Biochemical evidence for the presence of mixed membrane topologies of the severe acute respiratory syndrome coronavirus envelope protein expressed in mammalian cells

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Abstract Coronavirus envelope (E) protein is a small integral membrane protein with multi-functions in virion assembly, morphogenesis and virus–host interaction. Different coronavirus E proteins share striking similarities in biochemical properties and biological functions, but seem to adopt distinct membrane topology. In this report, we study the membrane topology of the SARS-CoV E protein by immunofluorescent staining of cells differentially permeabilized with detergents and proteinase K protection assay. It was revealed that both the N- and C-termini of the SARS-CoV E protein are exposed to the cytoplasmic side of the membranes ($N_{cyto}C_{cyto}$). In contrast, parallel experiments showed that the E protein from infectious bronchitis virus (IBV) spanned the membranes once, with the N-terminus exposed luminally and the C-terminus exposed cytoplasmically ($N_{exo(lum)}$-C_{cyto}). Intriguingly, a minor proportion of the SARS-CoV E protein was found to be modified by N-linked glycosylation on Asn 66 and inserted into the membranes once with the C-terminus exposed to the luminal side. The presence of two distinct membrane topologies of the SARS-CoV E protein may provide a useful clue to the pathogenesis of SARS-CoV.
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Keywords: SARS-CoV; Envelope protein; Membrane topology

1. Introduction

The acute respiratory syndrome coronavirus (SARS-CoV) is an enveloped virus with a single strand, positive-sense RNA genome of 29.7 kb in length. Similar to other coronaviruses, its envelope typically contains three major structural proteins: spike (S), membrane (M) and envelope (E), with functions including recognition of target cells and fusion, interaction with other viral components, and involvement in virion assembly and budding.

All coronavirus E proteins are small integral membrane proteins with a long hydrophobic stretch from 25 to 30 residues [2,4,26]. They vary in size from 76 to 109 amino acids. The protein is associated with the ER and Golgi complex, and can be translocated to the surface of the infected cells [4,16,23,26,27]. Functionally, coronavirus E protein plays a pivotal role in viral morphogenesis, assembly and budding. Co-expression of mouse hepatitis virus (MHV) E and M was shown to result in the production of virus-like particles, roughly the same size and shape as virions [18,31]. The same phenomena were also observed in other coronaviruses, such as SARS-CoV and infectious bronchitis virus (IBV) [5,9]. When the E protein was expressed in mammalian cells on its own, the E-containing vesicles can be produced and released to the culture medium [5,19]. Furthermore, MHV and SARS-CoV E proteins were shown to induce apoptosis [1,32]. More recently, the SARS-CoV E protein was found to permeabilize bacterial cells as well as to form pores in artificial membranes [14,15,31]. It was also reported that MHV E protein could modify the membrane permeability in both *Escherichia coli* and mammalian cells [18].

Despite the similarities in biochemical properties and biological functions, the E protein from different coronaviruses shares very low homology in the primary amino acid sequence [18]. Another striking feature is that different coronavirus E proteins assume distinct membrane topologies. Based on cell surface staining with a C-terminus-specific antibody, transmissible gastroenteritis virus (TGEV) E protein was demonstrated to have a $C_{exo}N_{cyto}$ membrane topology [8]. The IBV E protein is accessible to a C-terminus-specific antibody in the presence of either digitonin or Triton X-100 but is accessible to an N-terminus-specific antibody only in the presence of Triton X-100, suggesting that IBV E protein may possess an $N_{exo}C_{cyto}$ topology [4]. Immunofluorescence analysis of cells expressing MHV E protein demonstrated that its transmembrane domain spans the lipid bilayer twice, indicating that both the N- and C-terminal regions are exposed to the cytoplasm ($N_{cyto}C_{cyto}$) [20,26]. The putative transmembrane domain of SARS-CoV E protein has been reported to adopt a highly unusual topology, consisting of a very short transmembrane helical hairpin [2], with Phe23 as the center of inversion of the hairpin. This residue was suggested to be located at the center of the bilayer [2], although a more recent report places this residue adjacent to the polar head groups of the lipids [11]. However, in silico [28] and in vitro NMR and infrared dichroic data (unpublished results) are in contrast with this model and are consistent with a normal transmembrane helix. In addition, the membrane orientation of the protein is yet to be determined.

In this report, the membrane topology of SARS-CoV E protein was determined by immunofluorescent staining of cells differentially permeabilized with detergents and by limited proteinase K digestion of microsomal membranes. These studies have revealed that both the N- and C-terminal regions of

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the protein are located in the cytoplasm ($N_{cyto}C_{cyto}$). However, a minor proportion of the protein is found to be posttranslationally modified by N-linked glycosylation on the asparagine 66 residue. It suggests that a certain proportion of the protein may also adopt either an $N_{cyto}C_{exo}$ or $N_{exo}C_{exo}$ topology. This is the first coronavirus E protein with two distinct membrane topologies.

2. Material and methods

2.1. Polymerase chain reaction and site-directed mutagenesis

Amplification of respective template DNAs with appropriate primers was performed with Pfu DNA polymerase (Strategene) with 2 mM $MgCl_2$. The PCR conditions were 35 cycles of 94 °C for 45 s, 46–58 °C for 45 s, and 72 °C for 30 s. The annealing temperature and extension time were subjected to adjustments according to the melting temperatures of the primers used and the lengths of the PCR fragments synthesized.

Site-directed mutagenesis was carried out with two rounds of PCR and two pairs of primers.

2.2. Transient expression of SARS-CoV sequence in mammalian cells

HeLa cells were grown at 37 °C in 5% CO_2 and maintained in Glasgow's modified Eagle's medium supplemented with 10% fetal calf serum. SARS-CoV E and mutants were placed under the control of a T7 promoter and transiently expressed in mammalian cells using a vaccinia virus/T7 expression system described by Fuerst et al. [7]. Briefly, 60–80% confluent monolayers of HeLa cells grown on dishes (Falcon) were infected with 10 plaque-forming units/cell of a recombinant vaccinia virus (vTF7-3) that expresses T7 RNA polymerase. Two hours later, cells were transfected with plasmid DNA mixed with Effectene according to the instructions of the manufacturer (Qiagen). Cells were harvested at 12–24 h post-transfection.

