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RAPID COMMUNICATION

Membrane Topology of Coronavirus E Protein

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Coronavirus small envelope protein E has two known biological functions: it plays a pivotal role in virus envelope formation, and the murine coronavirus E protein induces apoptosis in E protein-expressing cultured cells. The E protein is an integral membrane protein. Its C-terminal region extends cytoplasmically in the infected cell and in the virion toward the interior. The N-terminal two-thirds of the E protein is hydrophobic and lies buried within the membrane, but its orientation in the lipid membrane is not known. Immunofluorescent analyses of cells expressing biologically active murine coronavirus E protein with a hydrophilic short epitope tag at the N-terminus showed that the epitope tag was exposed cytoplasmically. Immunoprecipitation analyses of the purified microsomal membrane vesicles that contain the same tagged E protein revealed the N-terminal epitope tag outside the microsomal membrane vesicles. These analyses demonstrated that the epitope tag at the N-terminus of the E protein was exposed cytoplasmically. Our data were consistent with an E protein topology model, in which the N-terminal two-thirds of the transmembrane domain spans the lipid bilayer twice, exposing the C-terminal region to the cytoplasm or virion interior.

The enveloped virus, the coronavirus, contains a large positive-stranded RNA genome. Its envelope typically contains three virus-specific proteins: S, M, and E. The S protein forms 180- and 90-kDa peplomers that bind receptors on coronavirus-susceptible cells and induce cell fusion. M protein is a transmembrane protein, which is the most abundant glycoprotein in the virus and in its infected cells. The scant E protein is present only in minute amounts in infected cells as well as in the viral envelope (9, 10, 17, 20). The viral envelope, embedded with S, M, and E, surrounds a helical nucleocapsid consisting of just viral RNA and N protein (16).

E protein has two major biological functions. Mouse hepatitis virus (MHV) E protein can induce apoptosis in E-protein-expressing cells (1). Overexpression of Bcl-2 oncoprotein suppresses MHV E-protein-induced apoptosis, indicating that initiation of the apoptotic pathway begins upstream of Bcl-2 (1). Another major biological function of E protein is its association with coronavirus envelope formation. Coexpression of coronavirus M protein and E protein results in the production of virus-like particles (VLPs), while expression of M protein alone does not produce VLPs (2–4, 11, 18). Furthermore, expression of E protein alone results in production and release of membrane vesicles containing E protein (E protein vesicles) (4, 11). These E protein vesicles are also released from MHV-infected cells (11). E protein shapes coronavirus; MHV mutants encoding mutated E protein are morphologically aberrant compared to wild-type (wt) MHV (7). In spite of affecting morphology, the E protein is not involved directly in packaging coronavirus RNA, because interaction of the nucleocapsid with M protein, which represents the packaging process, occurs without E protein (12).

Added to these two major biological functions is the observation that smooth, convoluted membranes, apparently derived from pre-Golgi membranes, appear in E-protein-expressing cells (14). Similar structures are hallmarks of MHV-infected cells (5). This unique membrane structure in the MHV-infected cell, which is apparently E-protein induced, likely will be shown to be significant biologically.

E protein is an integral membrane protein whose highly hydrophobic N-terminal two-thirds region is a transmembrane domain. Raamsman *et al.* (14) and Corse and Machamer (4) characterized the E proteins of MHV and infectious bronchitis virus (IBV), respectively. Both of those convincing studies showed that E protein localizes in intracellular membranes with its C-terminal region extending into the cytoplasm or the viral interior. Raamsman *et al.* (14) treated MHV particles with proteinase K in

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the absence of detergent and found that this treatment did not alter the molecular weight of E protein. The authors stated that no part of the E protein is detectably exposed on the virion exterior, and they speculated that the N-terminus of E protein may be oriented on either side of the membrane (14). Corse and Machamer showed that after treatment of E-protein-expressing cells with Triton X-100, IBV E protein is accessible to antibodies that specifically recognize the E protein N-terminal region, but E protein is not accessible after treatment with digitonin, which selectively permeabilizes the plasma membrane, leaving intracellular membranes intact (4). This observation led the authors to propose that IBV E protein possesses a luminal N-terminus, a cytoplasmic C-terminus, and a single transmembrane domain (4). Their data, however, can be interpreted differently; if the entire N-terminus of IBV E protein is buried within the intracellular membrane, then those antibodies that specifically recognize the N-terminal region would not recognize E protein after digitonin treatment of the E-protein-expressing cells. Perhaps no part of the IBV E protein N-terminus is exposed luminally or outside of virions, and possibly the transmembrane domain spans the membrane twice. Orientation of the N-terminus of the coronavirus E protein in the membrane is an open question.

