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The envelope protein of severe acute respiratory syndrome coronavirus interacts with the non-structural protein 3 and is ubiquitinated

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ABSTRACT

To analyze the proteins interacting with the severe acute respiratory syndrome coronavirus (SARS-CoV) envelope (E) protein, a SARS-CoV was engineered including two tags associated to the E protein. Using this virus, complexes of SARS-CoV E and other proteins were purified using a tandem affinity purification system. Several viral and cell proteins including spike, membrane, non-structural protein 3 (nsp3), dynein heavy chain, fatty acid synthase and transmembrane protein 43 bound E protein. In the present work, we focused on the binding of E protein to nsp3 in infected cells and cell-free systems. This interaction was mediated by the N-terminal acidic domain of nsp3. Moreover, nsp3 and E protein colocalized during the infection. It was shown that E protein was ubiquitinated in vitro and in cell culture, suggesting that the interaction between nsp3 and E protein may play a role in the E protein ubiquitination status and therefore on its turnover.

Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) was identified as the etiological agent of a respiratory disease that emerged in Guangdong Province, China, in late 2002, and rapidly spread to 32 countries (Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Marra et al., 2003; Peiris et al., 2003; Rota et al., 2003). After July 2003, only a few community-acquired and laboratory-acquired SARS cases were reported (http://www.who.int/csr/sars/en/). The disease causes an atypical pneumonia with an average mortality of 10%. No clearly defined efficacious treatment is available to prevent or treat SARS.

SARS-CoV is an enveloped, single-stranded positive-sense RNA virus with a genome of 29.7 kb that belongs to genus β of the *Coronavirinae* subfamily (de Groot et al., 2008; Enjuanes et al., 2008; Gorbalenya, 2008). The replicase gene is encoded within the 5′ two-thirds of the SARS-CoV genome, including two overlapping open reading frames (ORF) named ORFs 1a and 1b, the latter being translated by a ribosomal frameshift upstream of the ORF 1a stop codon (Brierley et al., 1989; Thiel et al., 2003; Ziebuhr, 2005). Translation of both ORFs in the cytoplasm of infected cells, results in the synthesis of two polyproteins, pp1a and pp1ab, that are processed by two viral proteinases to yield 16 functional non-structural proteins (nsps) (Ziebuhr, 2005; Ziebuhr et al., 2000). These non-structural proteins are the components of the membrane-anchored replication–transcription complex (Stertz et al., 2007). The largest non-structural protein of SARS-CoV is the multifunctional nsp3 protein that comprises 1922 amino acid residues. It has been proposed that nsp3 could act as a replication/transcription scaffolding protein (Imbert et al., 2008). At least seven domains have been identified in nsp3 based on amino acid secondary structure prediction, phylogenetic conservation, structure and functional analysis (Snijder et al., 2003; Thiel et al., 2003). These domains are: (i) the N-terminal acidic domain, called nsp3a, that adopts a ubiquitin-like globular fold (Serrano et al., 2007); (ii) the ADP-ribose-1″-phosphatase (ADRP), named nsp3b (Saikatendu et al., 2005); (iii) the SARS unique domain (SUD), also called nsp3c, that binds RNA G quadruplexes (Tan et al., 2009); (iv) the nsp3d domain that contains two subdomains involved in the papain-like proteinase ($PL2^{pro}$) activity (Harcourt et al., 2004), and another one with a ubiquitin-like fold; and (v) the additional domains (nsp3e–g) that include a RNA binding domain, a transmembrane (TM) region and a zinc finger motif. In a recent report nsp3 has been shown to be an interferon antagonist (Devaraj et al., 2007). In this sense, the papain-like protease domain has a deubiquitinating activity that shows a deISGylation activity that could be involved in the inhibition of interferon responses (Barretto et al., 2005; Devaraj et al., 2007; Frieman et al., 2009; Lindner et al., 2005, 2007). Posttranslational modification of proteins by ubiquitin and ubiquitin-like ligands is a multistep process, carried out by a well-defined enzymatic pathway, which is required to maintain physiological levels and functional activities of several cellular proteins. In addition, the reversal pathway, named deubiquitination, has been recognized as an important process in the regulation of protein degradation by the proteasome. Many viruses have evolved mechanisms to modify host ubiquitination and deubiquitination

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machinery to modulate cell cycle, to interfere with innate and adaptative immune responses and to enhance virus replication and egress (Isaacson and Ploegh, 2009). In this sense, it has been shown that several viral proteins are directly modified by ubiquitin or ubiquitin-like proteins during the virus cycle. Moreover, some viruses encode their own ubiquitinating or deubiquitinating enzymes that may alter the ubiquitination level of cellular and viral targets (Isaacson and Ploegh, 2009).

The CoV replicase complex is involved in genome replication and transcription of a nested set of subgenomic mRNAs (sgmRNAs) encoding structural proteins, such as the spike (S), envelope (E), membrane (M), and nucleocapsid (N). In addition, SARS-CoV sgmRNAs encode a set of group specific proteins, whose sequence and number differ from other coronavirus species (Enjuanes et al., 2008). In the case of SARS-CoV, some of these proteins (3a, 6, 7a, and 7b) also are structural proteins (Huang et al., 2006, 2007; Ito et al., 2005; Schaecher et al., 2007; Shen et al., 2005). The function of most group specific proteins is still unclear. However, these proteins may influence the virus–host interaction and viral pathogenesis.

SARS-CoV E protein is a small integral membrane protein that is 76 amino acids in length and contains a short hydrophilic amino terminus followed by a hydrophobic region and a hydrophilic carboxy terminus (Torres et al., 2007; Wilson et al., 2004). It has been shown that SARS-CoV E protein plays an important, but not well-defined role in virus assembly and budding. The SARS-CoV E gene has previously been shown to be a non-essential gene, although deletion of this gene from the viral genome reduces virus titers in cultured cells by 20- to 200-fold in relation to the wild-type virus (SARS-CoV-wt), depending on the cell type infected (DeDiego et al., 2007). SARS-CoV lacking the E gene is attenuated in the highly sensitive hACE-2 transgenic mouse model and in hamsters, and it has been proposed that the E gene is a virulence factor (DeDiego et al., 2007, 2008).

The identification of proteins interacting with SARS-CoV E protein is relevant to understand the mechanisms of action of this protein during the viral cycle. In this article, we report the construction of a recombinant SARS-CoV expressing E protein linked to two tandem affinity tags [influenza haemmaglutinning (HA) and a FLAG epitope] spaced by a tobacco etch virus (TEV) cleavage site throughout the C terminus of E protein. This system allowed the rapid purification of E and associated proteins from SARS-CoV-infected cells. Using this system, the interaction of SARS-CoV envelope protein with structural proteins S and M, and with the multifunctional protein nsp3 was identified. In addition, the binding of E protein to cellular proteins including dynein heavy chain, fatty acid synthase, aminopeptidase puromycin sensitive, transmembrane protein 43 and lactate dehydrogenase A is shown. In this paper, we focused on E-nsp3 binding that was mediated through the N-terminal ubiquitin-like domain-1 of nsp3 in the absence of other viral proteins. Moreover, these two proteins colocalized in the cytoplasm of SARS-CoV infected cells. In this report, evidence showing that E protein is ubiquitinated in cells is also provided. Taken together these data showed an interaction of SARS-CoV structural E protein with a replicase component (nsp3), that could be implicated in the virus life cycle influencing E protein ubiquitination and turnover.

Results

Generation of a recombinant SARS-CoV expressing a tagged E protein

To identify the proteins that interact with SARS-CoV E protein, a recombinant SARS-CoV expressing the E protein fused to a tag on its carboxy terminus was engineered as a BAC (pBAC-SARS-CoV-EtagCt) (Fig. 1A). The tag contains the FLAG epitope followed by a TEV protease site, and the HA epitope. Vero E6 cells were transfected with this plasmid or with a parental plasmid encoding the wt SARS-CoV (pBAC-SARS-CoV^{FL}) that lacks E protein associated tags. Recombinant viruses rSARS-CoV-EtagCt and rSARS-CoV-wt were recovered from both plasmids with high titers and showed the characteristic cytopathic effect induced by SARS-CoV infection (data not shown). The recombinant virus expressing tagged E protein was amplified, cloned by three rounds of plaque isolation, and passaged twice to generate a virus stock.

