The SARS Coronavirus E Protein Interacts with PALS1 and Alters Tight Junction Formation and Epithelial Morphogenesis

Kim-Tat Teoh,*† Yu-Lam Siu,*‡ Wing-Lim Chan,*§ Marc A. Schlüter,** Chia-Jen Liu,¶ J. S. Malik Peiris,**** Roberto Bruzzone,* Benjamin Margolis,¶†† and Béatrice Nal*‡‡

*HKU-Pasteur Research Centre, Pokfulam, Hong Kong S.A.R. China; Departments of †Pediatrics and Adolescent Medicine, ‡Pathology, **Microbiology, and ††Anatomy, The Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong S.A.R., China; †Medizinische Klinik D, Universitätsklinikum Münster, D-48149 Münster, Germany; Departments of ¶Internal Medicine and ‡‡Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109

Submitted April 22, 2010; Revised August 16, 2010; Accepted September 15, 2010
Monitoring Editor: Asma Nusrat

Intercellular tight junctions define epithelial apicobasal polarity and form a physical fence which protects underlying tissues from pathogen invasions. PALS1, a tight junction-associated protein, is a member of the CRUMB3-PALS1-PATJ polarity complex, which is crucial for the establishment and maintenance of epithelial polarity in mammals. Here we report that the carboxy-terminal domain of the SARS-CoV E small envelope protein (E) binds to human PALS1. Using coimmunoprecipitation and pull-down assays, we show that E interacts with PALS1 in mammalian cells and further demonstrate that the last four carboxy-terminal amino acids of E form a novel PDZ-binding motif that binds to PALS1 PDZ domain. PALS1 redistributes to the ERGIC/Golgi region, where E accumulates, in SARS-CoV–infected Vero E6 cells. Ectopic expression of E in MDCKII epithelial cells significantly alters cyst morphogenesis and, furthermore, delays formation of tight junctions, affects polarity, and modifies the subcellular distribution of PALS1, in a PDZ-binding motif-dependent manner. We speculate that hijacking of PALS1 by SARS-CoV E plays a determinant role in the disruption of the lung epithelium in SARS patients.

INTRODUCTION

The SARS coronavirus (SARS-CoV) is an enveloped virus with a positive single strand RNA genome, which has emerged in the human population during winter 2002–2003 causing an outbreak of severe acute respiratory infections with a 10% mortality rate (Peiris et al., 2004). The reasons for the severity of illness in SARS-CoV infected patients are still not clearly understood. The SARS-CoV mainly targets epithelial cells, the respiratory tract being the primary site of infection (Nicholls et al., 2003; Nicholls et al., 2006). One of the major pathological features of SARS-CoV infection is diffuse alveolar damage (DAD) of the human lung, more prominent in the terminal stage, with occasional extensive damage of the lung epithelium (Kuiken et al., 2003; Nicholls et al., 2003). Several hypotheses have been made to explain DAD, invoking either intrinsic cytopathic effect of the virus or dysfunction of the immune system (Chen and Subbarao, 2007; Perlman and Netland, 2009; Yoshikawa et al., 2009). Another clinical feature is the extrapulmonary dissemination of the virus with other organ dysfunction including lymphoid tissues, liver, intestine, and kidney (Farcaş et al., 2005; Gu et al., 2005). One possibility is that viral proteins disrupt mucosal integrity by interfering with the regulation and maintenance of specialized epithelial functions, such as intercellular junctions and apicobasal polarity and, as a consequence, induce viral dissemination. In this line, recent studies using a recombinant virus lacking the SARS-CoV E gene suggest that E envelope protein is a virulence factor influencing replication level, virus dissemination, and pathogenicity of SARS-CoV in animal models (DeDiego et al., 2007; DeDiego et al., 2008). However, the molecular mechanism involving E in pathogenesis is not known. Interestingly, in vitro studies on monolayers of human airway epithelial cells have shown that morphology of cells was affected at late time points following infection (72 and 120 h) and suggested that cellular junctions and polarity were altered (Sims et al., 2005).
Cell polarity is maintained by three protein complexes, the CRB, PAR, and SCRIB complexes, that act in concert to control the functional development of a polarized epithelium (reviewed in Shin et al., 2006; Suzuki and Ohno, 2006; Yamanaka and Ohno, 2008). The CRB complex consists of CRUMBS3 (CRBS3), PALS1, and PATJ (Roh et al., 2002; Hurd et al., 2003; Roh et al., 2003). PALS1 (Protein Associated with Caenorhabditis elegans Lin-7 protein 1) belongs to the group of PDZ (Post-synaptic density protein-95/Discs Large/Zonula occludens-1) domain-containing proteins that function as scaffolds for signaling proteins and are involved in diverse cellular functions (reviewed in Schuehler and Lynch, 2004; Miyoshi and Takai, 2005; Shin et al., 2006; Wang and Margolis, 2007). PALS1 is the central component of the CRB complex and is the ortholog of Drosophila melanogaster Stardust, key regulator of cellular polarity during embryogenesis (Kamberov et al., 2003). It is a member of the membrane-associated guanylate kinase (MAGUK) protein family and is also known as MPP5 (membrane protein, patimothylated 5) in humans. PALS1 contains 675 amino acids organized in the following functional domains: two L2 motifs, one PDZ, one SH3 (Src Homology 3), one band 4.1, and one Guk (Guanylate kinase) domains (Kamberov et al., 2000).

The CRB polarity complex plays a major role in establishment, regulation, and maintenance of apical polarity in epithelial cells (Tepass, 1996; Roh et al., 2003; Shin et al., 2006). CRB3, ubiquitously expressed in epithelial cells, docks the CRB complex at the apical domain of the plasma membrane where it is specifically anchored (Makarova et al., 2003). PALS1 PDZ domain binds to CRB3 carboxy-terminal PDZ domain-membrinding motif (PBM), which consists of four terminal amino acids, E-R-L-I (Roh et al., 2002; Hurd et al., 2003; Roh et al., 2003). In MDCKII cells, knockdown of PALS1 expression by small interfering RNA leads to concurrent loss of expression of PATJ, decrease of transepithelial electrical resistance (TER), and disruption of cell polarity, as illustrated by formation of cysts with multiple lumens when MDCKII are grown in a collagen gel matrix (Straight et al., 2004). Wang and coworkers have further demonstrated that depletion of PALS1 in MDCKII cells alters the intracellular trafficking of E-cadherin, which is retained as intracellular puncta at the cell periphery and is not effectively delivered to the cell surface. As a result, both TJ and adherens junction (AJ) formation are disrupted (Wang et al., 2007). In summary, PALS1 functionally regulates and maintains the integrity of TJ and AJ, and as a consequence cell polarity in epithelial cells.

We have hypothesized that the carboxy-terminal (CT) domain of SARS-CoV structural proteins interact with cytosolic cellular machineries in infected epithelia and that such interactions may be involved in virus-induced pathogenesis. Here we show that the CT of the SARS-CoV envelope protein E binds the human TJ protein PALS1 in human epithelial cells. E is a small hydrophobic integral membrane protein of 76 amino acid residues, which plays a major but not fully understood role in virus morphogenesis and budding (Liu et al., 2007; Siu et al., 2008). In transfected and infected cells, it is known that E localizes at the membranes of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and Golgi apparatus, where virus assembly occurs (Nal et al., 2005; Liu et al., 2007; Siu et al., 2008). In this report we study the biochemical interaction between SARS-CoV E and human PALS1 and investigate the consequences of E expression in MDCKII epithelial cells on cyst morphogenesis and tight junction formation. We propose a model which places E as a virulent factor that hijacks the TJ-associated protein PALS1, causing severe damage to the epithelial barrier. Our data provide new insight into the molecular mechanisms that contribute to SARS-CoV-induced pathogenesis in infected epithelia.