Our study looked at whether the N-terminus of the MHV E protein is oriented cytoplasmically or luminally. We began by attempting to produce three antibodies that separately could recognize the entire region, the N-terminus, and the C-terminus of the A59 strain of the MHV E protein. Three cDNAs, one corresponding to the entire 83 residues, one to the N-terminal 27 residues, and one to the C-terminal 31 residues, were independently cloned into a GST gene fusion vector, pGEX-3X (Pharmacia). The GST fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using GST purification modules (Pharmacia). Of the three fusion proteins, only the fusion protein containing the C-terminus was isolated in a soluble form. Antibodies (anti-EC antibody) against the E protein C-terminal region were elicited by injection of the fusion protein into a rabbit.

For estimation of the orientation of the N-terminus of E protein in the membrane, we used a Sindbis virus expression vector, pSINRep5 (Invitrogen, San Diego, CA), to clone and express E proteins with a hydrophilic FLAG epitope tag (D-Y-K-D-D-D-D-K). Two Sindbis virus expression vectors, one expressing E protein with a FLAG epitope tag at the N-terminus (N-FLAG E protein) and another expressing E protein with the FLAG epitope tag at the C-terminus (C-FLAG E protein), were constructed. Three Sindbis virus pseudovirions, SinE, expressing wt E protein (11), SinE-N-FLAG, expressing N-FLAG E protein, and SinE-C-FLAG, expressing C-FLAG E protein, were prepared as described previously (11). DBT cells were independently infected with the pseudovirions SinE,

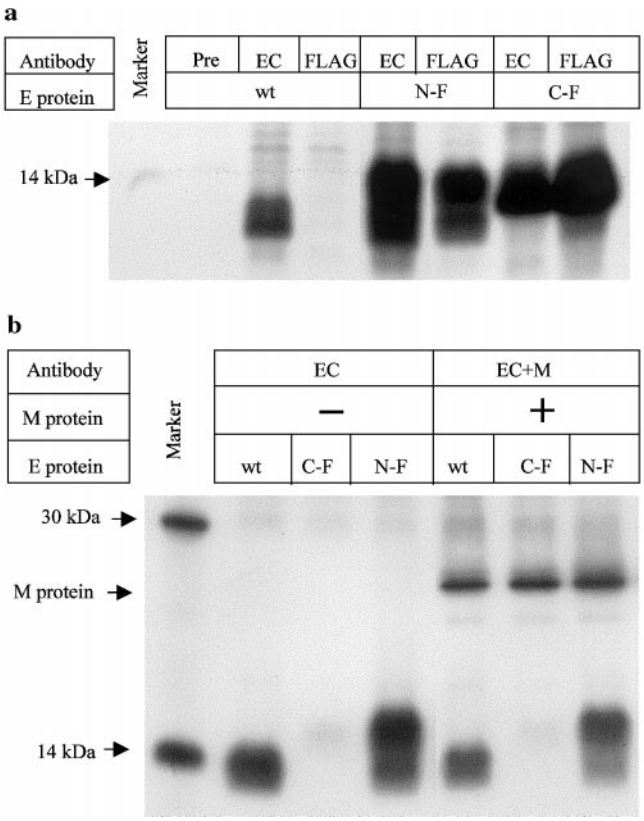

FIG. 1. Intracellular accumulation of wt E protein, N-FLAG E protein, and C-FLAG E protein in expressing cells (a) and E protein vesicles and VLPs in the supernatants of expressing cells (b). (a) DBT cells were independently infected with SinE pseudovirions (wt), SinE-N-FLAG pseudovirions (N-F), or SinE-C-FLAG pseudovirions (C-F). Cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i., and intracellular proteins were extracted. Cell extracts were incubated with anti-EC antibody (EC), preimmune serum (Pre), or anti-FLAG antibody (FLAG). Immunoprecipitated proteins were analyzed by SDS–PAGE. Marker, ^{14}C-labeled protein size marker. (b) DBT cells expressing wt E protein (wt), C-FLAG E protein (C-F), N-FLAG E protein (N-F), wt E protein and M protein, C-FLAG E protein and M protein, or N-FLAG E protein and M protein were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i. Culture fluid was harvested at 9 h p.i., and E protein vesicles and VLPs were partially purified by discontinuous sucrose-gradient centrifugation. Partially purified E protein vesicles and VLPs were disrupted with detergent and then immunoprecipitated with anti-EC antibody or a mixture of anti-EC antibody and anti-M-protein monoclonal antibody J1.3. Marker, ^{14}C-labeled protein size marker.

