasma membrane, CoVs assemble by budding into the lumen of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (16, 20). The ERGIC overlaps extensively with the Golgi region and is also called the *cis*-Golgi network (2), which is distinct from the first cisternae of the Golgi stack (*cis*-Golgi). This dynamic compartment is important for the transport and sorting of exocytic cargo. After budding into the ERGIC lumen, virions are released from the cell by exocytosis. A major unanswered question is what advantage intracellular assembly at the ERGIC provides for coronaviruses.

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a novel coronavirus implicated in the 2003 global outbreak of progressive respiratory failure that resulted in 10% mortality (44). SARS-CoV is thought to have entered the human population by transmission from a horseshoe bat reservoir through masked palm civet cats in Southern China (13, 37). Although the bat reservoir is extensive (23), the highly infectious SARS-CoV has not reemerged to date.

CoVs encode four main structural proteins: the membrane

(M), spike (S), nucleocapsid (N), and envelope (E) proteins. The N protein packages the viral RNA genome after its replication in the cytoplasm. The M protein, which is the most abundant protein in the viral envelope, forms a lattice into which the S and E proteins are incorporated. The S protein mediates virus binding and fusion with host target cells. The E protein makes up only a small percentage of the viral envelope but plays an important yet incompletely defined role in virus production (12, 16, 22, 39).

The SARS-CoV E protein is a small integral membrane protein (76 amino acids) that has a short N-terminal domain, a hydrophobic domain, and a C-terminal tail that is present in the cytoplasm. At least two different membrane topologies of SARS-CoV E have been reported: single spanning and membrane hairpin (3, 40, 51). When expressed from cDNA, epitope-tagged SARS-CoV E protein was reported to localize to the endoplasmic reticulum (ER), ERGIC, or Golgi complex (1, 26, 30, 46).

The observation that the E protein was sufficient to induce the formation of virus-like particles (VLPs) in cells expressing mouse hepatitis virus (MHV) M protein led to the idea that the insertion of E into a lattice of M results in budding (47). The minimal requirements for VLP assembly are controversial, however. SARS-CoV VLPs were observed intracellularly when M and N were coexpressed (18); more evidence indicates that the N protein, along with M and E, is required for efficient VLP formation (17). The requirement for E protein in virus assembly appears to be more stringent for some coronaviruses than for others. Transmissible gastroenteritis virus (TGEV) (39) and, probably, infectious bronchitis virus (IBV) (unpublished data) require the E protein for the production of infectious virus. SARS-CoV and MHV lacking E are replication

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competent but severely compromised (11, 22). It is possible that one or more of the accessory proteins encoded by coronaviruses can compensate for the loss of E in some coronaviruses. Recently, a duplicated and truncated version of MHV M lacking the C terminus was shown to partially rescue a ΔE mutant, suggesting that MHV E might promote membrane curvature by mediating interactions between the transmembrane domains of MHV M monomers (21).

The CoV E protein may have additional roles outside of its proposed role in viral assembly. One potential function is alteration of host cell membrane permeability by forming oligomeric ion channels. Other small viral membrane proteins have ion channel activity, which can modify host cell membranes and, ultimately, organelle microenvironments. Influenza virus M2 is the best characterized; its ion channel activity is essential for acidifying the virion during entry (41). IBV E, MHV E, and SARS-CoV E protein have each been shown to behave as viroporins that form membrane channels and alter membrane permeability (25, 33, 49, 50). For MHV E, ion channel activity may be important for virus replication (49). IBV E promotes exocytosis of infectious virus, which requires its hydrophobic domain (32, 45). TGEV E protein promotes trafficking and maturation of virions in the secretory pathway (38). Another potential function for the SARS-CoV E protein was recently suggested through its interaction with PALS1 (46). PALS1 is a cellular protein involved in maintaining tight junctions in epithelial cells. Interaction of the C terminus of SARS-CoV E protein with PALS1 induces relocation of PALS1 to the virus assembly site and disrupts tight junctions. Clearly, understanding the role(s) of the E protein in coronavirus infection requires further work.

Cellular Golgi proteins are predominantly localized by targeting signals present in their transmembrane and/or cytoplasmic domains, although the mechanisms of targeting are not very well understood (reviewed in reference 7). Studies of the cytoplasmic tail of IBV E protein showed that the C-terminal cytoplasmic tail directs Golgi complex targeting (8). However, precise targeting signals in IBV E or the mechanism of targeting have not been determined. To understand how this important viral protein contributes to viral assembly, the mechanism by which the CoV E protein is targeted to the Golgi region must be determined. Here, we identify a sequence motif in the SARS-CoV E cytoplasmic tail that contains Golgi complex targeting information. The motif is conserved in E proteins of beta and gamma coronaviruses, where it also functions as a Golgi complex targeting signal.