2.3. Western blot analysis

Total proteins extracted from HeLa cells were lysed with 2× SDS loading buffer in the presence of 200 mM DTT plus 10 mM of iodoacetamide and subjected to SDS–PAGE. Proteins were transferred to PVDF membrane (Stratagene) and blocked overnight at 4 °C in blocking buffer containing 5% fat free milk powder in PBST buffer pH 7.5 (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20). The membrane was incubated with 1:2000 diluted primary antibodies in blocking buffer for 2 h at room temperature. After washing three times with PBST, the membrane was incubated with 1:2000 diluted anti-mouse or anti-rabbit IgG antibodies conjugated with horseradish peroxidase (DAKO) in blocking buffer for 1 h at room temperature. After washing for three times with PBST, the polypeptides were detected with a chemiluminescence detection kit (ECL, Amersham Biosciences) according to the instructions of the manufacturer.

2.4. Indirect immunofluorescence

HeLa cells expressing Flag-tagged SARS-CoV or IBV E protein were fixed with 4% paraformaldehyde for 10 min at 16 h post-transfection, washed three times with 1× PBS, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and washed three times with 1× PBS. Monoclonal anti-Flag antibody was used to detect E protein and mutants. To selectively permeabilize the plasma membrane, HeLa cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 5 μg of digitonin per ml for 5 min at room temperature. Staining was as described above. Images were collected with a META 510 confocal laser-scanning microscope (Zeiss).

2.5. Glycosylation study of E protein

HeLa cells expressing E protein were treated with glycoprotein denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) at 100 °C for 10 min. The denatured proteins were incubated with 1 μl glycosidase PNGaseF (Research Biolabs) in the reaction buffer (50 mM sodium phosphate, pH 7.5) supplemented with 1% NP40 at 37 °C for 1 h. The deglycosylated proteins were analyzed by western blot.

2.6. Subcellular fractionation

HeLa cells were resuspended in hypotonic buffer (1 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 15 mM NaCl) containing 2 μg of leupeptin per ml and 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and broken by 20 strokes with a Dounce cell homogenizer. Cell debris and nuclei were removed by centrifugation at $1500 \times g$ for 10 min at 4 °C. The cytosol fraction and membrane fraction (postnuclear fraction) were separated by ultracentrifugation through a 6% sucrose cushion at $150000 \times g$ for 30 min at 4 °C. Membrane pellets were resuspended in hypotonic buffer, treated with 1% Triton X-100, 100 mM Na_2CO_3 or 1 M KCl for 30 min, and further fractionated into supernatant (S) and pellet (P) fractions by ultracentrifugation at $150000 \times g$ for 30 min at 4 °C.

2.7. Proteinase K protection assay

HeLa cells grown in 60 mm dishes were transfected with SARS-CoV E and IBV E tagged with the Flag epitope at either N- or C-terminus, respectively, by using the vaccinia/T7 virus (vTF7-3) system. After incubation for 18 h, the cells were washed twice with ice-cold PBS, scraped off the dish, and homogenized with 20 strokes in a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 1200 rpm for 15 min at 4 °C. Individual samples with the tagged SARS-CoV E or IBV E proteins were split into three microcentrifuge tubes. One tube was taken as control, the remaining two tubes in the absence or presence of 1% Triton X-100 were subjected to 20 μg/ml proteinase K digestion for 40 min on ice. The reaction was stopped by adding 4 mM PMSF. The samples were incubated at 100 °C for 15 min in the Laemmli sample buffer and then analyzed on SDS–PAGE.

2.8. Metabolic radiolabeling, immunoprecipitation and SDS–PAGE

HeLa cells in 100 mm dish were transfected with appropriate plasmid DNA, treated with different concentrations of hygromycin B (Sigma) for 30 min in methionine/cysteine free medium at 12 h post-transfection, and 25 μCi/ml of [^{35}S] methionine/cysteine (Amersham) were added to the culture medium. The cells were incubated at 37 °C for 3 h in the presence or absence of hygromycin B, harvested and lysed in RIPA buffer containing 1.0 mM PMSF and 10 μg/ml each aprotinin and leupeptin (Roche Applied Science). The cell extracts were clarified at 13000 rpm at 4 °C for 10 min, and the proteins were immunoprecipitated with appropriate antibodies for 1 h at 4 °C, incubated with 20 μl of protein A–agarose overnight at 4 °C, and washed three times with RIPA buffer. The proteins were analyzed by 15% SDS–PAGE.

2.9. Construction of plasmids

Plasmid Flag-SARS E, which covers the SARS-CoV E sequence, was constructed by cloning an EcoRV-/EcoRI-digested PCR fragment into EcoRV-/EcoRI-digested pKT0-Flag. The PCR fragment was generated using primers 5′-GCAAGATATCCTACTCATTCGTTTCGG AA-3′ and 5′-CCGGAATTCTTAGACCAGAAGATCAG-3′. Plasmid SARS E-Flag was created by cloning a BglII-/EcoRI-digested PCR fragment into BglII-/EcoRI-digested pKT0. The PCR fragment was generated using primers 5′-TGGAAGATCTCCACCATGTACT-CATTCGTT TCGGAA-3′ and 5′-CGGAATTCTTACTTGT-CATCGTCGTCCTTGTAATCGACCAGAAG ATCAGGAA-3′. Plasmid Flag-IBV E was generated by cloning an EcoRV-/EcoRI-digested PCR fragment into EcoRV-/EcoRI-digested pKT0-Flag. The two primers used to generate the PCR fragment are 5′-GCAAGA-TATCCAATTTATTGAATAAGTCG-3′ and 5′-CCGGAATTC TCA AGAG TACAATTTGTC-3′. Plasmid IBV E-Flag was created by cloning a BglII-/EcoRI-digested PCR fragment into BglII-/EcoRI-digested pKT0. The PCR fragment was generated using primers 5′-TGGAAGATCTCCACCATGAATTTATTGAATAA-3′ and 5′-CCGGAATTCTGTCA TCGTCGTCCTTGTAATCGAGAG-TACAATTTGTCTCG-3′. Plasmid pKT-SARS E was constructed by cloning a BglII-/EcoRI-digested PCR fragment into BglII-/EcoRI-digested pKT0. The PCR fragment was generated using primers 5′-TGGAAGATCTCCACCAT GTACTCATTCGTTTCGGAA-3′ and 5′-CCGGAATTCTTAGACCAGAAGATCAG-3′. Plasmid pKT-IBV E was constructed by cloning a BglII-/EcoRI-digested PCR fragment into BglII-/EcoRI-digested pKT0. The PCR fragment was generated using primers 5′-TGGAAGAT CTCCACCATGAATTTATT GAATAA-3′ and 5′-CCGGAATTCTCAAGAGTACAATTTG TC-3′. The underlined nucleotides represent the restriction sites introduced into each primer.