SinE-C-FLAG, or SinE-N-FLAG and were radiolabeled with Tran^{35}S-label from 5 to 9 h postinfection (p.i.), and the intracellular proteins were extracted as described previously (11). Radioimmunoprecipitation analysis revealed that anti-EC antibody immunoprecipitated all three E proteins, while anti-FLAG M2 monoclonal antibody (Sigma) (anti-FLAG antibody) immunoprecipitated C-FLAG E protein and N-FLAG E protein (Fig. 1a). These data demonstrated that a signal peptidase did not remove the FLAG epitope tag added at the N-terminus of the E protein during translation. Raamsman *et al.* also demonstrated that MHV E protein integrates into the membrane without cleavage of a signal sequence (14).

N-FLAG E protein appeared as two major signals, both of which were recognized by anti-FLAG antibody. The faster migrating band of N-FLAG E protein comigrated with wt E protein, while C-FLAG E protein migrated more slowly than wt E protein. Currently it is unclear why N-FLAG E protein appeared as two major signals.

To determine whether E protein with the FLAG epitope tag retained its biological activity, we examined whether tagged E protein can produce E protein vesicles (11). DBT cells were independently infected with SinE, SinE-C-FLAG, or SinE-N-FLAG pseudovirions. The infected cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i., and the culture fluid was harvested at 9 h p.i. and briefly centrifuged to remove cell debris. Culture media were applied onto a discontinuous sucrose gradient consisting of 20 and 60% sucrose in NTE buffer [100 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 1 mM EDTA]. After centrifugation at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor, the interface between 20 and 60% sucrose was collected and an equal amount of 2× lysis buffer [1× lysis buffer: 50 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride] was added. Production of E protein vesicles was examined by using immunoprecipitation analysis with anti-EC antibody (Fig. 1b). Large amounts of wt E protein and N-FLAG E protein and a much smaller amount of C-FLAG E protein were released into the culture fluid. The low radioactivity of C-FLAG E protein was not due to the poor binding of antibody to this protein because direct SDS–PAGE analysis of the sucrose fraction between 20 and 60% sucrose also showed poor production of C-FLAG E protein vesicles (data not shown). These results were observed in repeated experiments and demonstrated that expression of both tagged E proteins resulted in the production of the E protein vesicles.

To determine whether tagged E proteins can produce VLPs, three sets of DBT cells all infected with SinM pseudovirions that express MHV M protein (11) were doubly infected with SinE, SinE-C-FLAG, or SinE-N-FLAG pseudovirions. Cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i.. Culture fluid was harvested at 9 h p.i., and VLPs were purified and solubilized with lysis buffer as described above. Production of VLP was detected using a mixture of anti-M-protein monoclonal antibody J1.3 (8) and anti-EC antibody (Fig. 1b) for immunoprecipitation analyses. In all cases, similar amounts of M protein were detected in the culture fluid, demonstrating that both tagged E proteins functioned to produce VLPs when they were coexpressed with M protein. The amounts of E protein present in the samples were similar to those found in the supernatants of cells expressing E protein only. As demonstrated previously (11), production of VLP was not detected in the cells infected with SinM pseudovirions alone in repeated experiments (data not shown). These data revealed that the N-FLAG E and C-FLAG E proteins, like the wt, contained activities for production of VLPs and E protein vesicles. The topology of the tagged E proteins retaining MHV envelope formation biological activity and the topology of wt E protein in the membrane are unlikely to be drastically different. We assumed that the topology of tagged E proteins in membrane was the same as that of wt E protein and thus ventured into extended experiments.

The N-terminal two-thirds of wt E protein is highly hydrophobic, but according to hydropathy profile analysis, the N-terminus of the N-FLAG E protein was hydrophilic because of the added hydrophilic FLAG epitope tag (data not shown). Although the entirety of the N-terminal two-thirds of wt E protein may be buried within the membrane, we speculated that the FLAG epitope tag at the N-terminus of N-FLAG E protein might protrude from the lipid membrane due to its hydrophilic nature. If so, identification of the FLAG epitope tag in the membrane would suggest the localization of the N-terminus of E protein within the membrane.

