Short Communication

Two palmitylated cysteine residues of the severe acute respiratory syndrome coronavirus spike (S) protein are critical for S incorporation into virus-like particles, but not for M–S co-localization

Makoto Ujike,[1] Cheng Huang,[2] Kazuya Shirato,[3] Shutoku Matsuyama,[3] Shinji Makino[2] and Fumihiro Taguchi[1]

Correspondence
Makoto Ujike
ujike@nvlu.ac.jp

[1]Laboratory of Virology and Viral Infections, Faculty of Veterinary Medicine, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-8602, Japan

[2]Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston, Galveston, TX 77555-1019, USA

[3]Department of Virology III, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan

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The endodomain of several coronavirus (CoV) spike (S) proteins contains palmitylated cysteine residues and enables co-localization and interaction with the CoV membrane (M) protein. Depalmitylation of mouse hepatitis virus S proteins abolished this interaction, resulting in the failure of S incorporation into virions. In contrast, an immunofluorescence assay (IFA) showed that depalmitylated severe acute respiratory syndrome coronavirus (SCoV) S proteins still co-localized with the M protein in the budding site. Here, we determined the ability of depalmitylated SCoV S mutants to incorporate S into virus-like particles (VLPs). IFA confirmed that all SCoV S mutants co-localized with the M protein intracellularly. However, the mutants lacking two cysteine residues ($C^{1234/1235}$) failed to incorporate S into VLPs. This indicated that these palmitylated cysteines are essential for S incorporation, but are not involved in S co-localization mediated by the M protein. Our findings suggest that M–S co-localization and S incorporation occur independently of one another in SCoV virion assembly.

Severe acute respiratory syndrome (SARS) is a life-threatening disease caused by the SARS coronavirus (SCoV) (Drosten *et al.*, 2003; Fouchier *et al.*, 2003; Ksiazek *et al.*, 2003). SCoV belongs to the family *Coronaviridae*, which contains enveloped viruses with positive-sense ssRNA genomes. The virion of CoV carries four structural proteins: spike (S), membrane (M), small envelope (E) and nucleocapsid (N) (Rota *et al.*, 2003); some CoVs contain an additional membrane protein, haemagglutinin–esterase (HE).

CoVs assemble intracellularly, bud into the lumen of the endoplasmic reticulum (ER) Golgi intermediate compartment (ERGIC), and then are released from cells by exocytosis (Klumperman *et al.*, 1994). For efficient virus assembly, the structural proteins target and accumulate in the ERGIC. The M and E proteins of many CoVs harbour Golgi-targeting signals that localize them near the assembly site (Corse & Machamer, 2000, 2002). Some CoV S proteins contain ER-retrieval signals, whereas others do not (Lontok *et al.*, 2004; McBride *et al.*, 2007; Schwegmann-Wessels *et al.*, 2004; Shirato *et al.*, 2010, 2011; Winter *et al.*, 2008). However, previous studies have shown that even SCoV S proteins with weak ER-retrieval signals localize predominantly to the cell surface (McBride *et al.*, 2007). It is only when they are co-expressed with SCoV M protein that S proteins can be retained intracellularly and co-localize with M proteins. The N and HE proteins, which do not have such signals, also localize to the assembly site by interacting with the M protein. As M is necessary for virus-like particle (VLP) formation (Corse & Machamer, 2000; Huang *et al.*, 2004; Siu *et al.*, 2008) and interaction with other structural proteins can mediate its incorporation into virions and VLPs (de Haan *et al.*, 1999; Haijema *et al.*, 2003; Kuo & Masters, 2002; McBride & Machamer, 2010b; Narayanan & Makino, 2001; Nguyen & Hogue, 1997; Opstelten *et al.*, 1995; Vennema *et al.*, 1996), M is a key player in virion assembly (McBride *et al.*, 2007; Nguyen & Hogue, 1997; Opstelten *et al.*, 1995).