**MATERIALS AND METHODS**

**Two-Hybrid Assay in Yeast**

Bait cloning and yeast-two-hybrid screening were performed by Hybrigenics, S.A., Paris, France. In brief, the cDNA coding for the CT domain of SARS-CoV E was amplified by PCR and cloned into the pP2B vector enabling fusion with the LeuA binding domain. The bait construct was checked by sequencing the entire insert and was subsequently introduced into the L4oAGAl4 yeast strain (Fromont-Racine et al., 1997). A random-primed human placenta cDNA library that consists of ten million independent fragments was transformed into the Y187 yeast strain, and both strains were allowed to mate. In the screening, 70 millions interactions were tested with E CT. After selection on medium lacking leucine, tryptophane, and histidine, 146 positive clones were picked, and the corresponding prey fragments were amplified by PCR and sequenced at their 5' and 3' terminal ends. Sequences were then filtered and contiged as described previously (Formstecher et al., 2005) and compared with the latest release of the Genbank database using BLASTN. A Predicted Biological Score (PBS) was attributed to assess the reliability of each interaction, as described earlier (Rain et al., 2001; Formstecher et al., 2005).

**Cell Culture and Mammalian Cell Transfection**

Madin-Darby Canine Kidney II (MDCKII) epithelial cells were previously characterized (Gaush et al., 1966). Vero E6 African Green Monkey kidney (ATCC, Manassas, VA, USA), Human Embryonic Kidney (HEK) 293T (ATCC), and MDCKII epithelial cell lines were cultured in Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in a 5% O2/5% CO2 atmosphere. Well-differentiated normal human bronchial epithelial (wd-NHBE) cells were cultured as described previously (Chan et al., 2010).

For pull-down and coimmunoprecipitation assays, 8 × 106 cells plated per dish were seeded onto 100-mm and 150-mm diameter culture dishes, respectively. Cells were then incubated overnight at 37°C before transfection using either FuGene 6 transfection reagent (Roche Diagnostic GmbH, Mannheim, Germany) or CalPhos Mammalian transfection kit (Clontech Laboratories, Mountain View, CA, USA), essentially as described by the manufacturer, with only minor modifications.

**Plasmid Constructions**

The plasmids were previously described: pEYFP-PALS1 (Kamberov et al., 2000), pSecTag2B-Myc-CRB3 (Makarova et al., 2003), pHiT/G and pHT/gag-pol (Soneoka et al., 1995; Fouchier et al., 1997), and pcDNA-E (wt) (Siu et al., 2008). The pcDNA-Flag-PALS1 construct was generated by cloning the human PALS1 cDNA derived from purified RNA of wd-NHBE cells (Chan et al., 2010). Wd-NHBE cells were lysed and total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA). The total RNA was reverse-transcribed to single-strand cDNAs using random hexamer oligonucleotides and the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). The cDNAs were further amplified by PCR using specific sense and antisense primers (Supplementary Table 1) for human PALS1 allowing fusion of a Flag tag in 5' position and containing the BomHl and Xhol restriction sites for subcloning into the pcDNA3.1 vector (Invitrogen). The pcDNA plasmids containing either the wild-type HA-E (wt) or a truncated mutant with a deletion of the last four amino acids (ΔPBM) were prepared using a similar strategy. The pPCRScript-E (codon optimized) plasmid (GeneArt, Regensburg, Germany) was used as template for PCR amplifications with specific sense and antisense primers, and hemagglutinin (H4) tag was introduced in frame upstream of E sequences (Supplementary Table 1).

For expression of G5F-PALS1 fusion proteins, PALS1 cDNA inserts were subcloned into the pGEX-4T1 vector (Amersham Biosciences, Uppsala, Sweden). Briefly, PALS1 inserts were amplified by PCR from the pcP6-HK4T-IpB27 plasmid obtained from Hybrigenics (clone 131) and inserted in frame in between BamHI and XhoI restriction sites of the pGEX-4T1 vector, downstream of the glutathione S-transferase (GST) cDNA. Sense and antisense primers used to amplify PALS1 cDNA inserts were described as Supplemental Table 1.

For production of the MDCKII stable cell line expressing eGFP-PALS1, the pP6-HK4T-IpB27 plasmid was introduced into the eGFP retrovector plasmid pLEGFP-C1 (BD Biosciences, San Jose, CA) as previously described (Kamberov et al., 2000). For production of MDCKII stable cell lines expressing the HA-E (wt) and (ΔPBM), retrovector constructs were generated by inserting full-length HA-E nucleotides or truncated (Δ4) nucleotides as sequences from pcDNA by digestion with BamHI and XhoI, into the pCHMWS-eGFP-Hygro retrovector (kindly provided by Dr. Rik Gijbers, Molecular Medicine at the Katholieke Universiteit Leuven, Belgium) after removal of the eGFP gene.
All the pGEX-4T1 constructs were transformed into BL21-Gold (DE3) pLys bacteria strain (Strategene-Agilent Technologies, La Jolla, CA) for protein production. pHIT/G, pHIT/gag-pol, pLEFFP, pcDNA, and pCHMWS constructs were transformed into DH5α bacteria strain for plasmid amplification. All constructs were sequenced at the Genome Research Centre of the University of Hong Kong to verify that the procedures had not generated unwanted mutations.

Antibodies

The rabbit polyclonal antibody against the carboxy-terminal domain of the E protein was previously described (Siu et al., 2008). The mouse polyclonal antibody against the SARS-CoV RNA-dependent RNA polymerase was generated by immunization with mice with purified ORF3a proteins expressed in mammalian cells as described (Kam et al., 2007). The mouse monoclonal anti-ERGIC 53 (Schweizer et al., 1998) was a generous gift from Hans-Peter Hauri (Biozentrum, University Basel). The rabbit polyclonal antibodies against CRB3 antisera were produced as described (Kamberov et al., 2000; Makarova et al., 2003). The antibodies anti-Calnexin and Golgin-97 were mouse monoclonal antibodies from Abnova (Taipei City, Taiwan) and Invitrogen, respectively. The rabbit polyclonal antibody anti-Giantin was from Covance, Inc., (Chantilly, VA) and were dissolved in 4% dimethyl sulfoxide (DMSO, Sigma Aldrich).

Production and Purification of Retroviral Particles and Establishment of MDCKII Stable Cell Lines

HEK 293T cells were used as packaging cells to produce replication-defective retroviral particles. For this experiment, 5 × 106 cells were seeded in 100-mm diameter culture dishes and grown for 20 h. For production of the particles pseudotyped with the VSV-G envelope glycoprotein as vectors for eGFP-PAL5, PALS1 or eGFP expression (VSV-Gpp-eGFP-PALS1 and VSV-Gpp-eGFP-C1), two packing plasmids, namely pHIT/G (80 μg of either pCHMWS-HA-E (wt) or pCHMWS-HA-E (ΔPBM)), and HcRed (PVDF) membranes (Amersham Biosciences). The membranes were blocked overnight at 4°C in 10% skimmed milk prepared in 1X phosphate buffered saline (PBS), 0.1% Tween-20. To detect Flag-PALS1 membranes were hybridized with mouse IgG1 monoclonal anti-Flag M2 HRP-conjugated antibody; E, HA-E (wt), and HA-E (ΔPBM) proteins were detected by hybridization of the membranes with primary antibodies against rabbit anti-E and mouse IgG1 monoclonal anti-HA tag (Sigma Aldrich), respectively, followed by HRP-conjugated secondary antibodies. Antibody solutions were prepared in 5% skimmed milk, 1X PBS, 0.1% Tween-20. To visualize the protein bands, membranes were hybridized with ECL Western blotting detection reagent (Amersham Biosciences).