To study the stability of the rSARS-CoV-EtagCt virus, the synthesis of sgmRNAs by the recombinant viruses after being passaged 8 times, was characterized by RT-PCR (Fig. 1B). Total RNA from mock-infected cells or cells infected with the SARS-CoV-wt, the virus lacking the E gene (rSARS-CoV-ΔE), or the SARS-CoV-EtagCt virus were isolated and amplified by RT-PCR. E, M and N gene sgmRNAs were amplified using a forward primer complementary to the leader sequence and reverse primers specific for each gene. A band corresponding to wild-type E mRNA was detected in cells infected with the parental SARS-CoV while no band was detected in SARS-CoV-ΔE-infected cells, as expected. In the case of cells infected with SARS-CoV-EtagCt, a PCR product of 340 bp corresponding to mRNA encoding the tagged E protein was identified (Fig. 1B). The sequence of this PCR product maintained the sequence introduced in the SARS-CoV-EtagCt virus. No differences in the PCR products derived from the mRNA encoding M and N proteins were detected (Fig. 1B). To study tagged E protein expression, extracts from mock-infected cells, or cells infected with SARS-CoV-wt, SARS-CoV-ΔE or SARS-CoV-EtagCt were analyzed by Western blot (Fig. 1C). A SARS-CoV E protein-specific monoclonal antibody generated in our laboratory was used to detect the expression of this protein (J. L. Nieto-Torres, M. L. DeDiego, E. Alvarez, and L. Enjuanes, CNB-CSIC, Madrid, Spain). A band corresponding to E protein was detected in SARS-CoV-wt-infected cells while no band was observed in cells infected with SARS-CoV-ΔE. As expected, a band of lower electrophoretic mobility, corresponding to tagged E protein, was detected in cells infected with SARS-CoV-EtagCt. The densitrometric scanning of those bands showed only ~5% difference between E-wt and E-tag, indicating that the expression of tagged E protein is unaffected by the extra sequence added to E. In addition, using FLAG or HA epitope specific monoclonal antibodies, a band corresponding to tagged E protein was observed in cells infected with SARS-CoV-EtagCt, whereas no bands were detected in both SARS-CoV-wt or SARS-CoV-ΔE-infected cells (Fig. 1C), indicating that the E-tagged protein maintained both epitopes. As a control of infection, SARS-CoV N protein was detected in all the viruses (Fig. 1C). No differences in N protein expression levels were detected between cells infected with SARS-CoV-wt, SARS-CoV-ΔE and SARS-CoV-EtagCt.

To analyze whether the expression of viral mRNAs downstream of E gene is unaffected by the tag sequence added to this gene, synthesis of genomic and sgmRNAs was quantified by Q-RT-PCR (Fig. 2). The quantifications showed that the amount of viral mRNAs in cells infected with SARS-CoV-EtagCt were within a twofold range relative to the corresponding control cells infected with SARS-CoV-wt or SARS-CoV-ΔE, indicating that the E protein tag does not affect significantly the expression of viral mRNAs.

Growth kinetics of SARS-CoV-EtagCt virus was analyzed both in Vero E6 and Huh-7 cells in comparison with the parental virus and SARS-CoV-ΔE (Fig. 3). SARS-CoV-EtagCt and wt viruses showed similar growth kinetics and virus titers in both cell lines. In contrast, the titer of the rSARS-CoV-ΔE virus was ~20- and ~200-fold lower than those of the recombinant wild-type and SARS-CoV-EtagCt viruses in Vero E6 and Huh-7 cells, respectively. This result suggested that the E protein fused to a tag is as functional as the wild-type one since a virus containing a tagged E protein grew to the same extent as the wild-type virus.

Purification and identification of SARS-CoV E protein ligands

Vero E6 cells were infected with SARS-CoV-EtagCt virus, and the E-tag protein complexes were purified by two affinity chromatography

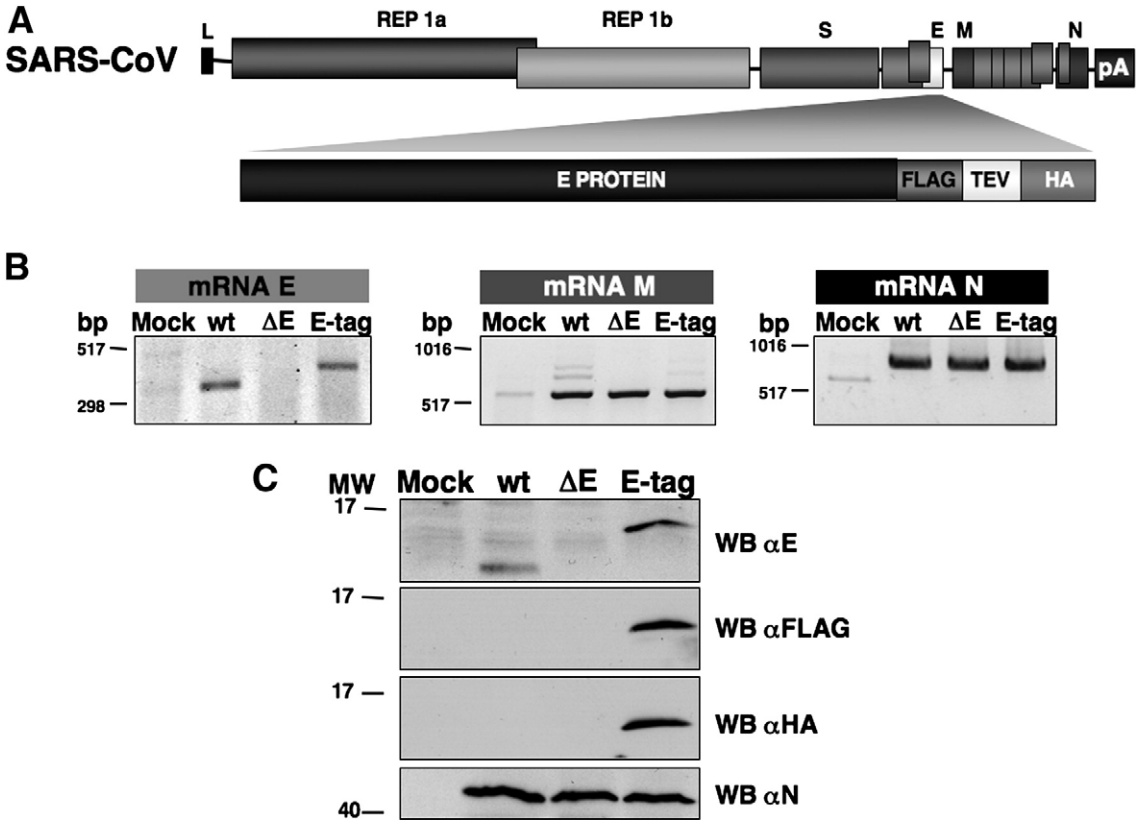

Fig. 1. Generation of a recombinant SARS-CoV expressing a tagged E protein. (A) Scheme of recombinant virus expressing tagged E protein. L, leader sequence; REP, replicase gene; S, spike protein, E, envelope protein; M, membrane protein; N, nucleocapsid protein; pA, poly(A) tail; FLAG, FLAG epitope; TEV, tobacco etch virus protease site; and HA, influenza haemmaglutinin epitope. (B) Vero E6 cells were mock infected (Mock) or infected with the recombinant wild-type (WT), the rSARS-CoV-ΔE (ΔE) or rSARS-CoV-EtagCt (E-tag) viruses. Viral mRNA expression was analyzed by RT-PCR using the oligonucleotides specific for sgmRNAs of E, M and N genes. (C) Western blot analysis of infected cell lysates using E, FLAG, HA and N protein-specific antibodies followed by peroxidase-labelled goat anti-rabbit or anti-mouse antibodies.

