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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

Table 1
Primes used for wild-type and truncated 3a constructs[a]

Construct name	Polarity	Sequence
3a/pEGFP-N1	Sense[b]	5′-CCGCTCGAGCGCCACCATGGATTTGTTTATGAGATTTTTTACTCTTG-3′
	Anti-sense[c]	5′-CGGAATTCCCAAAGGCACGCTAGTAGTCG-3′
3a/pEGFP-C1	Sense	5′-CCGCTCGAGCGATGGATTTGTTTATGAGATTTTTTAC-3′
	Anti-sense	5′-CGGAATTCTTACAAAGGCACGCTAGTAGTCG-3′
3a/pCMV-myc	Sense	5′-CGGAATTCGGATGGATTTGTTTATGAGATTTTTTAC-3′
	Anti-sense	5′-CCGCTCGAGTTACAAAGGCACGCTAGTAGTCG-3′
3a D1-147-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGATTTGTTTATGAGATTTTTTACTCTTG-3′
	Anti-sense	5′-CGGAATTCCAAGTAATGGGTTCTGGGATTTG-3′
3a D1-99-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGATTTGTTTATGAGATTTTTTACTCTTG-3′
	Anti-sense	5′-CGGAATTCCCGCCTCCATACCTGCAGCGAC-3′
3a D1-77-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGATTTGTTTATGAGATTTTTTACTCTTG-3′
	Anti-sense	5′-CGGAATTCCCTGGAAGCCGTTATAAAGGGCTAG-3′
3a D77-103-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGAGGGCTTCCAGTTCATTTGC-3′
	Anti-sense	5′-CGGAATTCCCGCCTCCATACCTGCAGCGAC-3′
3a D99-147-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGTGCTTGTCGCTGCAGGTATGG-3′
	Anti-sense	5′-CGGAATTCCAAGTAATGGGTTCTGGGATTTG-3′
3a D147-274EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGTTTATGATGCCAACTACTTTGTTTGC-3′
	Anti-sense	5′-CGGAATTCCCTTTGGCACGCTAGTAGTCG-3′
3a D99-274-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGTGCTTGTCGCTGCAGGTATGG-3′
	Anti-sense	5′-CGGAATTCCCTTTGGCACGCTAGTAGTCG-3′
3a D77-274-EGFP	Sense	5′-CCGCTCGAGCGCCACCATGGAGGGCTTCCAGTTCATTTGC-3′
	Anti-sense	5′-CGGAATTCCCAAAGGCACGCTAGTAGTCG-3′
M/pDsRed-N1	Sense[b]	5′-CCGCTCGAGCGCCACCATGGCAGACAACGGTACTATTACCGTTG-3′
	Anti-sense[c]	5′-CGGAATTCCCTGTACTAGCAGAGCAATATTGTC-3′
M/pCMV-myc	Sense	5′-CGGAATTCGGATGGCAGACAACGGTACTATTACCGTTG-3′
	Anti-sense	5′-CCGCTCGAGTTACTGTACTAGCAGAGCAATATTGTC-3′
Golgi/pDsRed-N1	Sense	5′-GCCG CTC GAG ATG AGG CTT CGG GAG CCG CTC CTG-3′
	Anti-sense	5′-GCC GGA TCC CG CGG CCG GGC CCC TCC GGT CCG GAG-3′

[a] Gene sequences are correspond to SARS-CoV (ZJ01).
[b] Underlined nucleotides represent restriction site and Kozak sequence before start codon (ATG).
[c] Underlined nucleotides represent restriction site, and delete the stop codon.

in 200 μl of PBS, 0.1 M Na_2CO_3 (pH 11.5), 0.5 M EDTA, 1 M NaCl, 4 M urea or 1% Triton X-100, incubated for 1 h on ice, and centrifuged for 1 h at 200,000 × *g* (at 4 °C). The supernatant fractions after freeze-dried and the pellet fractions, resuspended in 200 μl of one times lysis buffer, were analyzed by Western blotting with monoclonal anti-GFP antibody.

3. Results

3.1. Subcellular localization of SARS-CoV 3a protein

Previous studies revealed that SARS-CoV 3a encoded a protein of 274 amino acid that lacked significant similarities to any known protein. To characterize 3a, the gene in the SARS-CoV genome (ZJ01, AY297028) was inserted into an expression vector that allows the production of EGFP or myc fusion protein under the control of a CMV promoter (Fig. 1A). 3a/pEGFP-N1, pEGFP-N1, 3a/pEGFP-C1, pEGFP-C1, pCMV-myc and 3a/pCMV-myc plasmids were transfected into 293 cells separately. At 48 h after transfection, cell lysates were prepared, separated by SDS-PAGE, and transferred to PVDF membrane. Immunoblot analysis with anti-GFP antibody or anti-myc antibody demonstrated that the chimeric proteins were expressed and migrated at the expected molecular mass of approximately 56,000 Da or 28,000 Da separately (Fig. 1B).

In the next step, localization of 3a protein with different tags fused at N- or C-terminus was tested by fluorescence confocal microscopy. As shown in Fig. 1C, merged images (3, 6, 9 and 12) were represented regions of overlap between the EGFP fluorescence or myc-staining (green-) and Hoechest-labeling (blue-) image. The results showed that EGFP protein was distributed throughout the entire cell in both cytoplasm and nucleus, however, the pattern of 3a-EGFP was located in the cytoplasm and plasma membrane in a punctate pattern with condensing into discrete loci and spot fluorescence. The fluorescence distribution of EGFP-3a was similar to that of 3a in infected cells (Tan et al., 2004; Yu et al., 2004), and EGFP or myc tag at N- or C-terminus had little effect on the cellular localization (Fig. 1C). Similar patterns in immunofluorescence of 3a were observed in Vero, COS-7, A549 and 293 cells (Fig. 1D). Expression of 3a was emerged from 12 to 48 h after transfection in 293 cells (Fig. 1E). Published data showed that 3a was detected in Golgi complex with 58K protein and beta-COP staining in SARS infected cells (Yu et al., 2004). With fluorescence dyes – ER Tracker Blue–White DPX and BODIPY TR C_5-ceramide (Babia et al., 2001; Cole et al., 2000), which stained endoplasmic