Coimmunoprecipitation Assay

The pulldown and coimmunoprecipitated proteins were solubilized in LDS sample buffer (Invitrogen), 10 mM DTT, boiled at 95°C for five minutes, and separated by electrophoresis using NuPAGE Novex 4–12% Bis-Tris Mini gels and subsequently transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked overnight at 4°C in 10% skimmed milk prepared in 1X phosphate buffered saline (PBS), 0.1% Tween-20. To detect Flag-PALS1 membranes were hybridized with mouse IgG1 monoclonal anti-Flag M2 HRP-conjugated antibody; E, HA-E (wt), and HA-E (ΔPBM) proteins were detected by hybridization of the membranes with primary antibodies against rabbit anti-E and mouse IgG1 monoclonal anti-HA tag (Sigma Aldrich), respectively, followed by HRP-conjugated secondary antibodies. Antibody solutions were prepared in 5% skimmed milk, 1X PBS, 0.1% Tween-20. To visualize the protein bands, membranes were hybridized with ECL Western blotting detection reagent (Amersham Biosciences).

Electrophoresis and Immunoblotting

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Calcium Switch Assay and Transepithelial Electrical Resistance (TER) Measurement

Approximately 2.5 × 10^4 MDCKII cells were seeded onto 12-mm transwell membrane filters (Corning, Lowell, MA, USA) and cultured in culture medium containing 1.8 mM calcium until confluence. The monolayers were washed three times with 1X PBS and maintained overnight in low calcium medium (S-MEM, 0.1 mM dialyzed PBS [Invitrogen], 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.292 mg/ml L-glutamine) containing 5 mM calcium to dissociate intercellular junctions. On the following day, the low calcium medium was replaced with prewarmed normal medium (1.8 mM calcium), and transepithelial electrical resistance (TER) was measured at the specified time points post-calcium switch (t = 0, 1, 2, 4, 5, 6, 8, 17, 24, 120 h). In brief, TER was determined as described previously (Straight et al., 2004) using a Millicell-ERS volt-ohm meter (Millipore, Billerica, MA, USA).

Immunostaining of Vero E6 Epithelial Cells and MDCKII Cysts

BIOCHIP slides containing SARS-CoV-infected and uninfected Vero E6 cells (Euroimmun, Lübeck, Germany) were used according to the manufacturer's instructions. Cells were permeabilized with 0.2% Tween-20 in 1X PBS and then blocked with 10% normal goat serum (NGS, heat-inactivated, Zymed, Invitrogen) in 0.2% Tween-20, 1X PBS. Primary and secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas Red (Invitrogen) were prepared in 5% NGS in 0.2% Tween-20, 1X PBS. Cells were incubated at room temperature for 30 min with primary antibodies, washed three times, followed by incubation with secondary antibodies for an additional 30-min period. For Vero E6 cells transfected with pEYFP-PALS1 and pcDNA-HA-E (wt), cells were fixed with 4% PFA in 1X PBS for 15 min and subsequently immunostained as described above.

For MDCKII cysts, the medium was drained off, followed by three extensive washes in 1X PBS. Next, cysts were fixed with 4% PFA, 1X PBS, 0.02% sodium azide for one hour, permeabilized with 0.1% SDS, 1X PBS, 0.02% sodium azide for 30 min at room temperature and blocked as described above. MDCKII cysts were incubated with primary antibodies diluted in 2% NGS, 1X PBS, 0.02% sodium azide for 48 h and then extensively washed (3×) in the same medium. Alexa Fluor-conjugated secondary antibodies were added for 24 h at 4°C in a humidified chamber. For MDCKII cells grown on transwell membranes, cells were fixed, permeabilized, and immunostained as described above with minor modifications. Cell nuclei were labeled with 4′,6′-diamidino-2-phenylindole (DAPI). BIOCHIPs and Vero E6 cells were then mounted with anti-fading agent Mowiol 4–88 reagent (Merck KGaA, Darmstadt, Germany). Images were acquired using ZEISS LSM 510 Axiovert 200M inverted confocal microscope (Core Imaging Facility of The KGaA, Darmstadt, Germany). Images were acquired using ZEISS LSM 510/H11032, 6-diamidino-2-phenylindole (DAPI). BIOCHIPs and Vero E6 cells were transfected with pEYFP-PALS1 and pcDNA-HA-E (wt), cells were fixed with 4% PFA in 1X PBS for 15 min and subsequently immunostained as described above.

RESULTS

The Carboxy-Terminal (CT) Region of SARS-E Protein Interacts with Human PALS1 in Yeast-Two-Hybrid Assay

We applied a yeast-two-hybrid screening strategy to identify cellular proteins that could interact with the CT domain of E (Figure 1B, panel a). The yeast-two-hybrid system consists of the PALS1 PDZ domain.

PALS1 Is Present at the Virus Budding Site in SARS-CoV–Infected Cells

To study the relative subcellular localization of PALS1 and viral structural envelope proteins in SARS-CoV–infected Vero E6 cells, infected and control noninfected Vero E6 cells were processed for immunofluorescence microscopy. SARS-S, E, and PALS1 proteins were labeled with specific antibodies and their distribution was assessed with respect to the ERGIC compartment (stained with antibodies against the cellular lectin ERGIC-53), where viral structural proteins accumulate and virions assemble (Figure 3A).
immunoprecipitated with Flag-PALS1 from cotransfected cells (lane 5) whose input is shown in lane 1. The molecular mass (in kDa) and migration of protein standards are indicated on the left edge of each gel. Results shown are representative of two independent experiments.

As expected, the S and E proteins both localized at the ERGIC site in infected Vero E6 cells (Figure 3A, panels a–c, white arrow). PALS1 was mainly localized at cell–cell junctions in monolayers of non infected Vero E6 cells (Figure 3A, panels d and e, red arrow). Strikingly, in infected cells, PALS1 was found in the perinuclear region, where it partially colocalized with SARS-S protein (Figure 3A, panels d–e, white arrowhead). Moreover, colabeling of PALS1 and ERGIC-53 in infected samples indicated the presence of PALS1 in this later compartment (Figure 3A, panel f, white arrowhead). These data suggest that PALS1 is retained at the virus-assembly site in SARS-CoV infected cells where it may interact with E.

To further document the subcellular localization of PALS1 in SARS-CoV-infected cells, specific antibodies against Calnexin, an endoplasmic reticulum (ER)-resident protein and Golgin-97, a trans-Golgi marker, were used in combination with anti-PALS1 antibodies (Figure 3B, panels a and b). Our data show that PALS1 does not accumulate in the ER and is present in the Golgi compartment.

We then investigated whether another TJ protein, the ZO-1 protein, could also be relocalized to the virus assembly site in SARS-CoV infected cells (Figure 3B, panel c). Interestingly, PALS1 was present in an intracellular compartment, whereas ZO-1 was exclusively found at cell–cell contact zones. This result indicates that PALS1 is specifically recruited to the virus budding compartment (ERGIC/Golgi region) in infected cells.

Because both antibodies recognizing SARS-E and PALS1 were raised in rabbits, our data provide only indirect evidence for colocalization of these two proteins. Thus, in a separate series of experiments, we addressed this issue by transfecting Vero E6 epithelial cells with EYFP-tagged PALS1 and HA-E (wt). As expected, we observed colocalization of EYFP-PALS1 and HA-E (wt) in the perinuclear region in Vero E6 transfected cells (Supplemental Figure S1, panel A and B, white color arrow). Altogether, these findings implicate that E interacts with PALS1 and most likely retains PALS1 at the virus assembly site, both in infected and transfected cells.

