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# Subcellular localization and membrane association of SARS-CoV 3a protein

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**Abstract**

SARS-CoV 3a protein was a unique protein of SARS coronavirus (SARS-CoV), which was identified in SARS-CoV infected cells and SARS patients' specimen. Recent studies revealed that 3a could interact specifically with many SARS-CoV structural proteins, such as M, E and S protein. Expressed 3a protein was reported to localize to Golgi complex in SARS-CoV infected cells. In this study, it was shown that 3a protein was mainly located in Golgi apparatus with different tags at N- or C-terminus. The localization pattern was similar in different transfected cells. With the assay of truncated 3a protein, it was shown that 3a might contain three transmembrane regions, and the second or third region was properly responsible for Golgi localization. By ultra-centrifugation experiment with different extraction buffers, it was confirmed that 3a was an integral membrane protein and embedded in the phospholipid bilayer. Immunofluorescence assay indicated that 3a was co-localized with M protein in Golgi complex in co-transfected cells. These results provide a new insight for further study of the 3a protein on the pathogenesis of SARS-CoV.


*Keywords:* Severe acute respiratory syndrome; SARS coronavirus; 3a Protein; Golgi complex; Membrane association; Subcellular localization

## 1. Introduction

An outbreak of life-threatening atypical pneumonia, firstly appeared in GuangDong Province, People's Republic of China, has spread to North American, Europe, and other Asian countries. The syndrome is a new clinical entity and has been designated as severe acute respiratory syndrome (SARS). SARS has infected 8422 cases and caused 916 related deaths (Fan et al., 2004; Lee et al., 2003; Peiris et al., 2003; Tsang et al., 2003; WHO, 2003a,b). Vigorous research has been carried out world wide to find the cause for this disease, and a novel coronavirus, SARS conoravirus (SARS-CoV) identified and sequenced by various research groups, is distantly related to the established group 2 coronavirus (Snijder et al., 2003).

The SARS-CoV is a single-stranded, positive-sense RNA virus, 29,727 bp in length. The genomic organization is typical of coronaviruses, with the characteristic gene order [5′-replicase (rep), spike (S), envelope (E), membrane (M), nucleocapsid (N)-3′] and short untranslated regions at both termini. The SARS-CoV rep gene, comprising approximately two-thirds of the genome, is predicted to encode two polyproteins that undergo co-translational proteolytic processing. SARS-CoV contains four major structural proteins S, E, M and N, which are common to all known coronaviruses (Chan et al., 2003; Drosten et al., 2003; Holmes, 2003; Marra et al., 2003; Rota et al., 2003).

Coronaviruses also encode a number of non-structural proteins, whose open reading frames (ORFs) localize between S

*Abbreviations:* SARS, severe acute respiratory syndrome; PBS, phosphate-buffered saline; SARS-CoV, SARS-associated coronavirus; rep, replicase; S, spike; E, envelope; M, membrane; N, nucleocapsid; ORF, open reading frame; DMEM, Dulbecco's modified Eagel medium; EGFP, enhanced green fluorescent protein; ECL, enhanced chemiluminescence

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and E genes, or between M and N genes. These non-structural proteins vary widely among different coronavirus species. The genome of SARS-CoV contains ORFs for five potential non-structural proteins of greater than 50 amino acids in these intergenic regions. Among them, SARS 3a protein (CDS: 25,252–26,074), also referred as ORF3, X1 and U274 in other articles, was confirmed to express in the SARS-CoV infected cells and SARS patients' specimen (Tan et al., 2004; Yu et al., 2004; Zeng et al., 2004). It was reported that 3a was located in Golgi complex and plasma membrane in infected cells and interacted with many structural proteins, such as S, M and E protein, which suggested 3a was an important protein in viral life cycle.

In the present investigation, it was shown that 3a with different tags at C- or N-terminus was mainly located in the Golgi complex in transfected cells. And cellular localization of truncated 3a was revealed that 3a might contain three transmembrane regions, and the second or third region was properly reliable for Golgi localization. Ultra-centrifugation experiment with different extraction buffers demonstrated that 3a was an integral membrane protein and embedded in the phospholipid bilayer. Further, immunofluorescence assay was shown that 3a co-localized with M protein mainly in Golgi complex in co-transfected cells.