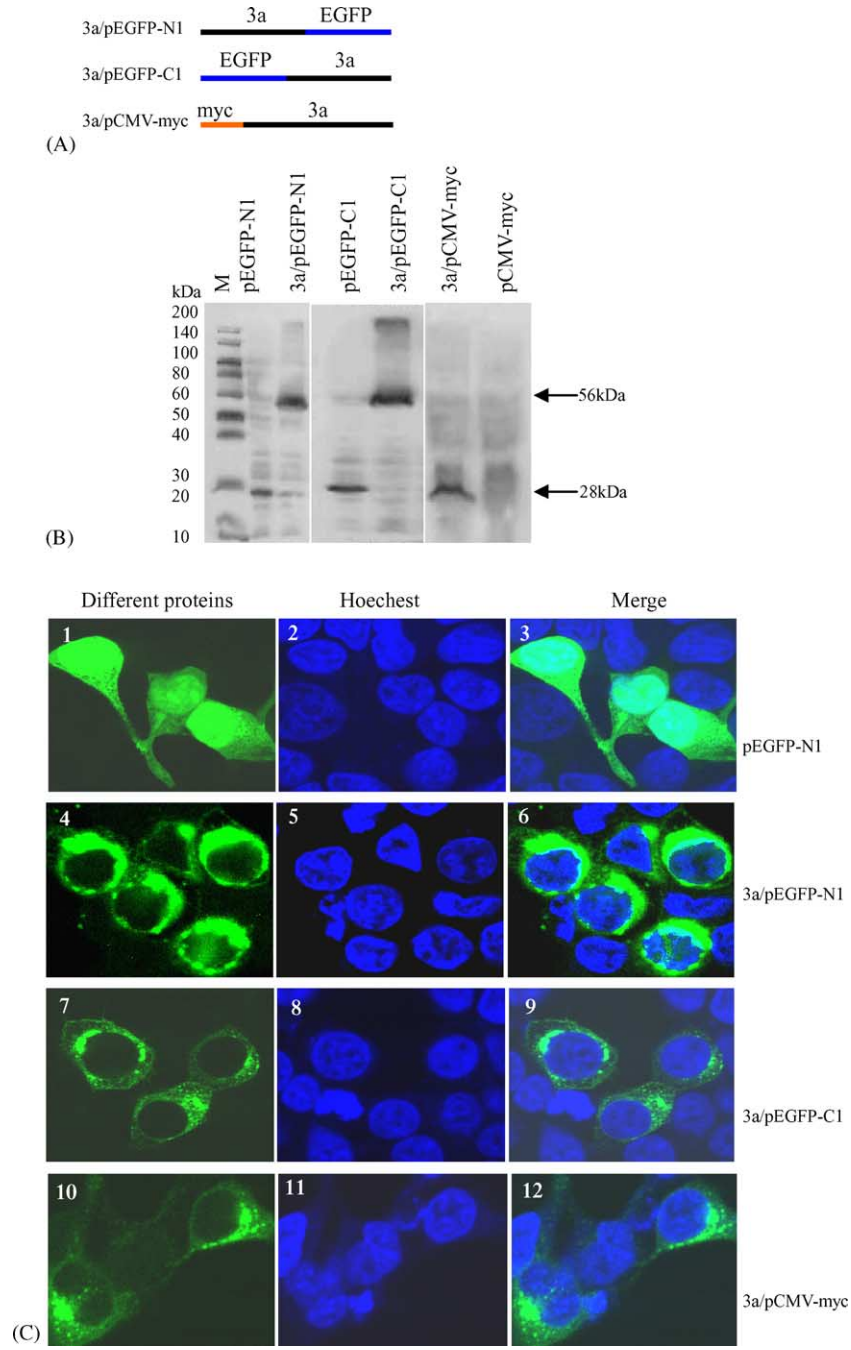


Fig. 1. Expression and cellular localization of 3a within transfected cells. (A) Construction of 3a/pEGFP-N1, 3a/pEGFP-C1 and 3a/pCMV-myc. 3a from the SARS-CoV genome was cloned as a C-terminal fusion to the pEGFP-N1 vector, and as N-terminal fusion to the pEGFP-C1 and pCMV-myc vectors. (B) Western blotting analysis. 293 cells were transiently transfected with pEGFP-N1, 3a/pEGFP-N1, pEGFP-C1, 3a/pEGFP-C1, pCMV-myc and 3a/pCMV-myc plasmids separately. Cell lysates were prepared at 48 h after transfection and separated by SDS-PAGE. Proteins transferred to PVDF membrane was probed with monoclonal anti-GFP or anti-myc antibody. Sizes (kDa) of molecular mass markers were indicated on the left. (C) Cellular localization of 3a protein with different tags. 293 cells were transfected with described plasmids separately. Cellular localization of 3a was observed by scanning fluorescence confocal microscopy. Green represented EGFP fluorescence (1, 4 and 7) from original EGFP, 3a-EGFP or EGFP-3a fusion protein; blue (2, 5, 8 and 11) represented Hoechest stained cell nuclei; 3, 6, 9 and 12 images represented overlapping green and blue fluorescence. In panels 10, cells were labeled with FITC conjugated goat anti-mouse antibody to show the expression of myc-3a. All three panels of a row had the same field of view. (D) Cellular localization of 3a protein in different transfected cells. COS-7, Vero and A549 cells were transiently transfected with 3a/pEGFP-N1 separately. Cellular localization of 3a was observed at 24 h after transfection. Green represented EGFP fluorescence from expressed 3a-EGFP protein in different cells; red represented propidium idodine (PI) stained cell nuclei; yellow (in the overlay image) represented overlapping green and red fluorescence. All three panels had the same field of view. (E) Cellular localization of 3a protein at different time after transfection. 3a/pEGFP-N1 was transfected into 293 cells. At 12 h, 24 h, 36 h and 48 h after transfection, the cellular localization of 3a was viewed with fluorescence confocal microscopy separately. Green represented EGFP fluorescence from expressed 3a-EGFP protein.