Figure 1. Interaction of SARS-E and PALS1 proteins. (A) Sequence of the cytoplasmic tail (CT) of SARS-E protein that was used as the bait for yeast-two-hybrid screening, NT, amino-terminus; TM1–2, transmembrane domains. (B) Schematic representation of clones 67, 131, and full-length PALS1. Functional domains are listed below and identified by different shapes. Among 28 PALS1 cDNA clones isolated, #67 (panel a) and #131 (panel b) encoded the smallest and largest PALS1 cDNA fragments, respectively. Numbers indicate amino acid position. (C) SARS-E binds to PALS1 in mammalian epithelial cells. Vero E6 cells were either mock transfected or transfected with plasmids expressing Flag-PALS1 and E protein, alone or in combination. Forty-eight hours post-transfection, cells were lysed and Flag-PALS1 was immunoprecipitated with anti-Flag M2 antibodies conjugated to agarose resin (lanes 2–5). Samples were separated by gel electrophoresis (4–12% acrylamide) and proteins revealed by immunoblotting (IB) using either mouse monoclonal anti-Flag M2 antibody (panel a) or rabbit anti-E serum (panel b). SARS-E protein was cotransfected with the plasmid expressing HA-E (wt) and PBM, to confirm this finding in human epithelial cells, coimmunoprecipitation experiments were performed with ly-

E Protein Possesses a PDZ Domain-Binding Motif at its Carboxy-Terminal End

Having demonstrated that E binds to the PDZ domain of PALS1 and that PALS1 is redistributed to the ERGIC and Golgi region in infected and transfected cells, we were interested in characterizing the amino acids in the E protein that are responsible for E-PALS1 interaction. To verify this hypothesis, we prepared a mutant in which the D-L-L-V motif, which is located at the carboxy-terminal PBM of CRB1 and 3 (E-R-L-I), the natural ligands of PALS1 PDZ domain. Indeed both sequences start with an acidic amino acid and end with two hydrophobic residues ([E,D]-X-[D,F]). Moreover, the four carboxy-terminal amino acids of E have characteristic of PDZ domain ligands (Beuming et al., 2005; Tonikian et al., 2008). Therefore, we hypothesized that the D-L-L-V carboxy-terminal peptide of E is a PDZ domain-binding motif that binds PALS1 PDZ domain.

To verify this hypothesis, we prepared a mutant in which these four amino acids were deleted and performed GST-pull down assays in which GST-PALS1 fusion proteins were incubated with cell lysates containing either E wild type or a truncation mutant of E, named E (wt) and E (ΔPBM), respectively. Strikingly, both GST-PALS1 fusion proteins (clone 131 and PDZ) efficiently pulled down HA-E (wt), but not HA-E (ΔPBM) (Figure 4A, panels a and b, lanes 3–6). Of note, the HA tag on SARS-E did not affect the strength of the interaction with GST-PALS1 fusion proteins (data not shown). We also did not observe any nonspecific binding with beads, GST protein (data not shown), and GST-PALS1 (SH3) fusion protein (Figure 4A, panels a and b, lanes 7–8), which were included as negative controls. These results indicate that the D-L-L-V motif, which is located at the carboxy-terminal extremity of E protein, is a PDZ domain-binding motif that enables interaction with PALS1 PDZ domain in vitro.

To confirm this finding in human epithelial cells, coimmunoprecipitation experiments were performed with ly-
whereas no signal was seen in mock-transfected cells or when control beads were used (a). Results shown are representative of two
structs containing the PDZ domain of PALS1, pulled down only by beads linked to con-
cwashed five times with cell lysis buffer and E protein was analyzed by SDS-
PAGE and immunoblotting (IB) using a rabbit anti-E serum. The molecular mass (in kDa)
E (amino acids 34-76) and CRB3 (amino acids 80-120) and
We then hypothesized that E CT could compete with CRB3 CT in vitro for binding to
motif is a PDZ domain-binding motif, which mediates E
The inhibitory complexes (GST-PDZ fusion proteins bound with E or CRB3 peptides) formed were not washed
in cell lysates containing either myc-CRB3 or HA-E (wt) proteins were added immediately, followed by overnight
At the completion of the incubation, proteins were added immediately, followed by overnight
SARS-E protein to PALS1 was reduced by ~85% by
deloting the putative PBM. As expected, the HA-E (wt) protein was
efficiently coprecipitated with Flag-PALS1 (Figure 4B, panels a and b). All proteins
in cells coexpressing HA E (wt) (Figure 4B, panel a, compare lanes 4, 5, and 6). Proteins were immunoprecipitated from
cytoplasmic tail (PBM) mutant protein were coimmunoprecipitated from the negative
control cell lysates (Figure 4B, panels c and d, lanes 2–3). Altogether, these findings strongly suggest that the D-L-L-V motif is a PDZ domain-binding motif, which mediates E binding to the PALS1 PDZ domain, both in vitro and in vivo.
It also indicates that internal motifs other than the PBM sequence could mediate binding to either PALS1 or other proteins that interact with PALS1 in human epithelial cells, albeit with lower affinity.

**Figure 2.** Characterization of PALS1 functional domains that interact with SARS-E protein. (A) Schematic representation of PALS1 clone 131 and truncation mutants fused to glutathione-S-transferase (GST) at the N-terminus. Functional domains are depicted by different shapes as in Figure 1B. (B) The PDZ domain of PALS1 is necessary and sufficient to bind to SARS-E in vitro. Purified GST-PALS1 (clone 131) and its truncation mutants linked to sepharose beads were incubated overnight at 4°C with cell lysates of Vero E6 cells transiently expressing E protein. Two assays were performed in parallel for each GST-PALS1 construct with increasing amounts (0.5 μg or 1.0 μg) of fusion protein, as indicated by the triangles above each blot. Beads were washed five times with cell lysis buffer and E protein was analyzed by SDS-

**E CT Competes with CRB3 CT in Vitro for Binding to PALS1 PDZ Domain**

We then hypothesized that E CT could compete with CRB3 CT, a natural ligand of PALS1 PDZ domain, and affect CRB3-PALS1 interaction. To verify this hypothesis, we designed two peptides corresponding to the CT tails of E (amino acids 34-76) and CRB3 (amino acids 80-120) and tested their capacity to interfere with the interaction between CRB3 and PALS1 PDZ domain in a GST-pull down assay (Figure 4C). Briefly, the peptides (0.2–1 mM in DMSO) were incubated with 1 μg of GST-PDZ fusion protein for six hours at 4°C. As control, GST-PDZ fusion proteins were incubated with DMSO in absence of peptide. The inhibitory complexes (GST-PDZ fusion proteins bound with E or CRB3 peptides) formed were not washed and cell lysate containing either myc-CRB3 or HA-E (wt) proteins were added immediately, followed by overnight incubation at 4°C. The precipitated interacting proteins were determined by immunoblotting using a rabbit anti-CRB3 serum (Figure 4C, panel a) and a mouse IgG1 monoclonal anti-HA antibody (Figure 4C, panel b), respectively.

As expected, both the myc-CRB3 and the HA-E (wt) proteins were pulled down by GST-PDZ in absence of peptide (Figure 4C, panels a and b, lane 3). By contrast, the CT peptide of E drastically inhibited the interaction of myc-CRB3, but not HA-E (wt), with PALS1 PDZ in a dose-
dependent manner (Figure 4C, panels a and b, lanes 4–5). Conversely, the CRB3 CT peptide could abrogate the interaction of both myc-CRB3 and HA-E (wt) interaction with PALS1 PDZ (Figure 4C, panels a and b, lanes 6–7). These data indicate that the carboxy-terminal of E can compete out the interaction of CRB3 with the PDZ domain of PALS1 in vitro.