steps as described in the Materials and methods section. As control, the same purification was performed with mock- or SARS-CoV-wt-infected cell extracts. Cell lysates were applied to an anti-HA resin and bound proteins were eluted by proteolytic cleavage of the tag at the TEV protease site. Eluates from the proteolytic cleavage on the anti-HA resin were incubated with an anti-FLAG agarose and proteins bound to matrix were eluted by specific competition with FLAG peptide. To identify proteins that copurified with E-tag, the eluted proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. In the case of purifications from cells infected with SARS-CoV-EtagCt, several protein bands were detected, whereas purifications in parallel using extracts from mock-infected cells or cells infected with the wild-type virus did not reveal any detectable protein band (Fig. 4A). These data indicated that the proteins identified specifically bound to E-tag protein. Some of the bands were excised, digested with trypsin, and subjected to MALDI TOF/TOF mass spectrometry to identify the corresponding proteins by screening against the available NCBI protein database. A set of 12 proteins was reproducibly identified in at least two out of four experiments with significant MASCOT scores ($p>0.05$) (Table 1). Among the identified proteins binding to E, the viral proteins nsp3, S and M were identified as E protein interacting partners. In addition, the proteins dynein heavy chain, fatty acid synthase, aminopeptidase puromycin sensitive, phosphofructokinase platelet, tubulin alpha and beta, actin beta, transmembrane protein 43 and lactate dehydrogenase A, were identified as cellular proteins interacting with E protein. This paper is focused on the interaction of E protein with nsp3.

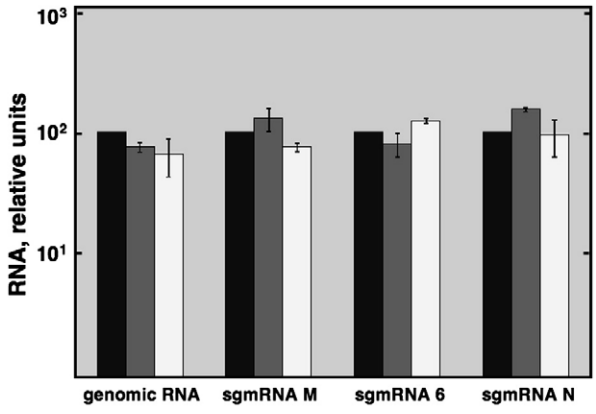

Fig. 2. Virus RNA synthesis in infected Vero E6 cells. Vero E6 cells were infected with rSARS-CoV-wt (black boxes), rSARS-CoV-ΔE (grey boxes) or rSARS-CoV-EtagCt (white boxes) at an moi of 0.5. Total RNA was extracted at 16 h post-infection and the accumulation of viral genomic or subgenomic messenger RNA (sgmRNA) of genes M, 6 and N was quantified by Q-RT-PCR. Levels of viral RNAs are represented in comparison to reference levels from cells infected with SARS-CoV-wt. The experiment was performed three times and the data represent the average of triplicates. Standard deviation is indicated as error bars.

Interaction between E and nps3 proteins

The interaction between nsp3 and E protein detected with the tandem affinity purification was confirmed by coimmunoprecipitation. To this end, protein complexes formed in Vero E6 cells infected with SARS-CoV-wt, SARS-CoV-ΔE, or mock-infected cells were pulled down using antibodies specific for E and nsp3 proteins. Immune precipitates were analyzed by Western blot using the anti-E and anti-nsp3 antibodies (Fig. 4B). Using an E protein-specific antibody, nsp3 was coimmunoprecipitated in samples derived from wild-type infected cells but not in extract derived from mock or

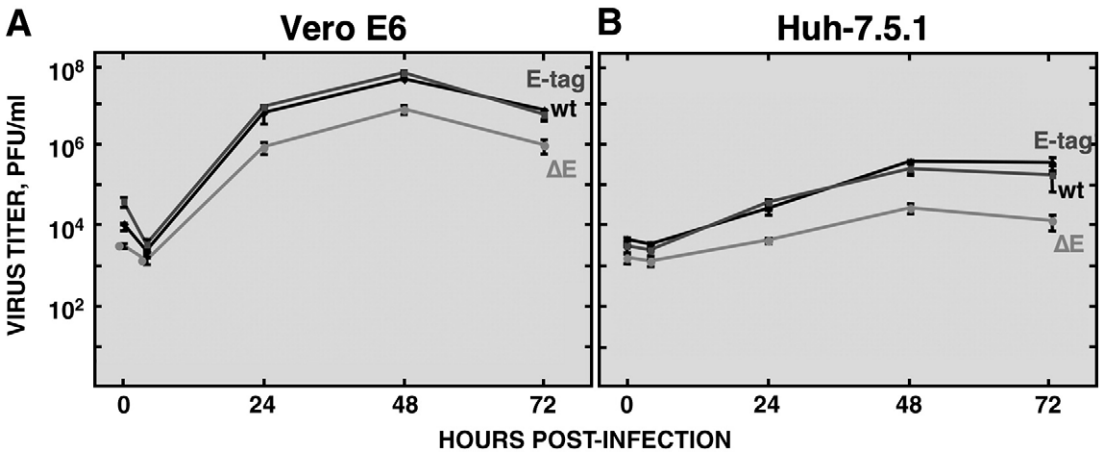

Fig. 3. Growth kinetics of the recombinant viruses in monkey and human cells. Vero E6 (A), and Huh-7.5.1 (B) cells were infected at an moi of 0.5 with the recombinant wild-type virus (WT), the rSARS-CoV-ΔE (ΔE) or the rSARS-CoV-EtagCt (E-tag). At different times post-infection, virus titers were determined by plaque assay on Vero E6 cells. Error bars represent standard deviations of the mean of results from three experiments.

SARS-ΔE-infected cells. Conversely, using anti-nsp3 antibody to pull-down nsp3, E protein coimmunoprecipitated in extracts from cells infected with SARS-CoV-wt, but not in those from mock-infected cells, or cells infected with SARS-CoV-ΔE. In contrast, no specific bands were observed in control experiments where the immunoprecipitations were performed with a monoclonal antibody specific for the N protein of transmissible gastroenteritis virus (TGEV). The amount of E or nsp3 proteins coimmunoprecipitated by antibodies specific for nsp3 or E proteins respectively was relatively low, suggesting that in the experimental conditions used, the binding between these two proteins was relatively weak. Overall, these results indicated that E protein forms complexes with nsp3 protein.