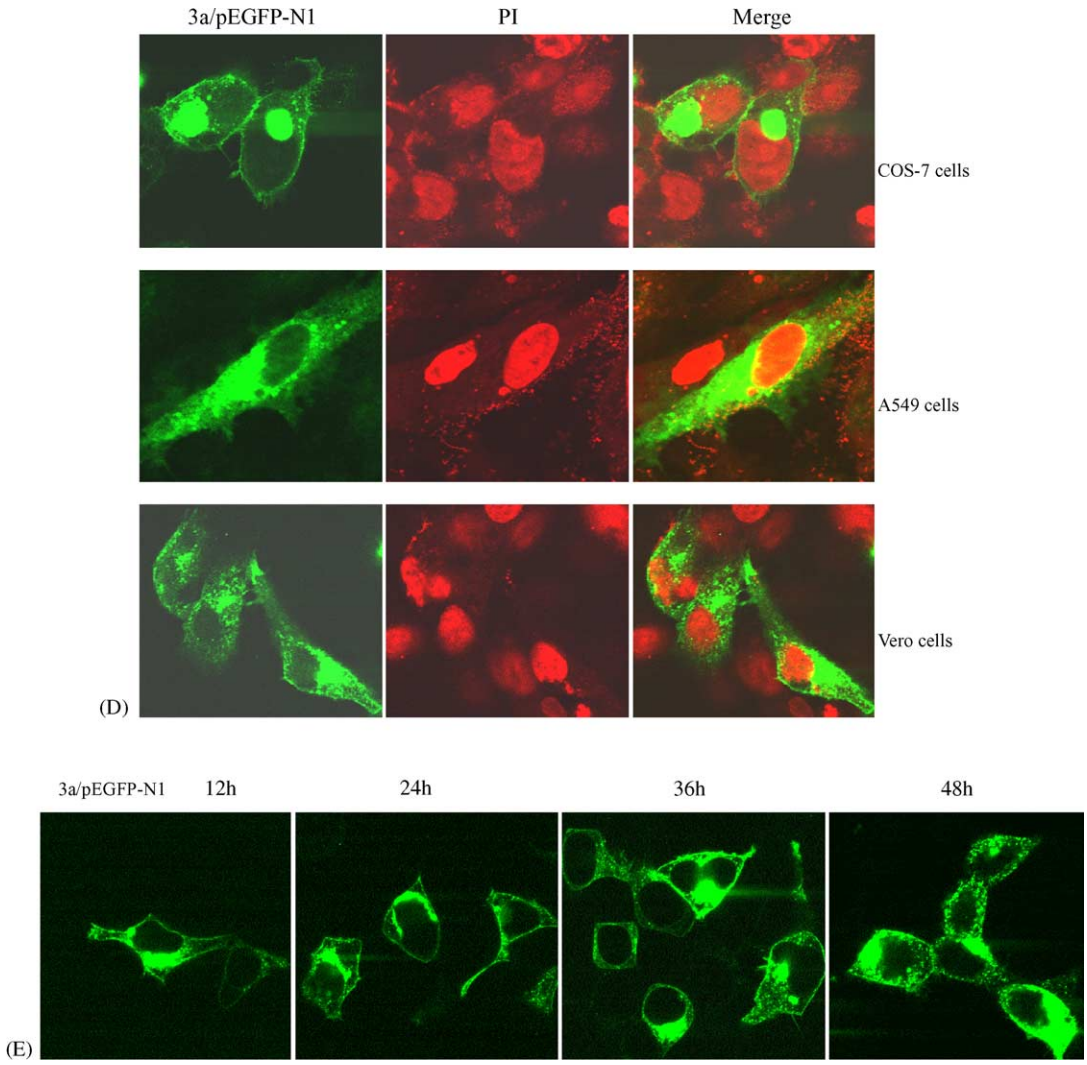


Fig. 1. (*Continued.*)

reticulum (ER) and Golgi complex, respectively, it was shown that 3a-EGFP was mainly distributed in Golgi apparatus, and partially in ER (Fig. 2A). Further, with the Golgi vector marker – Golgi/pDsRed-N1, it was confirmed 3a was localized well in Glogi complex (Fig. 2B). Using COS-7 cells, similar results were obtained (data not shown). These results suggested that the Golgi distribution of 3a protein was a conserved attribute.

3.2. Cellular localization of truncated 3a protein

Bioinformatics analysis showed that 3a protein might contain three transmembrane regions spanning approximately residues from 34 to 56, 77 to 99, and 103 to 125 (Marra et al., 2003; Rota et al., 2003) (Fig. 3A). To further characterize the region responsible for Golgi localization, a series of truncated 3a were constructed according to the bioinformation results. This approach was used in an attempt to avoid major disruption in protein folding that could result in cutting the protein in all transmembrane domains. Different truncated 3a proteins were expressed and migrated at the expected molecular mass separately (Fig. 3B). Cellular localization of truncated 3a protein was performed as before. As shown in Fig. 3C (A–F and V–X), the fluorescent pattern of 3a D1–147, D1–103 and D 77–274, containing two or three functional regions, was similar to that of wild-type 3a protein (Fig. 1C), distributing in the cytoplasm and somewhere condensing to spots fluorescence. However, construct 3a D147–274 gave rise to preservation of EGFP in the cytoplasm and nuclear (Fig. 3C (P–R)) as did pEGFP-N1 (Fig. 1C). These results suggested that the N-terminal 147 amino acids of 3a were mainly responsible for the Golgi localization. Furthermore, as shown in Fig. 3C (G–O and S–U), constructs, containing only one transmembrane region of 3a, all localized in the cytoplasm, but more disperse than that of wild-type 3a protein. Closer investigation revealed that the fluorescence from first region was distributed homogeneously over the cytoplasm. However, the ones from second or third region was some-