Mutations were introduced into the SARS-CoV E gene by two rounds of PCR. The PCR amplified fragments were cloned into *Eco*RV- and *Eco*RI-digested pFlag. All plasmids and the introduced mutations were confirmed by automated DNA sequencing.

3. Results

3.1. Prediction of the hydrophobicity and membrane topology of SARS-CoV E protein

The hydrophobicity of the SARS-CoV E protein is shown as a Kyte–Doolittle hydropathy plot in Fig. 1B. The protein is largely hydrophobic in the region from amino acids 9 to 59. In both N- and C-terminal regions, some hydrophilic amino acid stretches were found.

Three computer programs, i.e., TMHMM [13], HMMTOP [29], and MEMSAT [10], were used to predict the membrane topology of the SARS-CoV E protein. All three programs predicted that SARS-CoV E protein contains one transmembrane domain. Both TMHMM and MEMSAT predicted that SARS-CoV E protein may assume an $N_{cyto}C_{exo}$ topology, whereas HMMTOP indicated that SARS-CoV E protein may adopt a $C_{cyto}N_{exo}$ topology.

3.2. Analysis of the membrane topology of SARS-CoV E protein by immunofluorescence microscopy

To test these predictions and to experimentally determine the membrane topology of the SARS-CoV E protein, both SARS-CoV and IBV E proteins were tagged with Flag at N- and C-termini, respectively, and expressed in HeLa cells. Cells were then permeabilized with either 0.5% Triton X-100 or 5 µg/ml digitonin, and the expression of the Flag-tagged E protein was detected by indirect immunofluorescent staining using anti-Flag monoclonal antibody. Treatment of cells with digitonin at low concentrations selectively permeabilizes the plasma membrane but leaves the intracellular membranes intact, while Triton X-100 treatment permeabilizes both plasma and intra-

cellular membranes [25]. If the Flag tag is exposed to the cytoplasmic side, the epitope can be recognized by anti-Flag antibody after digitonin treatment. On the other hand, if the Flag tag is exposed luminally, the epitope cannot be recognized by the anti-Flag antibody after digitonin treatment. It can only be recognized after Triton X-100 treatment.

As shown in Fig. 2, clear detection of the N- and C-terminally tagged SARS-CoV E protein at perinuclear regions was obtained in HeLa cells permeabilized with either 0.5% Triton X-100 or 5 µg/ml digitonin (Fig. 2), suggesting that both N- and C-termini of the protein may be located in the cytoplasmic side. However, in cells expressing the N-terminally tagged IBV E protein, expression of the protein was observed in cells permeabilized with 0.5% Triton X-100 only (Fig. 2). No detection of the protein expression in cells permeabilized with 5 µg/ml digitonin was obtained (Fig. 2). It is also noted that no significant difference in the staining intensity was observed between cells treated with digitonin and Triton X-100. In cells expressing the C-terminally tagged IBV E protein, the protein was detected in cells permeabilized under either condition (Fig. 2). These results are consistent with the topology of IBV E protein established before [4], and justify the experimental conditions used. As a control, PDI, a host protein residing in the ER lumen, was detected in cells permeabilized with 0.5% Triton X-100, but was not detected in cells permeabilized with 5 µg/ml digitonin (Fig. 2), confirming that the ER membrane was still intact after treatment with digitonin (Fig. 2). Taken together, these results indicate that both the N- and C-termini of the SARS-CoV E protein may be located in the cytoplasmic side of the cell, whereas IBV E protein adopts an $N_{exo}C_{cyto}$ topology.

3.3. Analysis of the membrane topology of SARS-CoV E protein by differential permeabilization of the plasma membrane and limited proteinase K treatment