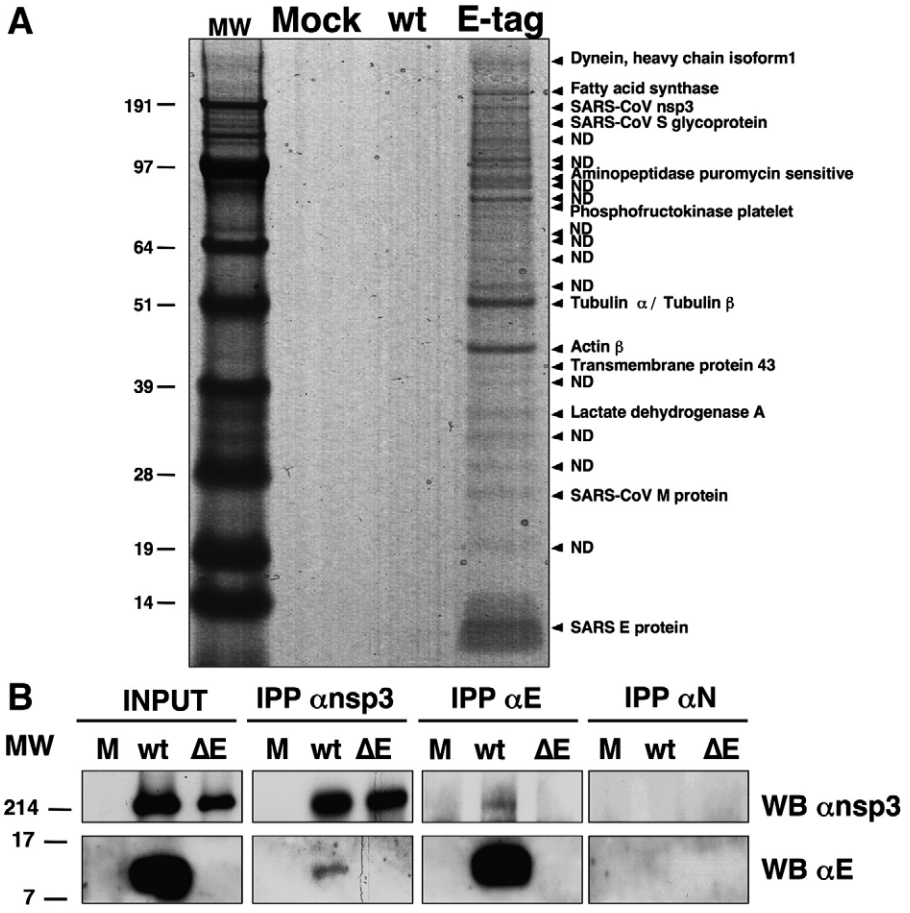

Fig. 4. Identification of viral proteins interacting with SARS-CoV E protein. (A) Purification of proteins interacting with E protein using a tandem affinity purification method. Vero E6 extracts from mock-infected cells or cells infected with rSARS-CoV-wt (wt) or rSARS-CoV-EtagCt (E-tag) were used in a double affinity chromatography. Purified proteins were detected by staining gels with Coomassie blue dye. Bands were excised from gels and were identified by mass spectrometry. (B) Coimmunoprecipitation assays of nsp3 and E protein. Extracts from Vero E6 cells infected with rSARS-CoV-wt or rSARS-CoV-ΔE were immunoprecipitated with nsp3, E or TGEV N protein-specific antibodies. Immunoprecipitations were analyzed by Western blot using E and nsp3 protein-specific antibodies followed by peroxidase-labelled goat anti-rabbit or anti-mouse antibodies.

Table 1
Proteins isolated with SARS-CoV E-tagged protein.

Accession no.	Protein name	Mr	Function[a]	Masses matched/searched	Score/threshold/database[b]	N[c]
gi|34555776	Dynein, heavy chain isoform 1	488,642	Microtubule-based transport	8/16	**73**/65/nr	3
gi|41584442	Fatty acid synthase	275,900	Lipid metabolism	14/33	**99**/80/nr	2
gi|34555776	SARS-CoV non-structural protein (nsp3)	216,607	Virus protein	30/49	**315**/80/nr	3
gi|30027620	SARS-CoV spike glycoprotein (S protein)	141,323	Virus protein	15/26	**247**/80/nr	3
gi|15451907	Aminopeptidase puromycin sensitive	99,125	Cell cycle	24/75	**187**/80/nr	3
gi|119606901	Phosphofructokinase platelet	93,725	Glycolysis	17/55	**83**/65/Hs	2
gi|14389309	Alpha tubulin	50,548	Cytosqueleton	27/94	**566**/80/nr	4
gi|18088719	Beta tubulin	50,096	Cytosqueleton	35/85	**558**/70/m	3
gi|4501885	Beta actin	42,052	Cytosqueleton	26/78	**516**/80/nr	4
gi|13236587	Transmembrane protein 43	44,904	Nuclear membrane	24/77	**453**/80/nr	3
gi|62897717	Lactate dehydrogenase A	36,951	Metabolism	13/93	**131**/80/nr	3
gi|30027623	SARS-CoV membrane protein (M protein)	25,070	Virus protein	1/83	**42**/36/Vs	3
gi|29836499	SARS-CoV E protein	8360	Virus protein	1/80	**58**/37/Vs	1

[a] Biological process according to Gene Ontology.
[b] Mascot scores are given in boldface. Mascot threshold scores indicate the limit score from which the identification was significant ($p<0.05$). Searches were performed against the NCBI database without restrictions (nr). In the indicated cases taxonomy was restricted to humans (Hs), mammals (m) or viruses (Vs).
[c] Number of times that the protein was identified.

To further confirm the interaction between nsp3 and E protein, a coimmunoprecipitation assay was performed using nsp3-derived fragments expressed in a cell-free coupled transcription–translation system (Fig. 5A). Biotinylated Lys-tRNA was used in translation reactions for subsequent detection of the expressed proteins with streptavidin conjugated to peroxidase (Fig. 5B). Recombinant purified SARS-CoV E protein was added to translation lysates and then E protein was pulled down using the specific monoclonal antibody indicated above. Fragment 1 of nsp3 was coimmunoprecipitated together with E protein, while no bands were detected in a control experiment in which the porcine reproductive and respiratory syndrome virus (PRRSV) glycoprotein 5 (Gp5) was added to translation lysates and pulled down with anti-Gp5 antibody (Fig. 5B). Overall, these results indicated that the interaction between nsp3 and E protein is independent of the presence of other SARS-CoV proteins, and that the interaction site in nsp3 is located in the N-terminal domain of the protein.

Mapping the nsp3 region required to interact with E protein in cell-free systems

To further delimit the domain of nsp3 involved in the interaction with E protein, the N-terminal acidic domain (UB1-AC), the ADRP, and the SUD domains of nsp3 protein were expressed in cell-free systems as described above (Fig. 6A). Purified E or Gp5 proteins were mixed with each of the nsp3 N-terminal biotinylated polypeptides and

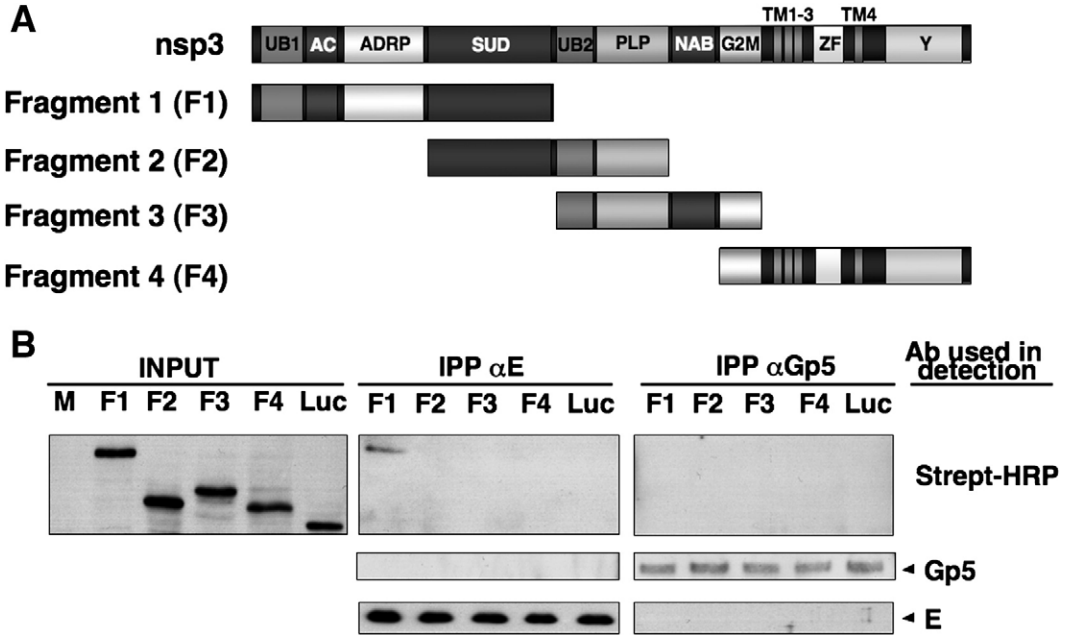

Fig. 5. Coimmunoprecipitation assays of nsp3 fragments. (A) Scheme of nsp3-derived fragments synthesized in TNT® coupled reticulocyte lysate systems (F1 to F4). The nsp3 domains are shown in the scheme: UB1, ubiquitin-like domain 1; AC, acidic hypervariable domain; ADRP, ADP-ribose-1″-phosphatase; SUD, SARS unique domain; UB2, ubiquitin-like domain 2; PLP, papain-like protease; NAB, group II-specific domain; G2M, group II-specific maker; TM, transmembrane motif; ZF, putative metal-binding region; and Y, Y region. Synthesized fragments were mixed with recombinant E or Gp5 proteins and then were used in immunoprecipitation assays using E or Gp5 protein-specific antibodies. Immunoprecipitations were analyzed by Western blot using E and Gp5 protein-specific antibodies followed by peroxidase-labelled goat anti-rabbit or anti-mouse antibodies. Luciferase (Luc) was used as a control.