MATERIALS AND METHODS

Plasmid construction. The coding sequence for SARS-CoV E from the Tor2 strain (GenBank sequence accession number NP_828854) was originally obtained from entry vector pDONR221 from the Pathogen Functional Genomics Resource Center of the Institute for Genomic Research (sponsored by NIAID). The coding sequence was amplified by PCR with a 5' BamHI site and a 3' XhoI site and then cloned into pCDNA3.1/Hygr⁺ (Invitrogen, Grand Island, NY). The coding sequence was then transferred into pCAGGS-MCS using the KpnI and XhoI sites. QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis was used according to the manufacturer's protocol to introduce mutations in pCDNA3.1/SARS-CoV E using primers that introduced a BamHI site at the junction of the hydrophobic and cytoplasmic tail (introducing a change of Ala32 to Gly) or the alanine replacements indicated below. The sequenced mutations were then subcloned into the mammalian expression vector pCAGGS-MCS (36) to increase expression levels. For the pCAGGS/G-E chimera, the sequence

encoding the ecto- and transmembrane domains of vesicular stomatitis virus glycoprotein (VSV-G) was excised with restriction enzymes EcoRI and BamHI from pBS/VSV-G_{TMB} (42) and the sequence encoding amino acid residues I33-V76 of SARS-CoV E was excised from pCDNA/SARS-E_{TMB} using BamHI and XhoI. The fragments were ligated into the pCAGGS-MCS vector at the EcoRI and XhoI sites.

The pBS/IBV E_{TMB} plasmid (8) was used as the template in PCR mutagenesis to generate an IBV E tail mutant with four alanine replacements. The wild-type or mutant IBV E tail was ligated with the VSV-G fragment (as described above) and pCAGGS to generate pCAGGS/G-E_{IBV}.

A plasmid encoding MHV-A59 E (pM54) was kindly provided by P. Masters (Wadsworth Center, Albany, NY). The coding sequence was PCR amplified with a 5' EcoRI site and a 3' KpnI site and cloned into pBS/SK⁺ (Stratagene). pBS/MHV E was used as template in PCR mutagenesis to introduce a BamHI site at the junction of the MHV E hydrophobic and tail domains to generate pBS/MHV_{TMB}. QuikChange PCR mutagenesis was used to introduce three alanine replacements in pBS/MHV_{TMB}, and the mutations were verified by sequencing. The MHV-E tail mutant was digested with BamHI and KpnI. The VSV-G insert was cut from the pCAGGS/G-E plasmid using EcoRI and BamHI. The G fragment, MHV E fragment, and linear pCAGGS-MCS cut with EcoRI and KpnI were ligated together to generate pCAGGS/G-E_{MHV}.

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

Transient transfections. Fugene6 (Roche, Indianapolis, IN) was used to transiently transfect HeLa cells with cDNA according to the manufacturer's instructions. HeLa cells (2×10^5) were plated onto 35-mm dishes. One day after being plated, 50% confluent HeLa cells were transfected with a total of 2 μ g of DNA. Fugene6 was first diluted in Opti-MEM (Invitrogen) before 2 μ g plasmid DNA was added. All mutant cDNAs were analyzed by indirect immunofluorescence microscopy at 18 to 22 h posttransfection or at 24 h posttransfection for biochemical assays.

Antibodies. The rabbit polyclonal antibody to SARS-CoV E used in the indirect immunofluorescence assay was generated by immunizing rabbits with a conjugate of keyhole limpet hemocyanin and a synthetic peptide corresponding to the C-terminal 14 amino acids of SARS-CoV E, with an N-terminal cysteine added for coupling (CKNLNSSEGVDPDLLV), by Covance, Inc. (Denver, PA). The hybridoma for mouse anti-VSV-G monoclonal antibody was grown as described previously (24). Rabbit anti-IBV E antibody (10) and rabbit anti-golgin 160 antibody (14) were described previously. Affinity-purified rabbit-anti-VSV-G tail (anti-CTG) was also previously described (31). Rabbit anti-MHV E (21) was generously provided by P. Masters. Mouse anti-GM130 and mouse anti-p230 were from BD Biosciences (San Diego, CA). Mouse monoclonal anti-ERGIC53 antibody was a kind gift from H. P. Hauri (Basel, Switzerland). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was from Amersham/GE Healthcare (Piscataway, NJ). Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG were from Invitrogen/Molecular Probes (Eugene, OR).