The topology of SARS-CoV E protein on cellular membranes was further analyzed by limited proteinase K digestion
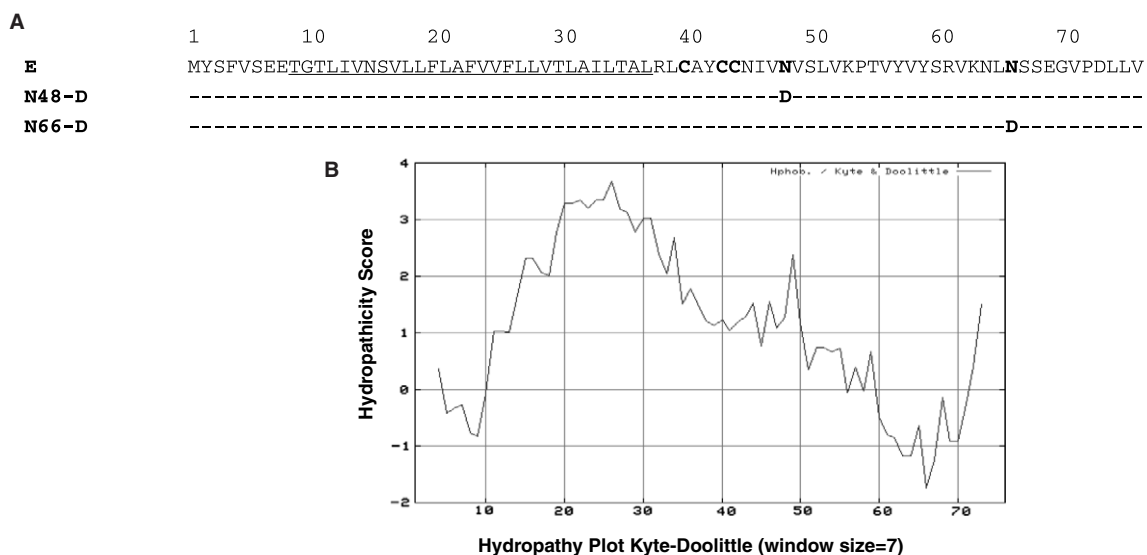
Fig. 1. Diagram of wild type and mutant SARS-CoV E constructs, and hydrophathicity score of SARS-CoV E protein. (A) Amino acid sequences of wild type and mutant SARS-CoV E protein. The amino acid sequence of the putative transmembrane domain is underlined, the three cysteine residues and the two potential glycosylation sites are indicated in bold. Also shown are the mutations introduced into each of the mutant constructs. (B) The hydropathy profile of SARS-CoV E protein determined by Kyte and Doolittle with a 7-residue window. It displays the highly hydrophobic character of this protein.
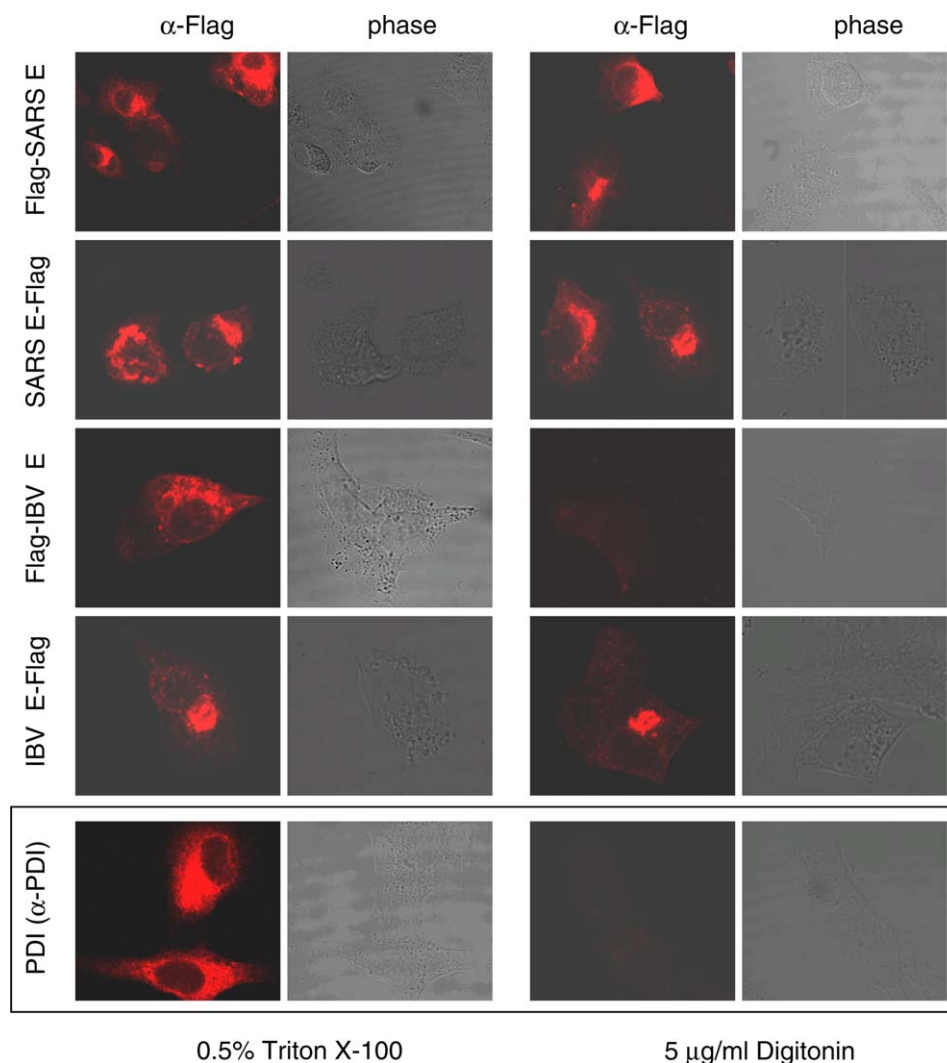
Fig. 2. Cytoplasmic exposure of the Flag epitope tagged at the N- and C-terminus of the SARS-CoV E protein and the C-terminus of the IBV E protein. HeLa cells expressing Flag-SARS E, SARS E-Flag, Flag-IBV E and IBV E-Flag, respectively, were permeabilized using either 5 μg/ml digitonin or 0.5% Triton X-100 and immunostained with anti-Flag antibody as the primary antibody and TRITC-conjugated anti-mouse IgG antibody as the secondary antibody. Untransfected HeLa cells were treated with digitonin or Triton X-100 and immunostained with anti-PDI antibody and TRITC-labeled anti-rabbit IgG secondary antibody.

of the membrane fraction prepared from cells expressing the N- or C-terminally tagged E protein. Digestion with the non-specific proteinase K would degrade proteins (or a portion of the protein) protruding from the exterior face of the micro-somal membranes, while proteins (or a portion of the protein) orientated towards the lumen are protected. For this purpose, HeLa cells expressing the Flag-tagged SARS-CoV E protein at either N- or C-terminus were broken by homogenization, and the membrane fraction was collected. The membrane fraction was then divided into three aliquots: one was treated with both 1% Triton X-100 and 20 μg/ml proteinase K, one treated with 20 μg/ml proteinase K only, and one without any treatment. The total proteins were then separated by SDS–PAGE and analyzed by Western blot with anti-Flag antibody. As shown in Fig. 3, efficient detection of both N- and C-terminally tagged SARS-CoV E protein was obtained from the untreated membrane fraction (Fig. 3, lanes 1 and 4). Upon treatment of the membrane fraction with 20 μg/ml proteinase K in the pres-ence of 1% Triton X-100, no detection of the SARS-CoV E

protein was observed from cells expressing either the N- or C-terminally tagged E protein (Fig. 3, lanes 3 and 6), demon-strating that treatment with proteinase K led to the removal of the Flag tag from either terminus of the SARS-CoV E protein. Similar results were observed after treatment of the membrane fraction with 20 μg/ml proteinase K in the absence of 1% Tri-ton X-100 (Fig. 3, lanes 2 and 5). However, prolonged expo-sure of the same gel showed detection of minor amounts of the E protein in the same fractions (data not shown).