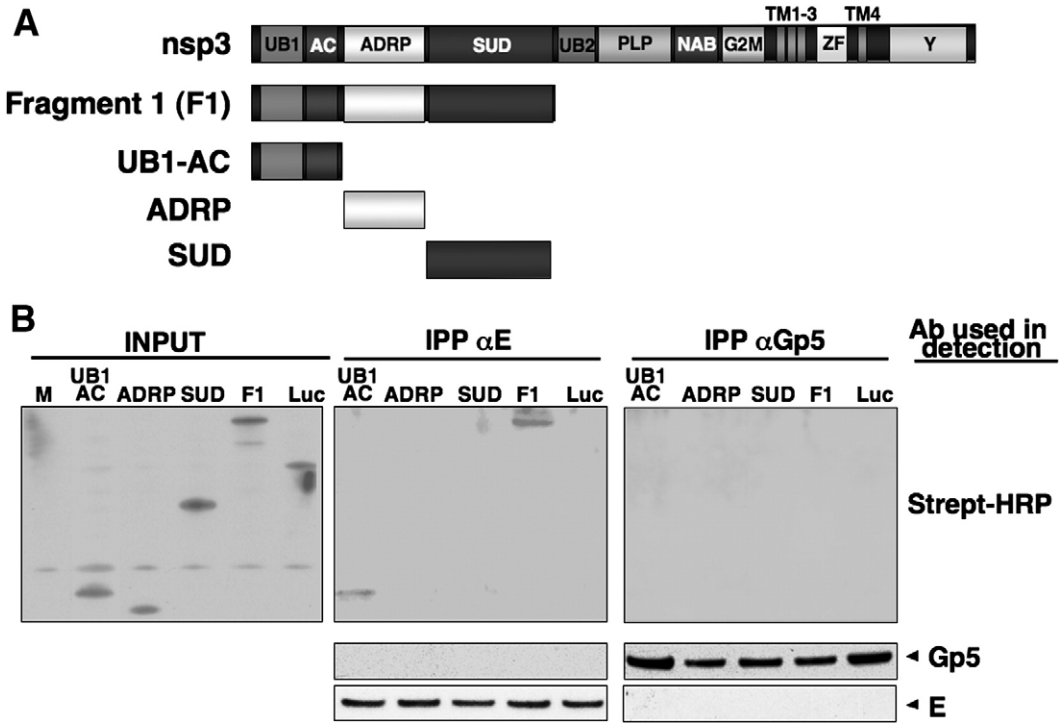

Fig. 6. Mapping the nsp3 region required to interact with E protein. (A) Scheme of nsp3-F1-derived fragments synthesized in TNT® coupled reticulocyte lysate systems (UB1-AC, ADRP and SUD). The nsp3 domains are shown in the scheme: UB1, ubiquitin-like domain 1; AC, acidic hypervariable domain; ADRP, ADP-ribose-1″-phosphatase; SUD, SARS unique domain; UB2, ubiquitin-like domain 2; PLP, papain-like protease; NAB, group II-specific domain; G2M, group II-specific marker; TM, transmembrane motif; ZF, putative metal-binding region; and Y, Y region. Synthesized fragments were mixed with recombinant E or Gp5 proteins and then were used in immunoprecipitation assays using E or Gp5 protein-specific antibodies. Immunoprecipitations were analyzed by Western blot using E and Gp5 protein-specific antibodies followed by peroxidase-labelled goat anti-rabbit or anti-mouse antibodies.

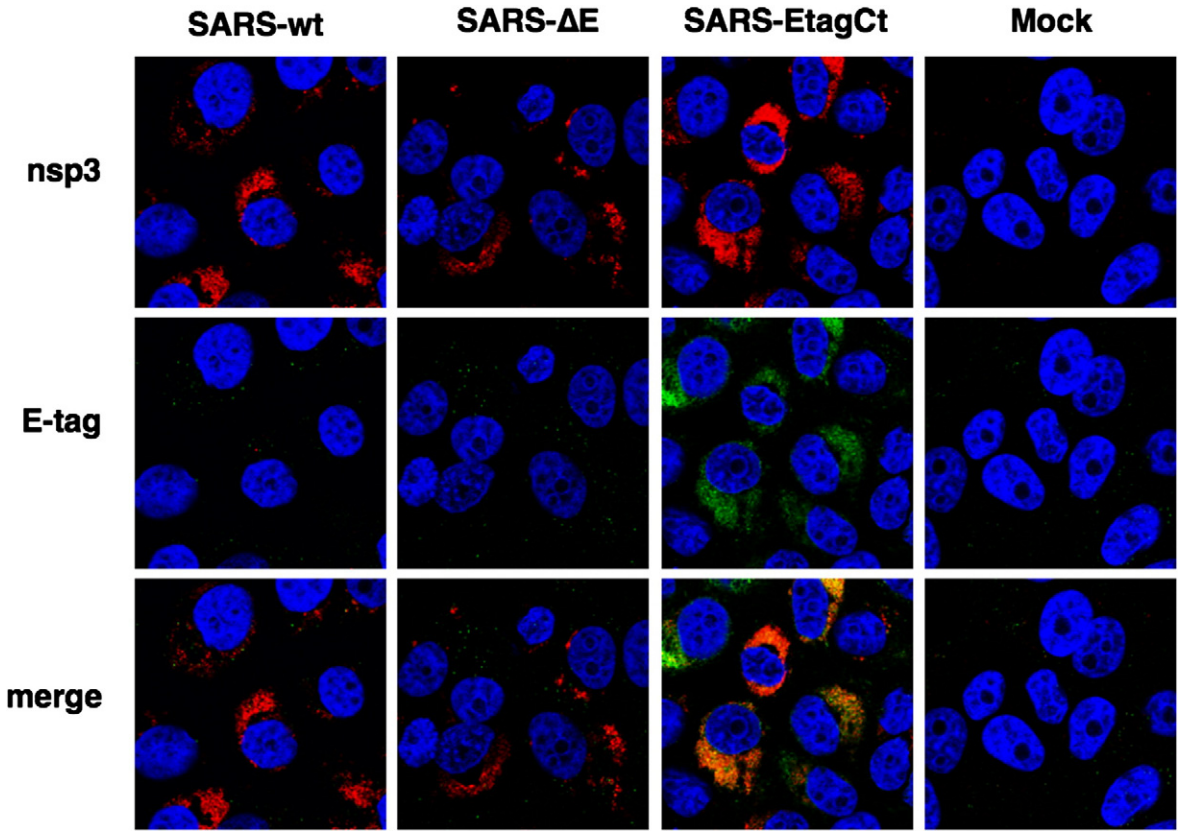

Fig. 7. Colocalization of nsp3 and E proteins. Vero E6 cells grown on glass coverslips were infected with rSARS-CoV-wt, rSARS-CoV-ΔE or rSARS-CoV-EtagCt at an moi of 0.5. At 15 h post-infection the cells were fixed with 8% paraformaldehyde. Cells were labelled with nsp3 (red) or HA (green) specific antibodies.

immunoprecipitated using antibodies against E protein or the unrelated protein Gp5, as a negative control (Fig. 6B). While no bands were detected in a control experiment when Gp5 was used as bait, a band corresponding to UB1-AC domain was observed in samples in which purified E protein was added to the translation mixtures and subsequently immunoprecipitated with E specific antibody (Fig. 6B). Moreover, bands corresponding to ADRP and SUD domains were not coimmunoprecipitated together with E protein (Fig. 6B). This data indicates that nps3 interacts with E protein through the ubiquitin-like domain-1 located in the N-terminus of the protein.