Our data seem to indicate that CRB3 C-terminal peptide has a stronger affinity to PALS1 PDZ than the E C-terminal peptide. However, one has to consider that in infected cells E expression levels are high and thus E might be able to compete out CRB3 binding to PALS1 or at least to retain significant levels of PALS1 in the secretory pathway.

We have recently performed a similar assay with a peptide corresponding to the E C-terminal sequence lacking the DLLV motif and no competition with HA-E or myc-CRB3 was observed (data not shown). This result indicates that the competition of the E CT peptide with the CRB3 CT peptide for binding to PALS1 PDZ depends on E PBM.
Expression of E Causes Defect in Morphogenesis of MDCKII Cysts

To investigate the functional consequences of E expression on epithelial cell morphogenesis, we chose to use the MDCKII cysts model, which has been widely used to establish molecular and cellular mechanisms that regulate epithelial polarity and morphogenesis (O’Brien et al., 2001; Straight et al., 2004; Shin et al., 2005; Horikoshi et al., 2009; Schlüter et al., 2009). To this end, we first generated MDCKII stable cell lines ectopically expressing both eGFP-PALS1 and HA-E (wt) or HA-E (ΔPBM). In addition, two control cell lines, MDCKII eGFP-PALS1 and MDCKII eGFP-PALS1, HcRed were included.

In these experiments, suspensions of predominantly single cells of the specified cell lines were embedded into DMEM medium supplemented with 4% GelTrex on a thin bottom of solid GelTrex, and the cells were maintained at 37°C with 5% CO2 for 5 days to form cysts. Then, the cysts were fixed, permeabilized, and colabeled with appropriate antibody combinations, followed by confocal microscopy. To perform a phenotypic cyst assay, we have counted 870–885 cysts for each cell line and the phenotypes observed were categorized into cysts with either a single apical lumen or multiple lumens. Subsequently, a Student’s t test was used to analyze whether defect of cysts morphogenesis significantly correlated with ectopic expression of E protein.

We initially verified that the majority (61%) of MDCKII-eGFP-PALS1 cells (540/885) formed single lumen cysts with distinct apico basal polarity (Figure 5, A and C). As expected, control MDCKII cysts expressing either eGFP or eGFP-PALS1 and HcRed fluorescent protein showed a similar percentage of cysts with single lumens and maintained polarity (data not shown). In both MDCKII-eGFP-PALS1 and MDCKII-eGFP-PALS1, HcRed cells, eGFP-PALS1 was found on apical membranes of the cysts, facing the internal lumen and present at TJ as indicated by its colocalization with the tight junction associated protein ZO-1 (Figure 5A and data not shown). HcRed fluorescent protein was diffusely distributed in the cytoplasm of the latter cells (data not shown). Together, these results strongly suggest that epithelial polarity in the two control cell lines, MDCKII eGFP-PALS1 and MDCKII eGFP-PALS1, HcRed, is maintained.

Strikingly, we observed that ectopic expression of HA-E protein in MDCKII-eGFP-PALS1 cells was associated with formation of cysts with either two or multiple lumens. HA-E protein was distributed in the subapical region of the cysts, as shown by its localization underneath the GP135 apical protein, which is consistent with localization in the ERGIC/Golgi region (Figure 5B). Quantitative analysis showed that only 21% (187/874) of cysts expressing HA-E (wt) proteins formed correctly (Figure 5C), a threefold decrease compared with control cell lines (p < 0.001, unpaired t test).

Expression of HA-E (ΔPBM) also led to a severe phenotype with only 24% (208/863) of the MDCKII-eGFP-PALS1, HA-E (ΔPBM) cysts displaying a single lumen (p < 0.001, unpaired t test) (Figure 5B and C). The defect of cyst morphogenesis observed in MDCKII cells expressing HA-E (ΔPBM) mutant protein suggests that the DLLV motif is not involved in this phenomenon and that other domains in E may interact with other cellular pathways implicated in morphogenesis of cysts.

Figure 3. Infection with SARS-CoV causes redistribution of PALS1 proteins to the ERGIC/Golgi region in Vero E6 cells. (A) SARS-CoV–infected Vero E6 cells were fixed, permeabilized, and labeled with antibodies against the SARS-S (S), SARS-E (E), PALS1, and ERGIC-53 (ERGIC, a protein marker of this compartment), as indicated. Images shown were single focal plane images and were acquired with a Zeiss LSM 510 Axiovert 200M confocal microscope. SARS-S and SARS-E colocalized in the ERGIC (panels a–c, white arrows). PALS1 was mainly present at cell-cell contacts (panels d–f, red arrows) and at the ERGIC (panel f, white arrowhead), where it colocalized with S protein (arrowheads in panels d and e) in infected cells. This cytoplasmic distribution of PALS1 was conspicuously absent from cells expressing little or no SARS-S (asterisks in panels d and e). (B) SARS-CoV-infected Vero E6 cells were fixed, permeabilized, and labeled with antibodies against the PALS1, Calnexin (ER), Golgin-97 (trans Golgi), and ZO-1 (tight junctions), as indicated. Images were acquired with a Zeiss Axio Observer Z1 fluorescent microscope. PALS1 did not redistribute to the ER (panel a). However, it colocalized with Golgin-97 (panel b, white arrows)

 whereas ZO-1 remained at cell to cell contacts (panel c, white arrowhead) and did not colocalize with the cytoplasmic accumulated PALS1 (panel c, white arrow). Bar, 10 μm.
Figure 4. SARS-E protein possesses a PDZ domain-binding motif (PBM) at its carboxy-terminus. (A) Pull-down assay. Purified GST-PALS1 fusion proteins (clone 131, PDZ and SH3 domains; see Figure 2A) linked to sepharose beads were incubated overnight at 4°C with lysates of HEK 293T human epithelial cells that transiently expressed either full-length (wt) or a truncated (ΔPBM) SARS-E protein containing a hemagglutinin (HA) tag at the N-terminal position. Two assays were performed in parallel for each construct with increasing amounts (0.5 μg or 1.0 μg) of fusion protein, as indicated by the triangles above each blot. Beads were washed five times with cell lysis buffer and E protein was analyzed by SDS-PAGE and immunoblotting (IB) using either anti-Flag M2 or anti-HA mouse monoclonal antibodies. Deletion of PBM abolished the interaction with PALS1 (cf. lane 5–6 in panel d). The SH3 construct was used as the negative control. (B) Coimmunoprecipitation assay. Cells were transfected with the combination of plasmids indicated on top. Forty-eight hours post-transfection, cells were lysed and proteins immunoprecipitated with anti-Flag M2 antibody conjugated to agarose resin (panels c and d). Samples were separated by gel electrophoresis (4–12% acrylamide), and proteins were analyzed by SDS-PAGE and immunoblotting (IB) using a mouse anti-HA serum. Deletion of PBM resulted in an almost complete disruption of the interaction between SARS-E and PALS1 (cf. lanes 5–6 in panel d). (C) Competition assay. 1 revealed by immunoblotting (IB) using either anti-Flag M2 or anti-HA mouse monoclonal antibodies. Deletion of PBM resulted in an almost complete disruption of the interaction between SARS-E and PALS1 (cf. lanes 5–6 in panel d).