The CoV S protein is a class I fusion protein, and protease treatment of S generates the S1 and S2 subunits (Kawase *et al.*, 2009; Matsuyama *et al.*, 2005; Ujike *et al.*, 2008). The S1 subunit contains a binding domain for a host-cell receptor (Dveksler *et al.*, 1993; Li *et al.*, 2005), while the S2 subunit is responsible for virus entry and cell fusion. The CoV S2 subunit is composed of a large ectodomain, a transmembrane

domain (TMD) and a cytoplasmic tail (CT), although the exact border between the TMD and CT has not yet been identified. A characteristic feature of the TMD and CT is the presence of a remarkably dense cluster of cysteine residues. This cysteine-rich domain of SCoV and mouse hepatitis virus (MHV) can be divided into two cysteine-rich motifs, CRM1 and CRM2 (Fig. 1a). Some cysteine residues within these motifs are modified with palmitic acid (Bos *et al.*, 1995; Petit *et al.*, 2007).

Palmitylation of CoV S proteins is important for fusion activity and virus assembly. Past studies of SCoV and MHV S proteins demonstrated that palmitylated cysteine residues in CRM1 are required for cell–cell fusion (Bos *et al.*, 1995; Chang *et al.*, 2000; Petit *et al.*, 2007). In contrast, underpalmitylation of MHV S proteins, achieved by using a specific inhibitor of palmitylation (2-bromopalmitate) in infected cells or by introducing cysteine-to-alanine substitutions in CRM2, showed reduced ability of M–S interactions and resulted in the failure of S incorporation into the virion (Shulla & Gallagher, 2009; Thorp *et al.*, 2006). These findings suggest a causal relationship between the M–S interaction and S incorporation and indicate that palmitylated cysteine residues in CRM2 are required for both of these processes.

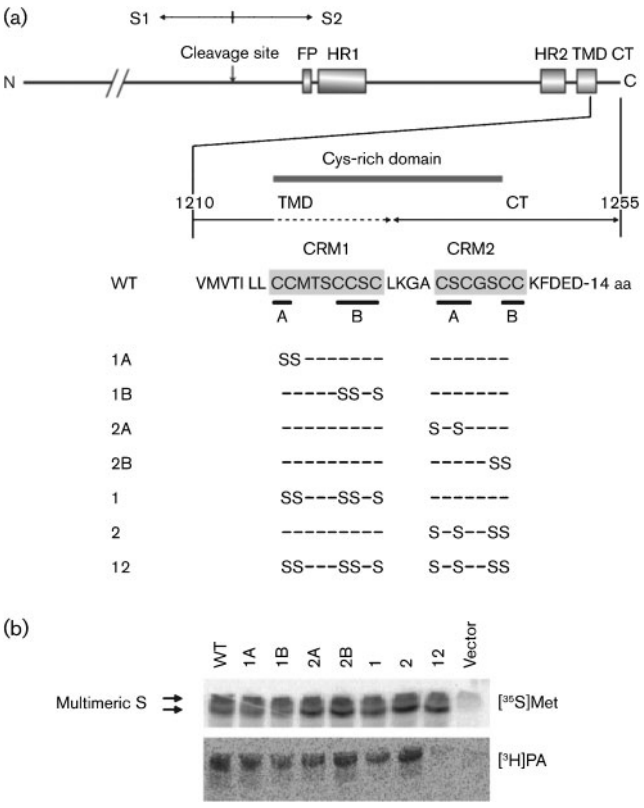

Fig. 1. WT and mutant SCoV S proteins and their incorporation of [^{35}S]methionine–cysteine or [^{3}H]palmitic acid. (a) Schematic of the WT and mutant (i.e. 1A, 1B, 2A, 2B, 1, 2, 12) S proteins. S contains two α-helical heptad repeats (HR), a putative fusion peptide (FP) and a trypsin-cleavage site. The expanded region shows the partial amino acid sequence of the TMD and CT. The cysteine-rich domain is indicated by a solid bar. The two cysteine-rich motifs (CRM1 and CRM2) are indicated by grey boxes; each motif is divided into two clusters, A and B. Mutant nomenclature was based on the polypeptide regions in which cysteine-to-serine substitutions were introduced. '14 aa' indicates the amino acid sequence DSEPVLKGVKLHYT. (b) S proteins were labelled with either [^{35}S]methionine–cysteine (Met) for 6 h or [^{3}H]palmitic acid (PA) for 20 h, recovered by immunoprecipitation and then analysed by SDS-PAGE.