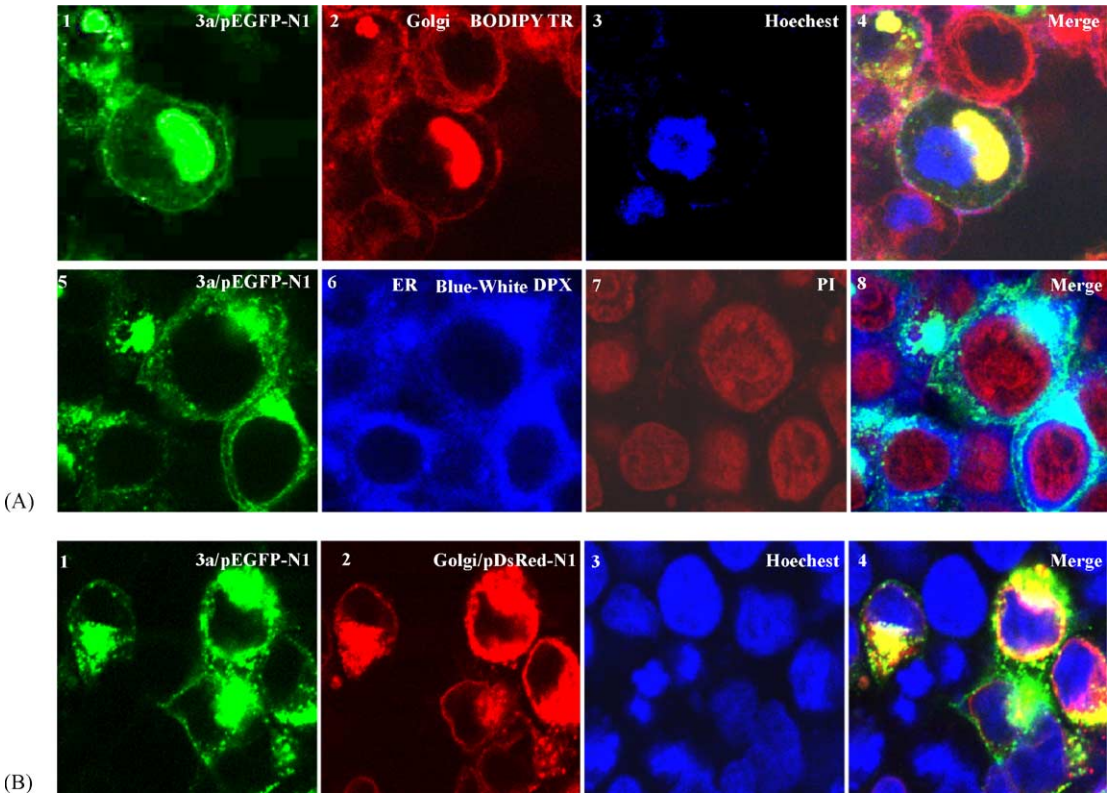


Fig. 2. Subcellular localization of 3a protein. (A) Subcellular localization of 3a protein with fluorescence marker. 293 cells transfected with the 3a/pEGFP-N1 plasmid were grown for 24 h, washed with PBS, and stained with Golgi or ER fluorescence marker. Subsequent, cells were fixed, and then nuclear was stained with Hoechest or PI. 3a-EGFP protein (green, 1 and 5), Golgi fluorescence marker BODIPY TR C_5-ceramide (red, 2), ER fluorescence marker ER-Tracker^{TM} Blue–White DPX (blue, 6) and merge image (4 and 8) were indicated. (B) Subcellular localization of 3a protein with Golgi-DsRed protein. 293 cells co-transfected with the 3a/pEGFP-N1 and Golgi/pDsRed-N1 plasmids. At 24 h after transfection, cells were fixed, and nuclear was stained with Hoechest. 3a-EGFP protein (green, 1), Golgi-DsRed marker (red, 2), DNA staining (blue, 3) and merge image (4) were indicated.

where emerged discrete loci fluorescence in the cytoplasm, which were partly co-localized with Golgi-DsRed protein (Fig. 3D). These results suggested that 3a protein might contain three transmembrane regions, which was consisted with that of bioinformation assay, and the second or third region might have a Golgi localization signal.

3.3. *Membrane association of 3a protein in vivo*

Many structural proteins of coronavirus are membrane-associated protein, which localized in Golgi apparatus or ER in infected cells. A method based on ultra-centrifugation was used to investigate the membrane association of 3a protein. With this experiment, membrane and cytoplasmic fractions were collected and analyzed by Western blotting from 293 cells transfected with either pEGFP-N1 or 3a/pEGFP-N1. As expected, the cytosolic EGFP protein was detected mainly in the supernatant fraction. When fused with 3a, however, it was detected mainly in the pellet fraction, and expressed at the expected molecular mass. Moreover, the cell lysate pretreated with NP-40 before ultra-centrifugation, 3a-EGFP protein was found exclusively in the supernatant fraction (Fig. 4A). These results were supportive of the suggestion that 3a protein was closely associated with membrane. Subsequently, the mode of membrane association was further investigated using different buffers to extract the membrane fraction. According to previous reports (de Jong et al., 2003): 1 M NaCl can increase the ionic strength of buffer; 4 M urea function as mild chaotropic salt conditions; 0.5 M EDTA can chelate divalent cations; 0.1 M Na_2CO_3 (pH 11.5) providing alkaline conditions, convert membrane to sheet and release soluble proteins that are trapped inside membrane vesicles. The cell lysate was first centrifugated to separate supernatant and pellet fractions, and the pellet fractions were then extracted with the indicated buffers for 1 h (Fig. 4B). As shown in Fig. 4B, after second ultra-centrifugation, 3a-EGFP protein remained attached to membranes on extraction with PBS (control) and the buffers that extract peripheral membrane proteins, such as 1 M NaCl, or 0.5 M EDTA. In addition, the protein was mainly detected in the pellet fraction when membranes were extracted using 0.1 M Na_2CO_3 (pH 11.5) and 4 M urea (de Jong et al., 2003), arguing for integral association attribute of 3a protein. While upon solubilization of membrane pellet using 1% Triton X-100, a non-ionic detergent that released integral membrane proteins, the 3a-EGFP protein was detected in the supernatant fraction. From these results, it was confirmed that 3a protein was integral membrane protein, which embedded in the phospholipid bilayer.