To ensure that the effects observed were not dependent on the clones selected or PALSI overexpression, the same set of experiments was performed on MDCKII cells expressing either form of E on an endogenous PALSI background. We found that 72%, 8%, and 14% of cysts had a normal morphology with a single lumen for MDCKII, MDCKII HA-E (wt) and MDCKII HA-E (ΔPBM) cells, respectively (Supplemental Figure S3).

Together, our observations demonstrate that E expression is responsible for alteration of morphogenesis of MDCKII cysts and that its DLLV motif is not essential to induce such a defect.

E Delays Formation of TJ in MDCKII eGFP-PALS1 Cells in a PBM-Dependent Manner

To further study the functional consequences of E protein expression on PALSI-dependent TJ formation, we measured the establishment of transepithelial electrical resistance (TER) in confluent monolayers of MDCKII cells grown on membrane filters, using a calcium-switch assay. MDCKII eGFP-PALS1 control cells could rapidly form TJ (Figure 6), as indicated by a maximum TER value of ~400 Ω/cm² reached at 2 h post-calcium switch in the experiment shown. A similar time course of TJ formation was also recorded with MDCKII cells expressing eGFP-PALS1 together with HcRed fluorescent proteins (data not shown). At later time points, TJ were gradually loosing strength, with lower TER values of 100 Ω/cm² measured after 17 h post-calcium switch, as previously reported in two different studies (Straight et al., 2004; Latorre et al., 2005). Interestingly, ectopic expression of HA-E protein led to a significant delay in TJ formation, which started only 4 h post-calcium switch, with TER values gradually raising to attain a maximum of 700 Ω/cm² recorded at 17 h post-calcium switch (Figure 6). Conversely, monolayer cells of the MDCKII eGFP-PALS1 expressing HA-E (ΔPBM) reached maximum TER at 2 h post-calcium switch with a maximum of 700 Ω/cm² (Figure 6). Interestingly, two opposite effects were observed for cells expressing E (wt) and E (ΔPBM) when compared with control cells. Whereas E (wt) expressing cells presented a significant delay in establishment of TER, E (ΔPBM) cells could reach higher TER values more rapidly than control cells. These data suggest that both expression of E (wt) and E (ΔPBM) affect the kinetics of TJ formation and that the DLLV C-terminal motif of E is responsible for the delay in TJ establishment in MDCKII epithelial cells. A similar pattern was observed for clones of MDCKII cells expressing E (wt) or E (ΔPBM) on an endogenous PALSI background (Supplemental Figure 4).

We then decided to analyze the morphology and polarity of MDCKII monolayers at 2 h post-calcium switch, when the control and the HA-E (ΔPBM) expressing cell lines had reached their maximal TER values (Figure 7). Monolayers of MDCKII eGFP-PALS1, eGFP-PALS1, HA-E (wt), and eGFP-PALS1, HA-E (ΔPBM) were fixed, permeabilized, and stained with appropriate antibodies to study the subcellular distribution of GP135 (apical marker), E-cadherin (A) protein), ZO-1 (TJ marker), and HA-E wt and truncated proteins. Relative localization of eGFP-PALS1 was also analyzed. Confocal microscopy and Z-sectioning allowed three-dimensional analysis of samples and monitoring of TJ formation and polarity establishment. At 2 h post-calcium switch, MDCKII eGFP-PALS1 cells had formed a regular monolayer of cubical cells (Figure 7A, panels a and b). In these cells, eGFP-PALS1 was present at cell–cell contacts in
apical regions where it colocalized with ZO-1. Analysis of XZ and YZ dimensions showed that both proteins were present at TJ (Figure 7A, panel b, black arrowheads). The GP135 marker was found on apical surface of the cells (Figure 7A, panel a), whereas the E-cadherin protein was present at cell–cell junctions on lateral membranes, underneath eGFP-PALS1 and ZO-1, delineating AJ (Figure 7A, panels a and b). We concluded that at t/H11005 2 h post-calcium switch, MDCKII eGFP-PALS1 cells had correctly formed TJ and were polarized.

Conversely, at the same time point, MDCKII eGFP-PALS1, HA-E (wt) cells had not yet polarized (Figure 7B). Indeed, confocal microscopy analysis revealed that the majority of cells was flat, with round cells locally occasionally lying on top of the monolayer. The subcellular localization of eGFP-PALS1 was significantly affected. Although the protein was still found at the cell–cell contacts, it was present at lower levels. GP135 did not specifically distribute to the apical surfaces but localized diffusely to the cell cytoplasm (Figure 7B, panel a). ZO-1 was found at the cell–cell junctions, preferentially at apical regions of the cells where it only partially colocalized with eGFP-PALS1 (Figure 7B, panel b, white arrows). The HA-E (wt) protein was expressed in a perinuclear compartment, suggestive of localization to the ERGIC/Golgi compartment (Figure 7B). Of note, a partial colocalization of eGFP-PALS1 and HA-E (wt) is expressed as percentage of total count of cysts scored. Results are shown as means ± SEM of the specified number of replicates from three independent experiments. For each cell line, we have counted total of 870–885 cysts. ***p < 0.001 by the unpaired Student’s t test.
Figure 7. Tight junction formation is delayed in MDCKII cells expressing HA-E (wt) protein, but not in cells expressing the HA-E (ΔPB M) mutant protein. MDCKII, eGFP-PALS1 cells grown to confluence on polyester membrane filter were transferred into low calcium medium (5 μM) for twenty-four hours to disrupt cell–cell junctions and then switched to normal growth medium (1.8 mM Ca2+). Cells were fixed at different time points (t = 0, 1, 2, 4, 6, 8 h) post-calcium switch, permeabilized and stained with antibodies against ZO-1 (a marker of tight junctions), E-cadherin (a marker of adherens junction), the apical protein GP135 and hemagglutinin (HA) tag at the amino terminus of HA-E (wt/ΔPB M) proteins, as indicated. Images were acquired with a ZEISS LSM 510 Axiovert 200M confocal microscope. All images shown in this figure were taken from cells fixed at 2 h post-calcium switch. For A–C, two representative fields are shown. (A) PALS1 is localized at the tight junction, as confirmed by colocalization of ZO-1 (panel b, black color arrowhead). E-cadherin is at the lateral membrane of two adjacent cells, which marks the adherens junction. (B) SARS-E (wt) containing a hemagglutinin (HA) tag at its amino terminus position is localized at the perinuclear region (panels a and b). In these cells, PALS1 is partially localized at the cell–cell periphery, with little overlap.
was observed in some cells (Figure 7B, panel b, white arrowheads), suggesting a partial retention of PALS1 by E in intracellular compartments.

Lastly, analysis of MDCKII eGFP-PALS1, HA-E (ΔPBM) cells indicated that these cells did not present a notable polarity defect at 2 h post-calcium switch (Figure 7C). In these cells, both eGFP-PALS1 and ZO-1 were found at the TJ, whereas GP135 was enriched at the apical surfaces. HA-E (ΔPBM) was diffusely distributed in the cell cytoplasm, indicating a role of the DLLV motif in E accumulation in the perinuclear compartment. This result also demonstrates that the DLLV motif of E plays a major role in alteration of polarity in monolayers of MDCKII cells.

Together, these findings demonstrate that SARS-CoV E expression, most likely through its interaction with PALS1, had a profound effect on cell polarity by inducing a severe delay in TJ formation in monolayers of MDCKII epithelial cells.