We used immunofluorescent microscopy analysis to estimate the orientation of the N-terminus of MHV E protein in the membrane. BHK-21 cells expressing N-FLAG E or C-FLAG E protein were independently treated with Triton X-100 or digitonin. Triton X-100 treatment permeabilizes both the plasma membrane and the intracellular membranes, while digitonin treatment at low concentrations permeabilizes the plasma membrane, leaving intracellular membranes intact (13). Expressed E protein is known to be found at intracellular membranes (4, 14). Accordingly, if the FLAG epitope is exposed to the cytoplasmic side in N-FLAG-expressing cells, then anti-FLAG antibody should recognize the FLAG epitope tag after digitonin treatment of the cells. If the FLAG epitope is exposed luminally or is buried within the membrane, then anti-FLAG antibody should not recognize the FLAG epitope tag after digitonin treatment. MHV M protein and C-FLAG E protein served as controls because the topology of MHV M protein is well established (15) and because the C-terminus of E protein juts out into the cytoplasm (14). Triton X-100 treatment of the cells was used to ensure that the conditions for immunofluorescent methods were appropriate. BHK-21 cells were independently infected with SinM, SinE-C-FLAG, or SinE-N-FLAG pseudovirions. At 5.5 h p.i., cells were treated with Triton X-100 or digitonin according to the methods described previously (4). Immunofluorescent analysis using an anti-M-protein monoclonal antibody, J1.3, that recognizes the luminal N-terminal domain of M protein (6) as a first antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody as a second antibody detected M protein after treatment with Triton X-100, but not after digitonin treatment (Fig. 2). That these data agreed with the well-established pattern of MHV M protein orientation in the membrane and with previous stud-

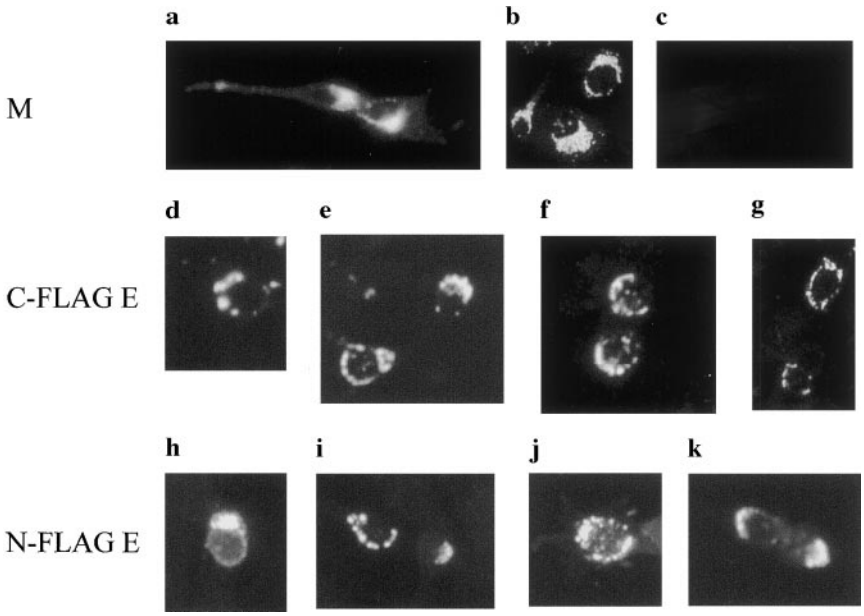

FIG. 2. Cytoplasmic exposure of the FLAG epitope of FLAG E protein in expressing cells. BHK-21 cells expressing M protein, C-FLAG E protein, or N-FLAG E protein were permeabilized by using either Triton X-100 (a, b, d, e, h, i) or digitonin (c, f, g, j, k). Immunofluorescent analyses were performed using anti-M-protein monoclonal antibody J1.3, which recognizes the luminal N-terminal domain of M protein as a first antibody and FITC-conjugated anti-mouse IgG antibody as a second antibody (a, b, c). FITC-conjugated anti-FLAG antibody was used for detection of FLAG epitope of C-FLAG E protein (d–g) and N-FLAG E protein (h–k).

ies by others who took a similar experimental approach (4, 14) justified our experimental conditions with the digitonin and Triton X-100 treatments. FITC-conjugated anti-FLAG antibody recognized the FLAG epitope of C-FLAG E protein after treatment of C-FLAG E-protein-expressing cells with Triton X-100 or digitonin, supporting an earlier finding of cytoplasmic exposure of the C-terminus of E protein (4, 14). Anti-FLAG antibody also recognized N-FLAG E protein when cells expressing N-FLAG E protein were treated with Triton X-100 and digitonin (Fig. 2), pointing to a cytoplasmic location for the FLAG epitope of N-FLAG E protein. Both tagged E proteins usually showed a punctate pattern of staining; this pattern of E protein staining has been described by others (14). Also, no significant difference in the intensity of staining was observed between digitonin and Triton X-100 treatments for the two E proteins.