Subcellular localization of E and nsp3

To obtain complementary support for the interaction between nsp3 and E protein, Vero E6 cells infected with SARS-CoV-wt, SARS-CoV-ΔE and SARS-CoV-EtagCt were analyzed by confocal immunomicroscopy using HA (to detect E-tag) or nsp3 specific antibodies. All infected cells showing the presence of E protein also stained with the nsp3 specific antibody giving an identical pattern, consistent with a perinuclear distribution (Fig. 7). The localization of the tagged E protein was the same as untagged E in infected cells indicating that the tag sequence did not change the E protein localization (Supplementary Fig. 1). These results suggest that nsp3 and E protein colocalize in the cytoplasm of infected cells and that the absence of E protein did not change the subcellular distribution of nsp3.

Ubiquitination of SARS-CoV E protein

To analyze whether E protein is ubiquitinated, a plasmid expressing E protein was cotransfected with a plasmid expressing a modified ubiquitin (Ub-OK) fused to a His tag into VeroE6 cells. The modified ubiquitin prevents the formation of poly-ubiquitin chains and the subsequently degradation of poly-ubiquitinated proteins by the proteasome. The cells were lysed 24 h post transfection, the proteins were resolved by SDS-PAGE and E protein was detected by Western blotting using an antibody E protein specific (Fig. 8A). An additional band corresponding to monoubiquitinated E protein was detected only when E protein was expressed in presence of Ub-OK (Fig. 8A, lane 3). By contrast, samples from cells expressing E protein (Fig. 8A, lane 2) or Ub-OK (Fig. 8A, lane 1) alone did not show the novel band. The promyelocytic leukemia protein (PML) was expressed in parallel to serve as a positive control of ubiquitination (Fig. 8B). In this case, different lower migrating bands corresponding to different ubiquitination states of the protein were observed. This result indicates that SARS-CoV E protein was ubiquitinated in transfected cells.

To further analyze E protein ubiquitination, an in vitro assay was performed using recombinant purified E protein expressed using baculoviruses, followed by immunoblotting detection of E protein with an E specific antibody. A slow migrating E band was detected when E protein was incubated with presence of mono-ubiquitin or poly-ubiquitin (Fig. 8C, lanes 2 and 3), whereas no additional band was detected when E protein was incubated in the absence of ubiquitin (Fig. 8, lane 1), indicating that SARS-CoV E protein is also ubiquitinated in a cell-free system.

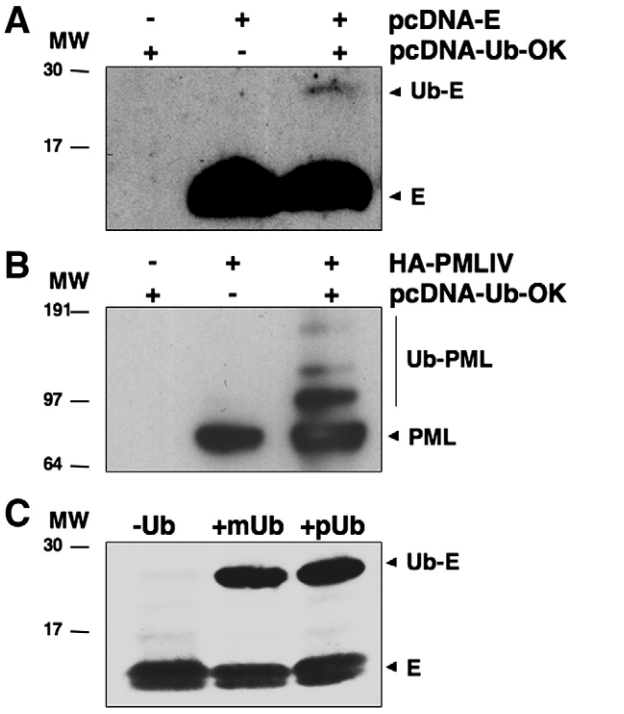

Fig. 8. Ubiquitination of SARS-CoV E protein. Ubiquitin conjugation to E protein. Vero E6 cells were transiently transfected with the plasmid mixtures shown in the figure. The control plasmid pcDNA was used to equalize the total amount of transfected plasmid in all cases. 24 h after transfection, the cells were lysed by Laemmli lysis buffer followed by boiling for 5 min. The denatured lysates were analyzed by SDS-PAGE and Western blot with an antibody against E protein (A) or HA epitope (B). (C) In vitro ubiquitination of SARS-CoV E protein. Baculovirus purified E protein was incubated with mono-ubiquitin (mUb) or poly-ubiquitin (pUb) in the presence of the all the enzymes of the ubiquitination process. The reactions were resolved by SDS-PAGE and transferred to nitrocellulose. The ubiquitinated (Ub-E) and non-ubiquitinated E protein was detected by Western blot using a specific antibody against E protein.

Discussion

Coronavirus E protein is present in a high copy number in the cytoplasm of infected cells but is a minor component of the virions (Godet et al., 1992; Liu and Inglis, 1991; Yu et al., 1994). It plays important roles in coronavirus assembly and morphogenesis (Fischer et al., 1998; Ortego et al., 2007, 2002), alteration of the cellular membrane permeability (Liao et al., 2004, 2006) and virus–host interaction (Liu et al., 2007). In this study, we used a TAP approach coupled to mass spectrometry to identify viral proteins interacting with SARS-CoV E protein that could be important to regulate its functions. This led to the observation of interactions between the E and other viral structural proteins such as S and M. Cellular proteins significantly binding to E protein were also identified, as dynein heavy chain, fatty acid synthase, aminopeptidase puromycin sensitive, phosphofructokinase platelet, tubulin alpha and beta, actin beta, transmembrane protein 43 and lactate dehydrogenase A. The interaction between E and M coronavirus proteins has already been reported in other CoV species, such as infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV), by coimmunoprecipitation in virus-infected or transfected cells (Corse and Machamer, 2003; Lim and Liu, 2001; Maeda et al., 1999). The role of SARS-CoV E protein in virus assembly has been analyzed studying the formation of VLPs both in mammalian and insect cells (Ho et al., 2004; Huang et al., 2004; Mortola and Roy, 2004; Siu et al., 2008), and by reverse genetics (DeDiego et al., 2007, 2008). Although there are conflicting reports on the proteins necessary for the formation of VLPs in SARS-CoV (Ho et al., 2004; Huang et al., 2004; Mortola and Roy, 2004), it has been clearly shown that a recombinant virus lacking E gene produced virions with the same morphology as the wt virus, indicating that E protein is not necessary to obtain infectious SARS-CoV (DeDiego et al., 2007, 2008).

In addition to the interaction of E protein with S and M, a novel interaction between E protein and the non-structural protein nsp3 was identified. The nsp3-E interaction was detected by reciprocal coimmunoprecipitation of lysates from SARS-CoV-infected cells. Furthermore, nsp3 and E protein colocalized in the perinuclear region of the cytoplasm of SARS-CoV-infected cells. Taken together, these data suggest that nsp3 and E protein may form a protein

complex in SARS-CoV-infected cells. The interaction among SARS-CoV proteins has been studied using different approaches (yeast two-hybrid assay, mammalian two-hybrid assay and coimmunoprecipitation) (Pan et al., 2008; von Brunn et al., 2007). Studies using a two-hybrid system have shown the interaction of E protein with nsp1, nsp8, nsp11, 3b, 7b and 9b proteins (von Brunn et al., 2007). Some of these interactions were confirmed by coimmunoprecipitation of proteins expressed in human HEK 293-transfected cells. In addition, interaction between E and 7a proteins was detected using a mammalian two-hybrid assay, although this interaction was not confirmed by coimmunoprecipitation nor pull-down approaches (Pan et al., 2008). Nevertheless, these articles did not describe the interaction between E protein and the structural proteins M and S, or the non-structural nsp3 protein. This was probably due to the limitation of two-hybrid systems in detecting proteins with transmembrane domains that could prevent the transfer of bait–ligand complexes to the cell nucleus, where these complexes lead to transcription. The system described here, allowed the identification of E protein interacting partners in the context of SARS-CoV infection overcoming potential limitations of two-hybrid assays. Interestingly, a recent report identifying the SARS-CoV structural proteins showed that, in addition to the well know structural proteins S, E, M, and N, nsp3 is a tentative novel minor virion component (Neuman et al., 2008). A network of protein–protein interactions within SARS-CoV components has been proposed (Neuman et al., 2008). In this network, the interaction between nsp3 and E protein could, in principle, be explained by an indirect link through the 9b protein in the virion. Nevertheless, we have demonstrated in an in vitro coimmunoprecipitation assay that nsp3 specifically interacts with E protein in the absence of other viral proteins. This data suggested that the interaction of nsp3 with E protein is most likely a direct interaction, although the requirement of proteins from the reticulocyte lysate assay could not be formally excluded.