E Expression Disturbs the Subcellular Localization of PALS1 and Alters the Formation of a Uniform Polarized Monolayer in a PBM-Dependent Manner

We then decided to perform the same experiment but with a more detailed time course post-calcium switch to analyze the consequences of SARS-CoV E expression on PALS1 distribution and monitor any potential change of the structure of the MDCKII monolayer upon time. TER measurement and confocal microscopy analysis were conducted in parallel from 0 to 120 h post-calcium switch (Figure 8). Control, HA-E (ΔPBM) and HcRed (data not shown) MDCKII eGFP-PALS1 cells presented a peak of TER at 3 h post-calcium switch whereas a delay was observed for the HA-E (wt) expressing cells (Figure 8A). For all time points, MDCKII HA-E (ΔPBM) cells showed higher TER values than control cells, suggesting that they establish junctions more rapidly and that mature TJ are tighter in these cells. For MDCKII HA-E (wt) cells, the maximal recorded value of TER was at 8 h post-calcium switch (400 Ω/cm²), although it is possible that resistance had risen further between the 8–24 h interval, for which we do not have TER values.

To monitor the differential changes of structure of monolayers expressing either HA-E (wt) or HA-E (ΔPBM) and analyze the relative subcellular distributions of E and PALS1, we have fixed cells at 2, 8, 24, and 120 h after calcium switch, permeabilized and labeled them with specific antibodies against the HA tag and either the Giantin (cis/median Golgi) or the Golgin-97 (trans Golgi) cellular proteins (Figure 8B and data not shown). For each panel we show a representative image of an apical confocal section (XY) as well as transversal XZ and YZ confocal sections.

At all time points and for both cell lines, a significant fraction of eGFP-PALS1 was observed at cell-cell contact, although with different patterns, indicating that either the protein had been retained at cell–cell contacts or had trafficked back to these areas after the calcium switch. As expected, HA-E (wt) partially colocalized with the Golgi markers (Figure 8B, white arrows), whereas HA-E (ΔPBM) did not but was present in the apical region of the cytoplasm. In HA-E (wt), but not HA-E (ΔPBM) MDCKII monolayers, round cells that expressed PALS1 in the cytoplasm and had lost structure of polarized cells were observed at all time points, indicating that proper localization of PALS1 and polarity were specifically affected in these cells (Figure 8B, red arrows). Interestingly, these round cells frequently showed a higher expression of E (wt) and a portion of eGFP-PALS1 colocalized with E (Figure 8B, panel e, white arrowhead).

At 2 h post-calcium switch, in HA-E (wt) monolayers, PALS1 appeared in confocal sections as a discontinuous line around cells, indicating that TJ might not be mature. Conversely, in HA-E (ΔPBM) cells PALS1 was uniformly present at cell-cell contacts. This result explains the significantly different TER values between the two cell lines at this time point (100 vs. 450 Ω/cm², Figure 8A). At 8 and 24 h, although TER values of both cell lines were similar (400–500 and 100–120 Ω/cm², respectively), differences could be observed. At 8 h, gaps between cells were occasionally observed close to round cells with high cytoplasmic PALS1 expression and so more frequently for the MDCKII eGFP-PALS1 HA-E (wt) cell line (Figure 8B, panel c). At 24 h, cells were in contact with each others. At this time, more round cells with higher expression of E (wt) and cytoplasmic localization of PALS1 were seen (Figure 8B, panel e, red arrow). PALS1 was mainly at apical cell–cell contacts in MDCKII eGFP-PALS1 HA-E (ΔPBM) cells, although cells had an

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Figure 7 (cont). with ZO-1 (panel b). Tight junction formation is disrupted, as indicated by discontinuous ZO-1 staining (panel b, white arrows). Interestingly, eGFP-PALS1 protein is partially colocalized with HA-E (wt) protein at the perinuclear region (panels a and b, white arrowhead). (C) HA-E (ΔPBM) mutant protein is diffused in the cytoplasm and localized at the subapical region (underneath GP135), as confirmed with the apical protein GP135 (panel a). This mutant protein did not colocalize with PALS1 in any subcellular compartment. PALS1 is distributed at the tight junction, as indicated by colocalization with ZO-1 (panel b, black color arrowhead). XZ and YZ are Z-section series along the X- and Y-axis, respectively.

Figure 8. E expression causes mis-localization of PALS1 and alters the structure of MDCKII monolayers in a PBM-dependent manner. MDCKII and eGFP-PALS1 cells grown to confluence on polyester membrane filter were transferred into low calcium medium (5 μM) for 24 h to disrupt cell–cell junctions and then switched to normal growth medium (1.8 mM Ca²⁺). (A) The restoration of cell junctions was monitored by measuring TER (Ohms/cm²) as a function of time. Cells expressing SARS-E (wt) perturbed the establishment of TJ. The maximal TER value was reached 3 h post-calcium switch for both control and SARS-E (ΔPBM) cells, whereas TER kept rising moderately for SARS-E (wt) cells. At 24 and 120 h post-calcium switch TER values had stabilized at low levels for all cell lines. Results are shown as means ± SEM of nine observations from one representative experiment and have been corrected for background.
irregular shape. This irregular shape was also observed in both cell lines at 120 h post-calcium switch.

Altogether, these data show that E expression disturbs the subcellular localization of PALS1, inducing the rounding up of the cells and altering the formation of a uniform polarized monolayer in a PBM-dependent manner.

**DISCUSSION**

The Last Four Carboxy-Terminal Amino Acids of SARS-CoV E Envelope Protein form a Novel PDZ Domain-Binding Motif

Here we demonstrate that the small envelope protein E of SARS-CoV interacts with the TJ-associated protein PALS1. The interaction was identified in a yeast-two-hybrid screen (Figure 1A-B). We have verified the E–PALS1 interaction in mammalian epithelial cell by coimmunoprecipitation (Figure 1C) and in vitro by GST-pull down assays (Figure 2B). Moreover, we have demonstrated that E possesses a novel PBM motif at its carboxy-terminal tail, which mediates binding of E to PALS1 PDZ domain (Figure 4, A and B), and that a CT peptide of E but not E (ΔPBM) competes against CRB3 interaction with the PDZ domain of PALS1 in vitro (Figure 4C and data not shown). This latter finding suggests that E-PALS1 association could possibly affect the interaction of PALS1 with CRB3 PBM in epithelial cells, leading to a disruption of TJ and apicobasal polarity.

In a recent large-scale genome-wide screen, Obenauer and coworkers have discovered the presence of a PBM at the CT of NS1 protein of the H5N1 avian influenza A virus (Obenauer et al., 2006). Presence of this PBM was shown to
be detrimental virulence factor in mice (Jackson et al., 2008). Interestingly, similar PBM have been characterized and identified at the CT of several viral oncoproteins, namely adenovirus type 9 E4-ORF1, human T-cell leukemia virus type 1 Tax1, and human papillomavirus E6. These viral PBM bind to PDZ domain-containing proteins involved in the regulation of epithelial cell polarity and induced epithelial-to-mesenchymal transformation in a PBM–dependent manner (reviewed in Javier, 2008; Liu and Baleja, 2008; Thomas et al., 2008; Tungteakkhun et al., 2008; Wise-Draper and Wells, 2008; Tomaic et al., 2009). Likewise, SARS-CoV may interfere with epithelial apicobasal polarity through molecular interactions involving the E CT domain.