3.4. Co-localization of 3a and M proteins

Recent data showed that 3a interacted with many structural proteins, such as S, M and E proteins (Tan et al., 2004; Zeng et al., 2004). Coronavirus M was a triple-spanning membrane protein, which was reported to mainly localize to the Golgi complex in infected or transfected cells (Rottier, 1995). The interactions of M protein with E and S proteins were important for virus formation and budding (de Haan et al., 1999; Klumperman et al., 1994). To characterize the localization of SARS M protein and to reveal the spatial interaction between SARS-CoV 3a and M proteins, the gene of M protein was cloned from SARS-CoV genome and inserted into pDsRed-N1 and pCMV-myc vector. After transfected into 293 cells separately, the fluorescence of M-DsRed and myc-M were mainly located in the cytoplasm and condensed into discrete loci and spot fluorescence, somewhat like Golgi complex localization (Fig. 5A), confirming that the M fusion proteins with myc or DsRed tag at its N- or C-terminus had similar localization. To reveal the Golgi localization of SARS-M protein, M/pCMV-myc and Golgi/pDsRed-N1 were co-transfected into 293 cells. As shown in Fig. 5B, the fluorescent distribution of myc-M and Golgi-DsRed was similar and co-localized with Golgi-DsRed in Golgi complex region.

To confirm the spatial interaction of M and 3a proteins in cells, 3a/pEGFP-N1 and M/pDsRed-N1 were co-transfected into 293 cells. As shown in Fig. 5C, the fluorescence of 3a-EGFP and M-DsRed were distributed in the cytoplasm and co-localized well along the Golgi region. Similar result was also observed from the cells co-transfected with 3a/pEGFP-N1 and M/pCMV-myc. The results suggested that the interaction of 3a protein with M protein might play an important role in SARS-CoV assembling or budding.

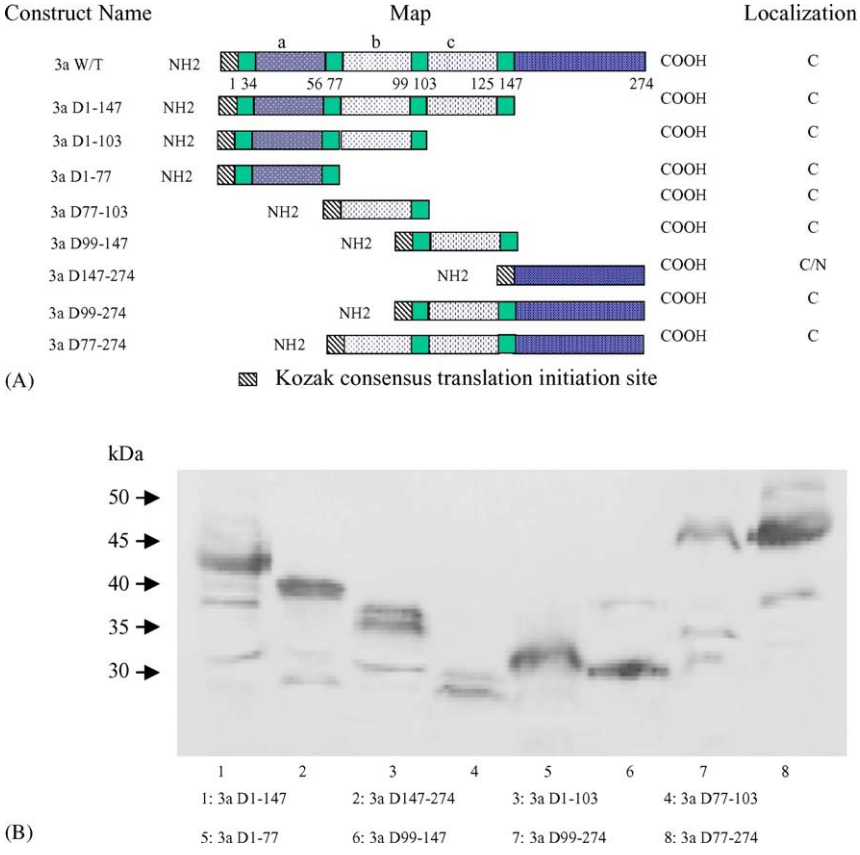
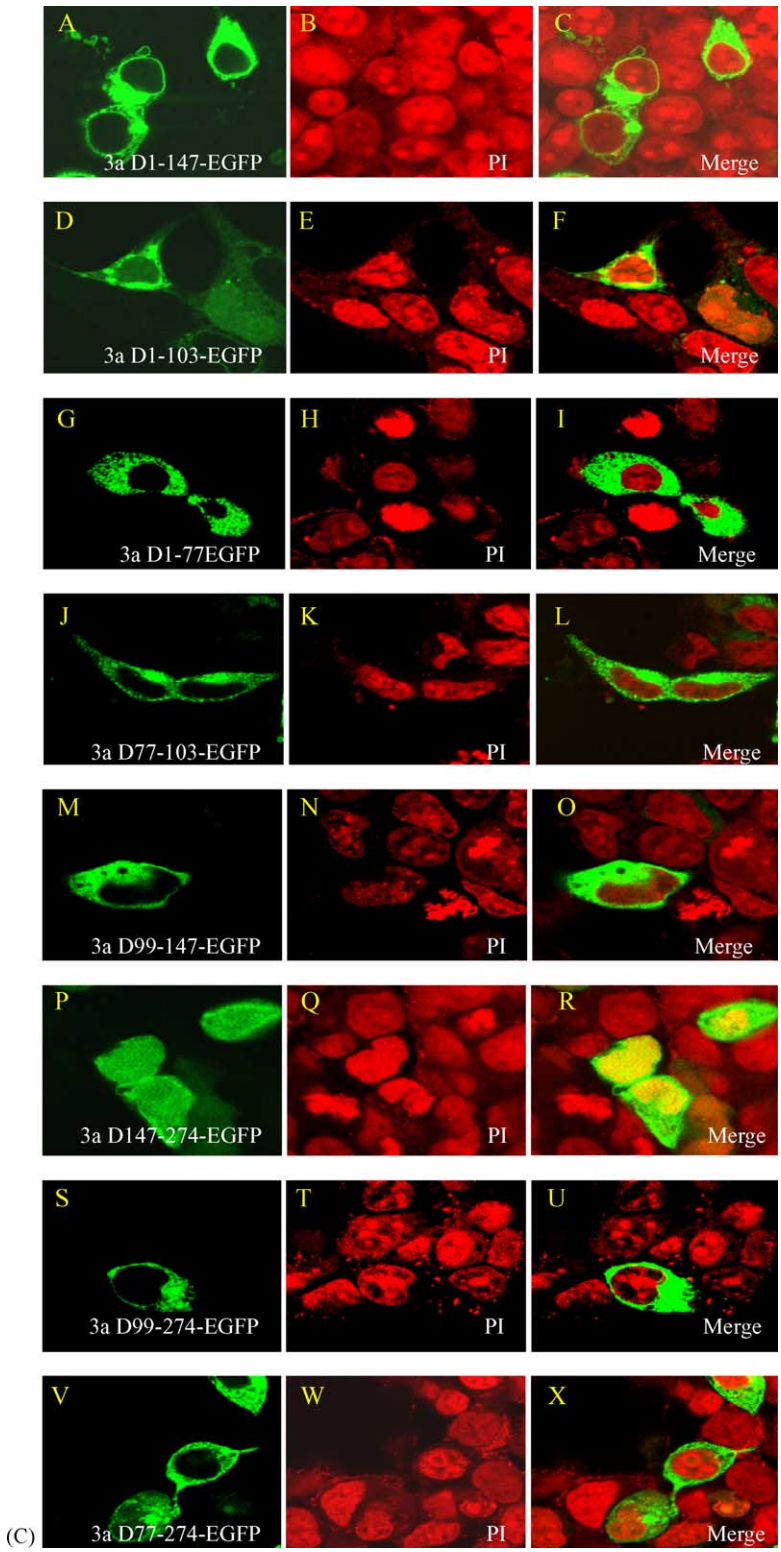


