



Short communication

## The SARS Coronavirus 3a protein binds calcium in its cytoplasmic domain



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### ABSTRACT

The Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) is a positive stranded RNA virus with ~30 kb genome. Among all open reading frames (orfs) of this virus, the orf3a is the largest, and encodes a protein of 274 amino acids, named as 3a protein. Sequence analysis suggests that the orf3a aligned to one calcium pump present in *Plasmodium falciparum* and the enzyme glutamine synthetase found in *Leptospira interrogans*. This sequence similarity was found to be limited only to amino acid residues 209–264 which form the cytoplasmic domain of the orf3a. Furthermore, this region was predicted to be involved in the calcium binding. Owing to this hypothesis, we were driven to establish its calcium binding property *in vitro*. Here, we expressed and purified the cytoplasmic domain of the 3a protein, called Cyto3a, as a recombinant His-tagged protein in the *E. coli*. The calcium binding nature was established by performing various staining methods such as ruthenium red and stains-all. <sup>45</sup>Ca overlay method was also done to further support the data. Since the 3a protein forms ion channels, we were interested to see any conformational changes occurring in the Cyot3a upon calcium binding, using fluorescence spectroscopy and circular dichroism. These studies clearly indicate a significant change in the conformation of the Cyto3a protein after binding with calcium. Our results strongly suggest that the cytoplasmic domain of the 3a protein of SARS-CoV binds calcium *in vitro*, causing a change in protein conformation.

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With the news pouring in about advances in infection by influenza viruses, the name of a closely related candidate called the coronavirus cannot be overlooked. Severe Acute Respiratory Syndrome (SARS) virus is one such member of the coronavirus family that spread viral respiratory illness in 2003 which attracted attention globally. After April 2004, no new cases of the SARS virus infection have been reported until September 2012, when a fresh case of viral respiratory illness got noticed with the causative agent belonging to the coronavirus family (Martina et al., 2003). SARS-CoV is a newly identified coronavirus in humans that leads to a dangerous acute inflammation and is more lethal than other human coronaviruses (Drosten et al., 2003). Among 14 orfs of this virus, the largest unique orf is the orf 3a which codes for 3a protein. This

protein has been proposed to contribute to increased virulence of the virus in humans (Pacheco et al., 2003). It is an important protein of this virus that needs to be studied extensively owing to its unique existence and multi-tasking behavior.