In cells expressing the N- and C-terminally tagged IBV E protein, the protein was clearly detected in the membrane frac-tion without treatment with Triton X-100 and proteinase K (Fig. 3, lanes 7 and 10). Treatment of the membrane fraction with 20 μg/ml proteinase K resulted in the detection of a frag-ment of approximately 10 kDa representing the N-terminal re-gion of the IBV E protein (Fig. 3, lane 8). Upon treatment of the membrane fraction with 1% Triton X-100 and 20 μg/ml proteinase K, the IBV E protein was no longer detected (Fig. 3, lane 9). In cells expressing the C-terminally tagged
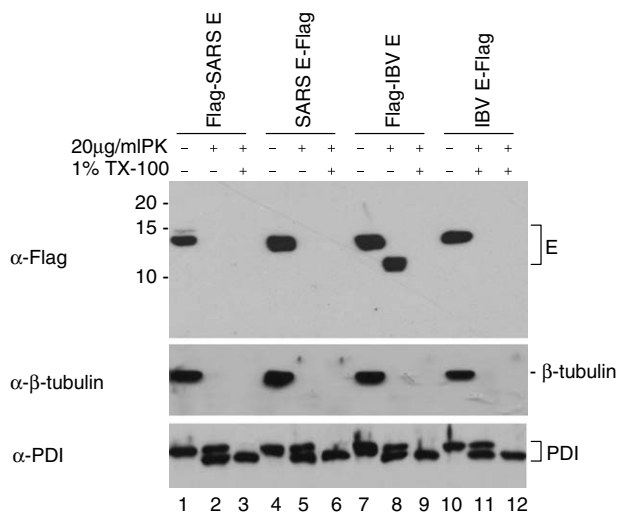
Fig. 3. Limited proteinase K digestion of microsomal membranes prepared from cells expressing the SARS-CoV and IBV E protein tagged with the Flag epitope at the N- and C-termini, respectively. Intact microsomes were isolated from cells expressing Flag-SARS E, SARS E-Flag, Flag-IBV E and IBV E-Flag, and subjected to digestion by proteinase K in the absence (lanes 2, 5, 8, 11) or presence (lanes 3, 6, 9, 12) of Triton X-100. The proteinase K-treated samples were separated on SDS–17.5% polyacrylamide gel and analyzed by Western blot. PDI (an ER luminal protein) was detected by anti-PDI antibody (Santa Cruz), and β-tubulin (a cytosolic protein) was detected by anti-β-tubulin antibody (Santa Cruz). Numbers on the left indicate molecular masses in kilodaltons.

IBV E protein, the protein was not detected after treatment of the membrane fraction with proteinase K in the presence or absence of Triton X-100 (Fig. 3, lanes 10–12). Consistent with the immunofluorescence data, these results reinforce the conclusion that the N-terminus of the IBV E protein was located in the lumen of the ER and the Golgi apparatus.

As an internal control for the integrity of microsomal membranes after limited proteolytic digestion, the detection of PDI was included in the experiment. As shown in Fig. 3, the full-length PDI was detected in the membrane fraction before and after treatment with 20 μg/ml proteinase K in the absence of 1% Triton X-100 (lanes 1, 2, 4, 5, 7, 8, 10 and 11). In addition, a shorter form of the protein was also detected in the membrane fraction after treatment with 20 μg/ml proteinase K in the absence of 1% Triton X-100 (lanes 2, 5, 8 and 11). In the presence of 1% Triton X-100, treatment of the membrane fraction with proteinase K showed that no full-length PDI was detected (Fig. 3, lanes 3, 6, 9 and 12). However, the shorter form of PDI was still detected (Fig. 3, lanes 3, 6, 9 and 12), suggesting that it may represent a proteinase K-resistant fragment of PDI. The fact that the shorter form of PDI was detected in the membrane fraction after treatment with 20 μg/ml proteinase K in the absence of 1% Triton X-100 suggests that the procedure used to prepare the membrane fraction may result in partial disruption of the microsomes. In the same experiment, β-tubulin was also included as an internal control for the degradation of cytosolic proteins. The protein was degraded by the treatment with 20 μg/ml proteinase K in the presence or absence of Triton X-100 (Fig. 3). Together with the immunofluorescence studies, these results confirm that both the N-terminus and C-terminus of SARS-CoV E are cytosolic with an orientation of $N_{cyto}C_{cyto}$.

3.4. Glycosylation of SARS-CoV E protein

Examination of the SARS-CoV E protein sequence showed the presence of two potential N-linked glycosylation sites on asparagine 48 (N48) and 66 (N66) (Fig. 1A). If the protein adopted a sole $N_{cyto}C_{cyto}$ topology, glycosylation at the two positions would not occur. However, multiple bands were usually detected when the protein was expressed in different systems, suggesting that it may undergo posttranslational modifications. To address the possibility that the protein may be modified by N-linked glycosylation, mutations were introduced into the E protein to change the predicted N-linked glycosylation sites from asparagine to aspartic acid. Two mutants, N48-D and N66-D, were generated and expressed (Fig. 1A). Western blot analysis of cells expressing wild type and the N48-D mutant showed the detection of three bands on an SDS–17.5% polyacrylamide gel (Fig. 4A, lanes 1 and 2). These may represent three isoforms of the E protein. In cells expressing the N66-D mutant, only two bands were detected; the most slowly migrating species of the three isoforms was not observed (Fig. 4A, lane 3).