Indirect immunofluorescence microscopy. Between 18 and 20 h posttransfection, HeLa cells growing on glass coverslips were washed in phosphate-buffered saline (PBS) and fixed for 10 min in 3% paraformaldehyde in PBS. The fixative was quenched in PBS containing 10 mM glycine (PBS-gly). Cells were permeabilized for 10 min in 0.05% saponin in PBS-gly and then stained for 20 min with primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS-gly containing 0.05% saponin. Primary antibodies were used at the following dilutions: rabbit anti-SARS-CoV E (1:800), rabbit anti-IBV E (1:800), rabbit anti-MHV E (1:800), mouse anti-VSV-G hybridoma tissue culture supernatant (undiluted), mouse anti-GM130 (1:250), rabbit anti-golgin 160 (1:500), and mouse anti-p230 (1:500). Cells were then stained for 20 min with secondary antibodies in the following combination: Alexa Fluor 488-conjugated anti-rabbit (1:500) and Alexa Fluor 568-conjugated anti-mouse antibody (1:1,000). Cells were washed twice with PBS-gly after each step for a total of 5 min and mounted in glycerol containing 0.1 M N-propylgallate. Images were obtained with an Axioscop fluorescence microscope (Zeiss, Thornwood, NJ) equipped for epifluorescence using an Orca-03G charge-coupled-device camera (Hamamatsu, Japan) and iVision software (Biovision Technologies, Exton, PA).

Cell surface biotinylation. For biotinylation experiments, HeLa cells were seeded on poly-L-lysine-treated (2 mg/ml) tissue culture dishes to increase cell adherence. At 24 h posttransfection, HeLa cells were washed twice in PBS. Cells were incubated for 30 min on ice with 1 mg/ml biotin (EZ-Link sulfo-NHS-LC-biotin; ThermoScientific/Pierce, Rockford, IL) in PBS. Cells were washed twice and then incubated for 3 min in PBS containing 50 mM glycine to quench the

FIG. 1. The SARS-CoV E protein is targeted to the Golgi region. HeLa cells expressing SARS-CoV E were fixed, permeabilized, and double labeled with rabbit anti-SARS-CoV E and mouse anti-GM130, mouse anti-ERGIC53, or mouse anti-p230 and appropriate secondary antibodies. Merged images show SARS-CoV E protein in green and the Golgi complex marker (GM130, ERGIC53, and p230) in red. Bar = 10 μ m; white box is enlarged in right panel. Bar in enlargements = 1.5 μ m.

biotin. Cells were lysed in biotinylation lysis buffer (10 mM HEPES [pH 7.2], 0.2% NP-40, 150 mM NaCl) containing protease inhibitor cocktail (Sigma, St. Louis, MO) at 0°C for 10 min. Lysates were clarified at 16,000 $\times$ g for 10 min at 4°C. Washed streptavidin agarose resin (ThermoScientific/Pierce) was added to the lysate to bind surface-biotinylated proteins for 1 h at room temperature with rotation. Streptavidin agarose resin was pelleted at low speed, 4,000 $\times$ g, for 2 min. The lysate supernatant was removed; 10 percent was used for quantifying nonbiotinylated protein. Streptavidin beads were washed twice in lysis buffer, and biotinylated proteins were eluted at 100°C in 1 $\times$ sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.025% bromophenol blue) and 5% 2-mercaptoethanol. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for Western blotting. Membranes were blocked for 30 min in 5% nonfat dry milk made with Tris-buffered saline containing Tween (TBST) (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 0.05% Tween 20) and then washed three times in TBST. Membranes were incubated overnight at 4°C in rabbit anti-VSV-G antibody diluted 1:5,000 in 3% BSA and 0.02% NaN₃ in TBST. Membranes were washed three times in TBST and then incubated for 1 h in HRP-conjugated anti-rabbit IgG diluted 1:10,000 in 5% nonfat dry milk made with TBST. Membranes were washed three times in TBST and then treated with HyGlo chemiluminescence reagent (Denville Scientific, Metuchen, NJ) according to the manufacturer's instructions. Membranes were analyzed using a Versa Doc imaging station (Bio-Rad, Hercules, CA) and quantified using Quantity One software (Bio-Rad).