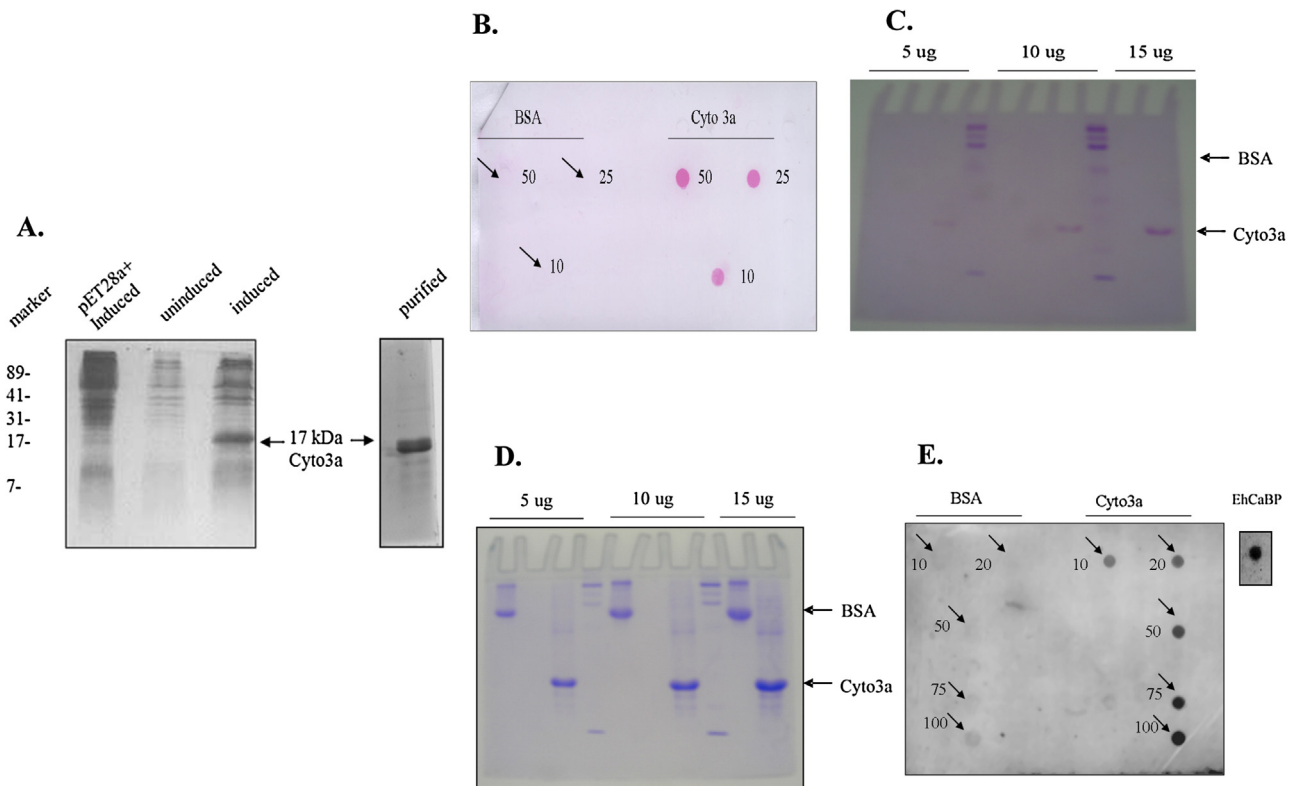
The 3a protein of SARS-CoV forms homo- as well as hetero-tetramers in transfected and possibly in infected cells (Yuan et al., 2005, 2007). This multi-meric pattern is commonly observed in most of the viral proteins that show ion channel activity commonly known as viroporins (Lu et al., 2006). Indeed, 3a protein was shown to form ion channels which are selective to potassium ions (Lu et al., 2006). However, till date, only a few ion channel proteins for viruses have been identified, and some of the important ones include Kcv protein of *Paramecium bursaria* chlorella virus (Plugge et al., 2000), M2 protein of influenza virus (Pinto et al., 1992) and Vpu and Vpr of HIV (Ewart et al., 1996; Piller et al., 1996). The functions of these ion channels vary. The Kcv is associated with virus replication (Plugge et al., 2000), and M2 protein is responsible for influenza A virus uncoating, which is a critical step in viral infection (Ciampor et al., 1995). The 3a protein shows similar function to those of Vpuprotein in HIV-1 (Lu et al., 2006). The formation of a pore structure in the virus-infected cell membrane makes the cell

**Abbreviations:** SARS-CoV, Severe Acute Respiratory Syndrome Coronavirus; orf, open reading frame.

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**Fig. 1.** (A) SDS-PAGE of un-induced and induced sample of *E. coli* BL21 DE3 cells expressing pET28a<sup>+</sup>-Cyto3a. The cytoplasmic domain of 3a was expressed as a protein of approximately 17 kDa. (B) Ruthenium red staining showing red stained blots of Cyto3a while no color with BSA. (C) Stains all staining shows purple band of Cyto3a protein. (D) Coomassie Brilliant blue stains both BSA and Cyto3a. (E) <sup>45</sup>Ca Overlay method shows signals for Cyto3a and a positive control EhCaBP3 whereas BSA does not give any signal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