Nsp3 is a multifunctional protein of the replication/transcription complex. It has recently been proposed that nsp3 may act as a replication/transcription scaffolding protein (Imbert et al., 2008). In this report, it has been shown that nsp3, in particular the PLP domain, interacted with a large number of partners of the replication/transcription complex. This data in combination with the presence of a transmembrane domain within nsp3 suggested that this protein could act bringing several proteins into close proximity, within the double membrane environment. In the present report, we showed that E colocalized with nsp3 in the cytoplasm during the infection. In this sense, nsp3 may bring the E protein into the vicinity of the replication/transcription complex.

It has been shown that ubiquitination and deubiquitination processes are important in the viral life cycle (Isaacson and Ploegh, 2009). Several viral proteins possess deubiquitinating activity. One of them is the PLpro domain of the SARS-CoV nsp3 protein that belongs to the USP family of deubiquitinases (DUBs) and has deubiquinatin activity in vitro (Lindner et al., 2005). Moreover, it has been proposed that the PLpro domain might act protecting the viral replication complex from proteasomal degradation via deubiquitination. However, the viral and cellular targets of the nsp3 DUB domain remain completely unknown. Here, we describe that SARS-CoV E protein is ubiquitinated in vitro and in cells, although the function of this post-translational modification in the course of infection is not known. Nps3-E interaction could have a role in controlling the ubiquitination status of E protein during the infection and, as a consequence, on its turnover and modulation of the innate and adaptative immune responses or virus replication and egress, similarly to what has been suggested in other viral systems (Isaacson and Ploegh, 2009).

In conclusion, our work describes a system to detect interactions between the E protein with other viral and cellular components in the infection context. We describe a novel protein–protein interaction between the structural protein E and the non-structural protein nsp3 and that this interaction was mediated through the N-terminal ubiquitin-like domain-1 of nsp3 in the absence of other viral proteins. This work also describes that SARS-CoV E protein is ubiquitinated both in vitro and in cells. Further investigation will be needed to clarify the role of E protein ubiquitination in viral cycle and virus–host interaction, and whether nsp3 plays a role in E protein ubiquitination.

Materials and methods

Cells and culture conditions

African green monkey kidney-derived Vero E6 cells and the Huh-7.5.1 clone derived from the human hepatome Huh-7 cells were kindly provided by E. Snijder (University of Leiden, The Netherlands) and F. V. Chisari (Scripps Research Institute, La Jolla, California, USA), respectively. In both cases, cells were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 25 mM HEPES and 10% fetal bovine serum (FBS, Biowhittaker, Berviers, Belgium). Virus growth and titrations were performed in Vero E6 cells following standard procedures previously described in detail (DeDiego et al., 2007). All work with infectious virus was performed in biosafety level (BSL) 3 facilities by personal wearing positive-pressure air purifying respirators (3 M HEPA AirMate, Saint Paul, MN).

Construction of plasmid pBAC-SARS-CoV-EtagCt

The pBAC-SARS-CoV-EtagCt plasmid encoding a rSARS-CoV expressing the E gene fused to a tag consisting on the FLAG and the HA epitopes separated by TEV cleavage site, was constructed from a previously generated full-length infectious cDNA clone (plasmid pBAC-SARS-CoV^{FL}) (Almazan et al., 2006). The tagged E gene was introduced by overlap extension PCR using the plasmid pBAC-SARS-CoV^{FL}. The oligonucleotides SARS-E-VS (5′-CTCTTCAGGAGTTGCTAATCCAG-CAATGG-3′) and SARS-EtagCt-RS (5′-GAGCTCACCCTGAAAATA-CAAATTCTCCTTATCGTCGTCATCCTTGTAATCGACCAGAAGATCAG-GAACTCC-3′), which includes the TEV site and the FLAG epitope followed by a sequence complementary from the nucleotides (nt) 26325 to 26348 of the SARS-CoV genome, were used to generate a PCR product from nt 26018 to 26348 of the SARS-CoV genome. The oligonucleotides SARS-EtagCt-VS (5′-GAGAATTTGTATTTTCAGGGT-GAGCTCTACCCATACGATGTTCCAGATTACGCTTAAGATCTTCTGGTC-TAAACGAACTAACTATTATTATTATTC-3′), which contains the TEV site and the HA epitope followed by a sequence from nt 26334 to 26372 of the SARS-CoV genome, and SARS-29794-RS (5′-CCAGGTCGGACCGC-GAGGAGGTG-3′) were used to generate a PCR product spanning nt 26334 to 29794 of the pBAC-SARS-CoV^{FL} plasmid. Both overlapping products were used as templates for PCR amplification using primers SARS-E-VS and SARS-29794-RS. The final PCR product was digested with the enzymes BamHI and RsrII and cloned into pBAC-SARS-CoV^{FL} digested with the same enzymes to generate the plasmid pBAC-SARS-CoV-EtagCt.

Transfection and recovery of infectious viruses from cDNA clones

Baby hamster kidney (BHK) cells were grown to 90% confluence in 12.5 cm^2 flasks and were transfected with 6 μg of pBAC-SARS-CoV-Etag-Ct or pBAC-SARS-CoV^{FL}, as a control, using 18 μg of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Recovered viruses were cloned by plaque titration as described in detail (Almazan et al., 2006; DeDiego et al., 2007).

Growth kinetics of recombinant viruses

Vero E6 or Huh-7.5.1 cells grown to 90% confluence were infected at a multiplicity of infection (moi) of 0.05 with the viruses rSARS-CoV-wt, rSARS-CoV-ΔE or rSARS-CoV-EtagCt. Culture supernatants were collected at different times post-infection, and virus titers were determined as previously described (DeDiego et al., 2007).

Western blot analysis

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane by wet immunotransfer and processed for Western blotting. The blots were probed with monoclonal antibodies specific for FLAG tag (dilution 1:1000; Sigma), HA tag (dilution 1:10,000; Sigma) or PRRSV Gp5 protein (dilution 1:1000; INGENASA, Madrid, Spain) or polyclonal antibodies against E (dilution 1:6000; kindly provided by Shen Shuo, Institute of Molecular and Cellular Biology, Singapore), nsp3 (dilution 1:2000; Rockland, Gilbertsville, PA), or N proteins (dilution 1:2000; Imgenex). Horseradish peroxidase-conjugated antibodies against the different species and the Immobilon Western chemiluminescent substrate (Millipore) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Agfa X-ray film.

Purification of proteins and generation of SARS-CoV E protein monoclonal antibody

Recombinant histidine-tagged SARS-CoV E and PRRSV Gp5 proteins were expressed in the baculovirus/insect cell system. Recombinant proteins were purified to near homogeneity by Ni^{2+}-NTA affinity chromatography (J. L. Nieto-Torres, M. L. DeDiego, E. Alvarez, and L. Enjuanes, CNB-CSIC, Madrid, Spain, unpublished results).

Purified E protein was the basis to generate the SARS-CoV E protein monoclonal antibody by immunizing BALB/c mice. Positive hybridoma clones were selected by ELISA, immunofluorescence and Western blot (J. L. Nieto-Torres, M. L. DeDiego, E. Alvarez, and L. Enjuanes, CNB-CSIC, Madrid, Spain, unpublished results).