Metabolic labeling and glycosidase digestion. At 24 h posttransfection, HeLa cells were pulse-labeled and chased as described previously (34). Briefly, HeLa cells were starved in methionine- and cysteine-free DMEM for 20 min, labeled for 20 min with 50 μ Ci of Expre³⁵S³⁵S (Perkin Elmer, Waltham, MA) [³⁵S]methionine-cysteine per dish in methionine- and cysteine-free DMEM, and then chased for various times in DMEM–10% FBS. The cells were washed in PBS and then lysed in detergent solution (50 mM Tris-HCl [pH 8.0], 1% NP-40, 0.4% deoxycholate [DOC], 62.5 mM EDTA) containing protease inhibitor cocktail. The lysates were clarified, SDS was added to a final concentration of 0.2%, and proteins were immunoprecipitated with anti-VSV-G antibody overnight at 4°C. Immune complexes were collected with washed *Staphylococcus aureus* Pansorbin cells (Calbiochem, San Diego, CA) and washed 3 times in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 50 mM Tris-HCl [pH 8.0], 1% DOC, 150 mM NaCl, 1% Triton X-100). Samples were eluted in 1% SDS in 50 mM Tris (pH 6.8) at 100°C and digested with 0.1 mU/ μ l endoglycosidase H (endo H)

(New England BioLabs, Beverly, MA) overnight at 37°C after 2-fold dilution with 150 mM sodium citrate (pH 5.5). Concentrated sample buffer was added to 1 $\times$, and samples were subjected to 10% SDS-PAGE. Labeled proteins were visualized by using a Molecular Imager FX phosphorimager (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

Secondary structure predictions. Secondary structure prediction software was used to analyze several CoV E tail sequences. The following programs were used: Self-Optimized Prediction Method with Alignment (SOPMA; http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html), Jpred secondary structure prediction server powered by the Jnet algorithm (<http://www.compbio.dundee.ac.uk/jpred/>), and Protein Homology/analogy Recognition Engine (Phyre; <http://www.sbg.bio.ic.ac.uk/~phyre/>). The Phyre consensus, based on psipred, jnet, and sspro, is shown in Fig. 8A.

RESULTS

The SARS-CoV E protein is targeted to the Golgi region. Other groups have examined the expression of epitope-tagged SARS-CoV E protein and reported its localization in the ER, ERGIC, or Golgi complex (1, 27, 30, 35, 51). To investigate the targeting of untagged SARS-CoV E protein to Golgi membranes, we expressed SARS-CoV E from cDNA in HeLa cells and examined its localization by indirect immunofluorescence microscopy. Double labeling with antibodies for endogenous proteins showed that SARS-CoV E protein was localized to the Golgi region (Fig. 1). The distribution of SARS-CoV E protein overlapped most extensively with that of the *cis*-Golgi marker, GM130. E protein overlap was less extensive with the ERGIC marker, ERGIC53, and the *trans*-Golgi marker, p230. We conclude that the SARS-CoV E protein is targeted to the *cis*-Golgi region when exogenously expressed in HeLa cells.

The cytoplasmic tail of the SARS-CoV E protein contains Golgi complex-targeting information. Since the C-terminal cytoplasmic tail of IBV E contains Golgi complex-targeting in-

FIG. 2. The cytoplasmic tail of the SARS-CoV E protein contains Golgi complex-targeting information. (A) HeLa cells expressing SARS-CoV-E, VSV-G, or chimeric G-E were fixed, permeabilized, and double labeled with either rabbit anti-SARS-CoV E and mouse anti-GM130 antibodies (E and G-E panels) or mouse anti-VSV-G and rabbit anti-golgin 160 (VSV-G panel) and appropriate secondary antibodies. HD, hydrophobic domain. Bar = 10 μ m. (B) HeLa cells expressing G-E were fixed, permeabilized, and double labeled with rabbit anti-SARS-CoV E and mouse anti-GM130, mouse anti-ERGIC53, or mouse anti-p230 and appropriate secondary antibodies. Merged images show G-E protein in green and the Golgi complex marker (GM130, ERGIC53, and p230) in red. Bar = 10 μ m; white box is enlarged in right panel. Bar in enlargements = 1.5 μ m.

formation (8), we first tested the tail portion of the SARS-CoV E protein. We constructed a chimeric protein, G-E, which includes the vesicular stomatitis virus glycoprotein (VSV-G) ectodomain and hydrophobic domains and the SARS-CoV E cytoplasmic tail (Fig. 2A). VSV-G is a well-characterized viral glycoprotein that traffics rapidly through the secretory pathway and localizes to the plasma membrane. HeLa cells expressing

the chimeric G-E protein were examined by indirect immunofluorescence microscopy. G-E was localized to the Golgi region, similar to the full-length SARS-CoV E protein (Fig. 2A). Further analysis showed the greatest overlap with GM130 (Fig. 2B), similar to the full-length protein. This suggests that the cytoplasmic tail of SARS-CoV E contains Golgi complex targeting information.