Fig. 3. Expression and cellular localization of truncated 3a protein. (A) DNA constructs of deletant 3a protein. The truncated SARS-CoV 3a proteins were cloned into pEGFP-N1 vector as C-terminal fusion to EGFP. The different mutants were constructed based on bioinformative studies of SARS-CoV 3a. Kozak consensus translation initiation site was indicated. The animo acid positions for 3a were given at the low (a, b and c represented the three transmembrane regions). C or N from localization represented the cytoplasm or nuclear localization. (B) Western blotting analysis. 293 cells were transiently transfected with described plasmids separately. Cell lysates were prepared at 48 h post-transfection and separated by SDS-PAGE. Proteins transferred to PVDF membrane was probed with anti-GFP antibody. Sizes (kDa) of molecular mass markers were indicated on the left. (C) Cellular localization of truncated 3a protein. At 24 h post-transfection with indicated mutants, 293 cells were analyzed by confocal fluorescence microscopy. Left row (green) showed fusion EGFP fluorescence; middle row (red) showed PI stained cell nuclei; right row (yellow) represented overlapping green and red fluorescence. The fluorescent pattern of 3a D1–147, D1–103 and D77–274 (A–F, V–X), like wild-type 3a, was distributed in the cytoplasm and somewhere condensed to spots fluorescence. And mutant 3a D1–77, D77–103, D99–147 and D99–274 were localized in the cytoplasm (G–O, S–U). However, construct 3a D147–274 gave rise to preservation of EGFP in the cytoplasm and nuclear (P–R). (D) Subcellular localization of deletant 3a protein with Golgi-DsRed protein. 293 cells co-transfected with the deletant 3a and Golgi/pDsRed-N1 plasmids. At 24 h after transfection, cells were fixed, and nuclear was stained with Hoechest. Deletant 3a-EGFP protein (green, 1, 5 and 9), Golgi-DsRed marker (red, 2, 6 and 10) and merge image (3, 7 and 11) were indicated.

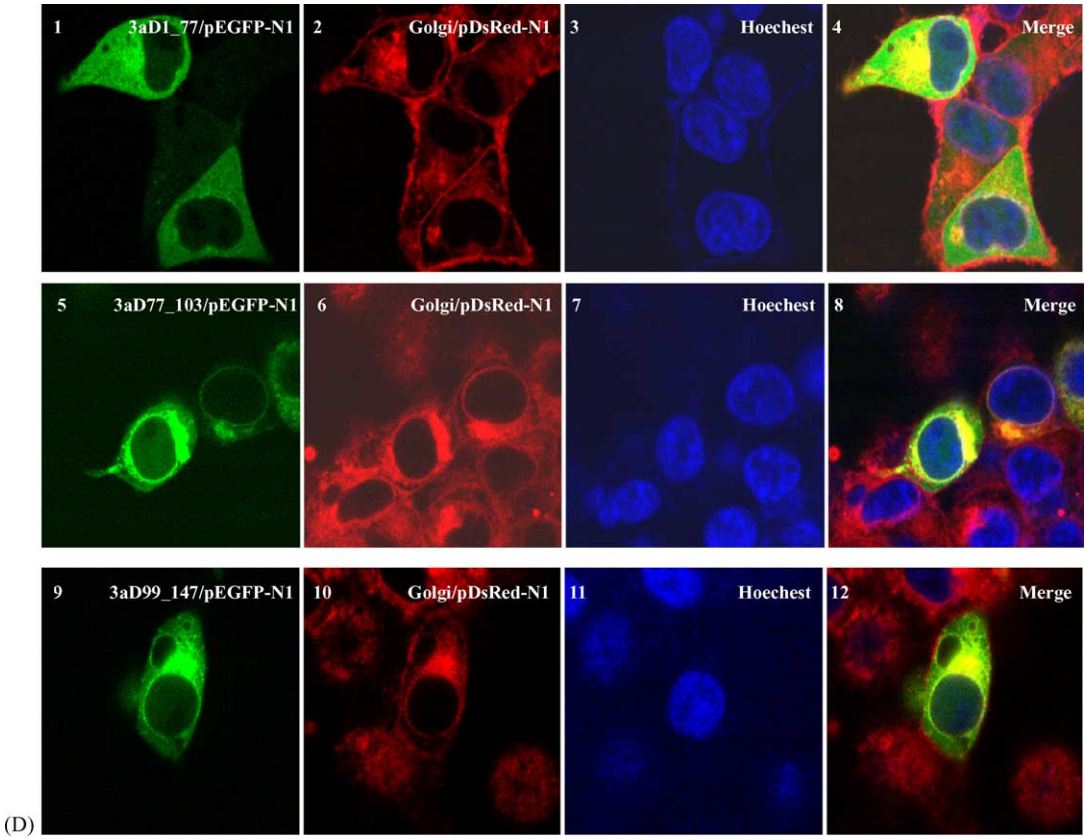


(C)

