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Subcellular location and topology of severe acute respiratory syndrome coronavirus envelope protein

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ABSTRACT

Severe acute respiratory syndrome (SARS) coronavirus (CoV) envelope (E) protein is a transmembrane protein. Several subcellular locations and topological conformations of E protein have been proposed. To identify the correct ones, polyclonal and monoclonal antibodies specific for the amino or the carboxy terminus of E protein, respectively, were generated. E protein was mainly found in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) of cells transfected with a plasmid encoding E protein or infected with SARS-CoV. No evidence of E protein presence in the plasma membrane was found by using immunofluorescence, immunoelectron microscopy and cell surface protein labeling. In addition, measurement of plasma membrane voltage gated ion channel activity by whole-cell patch clamp suggested that E protein was not present in the plasma membrane. A topological conformation in which SARS-CoV E protein amino terminus is oriented towards the lumen of intracellular membranes and carboxy terminus faces cell cytoplasm is proposed.

Introduction

The etiologic agent of severe acute respiratory syndrome (SARS) is a coronavirus (CoV), which is the responsible for the most severe human disease produced by a CoV (van der Hoek et al., 2004; Weiss and Navas-Martin, 2005). SARS-CoV emerged in Guangdong province, China, at the end of 2002 and during 2003 rapidly spread to 32 countries causing an epidemic of more than 8000 infected people with a death rate of around 10% (Drosten et al., 2003; Rota et al., 2003). Since then, only a few community-acquired and laboratory-acquired SARS cases have been reported (http://www.who.int/csr/sars/en/). Nevertheless, CoVs similar to SARS-CoV have been found in bats distributed in different regions all over the planet (Chu et al., 2008; Drexler et al., 2010; Muller et al., 2007; Quan et al., 2010), making the reemergence of SARS possible.

SARS-CoV is an enveloped virus with a single-stranded positive-sense 29.7 kb RNA genome, which belongs to *Coronavirinae* subfamily, genus β (Enjuanes et al., 2008) (http://talk.ictvonline.org/media/g/vertebrate-2008/default.aspx). Several proteins are embedded within the SARS-CoV envelope: spike (S), envelope (E), membrane (M), and the group specific proteins 3a, 6, 7a and 7b (Huang et al., 2006, 2007; Schaecher et al., 2007; Shen et al., 2005). Protected by the viral envelope, there is a helicoidal nucleocapsid, formed by the association of the nucleoprotein (N) and the viral genome (gRNA). The CoV infectious cycle begins when the S protein binds the cellular receptor, which in the case of SARS-CoV is the human angiotensin converting enzyme 2 (hACE-2) (Li et al., 2003; Wong et al., 2004), and the virus enters into the cell. Then, the virus nucleocapsid is released into the cytoplasm, and ORFs 1a and 1b are translated directly from the gRNA, generating two large polyproteins, pp1a and pp1ab, which are processed by viral proteinases yielding the replication–transcription complex proteins (Ziebuhr, 2005; Ziebuhr et al., 2000). This complex associates with double membrane vesicles (Gosert et al., 2002; Snijder et al., 2006) and is involved in viral genome replication and in the synthesis of a nested set of subgenomic messenger RNAs (sgmRNAs) through negative polarity intermediaries in both cases (Enjuanes et al., 2006; Masters, 2006; Sawicki and Sawicki, 1990; van der Most and Spaan, 1995; Zuñiga et al., 2010). CoV proteins M, S and E are synthesized and incorporated in the endoplasmic reticulum (ER) membrane, and transported to the pre-Golgi compartment where M protein recruits S protein and binds E protein (de Haan et al., 1999; Lim and Liu, 2001; Nguyen and Hogue, 1997). In parallel, N protein binds gRNA to

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generate the nucleocapsid that is incorporated into virions through the interaction of N and M proteins during an intracellular budding process (Narayanan et al., 2000). Assembled virions accumulate in vesicles that progress through the secretory pathway, and fuse with the plasma membrane to release viruses into the extracellular media (Tooze et al., 1987).

CoV E protein is a small integral membrane protein whose sequence varies between 76 and 109 amino acids (Arbely et al., 2004; Raamsman et al., 2000). Based on primary and secondary structure, the E protein can be divided into a short hydrophilic amino terminal stretch of between 7 and 12 amino acids, a hydrophobic zone of around 25 amino acids with an α-helix secondary structure that constitutes the transmembrane region of the protein, and a carboxy terminal domain, that comprises the majority of the protein (Torres et al., 2007). Nevertheless, a variety of E protein topologies have been described for different CoVs. Mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV) E proteins expose their carboxy terminal region towards the cell cytoplasm, whereas the amino terminal domain is located towards the luminal side of intracellular membranes for IBV or towards the cytoplasm for MHV (Corse and Machamer, 2000; Raamsman et al., 2000). Transmissible gastroenteritis virus (TGEV) E protein adopts a carboxy terminus luminal, amino terminus cytosolic conformation (Godet et al., 1992). In the case of SARS-CoV two alternative topologies have been proposed. In one of them, the transmembrane region forms a helical hairpin, with the amino and carboxy termini oriented towards the cytoplasm (Arbely et al., 2004; Yuan et al., 2006). In the other one, E protein establishes a single-pass transmembrane conformation with the carboxy terminal domain oriented towards the luminal side and the amino terminal domain remaining oriented towards the cytoplasm (Yuan et al., 2006). Therefore, the precise intracellular topology of SARS-CoV E protein is still under debate and needs to be clarified.