FIG. 3. A predicted structural motif in the SARS-CoV E tail is important for Golgi complex targeting. (A) Chimeric G-E mutants contain the N-terminal VSV-G sequence and hydrophobic domain (HD) fused to the C-terminal SARS-CoV E cytoplasmic tail sequence. The amino acid residues predicted to form beta-strands are underlined, alanine replacements within the predicted beta-strands are indicated by the letter "A" in boldface, and the highly conserved proline residue is indicated by the arrow. (B) HeLa cells expressing G-E and mutant G-E proteins (G-E_{PA}, G-E_{Ala4}, and G-E_{Ala6}) were fixed, permeabilized, and labeled with mouse anti-VSV-G.

A predicted structural motif in the SARS-CoV E tail is important for Golgi complex targeting. To further understand how the SARS-CoV E tail sequence contributes in Golgi complex targeting, we considered possible secondary structure. Several secondary structure prediction programs predicted two beta-strands within the SARS-CoV E cytoplasmic tail sequence (Fig. 3A). A proline residue, highly conserved in all CoV E proteins, is present between the predicted beta-strands in the SARS-CoV E protein tail. The predicted beta-strands fit the criteria for forming a beta-hairpin, which is a simple structural motif with two beta-strands linked by a short loop of two to five amino acid residues (19). The antiparallel beta-strands form hydrogen bonds to stabilize the hairpin structure.

To determine if the predicted beta-strands or the conserved proline residue were involved in Golgi complex targeting, we made alanine replacement mutations in the G-E chimeric protein. Proline 54 was mutated to alanine (G-E_{PA}), or residues in one or both beta-strands were replaced with alanines (G-E_{Ala4}

FIG. 4. Steady-state surface levels of protein confirm the importance of the predicted beta-hairpin. HeLa cells expressing VSV-G, G-E, G-E_{PA}, G-E_{Ala4}, and G-E_{Ala6} were surface biotinylated using a membrane-impermeant biotin reagent at 24 h posttransfection. Biotinylated proteins were collected from lysate using streptavidin agarose resin, washed, and eluted in sample buffer. The supernatant (10%) was collected to assess nonbiotinylated protein. After SDS-PAGE, proteins were detected with Western blotting with anti-VSV-G. S, surface protein; I, internal protein. Quantification of results from at least four independent experiments $\pm$ standard errors of the means are shown.

and G-E_{Ala6}), as indicated in Fig. 3A. To verify disruption of the predicted beta-strands, the Ala4 and Ala6 E tails were analyzed by secondary structure prediction programs and confirmed to no longer contain the second (Ala4) or both (Ala6) predicted beta-strands. As determined by indirect immunofluorescence microscopy, the localization of each of the mutant chimeric proteins was disrupted, with significant plasma membrane staining (Fig. 3B). We concluded that disrupting the predicted beta-strands and mutating the conserved proline residue disrupts the cytoplasmic Golgi complex targeting signal.

Steady-state surface levels of protein confirm the importance of the predicted beta-hairpin. To confirm the immunofluorescence assay results, we quantified the steady-state surface levels of each chimeric protein. HeLa cells expressing VSV-G or each of the chimeric proteins were surface biotinylated using sulfo-NHS-LC-biotin. Biotinylated molecules were captured on streptavidin-conjugated beads, eluted, and separated by SDS-PAGE along with a portion of the nonbiotinylated supernatant (Fig. 4). After blotting with anti-VSV antibody, the percentage of each protein at the surface was quantified. Corroborating our immunofluorescence data, the G-E chimera was not appreciably surface biotinylated. The three chimeric G-E mutants were all detected at the surface, although at lower levels than VSV-G. G-E_{Ala6} demonstrated a slightly higher surface level than G-E_{Ala4} and G-E_{PA}, although this was not statistically significant. Interestingly, G-E_{PA} migrates slightly faster in SDS-PAGE than G-E or the other chimeric mutants. This may indicate a conformational change that affects SDS binding, since there is only one amino acid difference in this protein compared to the sequence of G-E.