Recently, McBride & Machamer (2010a) created a non-palmitylated SCoV S mutant by substituting all of the cysteines with alanine residues. Indirect immunofluorescence microscopy showed that both wild-type (WT) and non-palmitylated SCoV S proteins localized to the cell surface when expressed individually; however, when either protein was co-expressed with the SCoV M protein, each co-localized with M in the budding site. These results implied that palmitylation of SCoV S protein was not necessary for its interaction with SCoV M, which is quite different from studies with the MHV S protein. From this result, they expected that both proteins would be incorporated efficiently into virions. However, whether the WT and non-palmitylated SCoV S proteins are different in their ability to be incorporated into virions was not addressed in that study.

In this study, we generated a comprehensive set of SCoV S mutants that lack cysteine residues and tested their ability to be incorporated into VLPs. Both CRM1 and CRM2 carry two cysteine-rich clusters, designated A and B (Fig. 1a). cDNAs encoding SCoV S, M, E and N proteins (Frankfurt strain) were subcloned into *Eco*RI sites within the pCAGGS expression vector. Site-directed mutagenesis was performed using standard PCR protocols with the overlap-extension technique (Horton & Pease, 1991).

To determine whether the SCoV S mutants were palmitylated, COS-7 cells transiently expressing the various S protein mutants were metabolically labelled with either [^{35}S]methionine–cysteine or [^{3}H]palmitic acid. Transient S expression was performed using *Trans*IT COS Transfection Reagent (Mirus Bio), according to the manufacturer's instructions. At 28 or 42 h post-transfection, S protein-expressing cells were labelled with 500 μCi (18.5 MBq) [^{3}H]palmitic acid (Perkin-Elmer) for 20 h or with 5.0 μCi (185 kBq) Tran[^{35}S]-label (MP Biomedicals) for 6 h, respectively. Labelled cells were lysed in RIPA (radioimmunoprecipitation assay) buffer [0.15 M NaCl, 50 mM Tris/HCl (pH 7.4), 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS], and the supernatants were immunoprecipitated overnight at 4 °C with anti-SCoV rabbit polyclonal antiserum (generously provided by Dr Morikawa) and protein G–Sepharose beads (GE Healthcare). The labelled immunocomplexes were released from the beads by incubating for 5 min at 70 °C in SDS sample buffer without reducing reagent (50 mM Tris, 2 %

SDS, 0.1 % bromophenol blue and 10 % glycerol). The resultant solution was subjected to SDS-PAGE and visualized by autoradiography using a FUJIX BAS 2500 system (Fujifilm). As shown in Fig. 1(b), multimeric forms of SCoV S proteins were predominantly detected under these conditions. All S mutant proteins were labelled with [^{35}S]methionine–cysteine to a similar extent as the WT S protein. The CRM1- and CRM2-mutated S proteins (i.e. mutants 1A, 1B, 2A, 2B, 1 and 2) were labelled with [^{3}H]palmitic acid, whereas mutant 12, which lacked all cysteine residues, was not labelled (Fig. 1b). Densitometer analysis revealed that mutant 1 reduced the incorporation of palmitic acid by approximately 70 %, consistent with a previous study (Petit *et al.*, 2007). Our results suggest that cysteine residues of both CRM1 and CRM2 are palmitylation sites.

We next wanted to determine whether depalmitylated SCoV S mutants could be incorporated efficiently into SCoV VLPs (Huang *et al.*, 2006). To do this, 293T cells in a 60 mm dish were cotransfected with 1.0 μg pCAGGS-SCoV S, 4.7 μg pCAGGS-SCoV E, 0.5 μg pCAGGS-SCoV M and 1.0 μg pCAGGS-SCoV N using *Trans*IT 293 Transfection Reagent (Mirus Bio). At 72 h post-transfection, VLPs released into the culture medium were harvested and pelleted though a 20 % sucrose cushion at 45 000 r.p.m. for 2 h (SW55Ti rotor; Beckman). The production of VLPs and the accumulation of SCoV proteins within the cells were detected by Western blot analysis. S, N and M proteins were detected with the anti-S antibody IMG-557 (Imgenex) or anti-SCoV N or M protein rabbit polyclonal antiserum (generously provided by Dr Mizutani), together with HRP-conjugated anti-rabbit IgG (Invitrogen). Protein bands were visualized using ECLplus (GE Healthcare) on an LAS-1000 instrument (Fujifilm).