We went on to further examine the membrane topology of E protein using a biochemical approach. The open reading frames of the M protein of MHV-JHM, the N-FLAG E protein, and the C-FLAG E protein were each inserted downstream of the T7 promoter in separate constructs. Capped RNA transcripts transcribed from the linearized plasmid DNA using T7 polymerase were used to independently translate M protein, N-FLAG E protein, and C-FLAG E protein in rabbit reticulocyte lysate in the presence of canine pancreatic microsomal membrane vesicles (19). In infected cells, the N-terminal domain of M protein is located on the luminal side of the intracellular membrane and translocates inside of microsomal vesicles (15). The C-terminus of the E protein is exposed to the cytoplasm (4, 14); hence, the FLAG epitope in the C-FLAG E protein should be exposed to the outside of microsomal vesicles. Because immunofluorescent analysis indicated that the FLAG epitope in N-FLAG E protein was exposed on the cytoplasmic side, we expected that the FLAG epitope in N-FLAG E protein would be exposed outside microsomal vesicles too. If the FLAG epitope of both tagged E proteins is exposed to the outside of microsomal vesicles, then anti-FLAG antibody should recognize the tagged E proteins that are integrated within the intact microsomal vesicles. In contrast, monoclonal antibody J1.3, which recognizes the luminal N-terminal domain of M protein, should not recognize M protein that is integrated within the intact microsomal vesicles.

Whether the FLAG epitope in N-FLAG E protein was exposed to the outside of microsomal vesicles was determined using radioimmunoprecipitation. For this analysis, microsomal vesicles containing translocated proteins were purified by way of flotation centrifugation (11). Sucrose crystals were added to *in vitro* translation samples at a final concentration of 67%, and the samples were placed in centrifuge tubes and then overlaid with 4 ml of 65% sucrose and 3 ml of 10% sucrose solutions, both in NTE buffer. The gradients were centrifuged at 35,000 rpm for 18 h at 4°C using a Beckman SW41 rotor, and the interface between 65 and 10% sucrose was collected. Free proteins remain in the bottom of the gradient, while membrane proteins associated with microsomal vesicles move to the interface between 65 and 10% sucrose (11). Individual purified membrane vesicle

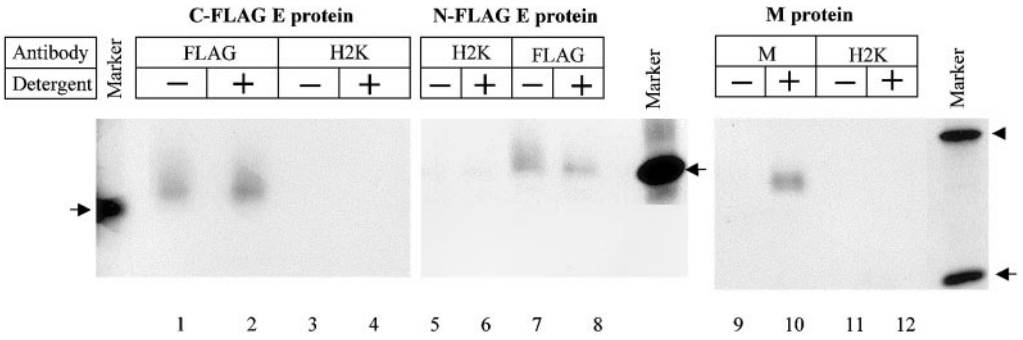

FIG. 3. Exposure of the FLAG epitope of N-FLAG E protein outside microsomal vesicles. Microsomal vesicles containing translocated C-FLAG E protein, N-FLAG E protein, or M protein were purified by using flotation centrifugation. Translocated proteins in the purified microsomal vesicles were immunoprecipitated with anti-FLAG antibody (FLAG), non-MHV antibody (H2K), or anti-M protein monoclonal antibody J1.3, which recognizes the luminal N-terminal domain of M protein (M) in the presence of lysis buffer or absence of detergent. Immunoprecipitated proteins were analyzed by SDS–PAGE. Marker, ^{14}C-labeled protein size marker. Arrow, 14-kDa size marker; arrowhead, 30-kDa size marker.