Chimeric G-E mutants move through the medial-Golgi at different rates. We next analyzed N-linked oligosaccharide processing to assess the rate of intra-Golgi complex trafficking. As a glycoprotein moves through the medial-Golgi, N-linked

FIG. 5. Chimeric G-E mutants move through the *medial*-Golgi at different rates. At 20 h posttransfection, HeLa cells expressing VSV-G and the indicated G-E chimeras were labeled with [³⁵S]methionine-cysteine for 20 min and then chased for the indicated times. After lysis, proteins were immunoprecipitated, denatured, and digested with endo H. Endo H-sensitive (*) and -resistant (**) forms are indicated. After electrophoresis and phosphorimaging, the percentage of endo H-resistant protein was quantified. The averages of the results of at least three independent experiments $\pm$ standard errors of the means are shown.

sugars become resistant to digestion with endoglycosidase H (endo H). Using pulse-chase labeling, glycoprotein trafficking rates can be inferred from the amount of endo H-resistant protein at increasing chase times.

HeLa cells expressing VSV-G, G-E, G-E_{PA}, G-E_{Ala4}, and G-E_{Ala6} were pulse-labeled with [³⁵S]methionine-cysteine and chased in nonradioactive medium for various times. After immunoprecipitation, labeled samples were denatured and digested with endo H. After 30 min, approximately 80% of pulse-labeled VSV-G protein was endo H resistant (Fig. 5). G-E_{Ala6} showed a similar rate of acquisition of endo H resistance, suggesting that it moves through the *medial*-Golgi at a rate similar to that of VSV-G. After 30 min, approximately 75% of pulse-labeled G-E_{Ala4} was endo H resistant, indicating that slightly less protein had reached the *medial*-Golgi. In contrast, both G-E and G-E_{PA} were less extensively processed. Even

FIG. 6. Mutations in the conserved regions of the full-length SARS-CoV E do not disrupt Golgi complex targeting. HeLa cells expressing each full-length SARS-CoV E mutant were fixed, permeabilized, and double labeled with rabbit anti-SARS-E and mouse anti-GM130 and appropriate secondary antibodies.

after 120 min, only $\sim$ 60% of G-E_{PA} and $\sim$ 40% of G-E had acquired endo H resistance.

The rapid acquisition of endo H resistance by G-E_{Ala6} and G-E_{Ala4} contrasts with their significantly lower steady-state surface levels compared with that of VSV-G (Fig. 4). Either G-E_{Ala6} and G-E_{Ala4} are exported slowly from the late Golgi complex or they are internalized from the cell surface more rapidly than VSV-G.

Mutation of the predicted beta-hairpin in full-length E protein does not disrupt Golgi complex targeting. To test whether the predicted beta-strands and conserved proline residue were necessary for targeting of the full-length SARS-CoV E, the same mutations were introduced into this protein using site-directed mutagenesis. HeLa cells expressing wild-type SARS-CoV E or mutant SARS-CoV E_{PA}, SARS-CoV E_{Ala4}, or SARS-CoV E_{Ala6} were analyzed by indirect immunofluorescence microscopy (Fig. 6). Surprisingly, none of the mutations disrupted the targeting of full-length SARS-CoV E to the Golgi region. Proteins that are rapidly turned over could also demonstrate steady-state Golgi complex localization. To test the stability of the full-length SARS-CoV E mutants, protein half-lives were determined using a cycloheximide chase (data not shown). We found that at 6 h after cycloheximide treatment, the wild-type SARS-CoV E and full-length mutants remained at the Golgi region, suggesting that they are not rapidly degraded. Together, these results suggest that additional targeting information may be present elsewhere in the SARS-CoV E protein.

FIG. 7. Targeting of a reciprocal chimera to the Golgi region reveals additional targeting information. Reciprocal chimera E-G contains the N-terminal SARS-CoV E sequence and hydrophobic domain fused to the C-terminal VSV-G sequence. HeLa cells expressing E-G were fixed, permeabilized, and double labeled with rabbit anti-VSV-G tail and mouse anti-GM130, mouse anti-ERGIC53, or mouse anti-p230 and appropriate secondary antibodies. Merged images show E-G in green and the Golgi complex marker (GM130, ERGIC53, and p230) in red. Bar = 10 μ m; white box is enlarged in right panel. Bar in enlargements = 1 μ m.