As shown in Fig. 2(a), co-expression of SCoV N, M, E and WT S proteins resulted in VLP release. Excluding the S protein plasmid from the transfection mixture resulted in the release of VLPs lacking the S protein. SCoV S mutant proteins with cysteine substitutions at the 2B cluster of CRM2 (i.e. mutants 2B, 2 and 12) were not detected in the released VLPs, in contrast to the other S mutants. As accumulation of intracellular S mutants 2B, 2 or 12 was similar to that of WT S (Fig. 2a), the lack of incorporation of these mutants into VLPs was not due to poor accumulation. In addition, S proteins were not detected in medium when only the S protein plasmid was transfected into 293T cells, demonstrating that S proteins in medium were not derived from secreted free S proteins (Fig. 2b). These results demonstrated that two palmitylated cysteine residues of CRM2B (i.e. C^{1234} and C^{1235}) are essential for S incorporation into SCoV VLPs.

Previous studies using indirect immunofluorescence microscopy demonstrated that depalmitylated SCoV S proteins co-localize with SCoV M proteins (McBride & Machamer, 2010a). These results imply the ability of S to interact with M proteins, so its lack of incorporation into

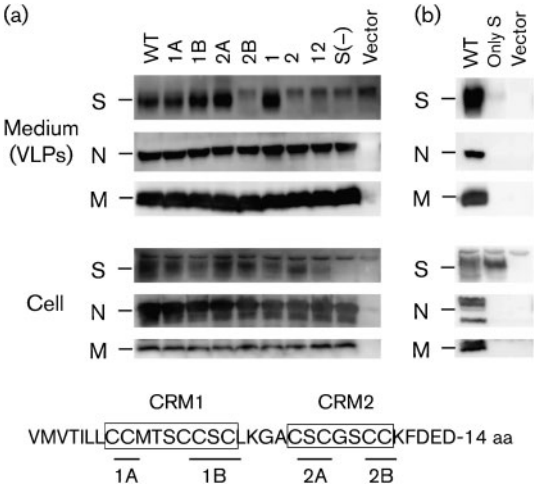

Fig. 2. WT and mutant S incorporation into SCoV VLPs. (a) Plasmids encoding S, M, N or E proteins were cotransfected into 293T cells. At 72 h post-transfection, released VLPs and cellular extracts were subjected to SDS-PAGE, electrotransfer of the separated proteins onto a transfer membrane and Western blot. S(−) VLPs were generated with empty pCAGGS vectors instead of pCAGGS-SCoV S. (b) 'Only S' indicates that plasmids encoding S and empty pCAGGS vectors were transfected into 293T cells.

VLPs was unexpected. To confirm whether the SCoV S mutants generated in this study could co-localize with SCoV M proteins, their subcellular locations within COS-7 cells were evaluated by indirect immunofluorescence microscopy under permeabilized and non-permeabilized conditions. COS-7 cells were cotransfected with 1.0 μg pCAGGS-SCoV S and 0.5 μg of either pCAGGS-SCoV M or empty pCAGGS vector. At 40 h post-transfection, SCoV S and M protein-expressing COS-7 cells were fixed with 4 % paraformaldehyde for 15 min at room temperature and permeabilized, or not, with 0.1 % Triton X-100 for 20 min at room temperature. The cells were then stained with anti-SCoV S mouse mAb (Ohnishi *et al.*, 2005) and anti-SCoV M rabbit polyclonal antiserum, using Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) and FITC-conjugated goat anti-rabbit IgG secondary antibodies. Images were obtained with an Axioscope microscope (Carl Zeiss). The representative SCoV S mutant 2B, which failed to be incorporated into VLPs, is shown in Fig. 3. When mutant 2B was expressed alone, it localized predominantly to the cell surface (Fig. 3a, left). In contrast, when it was co-expressed with SCoV M, mutant 2B was retained intracellularly (Fig. 3a, right) and co-localized with M (Fig. 3b). All of the other SCoV S mutants, including non-palmitylated mutant 12, stained similarly (data not shown), consistent with previous studies (McBride & Machamer, 2010a). Thus, all of the SCoV S mutants generated in this study co-localize efficiently with the SCoV M protein.