To analyze further the N-linked glycosylation of the E protein, cells expressing wild type (Fig. 5B, lanes 1 and 2) and mutant E (Fig. 4B, lanes 3–6) were lysed. The total cell lysates were first treated with the N-linked glycosidase PNGaseF, and analyzed by Western blot with anti-E polyclonal antibodies. In cells expressing wild type and the N48-D mutant E protein, the protein was separated into two major bands in the gel system used (Fig. 4B, lanes 1 and 3). Treatment of the same total cell lysates led to the removal of the upper band (Fig. 4B, lanes 2 and 4), confirming that it represents the glycosylated form of the protein. In this gel system, the two unglycosylated isoforms of wild type SARS-CoV E protein were not well separated, so three major bands were detected with Flag-SARS E but only two major bands detected with wild type SARS-CoV E. In addition, a minor species of approximately 20 kDa, representing the dimeric form of the protein, was observed in cells expressing the two constructs (Fig. 4B, lanes 1–4). In cells expressing the N66-D mutant, the glycosylated form was not detected either before or after treatment with PNGaseF (Fig. 4B, lanes 5 and 6). Interestingly, in cells expressing this mutant E protein, more dimeric form and a species representing trimers of the E protein were detected (Fig. 4B, lanes 5 and 6). It suggests that this mutant E protein tends to form multimers or aggregates. As a control, cells expressing IBV E protein were treated with the same glycosidase. The protein was detected as a single band in total cell lysates with or without PNGaseF treatment (Fig. 4B, lanes 7 and 8). In cells expressing the Flag-tagged E protein, this treatment resulted in the disappearance of the most slowly migrating species of the three isoforms (Fig. 4B, lanes 9 and 10), further confirming that it is the glycosylated form of the E protein. It was noted that the pattern of the Flag-tagged E protein present in this figure was different from that in Fig. 3. This is because that much more materials were loaded and prolonged electrophoresis of the gel was applied in order to separate well and to detect clearly the minor glycosylated form of the protein. These results demonstrate that a minor proportion of the SARS-CoV E protein is modified by N-linked glycosylation and the N66 residue is the site for this modification. More importantly, it suggests that this portion of the SARS-CoV E protein would assume a different membrane topology from the majority of
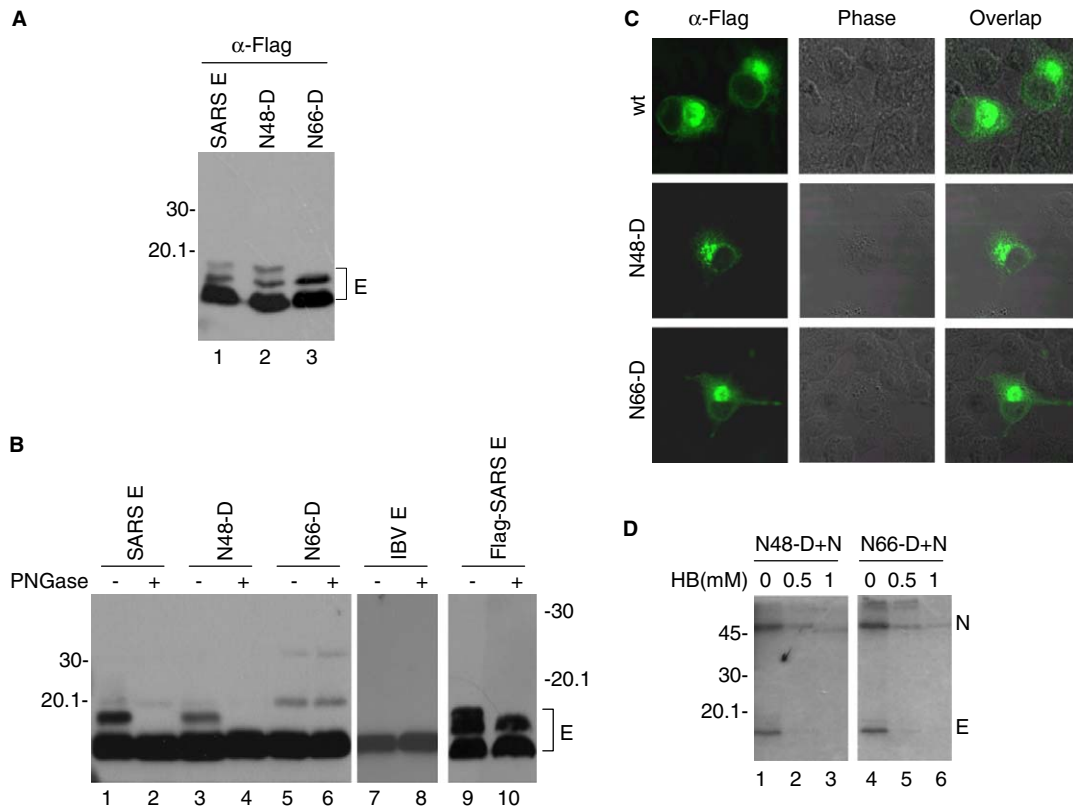
Fig. 4. N-linked glycosylation of SARS-CoV E protein. (A) HeLa cells were transfected with the Flag-tagged wild type (lane 1) and three mutant constructs containing mutations of the N48 (lane 2) and N66 (lane 3). Cell lysates were prepared 24 h posttransfection, polypeptides were separated by SDS–PAGE and analyzed by Western blot using the anti-Flag antibody. Numbers on the left indicate molecular masses in kilodaltons. (B) Total cell lysates prepared from HeLa cells expressing wild type SARS-CoV E (lanes 1 and 2), N48-D (lanes 3 and 4), N66-D (lanes 5 and 6), wild type IBV E (lanes 7 and 8) and the Flag-tagged SARS-CoV E (lanes 9 and 10) were treated either with (lanes 2, 4, 6, 8 and 10) or without (lanes 1, 3, 5, 7, and 9) PNGase F. Polypeptides were separated by SDS–PAGE and analyzed by Western blot using anti-SARS-CoV E antibodies (lanes 1–6), anti-IBV E (lanes 7 and 8) or anti-Flag (lanes 9 and 10). Numbers on the left indicate molecular masses in kilodaltons. (C) Subcellular localization of the Flag-tagged wild type SARS-CoV E, N48-D and N66-D. BHK cells expressing the Flag-tagged wild type and mutant E protein were stained with anti-Flag antibodies at 12 h posttransfection after permeabilizing with 0.2% Triton X-100. (D) Entry of hygromycin B into HeLa cells expressing wild type and mutant E proteins. HeLa cells expressing N48-D (lanes 1–3) and N66-D (lanes 4–6), respectively, were treated with 0, 0.5 and 1 mM of hygromycin B for 30 min at 12 h posttransfection, and radiolabelled with [35S] methionine–cysteine for 3 h. Cell lysates were prepared and the expression of E protein was detected by immunoprecipitation with anti-Flag antibody under mild washing conditions. SARS-CoV N protein was co-expressed with wild type and mutant E protein, and the expression of N protein was detected by immunoprecipitation with polyclonal anti-N antibodies. Polypeptides were separated by SDS–PAGE and visualized by autoradiography. Numbers on the left indicate molecular masses in kilodaltons.

the E protein, i.e., the C-terminal region of the protein would be located in the lumen of the ER and the Golgi apparatus.