The N terminus of SARS-CoV E protein contains targeting information. We made a reciprocal chimera, E-G, to test our hypothesis that the SARS-CoV E protein contains additional Golgi complex targeting information. The reciprocal chimera has the N-terminal and hydrophobic domains from SARS-CoV E fused to the C-terminal tail of VSV-G (Fig. 7). HeLa cells expressing E-G were analyzed by indirect immunofluorescence microscopy. Interestingly, the reciprocal chimera was also targeted to the Golgi region. HeLa cells expressing E-G were double labeled for E-G and Golgi complex markers (GM130, ERGIC53, and p230). The localization of E-G overlapped most with GM130, although not as extensively as that of the full-length E protein (Fig. 1). The additional Golgi complex targeting information in the N-terminal portion of SARS-CoV E protein probably explains the observation that mutations in the cytoplasmic tail did not disrupt targeting of the full-length protein.

Conservation of a predicted beta-hairpin structural motif within the cytoplasmic tail of other coronavirus E proteins. Sequence analysis of CoV E proteins indicates that the potential beta-hairpin motif surrounding the conserved proline residue in the cytoplasmic tail is present in beta and gamma CoV E proteins but not in those from alpha coronaviruses (Fig. 8A). To test whether the sequences contained within this motif were involved in Golgi complex targeting of other CoV E proteins, we examined VSV-G chimeric proteins containing the cytoplasmic tails of IBV-E (G-E_{IBV}) or MHV-E (G-E_{MHV}) with an intact predicted hairpin or with mutations to disrupt the struc-

ture (Fig. 8B). Both G-E_{IBV} and G-E_{MHV} were localized to the Golgi region, while the chimeric proteins with mutated tails (G-E_{IBV}Vala4 and G-E_{MHV}Vala3) were both localized to the plasma membrane (Fig. 8C). These results indicate that disrupting the potential beta-hairpin structural motif in other CoV E tails disrupts Golgi complex targeting and suggest that the Golgi complex targeting mechanism for MHV and IBV E proteins is conserved similarly to that of SARS-CoV E.

DISCUSSION

The SARS-CoV E protein plays an important role in virion assembly and morphogenesis and may alter host cell membrane permeability (16, 29). Several groups have shown that E and M are the basic components of virus-like particles (VLPs). The coexpression of E and M but not the expression of M protein alone forms VLPs that are morphologically similar to coronavirus virions (4, 5, 10, 15). Direct interaction of IBV E and M proteins has been reported (9, 28). The MHV E protein has been shown to induce the formation of tubular structures in ERGIC membranes (43). This observation suggests a potential function for coronavirus E protein in inducing membrane curvature or virion scission that contribute to virus budding. With all that is known about the function of CoV E proteins, many unanswered questions remain. A fundamental unanswered question is how the CoV E protein is targeted to the site of viral assembly. Identifying the targeting signal is a

FIG. 8. Mutations in the predicted beta-hairpin structural motif disrupt Golgi complex targeting of beta and gamma CoV E proteins. (A) A predicted beta-hairpin structural motif is conserved in the cytoplasmic tails of beta and gamma CoV envelope proteins but not those of alpha CoV. For each CoV E tail sequence, the first amino acid residue is indicated in parentheses, the highly conserved proline residue is indicated with an arrow, and the residues that form predicted beta-strands are underlined. (B) Residues within the predicted beta-strand downstream from the conserved proline were mutated to alanines for IBV E (G-E_{IBVVala4}) and MHV A59 E (G-E_{MHVVala3}). Alanine replacements are indicated by the letter “A” in boldface. (C) HeLa cells expressing the indicated chimeric proteins were fixed, permeabilized, and double labeled with mouse anti-VSV-G and rabbit anti-golgin 160. Bar = 10 μm.

prerequisite for understanding the mechanism of targeting and the role of E in virus assembly.

To address the issue of protein targeting, many groups have expressed epitope-tagged SARS-CoV structural proteins and analyzed the subcellular protein localization. Using a C-terminally tagged SARS-CoV E protein, Nal et al. reported that the SARS-CoV E protein localizes to perinuclear patches (35), colocalizing with ER markers. N-terminally hemagglutinin (HA)-tagged SARS-CoV E protein was assayed by Lopez et al. and found to overlap closely with the Golgi complex marker

giantin (30), while Liao et al. found that N-terminally FLAG-tagged SARS-CoV E showed only partial Golgi complex overlap (27). Alvarez et al. expressed C-terminally HA-tagged SARS-CoV E in the context of infection with recombinant virus and reported perinuclear localization (1).