samples with tagged E or M proteins were split into four microcentrifuge tubes. We added equal amounts of NTE buffer to two of these tubes and to the remaining two tubes 2× lysis buffer. We expected that the microsomal vesicles would be solubilized in the presence of lysis buffer and would remain intact in the NTE buffer. For the samples of the microsomal vesicles containing the tagged E protein, anti-FLAG antibody was added to two tubes, one containing NTE buffer and the other containing lysis buffer. As a control, non-MHV monoclonal antibody $H2K^kD^k$ (anti-H2K antibody), which reacts with the major histocompatibility complex class I antigen, was added to the remaining two tubes. Similar experimental approaches were used for the samples of the microsomal vesicles containing M protein, except that J1.3 was used in place of anti-FLAG antibody. After incubation at 4°C for 2 h, protein A (Pansorbin cells, Calbiochem, La Jolla, CA), which had been washed with NTE buffer, was added to the samples. After subsequent incubation at 4°C for 2 h, the samples were washed with NTE buffer three times. The immunoprecipitated proteins were incubated at 37°C for 15 min in sample buffer and then analyzed on SDS–PAGE (Fig. 3). Anti-FLAG antibody immunoprecipitated C-FLAG E protein in the sample containing lysis buffer and in the sample lacking the detergent. These results were consistent with the known orientation of C-FLAG E protein in intracellular membranes (4, 14). Anti-H2K antibody did not precipitate C-FLAG E protein under either condition, eliminating the possibility that precipitation of C-FLAG E protein in the absence of detergent was an artifact of immunoprecipitation. Similarly, anti-FLAG antibody, but not anti-H2K antibody, immunoprecipitated N-FLAG E protein both in the presence and in the absence of the detergent, indicating that the FLAG epitope tag in N-FLAG E protein was also outside the microsomal vesicles. Anti-M protein J1.3 monoclonal antibody, which recognizes the N-terminal region of M protein, immunoprecipitated M protein in the presence of lysis buffer, but failed in the absence of lysis buffer, suggesting that the integrity of the majority of microsomal vesicles was maintained in the absence of lysis buffer. These immunoprecipitation analyses indicated that the FLAG epitope at the N-terminus of E protein was on the exterior of the microsomal vesicles. Both immunofluorescent and immunoprecipitation analyses indicated that the epitope tag at the N-terminus of the E protein was exposed cytoplasmically.

The present study looked at the topology of the MHV E protein in the cellular membrane. C-FLAG E protein, N-FLAG E protein, and wt E protein all had the biological activity needed to produce VLPs (Fig. 1). Consistent with previous studies of MHV E protein (14) and IBV E protein (4), immunofluorescent and radioimmunoprecipitation analyses of C-FLAG E protein revealed cytoplasmic exposure of the C-terminus region of E protein. Characterization of N-FLAG E protein indicated that the hydrophilic FLAG epitope at the N-terminus was also exposed cytoplasmically. Hydropathy profile analysis showed that the very N-terminus of N-FLAG E protein was hydrophilic, while the very N-terminus of wt E protein was hydrophobic (14). Due to the hydrophilic nature of the very N-terminus of wt E protein, this region is probably buried completely in the membrane. Based on the present data and those of others (4, 14, 20), we propose an E protein topology model in which E protein spans the membrane twice with its very N-terminus near the cytoplasmic side (Fig. 4).

We used N-FLAG E protein to estimate the location of the N-terminus of wt E protein in the membrane; hence, we could not exclude the possibility that wt E protein has a topology in the transmembrane domain different from that of N-FLAG E protein. For example, the N-terminus of wt E protein may adopt a different orientation and the transmembrane domain may span the membrane once. In that case, wt E protein spanning the membrane once and N-FLAG E protein spanning the membrane twice have the same biological function for VLP production (Fig. 1). VLP production probably requires M protein–E

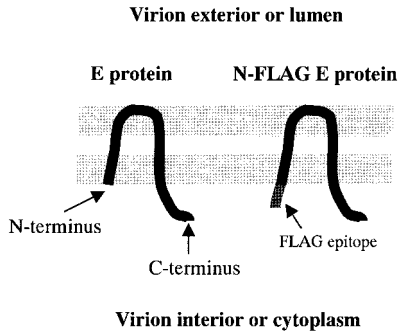

FIG. 4. Membrane topology models of wt E protein and N-FLAG E protein.

protein interaction as well as E protein–E protein interaction. It is difficult to imagine that these expected macromolecular interactions could be carried out equally efficiently by N-FLAG E protein and by a wt E protein that spans the membrane only once. It is more likely that wt E protein also spans the membrane twice with its very N-terminus near the cytoplasmic side (Fig. 4).