Fig. 3. (*Continued.*)

4. Discussion

Laboratory studies of SARS patients and experimental infections of macaques proved that SARS-CoV was the primary cause of SARS. SARS-CoV is a new member of coronavirus family. As shown from previously known coronaviruses, coronaviruses are enveloped positive-strand RNA viruses, which acquired the membrane envelope by budding



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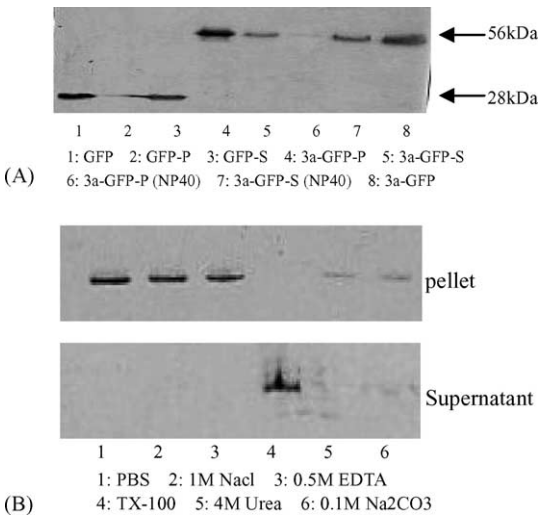

Fig. 4. Membrane association of 3a protein. (A) Analysis of cytoplasmic and membrane fractions. Cells transfected with the pEGFP-N1 or 3a/pEGFP-N1 were harvested at 48 h post-transfection, and lyzed in Dounce homogenizer. Cytoplasmic (supernatant, C) and membrane (pellet, P) were prepared and analyzed by Western blotting with anti-GFP antibody. Sizes (kDa) of molecular mass markers were indicated on the right. (B) Mode of membrane association. At 48 h after transfection with 3a/pEGFP-N1, cells membrane fractions were separately extracted with the indicated buffer and centrifuged to obtain the pellet (in the top) and supernatant fractions (in the low), and then analyzed by Western blotting with anti-GFP antibody.

into the lumen of Golgi complex (de Haan et al., 1999). Lateral interactions between the coronavirus membrane proteins are thought to mediate the formation of virion envelope. The coronavirus envelope contains three main structural proteins M, S, and E. The S protein is a type I glycoprotein, which is transported to the plasma membrane when expressed on its own, and co-accumulates with the M protein on Golgi complex in coronavirus infected cells (Corse and Machamer, 2000). The M protein is the most abundant envelope protein of coronaviruses, and the physical nature of coronavirus envelope is largely determined by the characteristics of M protein. M protein accumulates with E and M proteins in the Golgi complex, where they assemble into virus particles in coronavirus infected cells or co-transfected cells. Indirect immunofluorescence and Western blotting assays confirmed the interactions of M protein with E and S proteins. The E protein is a minor but essential viral component. Many studies confirm that coronavirus M protein, interacting with E protein and nucleocapside, plays an important role in viral budding through the membrane (de Haan et al., 1998, 1999, 2000; Lim and Liu, 2001; Narayanan et al., 2000).

SARS-CoV 3a protein was identified in SARS-CoV infected cells and SARS patients' specimen (Tan et al., 2004; Yu et al., 2004). Antibody raised against recombinant 3a protein and the sera from SARS patients could specifically detect the recombinant SARS 3a protein expressed in *E. coli*