## 2. Materials and methods

### 2.1. Cell culture and transfection

293 (human embryonal kidney) cells, Vero (African green monkey kidney) cells and COS-7 (African green monkey kidney) cells were grown in Dulbecco's modified Eagel medium (DMEM) (Gibco BRL) supplemented with 10% FBS. A549 (human lung carcinomatous) cells were cultured in Ham's F12K medium with 10% FBS at 37 °C in a incubator supplied with 5% $CO_2$. When cell density in a culture plate reached 70% confluence, the cells were transfected with 1.5 μg/ml plasmid DNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturers' recommendation. The old medium was replaced with fresh medium 5 h after transfection and then incubation continued until experiment.

### 2.2. Construction of 3a/pEGFP-N1, 3a/pEGFP-C1, 3a/pCMV-myc, serial of truncated 3a protein and M/pCMV-myc, M/pDsRed-N1

The 3a and M genes used for this study were PCR amplified from the SARS-CoV (ZJ01, AY297028) genome by using *Taq* DNA polymerase (NEB). PCR was performed with a forward primer (containing a *Xho*I site) (Table 1) and a reverse primer (containing a *Eco*RI site) complementary to the 3′ end of the 3a gene but without the stop codon of 3a to allow for read-through. This product was cut with *Xho*I and *Eco*RI and cloned into the multiple cloning site (MCS) of the pEGFP-N1 vector (Clontech), producing a 3a/pEGFP-N1 plasmid. The 3a/pEGFP-C1, truncated 3a, 3a/pCMV-myc, M/pCMV-myc and M/pDsRed-N1 constructs were made in a similar fashion, and the oligonucleotide primers were listed in Table 1. The Golgi gene was PCR amplified from the pECFP-Golgi marker vector (Clontech) and cloned into pDsRed-N1 vector to product Golgi/pDsRed-N1 plasmid.

### 2.3. Expression of 3a/pEGFP-N1, 3a/pEGFP-C1 and 3a/pCMV-myc

The transfected cells were harvested at 48 h after transfection. The cell lysates were prepared, ran on SDS-PAGE, transferred to PVDF membrane, and incubated with monoclonal anti-GFP antibody (1:10,000) (Sigma) or anti-myc antibody (1:1000) (Santa Cruz). Membranes were washed with TBS buffer and incubated with corresponding secondary antibody (Santa Cruz) tagged with horseradish peroxidase for 1 h. Proteins were visualized with enhanced chemiluminescence (ECL) reagents (Cell Signaling). Biotinylated protein marker detection pack was purchased from Cell Signaling Tech.

### 2.4. Confocal microscopy of 3a protein

At 24 h after transfection, cells on glass cover slips were rinsed with phosphate buffered saline (PBS) and subjected to fixation using 3.7% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100. Fluorescence antibody staining was performed by incubating cells with appropriate antibody for 1 h, followed by secondary antibody for 30 min. Then the nuclear was stained with PI (50 μg/ml) (Sigmal) or Hoechest (0.25 μg/ml). Anti-myc antibody was used at 1:400.

To live-cell staining, cells were incubated with BODIPY TR $C_5$-ceramide (5 μM), ER-Tracker™ Blue–White DPX (1 μM) (Molecular Probes) for 30 min at 37 °C. After that, the cells were fixed with 3.7% formaldehyde for 10–20 min, and then permeabilized with 0.2% Triton X-100 for 10 min. The nuclear was stained with Hoechest. Images were viewed and collected with confocal fluorescence microscope connected to a Bio-Rad Radiance2100 laser scanner.

### 2.5. Membrane association assay

At 48 h post-transfection, 293 cells were washed with PBS and resuspended in ice-cold 1:10 250 μl of TES (20 mM Tris, pH7.4; 100 mM NaCl; 1 mM EDTA) supplemented with complete protease inhibitor cocktail (Roche). Cells were incubated for 30 min on ice and lysed by 30 strokes in a Dounce homogenizer, and then centrifuged at 5000 × *g* for 10 min (at 4 °C) to remove cell debris and nuclei. Postnuclear supernatants were treated with 1% Nonide P-40 or not treated, and followed to centrifuge at 200,000 × *g* for 1 h (at 4 °C) to separate membrane and cytoplasmic fractions. Supernatants were freeze-dried and resuspended in 200 μl of one times lysis buffer for further analysis. Pellet fractions were resuspended