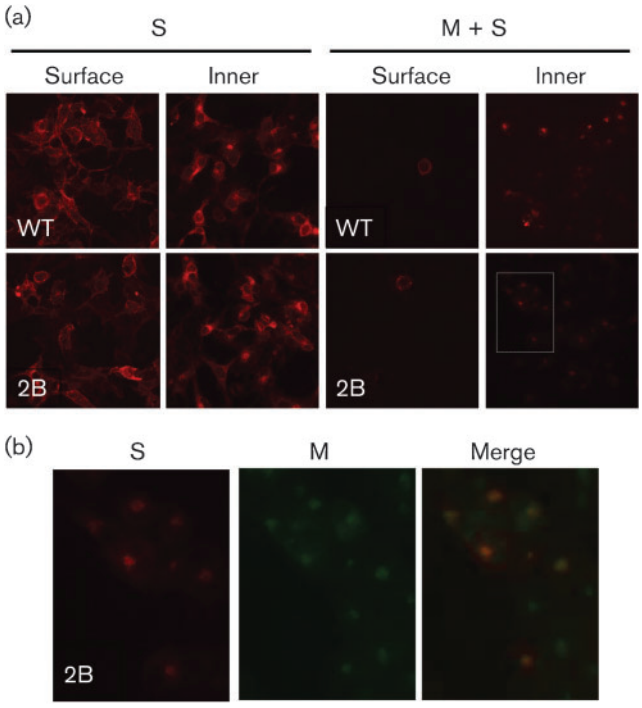

Fig. 3. Subcellular localization of WT and mutant SCoV S proteins with or without SCoV M proteins. COS-7 cells were cotransfected with pCAGGS-SCoV S and either pCAGGS-SCoV M or empty pCAGGS vector. (a) Subcellular localization of WT and mutant SCoV S proteins with (M+S) or without (S) the SCoV M protein. (b) Co-localization of S mutant 2B and M proteins. The dashed square in (a) was magnified. All SCoV S mutants showed similar co-localization staining results (data not shown).

In summary, we demonstrated that two palmitylated cysteines in CRM2B of the SCoV S protein are essential for S incorporation into VLPs, but not for the S co-localization mediated by the SCoV M protein in the budding site. This is in contrast to studies of the MHV S protein (Shulla & Gallagher, 2009; Thorp *et al.*, 2006). Palmitylation of MHV S protein was required for MHV M–S co-localization and interaction, which are thought to mediate MHV S incorporation into virions. C-terminal truncation mutants of MHV S, which lacked cysteine residues in CRM2, were unable to interact with MHV M, resulting in no S incorporation (Bosch *et al.*, 2005). Thus, M–S co-localization/interaction and S incorporation were both dependent on palmitylated cysteines within MHV S proteins. However, our study demonstrated that, in the case of SCoV S proteins, the absence of cysteine residues in CRM2B did not affect M–S co-localization, but failed to allow S incorporation, indicating the independence of these two functions. Importantly, these data also suggest that intracellular retention of S proteins within the virion-assembly site, which is mediated by M protein, is not sufficient for S incorporation and requires two palmitylated cysteines within the S protein.

It is unclear why SCoV M–S co-localization occurs independently of SCoV S incorporation. A few possible explanations are conceivable. As the M–S co-localization detected by indirect immunofluorescence assay does not necessarily reflect direct M–S interaction, we could not exclude the possibility that, despite M–S co-localization, depalmitylated SCoV S proteins might not interact directly with SCoV M proteins, resulting in a failure of S incorporation into VLPs. However, this seems unlikely because findings from an *in vitro* binding assay suggested that the recombinant SCoV S-tail domain purified from a bacterial expression system, which was not palmitylated, was able to interact with SCoV M proteins (McBride *et al.*, 2007). Given that depalmitylated SCoV S proteins could interact with the SCoV M protein, another possible explanation is as follows. During virion and VLP assembly, it is hypothesized that M proteins interact through multiple contact sites, creating a dense matrix of protein within the viral membrane (de Haan *et al.*, 2000). This dense matrix, formed by lateral M–M interactions, may limit the space available for membrane-spanning S proteins. Therefore, in addition to M binding, efficient S incorporation also requires that S adapts an appropriate structure to accommodate its endodomain in the limited space. Although we showed in mutant 1 that cysteines in only CRM2 (2A and 2B) were palmitylated, we could not rule out the possibility that cysteine(s) in the 2A rather than the 2B region were palmitylated. Thus, the two hydrophobic palmitic acids branching off CRM2B into the inner membrane, or the non-palmitylated cysteine(s) themselves, may contribute to formation of such a structure. Our data highlight the mechanistic differences of S incorporation between MHV and SCoV.

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