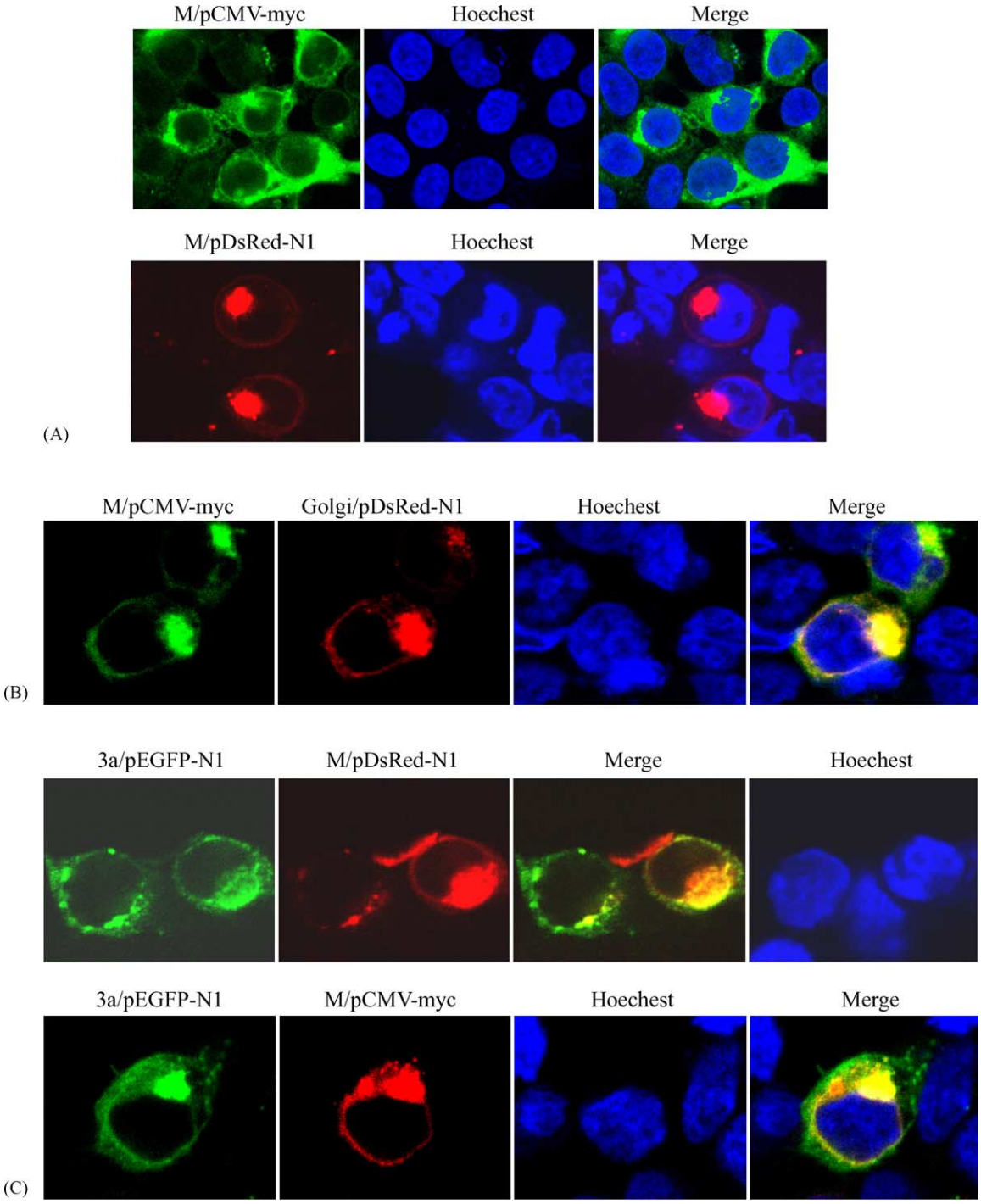

Fig. 5. Co-localization of 3a and M proteins. (A) Cellular localization of M protein. 293 cells were transiently transfected with M/pDsRed-N1 and M/pCMV-myc separately. Localization of the protein was observed by fluorescence confocal microscopy. Red fluorescence came from expressed M-DsRed protein, and green fluorescence was labeled by FITC conjugated goat anti-mouse antibody to show the expression of myc-M. The nuclear DNA was stained with Hoechest (blue). All three panels of a row had the same field of view. (B) Subcellular localization of M protein. The cells transfected with M/pCMV-myc and Golgi/pDsRed-N1 were labeled with FITC conjugated antibody to show the expression of myc-M, and nuclear DNA was stained with Hoechest. The merged image was represented region of overlap between the myc antibody (green-) and Golgi-DsRed (red-) image. All four panels of a row had the same field of view. (C) Co-localization analysis of 3a and M proteins. The cells transfected with 3a/pEGFP-N1 and M/pDsRed-N1 or 3a/pEGFP-N1 and M/pCMV-myc were analyzed by confocal fluorescence microscopy. The merged image was represented region of overlap between the 3a-EGFP (green-) and M-DsRed (red-) or 3a-EGFP (green-) and myc-M (red-) image. All four panels of a row had the same field of view.

and in Vero E6 cells. Previous research was shown that 3a could specifically interact with many SARS-CoV structural proteins such as M, S and E proteins, and was distributed mainly in Golgi complex in infected cells (Tan et al., 2004; Yu et al., 2004; Zeng et al., 2004). To further characterize the subcellular localization and reveal possible functions of 3a protein, 3a was fused with different tag at N- or C-terminus and transfected into different cells. The results showed that the localization of 3a with myc or EGFP tag was similar and mainly distributed in Golgi complex (Figs. 1 and 2). Golgi complex and rough endoplasmic reticulum (RER) were the sites of coronavirus assembling and budding. Many coronavirus structural proteins, such as M and E proteins, were targeted to the Golgi complex when expressed alone or in coronavirus infected cells (Corse and Machamer, 2003; de Haan et al., 2000; Locker et al., 1992). These proteins are demonstrated to membrane-associate proteins, having one or more transmembrane regions. Ultra-centrifugation assay was a common method to analyze the interaction of membrane proteins. With this approach, 3a was revealed to be a membrane associated protein, which was resistant to 0.1 M Na_2CO_3 (pH 11.5) and 4 M urea buffer, but resolved in Triton X-100 buffer. This suggested that 3a was an integral membrane protein, which embedded in the phospholipid bilayer. Bio-information assay predicted that 3a protein contained three transmembrane regions, which spanned from 34 to 56, 77 to 99 and 103 to 125 amino acid. According to the bioinformative results, a series of truncated 3a were constructed. Through analyzing the cellular or subcellular localization patterns of the constructs of 3a protein, 3a was confirmed to be a triple-spanning protein, and the second or third region was reliable for Golgi complex localization (Fig. 3). Topology analysis showed that 3a, like coronavirus M proteins, also had its NH2 terminus translocated to the lumen of intracellular membrane and its COOH terminus exposed in the Golgi complex (not published data). M protein is type III glycoprotein, which forms homogeneous or heterogeneous complex (with M, S, N and E proteins) in the cytoplasm and functions during host cell entry and virion morphogenesis and release, facilitating the assembly of the virion particles. As provided in this article, co-localization of 3a and M proteins mainly in Golgi complex further confirmed the spatial interaction of 3a and M proteins. These results suggested that 3a protein might take part in processes of SARA-CoV assembling or budding. In addition, Zeng et al. (2004) detected 3a protein in the crude virions with anti-3a antibody. Therefore, 3a protein might be a structural protein, which distributed in Golgi complex membrane or virion surface.

In conclusion, it was confirmed that 3a protein of SARS-CoV was localized mainly in Golgi complex, contained three transmembrane regions, and found the second and third regions were responsible for Golgi localization. Moreover, 3a protein was demonstrated to be an integral membrane protein, which embedded in the phospholipid bilayer. In additional, it was shown that 3a was co-localized with M protein along the Golgi complex. The results of 3a protein presented above provided a new insight for further studies on the potential role of 3a protein in viral budding and cell injury.

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