**E–PALS1 Interaction: Consequences for the Morphogenesis of Cysts and Integrity of TJ**

We have further shown that in SARS-CoV–infected Vero E6 epithelial cells, PALS1 protein is enriched at the ERGIC/ Golgi region, where E proteins accumulate (Figure 3). We argue that in these cells, PALS1 trafficking is altered through mis-targeting to/or retention at the ERGIC/Golgi site. Conversely, the tight junction protein ZO-1 was maintained at intercell contact domains in SARS-CoV–infected cells, indicating the specificity of PALS1 mislocation. Additionally, in Vero E6 cells transiently expressing EYFP-PALS1 and E, we observed that these proteins also colocalized in the perinuclear region (Supplemental Figure S1). Interestingly, we observed that morphogenesis of MDCKII cysts is significantly disrupted when E is expressed (Figure 5 and Supplemental Figure S3). However, polarity was not affected at the time of analysis, as indicated by the apical distribution of GP135, ZO-1, PALS1, and CRB3 (Figure 5 and Supplemental Figure S3). Strikingly, similar findings were observed when E (ΔPBM) was expressed, but not a control HcRed protein (data not shown). Correct tissue morphogenesis is a very sensitive marker, which depends on intact polarity (Schlüter and Margolis, 2009) but also on cdc42-dependent spindle morphology (Jaffe et al., 2008; Qin et al., 2010) independently from tight junction formation. It is possible that E interacts with a cellular pathway that regulates cyst morphogenesis, independently of its PBM. It is reasonable to postulate that E contains more than one region that interacts with host cell factors. It is indeed well known that pathogens usually develop several strategies to ensure their optimal adaptation/interaction/virulence within the host cell environment.

Our data also showed that expression of E (wt), but not E (ΔPBM), delayed TJ formation in MDCKII cells in calcium switch assays (Figure 6). This is well illustrated by immunofluorescence and confocal microscopy analysis of these cells (Figures 7 and 8). Indeed, two hours post-calcium switch, E (wt) expressing cells present a strong defect of polarity with a mis-location of polarity markers, whereas control cells and E (ΔPBM) expressing cells are polarized, with TJ (ZO-1, PALS1), AJ (E-cadherin) and apical (GP135) markers correctly localized. These data indicate that E expression alters TJ formation in a PBM-dependent manner, and affects establishment of polarity. A significant number of E (wt) expressing cells were round with presence of PALS1 in the cytoplasm, occasionally found associated with E in the Golgi region. This was generally observed for cells with higher expression levels of E. However, TJ

**Figure 9.** Model of the potential consequences of SARS-CoV infection on polarity and intercellular junctions formed by alveolar epithelial cells. (A) The interior surface of human lung alveole is lined with a monolayer of polarized epithelial cells that organize themselves spherically around a central lumen. CRB and PAR polarity complexes are clustered to the apical domain to maintain and regulate apical polarity. Green, PALS1, a tight junction-associated protein. (B) A scheme illustrating a working model of sequential events that occur during SARS-CoV infection in alveolar epithelial cells. (a) Infection of alveolar epithelial cells by incoming viruses. The SARS-CoV virions attach to ACE2 receptors, which are localized at the apical surface. Virions are internalized into endosomes where the acidic pH triggers envelope fusion. The viral RNA (vRNA) is released into the cytoplasm and is transcribed to a set of subgenomic (sgmRNA) strands that encode for structural proteins S, M, N, E, and other accessory proteins. S, M, N, and E accumulate in the ERGIC compartment where virions assemble. At this stage, SARS-CoV virions with the structural proteins S, M, and N are released from the infected cell and enter the intercellular space, where they can infect adjacent cells. (b) Disruption of TJ and virus dissemination. Loss of PALS1 at TJ results in a progressive disruption of TJ, which leads to leakage between adjacent epithelial cells, loss of barrier function, and infiltration of SARS-CoV virions into underlying tissues. Eventually, viruses reach the systemic circulation and disseminate to distant organs. Hijacking of PALS1 by SARS-CoV in infected pneumocytes may explain the severe alveolar damages observed in post-mortem lung biopsies from SARS-CoV–infected patients.
could form, although with a delay of several hours, suggesting that the kinetics of trafficking of PALS1 was affected but not the process of TJ formation. At 120 h post-calcium switch, PALS1 was present at cell–cell contacts for both E (wt) and E (ΔPBM) expressing cells, with which are most likely back to a steady state with low TER values, common for this MDCKII cell line (Figure 8, panels g and h). However, round cells were still present in E (wt) expressing cells. Conversely, E (ΔPBM) expressing cells showed enhanced TER values, which indicated a rapid formation of junctions. E (ΔPBM) was not present in the Golgi region but was rather diffuse in the apical region of the cytosol. PALS1 was not mislocalized and did not colocalize with this truncated form of E. Therefore, we conclude that SARS-CoV E alters PALS1 distribution in monolayers of MDCKII cells and, as a consequence, disturbs TJ and polarity in a PBM-dependent manner.

**Implication of E-PALS1 Interaction in SARS-CoV–Induced Pathogenesis in Vivo**

Histopathological analysis of *post mortem* lung tissues of SARS-CoV–infected patients and cynomolgus macaques consistently demonstrates severe DAD, with massive infiltration of macrophages and monocytes in the alveolar space, thickening of epithelium wall, fusion of alveolar septa, and necrotic lesions at hemorrhagic septa (Kuiken et al., 2003; Peiris et al., 2003; Li et al., 2005). However, a molecular mechanism that contributes to the destruction of the alveolar walls remains unclear. Several studies have revealed that human airway epithelial cells produce chemokines and cytokines that contribute to the massive recruitment of leukocytes at the site of infection and strongly suggest that inflammation of the lung contributes to DAD (reviewed in Lau and Peiris, 2005; Thiel and Weber, 2008). Similarly, mechanisms leading to dysfunction of other organs, such as the kidney, at late times of infection, are unknown.

It is intriguing to speculate that upon infection E participates in SARS-CoV–induced pathogenesis and destruction of the epithelial barrier. We have verified that PALS1 and CRB3 are expressed in pneumocytes in immunohistochemistry experiments on post mortem lung biopsies (Kim Tat Teoh and John Nicholls, the University of Hong Kong). We hypothesize that E binds to PALS1 and alters its localization in infected cells in vivo. Loss of PALS1 at TJ could cause TJ and AJ disruption and contribute to the desquamation of the alveolar wall, as observed in lung biopsies from SARS-CoV–infected macaques and patients (Nicholls et al., 2003; Li et al., 2005). We propose that alteration of TJ and AJ would create a breach in the epithelial barrier allowing virions to reach the basal matrix and eventually the systemic circulation to disseminate to distant organs (Figure 9B, panel b).

Our data describe a novel interaction between the small envelope E protein of the SARS coronavirus and the PALS1 tight junction protein strongly impacting the structure of mammalian epithelial cells. These insights contribute to a better understanding of the molecular mechanisms responsible for the abrupt deterioration of the lung epithelium in individuals infected by this deadly virus.

**ACKNOWLEDGMENTS**

We thank Nadège Lagarde and Dr. Jean-Michel Garcia (HKU-Pasteur Research Centre) for treatment of images acquired by confocal microscopy, preparation of cysts, 3D models, and advice on statistical analysis, respectively. We thank Dr. Michael Chan and Dr. Renee Chan (Department of Microbiology, The University of Hong Kong) for providing the wt-NRBE cells and Dr. John Nicholls and Kevin Fung (Department of Pathology, the University of Hong Kong) for immunohistochemistry analysis of *post mortem* biopsies from SARS-CoV–infected patients. We acknowledge the Core Imaging Facility of the Faculty of Medicine of the University of Hong Kong. We also thank Professor Yu-lung Lau (Department of Pediatrics and Adolescent Medicine, The University of Hong Kong) for his continuous encouragement during this project. This work was supported by the Research Fund for the Control of Infectious Diseases (Grant Ref#08070992), the RISPARJ project of the International Network of Pasteur Institutes, and the Area of Excellence Scheme of the University Grants Committee (Grant AoE/M-12/06) (to B.N.) and National Institutes of Health grants (DK58208, and DK69605) (to B.M.). K.-T.T was a Ph.D. student supported by The University of Hong Kong.

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