The effects of these mutations on the subcellular localization of E protein were then analyzed. As shown in Fig. 4C, expression of the Flag-tagged SARS-CoV E protein in BHK cells stably expressing the T7 RNA polymerase [3] showed that the protein mainly located in the perinuclear region. The two mutant proteins also displayed very similar localization patterns (Fig. 4C). These results demonstrated that mutation of the glycosylation site of the protein does not affect the membrane association and subcellular localization patterns of the protein.

As the N66-D mutant tends to form multimers or aggregates, it would be interesting to test if this mutation could affect the membrane-permeabilizing activity of the SARS-CoV E protein. For this purpose, the Flag-tagged constructs were expressed in HeLa cells. At 12 h post-transfection, cells were treated with two different concentrations of hygromycin B for 30 min, and then radiolabeled with [35S] methionine–cysteine for 3 h. Cell extracts were prepared and the expression

of E protein was detected by immunoprecipitation with anti-Flag antibody. As shown in Fig. 4D, this mutation did not obviously affect the membrane-permeabilizing activity of the E protein. A very similar degree of inhibition of protein synthesis was observed in cells expressing both N-48-D and N-66-D constructs (Fig. 4D).

3.5. Cell surface expression of the SARS-CoV E protein

Immunofluorescent staining of cells expressing the Flag-tagged SARS-CoV E protein at either N- or C-terminus was then carried out to test if the E protein translocated to the cell surface could be accessed by the antibody. As shown in Fig. 5, immunofluorescent staining of HeLa cells expressing the N-terminally Flag-tagged IBV E using anti-Flag antibody exhibit typical cell surface staining. In cells expressing SARS-CoV E protein with the Flag epitope tagged at either N- or C-terminus, no cell with obvious positive staining was detected (Fig. 5). However, cells with a few fluorescent dots on the surface were consistently observed (Fig. 5).
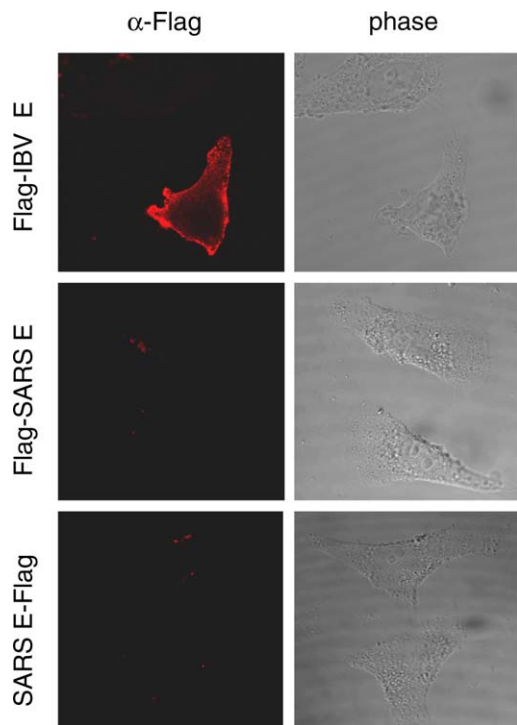
Fig. 5. Surface staining of HeLa cells expressing the SARS-CoV E protein with Flag tagged at the N- and C-terminus, respectively, and IBV E protein with the Flag tagged at the N-terminus. HeLa cells expressing Flag-SARS E, SARS E-Flag and Flag-IBV E, respectively, were immunostained with anti-Flag antibody as the primary antibody and TRITC-conjugated anti-mouse IgG antibody as the secondary antibody.

4. Discussion

Coronaviruses encode a small integral membrane protein that is associated with the viral envelope and plays important functions in virion assembly and morphogenesis [19,30]. Recently, the E protein from SARS-CoV and MHV was shown to enhance the membrane permeability of bacterial and mammalian cells to small molecules [14,15,18]. SARS-CoV E protein contains a putative long transmembrane domain of 29 amino acid residues. Based on the distribution of the charged amino acids flanking the transmembrane domain and the recent observation that the E protein is palmitoylated on all

three cysteine residues [15], the protein may insert into the ER and Golgi membranes with an $N_{exo(lum)}C_{cyto}$ orientation. In vitro results reported by Arbely et al. suggest that the transmembrane domain of SARS-CoV may adopt a highly unusual topology, consisting of a short transmembrane helical hairpin that forms an inversion about a previously unidentified pseudo-centre of symmetry [2], although this is in contrast with simulation results based on phylogenetic data [28]. In the present study, the membrane topology of the SARS-CoV E protein is systematically studied with N- and C-terminally Flag-tagged SARS-CoV E protein. As the Flag tag does not affect the release of E-containing vesicles and virus-like particles (data not shown), and the permeabilizing activity of the protein, it is assumed that the Flag-tagged E protein is functionally active. Immunofluorescent staining of cells differentially permeabilized with detergents and proteinase K protection assay revealed that both the N- and C-termini of the SARS-CoV E protein are exposed to the cytoplasmic side of the membranes ($N_{cyto}C_{cyto}$) (see Fig. 6A). Two potential forms are proposed (Fig. 6A, forms (1) and (2)). Based on Arbely et al. [2], the SARS-CoV E protein may assume the form (1) topology. However, we cannot rule out the possibility that the protein may cross the membrane twice and assume the form (2) topology, based on our biophysical and molecular simulation data [28]. Consistent with previous studies, parallel experimental data presented in this study demonstrated that the IBV E protein spans the membranes once with the N-terminus exposed luminally and the C-terminus exposed cytoplasmically ($N_{exo(lum)}C_{cyto}$) (see Fig. 6A).