Raamsman *et al.* treated MHV particles with proteinase K in the absence of detergent and found that this treatment did not alter the molecular weight of E protein (14). The authors concluded that no part of E protein is detectably exposed on the virion exterior; their observation agrees with the E protein topology model presented here. Corse and Machamer reported that IBV E protein is accessible to antibodies that specifically recognize the E protein N-terminal region after treatment of E-protein-expressing cells with Triton X-100, but not after treatment with digitonin (4). Based on this observation, the authors proposed that IBV E protein has a luminal N-terminus (4). The same experimental data, however, would be obtained if the entire N-terminal region of IBV E protein was buried within the intracellular membrane. Corse and Machamer's data (4) are compatible with our E protein topology model (Fig. 4). Both MHV E protein and IBV E protein have the same biological functions in coronavirus envelope formation (4, 11, 18), so we speculate that IBV E protein membrane topology is also as illustrated in Fig. 4. Yu *et al.* also aimed to examine the membrane topology of MHV E protein (20). The authors prepared two anti-peptide antibodies, each of which was produced after immunization of goats with a peptide corresponding to the N-terminal 12 amino acids and with a peptide corresponding to amino acids 60–71. Consistent with the topology model of E protein shown in Fig. 4, neither antibody neutralized MHV infectivity in the presence of complement. They showed that rabbit antibodies against a recA–E-lacZ fusion protein neutralized MHV infectivity in the presence of complement (20), indicating that a portion of E protein is exposed on the surface of the virion envelope. The possible exposure of a part of E protein on the surface of the virion conflicts with the data presented by Raamsman *et al.* (14), who found that treatment of MHV particles with proteinase K in the absence of detergent did not alter the molecular weight of E protein.

The C-FLAG E, N-FLAG E, and wt E proteins all efficiently accumulated in cells that expressed them (Fig. 1A). We detected similar amounts of M protein in the culture fluid of cells coexpressing wt E and M proteins, in cells coexpressing N-FLAG E and M proteins, and in cells coexpressing C-FLAG E and M proteins, implying that the numbers of VLPs produced from all these cells were similar. Production and release of wt E protein vesicles and N-FLAG E protein vesicles were easily detectable in the supernatant of the expressing cells, while only a low level of C-FLAG E protein vesicles was released into the supernatant (Fig. 1b). Similarly, the amount of E protein detected in the supernatant of cells coexpressing wt E and M proteins and in cells coexpressing N-FLAG E and M proteins was significantly higher than that in cells coexpressing C-FLAG E and M proteins. Although we could not rule out the possibility that wt E protein and N-FLAG E protein were incorporated into VLPs more efficiently than C-FLAG E protein, a straightforward interpretation of these data is that the majority of E protein signals detected in the culture fluid of coexpressing cells was derived from E protein vesicles. The three E proteins that we looked at had different activities for the production of E protein vesicles, yet all three were equally active in producing VLPs (Fig. 1b); this lack of correlation suggests that E protein's activities for E protein vesicle formation and VLP formation are distinct.

The E protein vesicles and VLPs are difficult to separate on sucrose gradients (11); sucrose fractions that contain purified VLPs are likely to contain E protein vesicles as well (4, 11). The source of E protein detected by using SDS–PAGE analysis of a purified VLP sample might possibly be from copurified E protein. Accordingly, it is unclear whether E protein is a structural component of VLPs. Characterization of highly purified coronavirus particles, however, indicates that E protein is indeed a structural component of the envelope of coronavirus particles (9–11, 14, 17, 20). If VLPs lack E protein, then E protein exerts its effect on VLP production exclusively within the cells; in which case, what is the biological significance of E protein in the coronavirus particle? E protein may have some role(s) early in infection that affects coronavirus replication; conceivably, E protein could affect the viral uncoating process.

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