Only a small fraction of the pool of CoV E protein generated during infection is incorporated in virions (Maeda et al., 2001; Raamsman et al., 2000), which suggests an important role of E protein within the cell. Apparently, CoV E protein is mainly distributed in intracellular membranes between ER and Golgi compartments (Lim and Liu, 2001; Nal et al., 2005; Raamsman et al., 2000), where it participates in virus assembly, budding and intracellular trafficking through a not fully understood mechanism. In the case of SARS-CoV, it has been shown that E protein is located in the ER or in the Golgi apparatus using cells expressing tagged versions of the protein (Liao et al., 2006; Nal et al., 2005), however, no studies have been performed using infected cells. Recently it has been reported that E protein displays ion channel activity in the plasma membrane when expressed in mammalian cells (Pervushin et al., 2009), which indirectly suggests the presence of SARS-CoV E protein on the cell surface. These data reinforce the need to clearly determine the subcellular location of SARS-CoV E protein in infected cells and specifically, to clarify whether this protein is located at the plasma membrane.

Different requirements of E protein for virus production have been described among different CoVs. TGEV (an α genus CoV) E protein is essential for the maturation and secretion of recombinant infectious viruses (Ortego et al., 2007, 2002). In contrast, a recombinant MHV (β genus CoV) lacking E gene was infectious although it showed lower titers in cell culture than the recombinant wild type virus (Kuo and Masters, 2003). Similarly, in the case of SARS-CoV, the E gene is not essential, although recombinant SARS-CoV lacking the E gene (rSARS-CoV-ΔE) grew from 20- to 200-fold lower than the wild-type virus (rSARS-CoV *wt*) in monkey or human cells, respectively (DeDiego et al., 2007). In addition, SARS-CoV lacking the E gene was attenuated in two animal models (DeDiego et al., 2007, 2008; Netland et al., 2010) indicating that SARS-CoV E gene may be a virulence factor.

Of the CoV E protein activities, the ion channel activity is one of the most remarkable. Several viral proteins with ion channel activity have been described for other RNA viruses, such as M2 from influenza A virus, p7 from hepatitis C virus, Vpu from human immunodeficiency virus (HIV), or 2B from enterovirus (de Jong et al., 2006; Ewart et al., 1996; Pinto et al., 1992; Wozniak et al., 2010). Nevertheless, the relevance of the SARS-CoV E protein ion channel activity and its possible relationship with virus trafficking and assembly is not known. In vitro studies using artificial lipid bilayers showed that HCoV-229E, MHV, SARS-CoV and IBV E proteins behaved as cation-selective ion channels (Torres et al., 2007; Wilson et al., 2006, 2004). A recent report determined that SARS-CoV E protein expressed in mammalian cells, displayed ion channel activity at the plasma membrane as determined by whole-cell patch clamp (Pervushin et al., 2009). Nevertheless, contradictory results have also been reported indicating that SARS-CoV E protein does not behave as an ion channel at the cell surface (Ji et al., 2009).

The clarification of the cellular localization and topology of the SARS-CoV E protein is a crucial issue to understand the activities of E protein. In this article, we report the generation and characterization of monoclonal and polyclonal antibodies specific for the SARS-CoV E protein as essential tools to address E protein subcellular location and topology. The data presented in this study showed that the SARS-CoV E protein essentially accumulated in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) when expressed both alone or after SARS-CoV infection. No evidence of E protein presence in the plasma membrane was found using immunofluorescence, immunoelectron microscopy or cell surface protein labeling and purification. In addition, whole-cell patch clamp assays revealed decreased current intensity in the plasma membrane of cells expressing E protein, which is not compatible with an E protein mediated voltage gated ion channel at the cell surface. All these data indicated that the E protein would carry out its direct functions from intracellular membranes. By using the specific antibodies generated in this work and selective permeabilization of plasma or intracellular membranes, we propose a topological conformation for SARS-CoV E protein in which this protein spans intracellular membranes only once, with the E protein amino terminus oriented towards the lumen of intracellular membranes and the E protein carboxy terminus exposed towards the cytoplasm.

Results

Generation and characterization of antibodies specific for SARS-CoV E protein

To determine the cellular localization and topology of SARS-CoV E protein, five independently derived hybridomas producing mAbs specific for SARS-CoV E protein were generated. SARS-CoV E protein is mainly hydrophobic and poorly immunogenic (Fig. 1A). Nevertheless, in silico analysis of the E protein amino acid sequence revealed the presence of two regions located in the amino (Ent) and carboxy terminus (Ect) of the protein with higher probability of inducing an immune response (Fig. 1A). To obtain mAbs recognizing different regions of the protein, three pairs of BALB/c mice were each immunized with affinity chromatography purified full-length SARS-CoV E protein expressed in bacteria, or in a baculovirus system, or with chemically synthesized E protein. Sera from immunized mice were analyzed by ELISA using the three sources of purified E protein, and by immunofluorescence using rSARS-CoV *wt*-infected Vero E6 cells and cells infected with a recombinant virus lacking E gene (rSARS-CoV-ΔE) as a negative control. ELISA and immunofluorescence assays showed that the sera from all immunized animals contained antibodies specific for SARS-CoV E protein (data not shown). However, three of the six mice (one of each pair), which developed the highest titers to E protein died or became sick prior to hybridoma generation. The remaining seropositive mice were boosted with the same antigen used in previous immunizations, and sacrificed to collect their spleens to generate hybridomas. Five hybridomas that produced mAbs positive by ELISA, immunofluorescence and Western