Interestingly, a small proportion of the SARS-CoV E protein was shown to be modified by N-linked glycosylation on the asparagine 66 residue. Glycosylation of the C-terminal region of the E protein was an unexpected finding, since no proteinase K-protected fragment was detected in the limited proteinase K assay. Nevertheless, the detection of the N-linked glycosylation at the C-terminal region and the inefficient synthesis of the glycosylated form suggest that the SARS-CoV E protein may have an alternative membrane topology. Two possible models for this alternative membrane topology of the SARS-CoV E protein were proposed (Fig. 6B). In the first case, the protein may span the membranes once with an $N_{cyto}C_{exo(lum)}$ orientation (Fig. 6B). Alternatively, both N- and C-termini may be exposed to the luminal side of the membranes ($N_{exo(lum)}C_{exo(lum)}$) (Fig. 6B). Once again, two potential forms are proposed (Fig. 6B, form (1) and (2)). At present, we
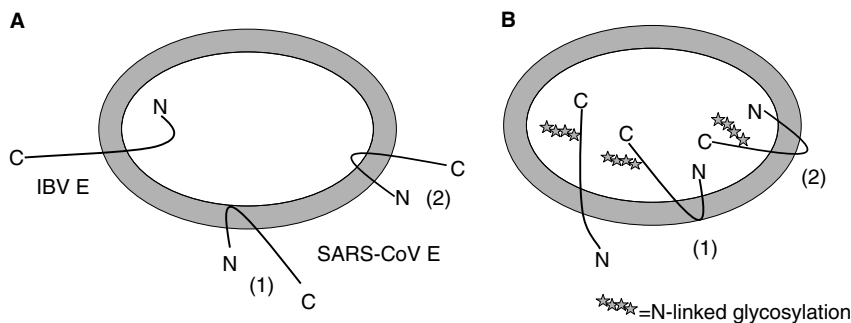

Fig. 6. Proposed membrane topologies of the unglycosylated form of the SARS-CoV E (A), the IBV E (A), and the glycosylated form of the SARS-CoV E protein (B). N and C represent the N- and C-termini of the proteins, respectively. The two potential forms of SARS-E when both the N- and C-terminal ends are located in the same side of the membrane are indicated by (1) and (2). The Asn-linked high mannose carbohydrate modification is shown at the C terminus of the two proposed forms by stars.

cannot sort out which form is more likely the membrane topology for this portion of the E protein, as neither the full-length nor a shorter form of the protein was detected in the limited proteinase K assay probably due to the reason that only a very small proportion of protein assumes this topology.

It is therefore established that the majority of the SARS-CoV E protein is inserted into cellular membranes with an $N_{cyto}C_{cyto}$ topology, but a small proportion of the protein is modified by N-linked glycosylation and inserted into the membranes with the C-terminus exposed to the luminal side. There are a few examples of viral proteins that adopt more than one membrane topology. These include the transmissible gastroenteritis coronavirus membrane protein [6], the fusion protein F from the Newcastle disease virus [21] and the adenovirus E3, a 6.7K protein [22]. As coronavirus E protein including SARS-CoV E protein is a multi-functional protein, it would be important to establish whether different topological forms are responsible for a distinct function. The SARS-CoV E protein was found to interact with Bcl-xL [32]. As this interaction was mediated by the C-terminal BH_3-like region of the E protein, it would require that the C-terminal part of the protein is exposed to the cytoplasm [32]. The C-terminal tail of the IBV E protein is important for its interaction with the M protein during virus budding [5]. It is therefore likely that the $N_{cyto}C_{cyto}$ form of the E protein may be the form that accomplishes these functions.

In the limited proteinase digestion assay, a proteinase K-resistant fragment of PDI was consistently observed. The same fragment as well as some smaller fragments was observed when PDI expressed in *E. coli* was treated with proteinase K [12], confirming that it represents a proteinase K-resistant fragment of PDI. The smaller fragments were not detected in this study may be due to fact that the antibody used was raised against the middle region of PDI from amino acids 211 to 370 only.

Most coronavirus E proteins could be translocated to the cell surface to facilitate budding and release of progeny viruses [16,17,19,27]. Recently, the E protein of SARS-CoV and MHV was found to modify membrane permeability, allowing entry of small molecules into cells and leading to cell lysis [14,15,18,31]. As cell surface expression of E protein would be essential for this function, it is quite certain that SARS-CoV must be translocated to the cell surface. In this study, however, we were unable to detect efficiently cell surface expression of the SARS-CoV E protein in cells expressing the protein under non-permeabilizing conditions. This would be an additional line of evidence that indirectly supports the conclusion that the majority of the SARS-CoV E protein adopts an $N_{cyto}C_{cyto}$ topology in cells. Interestingly, a few fluorescent dots were consistently observed in non-permeabilized cells expressing the protein. We are currently unclear if these represent the minor proportion of the SARS-CoV E protein that expresses on the cell surface and with both N- and C-termini located outside the cells.

Two of the three coronavirus membrane-associated structural proteins, M and S, are posttranslationally modified by either N- or O-linked glycosylation [23]. The fourth membrane-associated structural protein, the hemagglutinin-esterase (HE), in some coronaviruses is also a glycoprotein [23]. So far, the E protein is an apparent exception. In this study, we show that the SARS-CoV E protein is also modified by N-linked glycosylation. In N-linked glycosylation, the oligosaccharides are added to specific asparagine residues in the consensus sequence Asn-X-Ser/Thr. The minimum distance between a functional C-terminal glycosylation acceptor site and the luminal end of the transmembrane domain is 12–13 residues [24], suggesting that only the most C-proximal asparagine residue out of the two potential sites in the SARS-CoV E protein can be glycosylated. Consistent with this observation, mutagenesis studies present in this paper confirmed that only N66 is modified. Furthermore, we also show that a potential N-linked glycosylation site in the N-terminal region of the IBV E protein (N6), which is 6 amino acids upstream of the transmembrane domain, was not modified, although the N-terminal region of this protein is exposed to the luminal side of the membranes.

N-linked glycosylation affects most of the proteins present on the surface of the enveloped viruses. For this reason, it is likely to play a major role in the stability, antigenicity and other biological functions of the modified viral envelope proteins. In the early secretory pathway, the glycans also play a role in protein folding, quality control and certain sorting events. However, N-linked glycosylation seems not to affect the membrane-association and membrane-permeabilizing activity of the SARS-CoV E protein. To further characterize the functional significance of this modification, it would be interesting to test if N-linked glycosylation of SARS-CoV E protein can also be detected in virus-infected cells and in purified virions. As the N66 residue is not conserved in the E protein of other coronaviruses, it is likely that this modification may render unique features to the SARS-CoV E protein. Further exploration of the functions and effects of this modification on SARS-CoV E protein using an infectious cloning system may shed new light on the molecular biology and pathogenesis of SARS-CoV.

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