Analysis of viral RNA synthesis

Viral RNA synthesis was quantified by Q-RT-PCR. cDNAs were synthesized from 50 ng of total RNA extracted from SARS-CoV-wt, SARS-CoV-ΔE or SARS-CoV-EtagCt infected cells using specific reverse sense oligonucleotides to genomic or subgenomic messenger RNA (sgmRNA) of genes M, 6 and N (DeDiego et al., 2007). The High Capacity cDNA Transcription kit (Applied Biosystems) was used in reverse transcription following the manufacturer's instructions. To analyze viral RNA synthesis a SYBR green assay (Applied Biosystems) was used. PCRs were performed using a virus sense primer complementary to the leader sequence, and specific reverse oligonucleotides to genomic or sgmRNAs (DeDiego et al., 2007). Data were acquired with an ABI PRISM 7000 sequence detection system (Applied Biosystems) and analyzed with ABI PRISM 7000 SDS version 1.0 software. Levels of viral RNAs are represented in comparison to reference levels from cells infected with SARS-CoV-wt.

Cell extracts

Vero E6 were grown to 90% confluence and infected at an moi of 0.1 with rSARS-CoV-wt or rSARS-Co-EtagCt. After an adsorption period of 1 h the inocula medium was replaced with fresh DMEM containing 10% FBS. At 40 h post-infection the cells were washed twice with ice-cold phosphate-buffered saline (PBS), scrapped off the plates and lysed in a buffer containing 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.5 mM EDTA, 1% IGEPAL CA-630 (Octylphenyl-polyethylene glycol, Sigma) and protease inhibitor cocktail (Roche) and incubated at 4 °C for 20 min. The extracts were clarified by centrifugation at 10,000×*g* for 15 min. The supernatant was collected and the total protein concentration was determined with Coomassie Plus protein Assay (Pierce) and cell extracts were aliquoted to avoid repeated freeze/thaw cycles.

Tandem affinity purification

Cell extracts (4 ml) at a protein concentration of ~5 mg/ml were incubated with 30 µl of Red Anti-HA Affinity Gel (Sigma) overnight at 4 °C in an orbital shaker. Agarose beads were washed 10 times with 10 volumes of wash buffer containing 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.5 mM EDTA, and 0.1% IGEPAL CA-630 and then, protein complexes were eluted from the matrix by incubation with 500 U of AcTEV protease (Invitrogen) for 3 h at room temperature. Eluted complexes were incubated with 30 µl anti-FLAG M2 Affinity Gel (Sigma) overnight at 4 °C and then beads were washed 10 times with 10 volumes of wash buffer. Protein complexes were eluted by competition with FLAG peptide (Sigma) at a final concentration of 1 mg/ml diluted in a buffer containing 10 mM Tris–HCl pH 8, 300 mM NaCl, 0.5 mM EDTA, and 0.1% IGEPAL CA-630. Proteins were precipitated by trichloroacetic acid at a final concentration of 10 % (v/v) and then were resuspended in 30 µl of NuPage Sample buffer (Invitrogen) and incubated 10 min at 65 °C. Purified proteins were loaded into 1.0 mm NuPAGE 4–12% Bis-Tris gels (Invitrogen) and electrophoresis was performed at 100 V using the MES SDS running buffer from the same manufacturer. The gels were washed three times in deionized water, stained with Coomassie blue Simply Blue Safe Stain (Invitrogen), and the protein bands were excised from the gels for their identification by mass spectrometry.

Identification of proteins by mass spectrometry

Excised protein bands were in-gel digested with sequencing grade modified porcine trypsin (Promega). Peptides were extracted from gel bands in 0.5% trifluoroacetic acid, dried by speed vacuum centrifugation and resuspended in 4 µl of MALDI solution. A 0.8 µl aliquot of each digestion was deposited and dried onto a 2386-well OptiTOF™ plate (Applied Biosystems) and co-crystallized with 0.8 µl of matrix solution (3 mg/ml CHCA in MALDI solution). Samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in an ABI 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems) at the proteomic facility of the National Center of Biotechnology (CNB-CSIC, Madrid, Spain). Data were analyzed using the ABI 4000 series explorer Software v3.6 and searches were performed with the MASCOT software v2.1 (Matrix Science) against the MSDB from the non-redundant NCBI protein database with mass tolerance of 100 ppm. No restrictions were imposed for protein molecular weight, although in some cases taxonomy restrictions for the viral, human or mammalian databases were included.

Plasmid construction and in vitro transcription/translation

The plasmids used to generate the SARS-CoV nsp3 protein fragments were engineered by inserting the PCR products digested with EcoRI/XhoI in the same sites of pcDNA3 (+) plasmid. The corresponding nucleotides (nt) of each fragment in the SARS-CoV genome are the following: i) fragment 1 (F1), nt 2719–4896; ii) fragment 2 (F2), nt 3886–5829 ; iii) fragment 3 (F3), nt 4888–6672; iv) fragment 4 (F4), nt 6397–8484; v) fragment UB1-AC, nt 2719–3055; vi) fragment ADRP, nt 3269–3814 ; and vii) fragment SUD, nt 3815–4896. The in vitro transcription/translation reaction was carried out with the TNT® coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Biotinylated

Lys-tRNA (Promega) was used in translation reactions for subsequent detection of the expressed proteins with streptavidin conjugated to peroxidase (dilution 1:5000).

The pcDNA-E plasmid used to express the SARS-CoV E protein was engineered by inserting the PCR product digested with EcoRI/XhoI in the same sites of pcDNA3.1 (+) plasmid.

The pcDNA-UB-OK plasmid that expresses a mutated ubiquitin fused to His tag, which is unable to form poly-ubiquitin chains, was kindly provided by Manuel S. Rodriguez (CIC-BIOGUNE, Spain).

The HA-PMLIV plasmid, expressing the PML protein fused to HA epitope, and used as ubiquitination positive control in culture cells, was kindly provided by Jin-Hyun Ahn (Sungkyunkwan University School of Medicine, South Korea).

Immunoprecipitation

For immunoprecipitation assays Protein A/G Plate IP Kit (Pierce) was used following the manufacturer's instructions. Briefly, coated plates were incubated with the appropriate antibodies (polyclonal anti-nsp3 or monoclonal anti-E) diluted in the immunoprecipitation buffer (PBS, 1% Surfactant-Amps X-100) for 2 h at room temperature. Then, antigen samples were diluted with 1 volume of immunoprecipitation buffer and were incubated in the coated plates overnight at 4 °C. The wells were washed five times with 200 µl of immunoprecipitation buffer and then immune complexes were eluted with 50 µl of elution buffer. Analysis of precipitate complexes was carried out by SDS-PAGE and Western blotting.

Confocal microscopy

For confocal microscopy, Vero E6 cells grown on glass coverslips were infected with rSARS-CoV, rSARS-CoV-ΔE and rSARS-CoV-EtagCt at an moi of 0.5. At 15 h post-infection the growth medium was removed and cells were washed twice with PBS and fixed with 8% paraformaldehyde for 30 min at room temperature. Then, cells were washed twice in PBS, and permeabilized for 10 min with 0.2% Triton X-100 in PBS. All antibody incubations were carried out for 1 h in PBS containing 10% FBS. The immunofluorescence was done with monoclonal antibodies specific for HA tag (dilution 1:1000; Sigma), or polyclonal antibodies against E (dilution 1:2000; kindly provided by Shen Shuo, Institute of Molecular and Cellular Biology, Singapore) and nsp3 (dilution 1:500; Rockland, Gilbertsville, PA). Coverslips were washed three times with PBS between primary and secondary antibody incubations. Alexa 488- or Alexa 594-conjugated antibodies against the different species (dilution 1:500; Molecular Probes) were used as secondary antibodies. Coverslips were mounted in ProLong Gold anti-fade reagent (Invitrogen) and examined on a Leica SP5 confocal microscope (Leica Microsystems).

In vitro ubiquitination assay

For a standard reaction, 1 mg of the baculovirus produced SARS-CoV E protein was incubated in a 10 µl reaction including an ATP regenerating system (50 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), 10 ng ubiquitin, 50 ng human E1 and 500 ng human E2 (Ubch5). Reactions were incubated at 37 °C for 2 h. After terminating the reactions with SDS sample buffer containing mercaptoethanol, reaction products were fractionated by SDS-PAGE. Detection of ubiquitinated and non-ubiquitinated E protein was done by Western blot using an antibody specific for E protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.03.015.

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