Thus, there are inconsistencies in reports of SARS-CoV E protein localization. These inconsistencies may be a result of complications with epitope tags. In particular, N-terminal tags could interfere with the translocation of the N terminus into the ER lumen during biosynthesis. In addition, epitope tags on

either end could interfere with ER export and trafficking by blocking interactions with cellular machinery. In the current study, untagged SARS-CoV E protein was examined. Using indirect immunofluorescence microscopy, we showed that the SARS-CoV E protein is targeted to the Golgi region in HeLa cells and colocalizes most closely with GM130, which is a *cis*-Golgi protein (Fig. 1). Individually expressed CoV envelope proteins have been shown to move further in the secretory pathway than the assembly site (20). Localization of SARS-CoV E protein past the ERGIC suggests that interactions with other viral proteins are likely to fine tune its localization to the ERGIC.

To determine which portion of the SARS-CoV E protein was responsible for Golgi complex targeting, we constructed a chimeric G-E protein that contained the full-length SARS-CoV E tail sequence fused to the VSV-G ecto- and transmembrane domains. We found that the full-length SARS-CoV E tail was sufficient to redirect the VSV-G protein from the plasma membrane to Golgi membranes (Fig. 2). Mutating the highly conserved proline residue or residues critical for the predicted beta-hairpin secondary structure in the SARS-CoV E tail disrupted Golgi complex targeting of the chimeric protein (Fig. 3).

Disrupting both predicted beta-strands (G-E_{Ala6}) did not further disrupt targeting compared to that of a mutant with only one beta-strand disrupted (G-E_{Ala4}). Thus, either the hairpin or the primary sequence of the second beta-strand is the critical feature for G-E targeting. The proline residue appeared to contribute only modestly to the targeting of G-E. Based on the intra-Golgi complex trafficking rates (Fig. 5), we expected the surface levels of G-E_{Ala6} and G-E_{Ala4} to be similar to that of VSV-G (Fig. 4). Surprisingly, in spite of rapid trafficking through the Golgi complex, the steady-state surface levels of G-E_{Ala6} and G-E_{Ala4} were significantly lower than that of VSV-G. This suggests that Golgi complex export of these proteins may be slower than that of VSV-G or that they are efficiently endocytosed from the plasma membrane.

Surprisingly, a reciprocal chimera containing the SARS-CoV E N-terminal and hydrophobic domains with the VSV-G cytoplasmic tail was also targeted to the Golgi region (Fig. 7). These results suggest that additional targeting information exists within the SARS-CoV E protein. The presence of additional targeting information could explain the finding that cytoplasmic tail mutations within the full-length SARS-CoV E protein did not disrupt Golgi complex targeting (Fig. 6). Given the complication of at least two membrane topologies of SARS-CoV E, we did not attempt to dissect the targeting of E-G further. In the context of viral assembly, redundancy in Golgi complex targeting information may have evolved as a mechanism to ensure the proper subcellular localization of this important viral protein. Alternatively, different targeting mechanisms may be used for two different pools of SARS-CoV E protein, one that is incorporated into virions and one that is not.

Secondary structure predictions indicate that both beta and gamma but not alpha CoV E proteins contain the predicted beta-hairpin structure. We found that this predicted motif was also critical for Golgi complex localization of VSV-G chimeric proteins containing the cytoplasmic tails of MHV or IBV (Fig. 8C). These data suggest that a similar targeting mechanism

may be employed for both beta and gamma CoV E proteins. Evidence for functional similarities among E proteins from beta and gamma coronaviruses has been previously reported. Kuo et al. examined the extent to which heterologous E proteins could replace MHV E in the MHVΔE virus (21). They substituted E genes from other coronaviruses and showed that the E genes from SARS-CoV (formerly referred to as a group 2b CoV) and IBV (gamma CoV) were both able to functionally replace the MHV E gene. However, the E gene from the alpha coronavirus TGEV was unable to functionally replace the MHV E gene (21). These results support the hypothesis that E protein structure and not primary sequence dictates function.

Although the importance of protein sequence versus protein structure in targeting is unclear, we hypothesize that this potential motif promotes interaction with resident Golgi complex proteins. CoV E proteins contain cytoplasmic cysteine residues that are S palmitoylated (6, 8, 27). Depending on the particular E protein, two to four cysteine residues are located in the membrane-proximal portion of the cytoplasmic tail, just upstream of the predicted beta-hairpin motif. It is easy to imagine that the palmitate chains position the predicted beta-hairpin very close to Golgi membranes. We hypothesize that protein-protein interactions between the SARS-CoV E tail targeting motif and resident Golgi complex proteins would be promoted by proximity to the membrane. It will be interesting to screen for interactions of the SARS-CoV E Golgi complex-targeting motif with known Golgi complex-associated proteins.

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