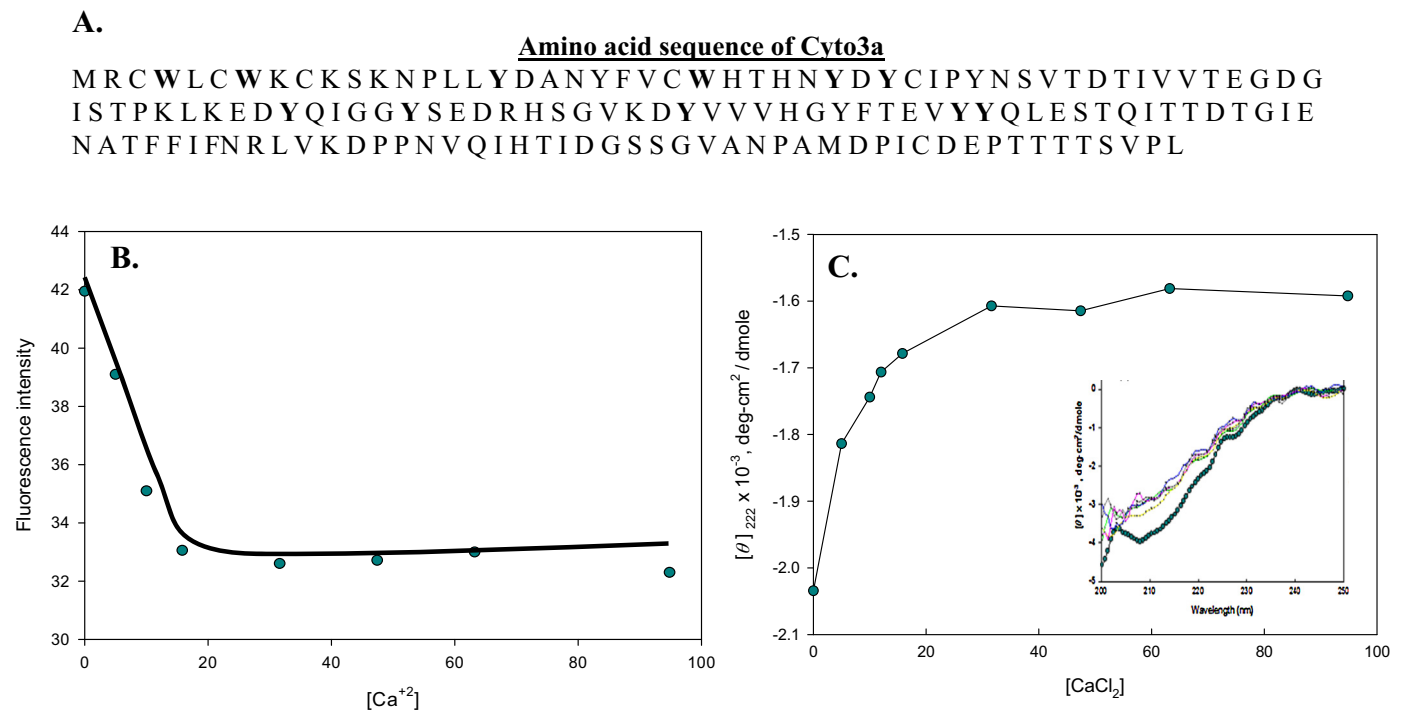
more permeable which could be an important factor in the SARS-CoV life cycle. The ion channel activity of the 3a protein is also linked to its pro-apoptotic function (Chan et al., 2009).

The 3a protein is predicted to contain three transmembrane regions (34–56, 77–99 and 103–125), and a C-terminal cytoplasmic domain of 149 amino acid residues (Rota et al., 2003). The 3a protein has been studied to form ion channel sensitive to potassium ions (Lu et al., 2006). Severe acute respiratory syndrome-associated coronavirus 3a protein forms an ion channel and modulates virus release. Moreover, in case of bacterial potassium channels, a portion of the protein functions as gate to the channel (Doyle et al., 1998; Jiang et al., 2002a,b). The binding of calcium to the cytoplasmic domain of these channels lead to conformational change that is shown to effect the pore lining (Schumacher and Adelman, 2002). Based on these evidences we speculated that calcium might be playing same kind of role as a ligand to the gated channel. It has been reported on the basis of bioinformatics analysis that the cytoplasmic domain of SARS 3a protein is a calcium-binding protein (Singh et al., 2004). Calcium binding motifs present in viral proteins are known to play an important role in virion assembly and stability (Gorbalenya et al., 1991; Garbutt et al., 1999; Ruiz et al., 2000). The calcium-binding pockets in 3a cytoplasmic domain shows a putative 'EF hand' motif commonly found in many calcium-binding proteins. We focused our study on the cytoplasmic domain, the Cyto3a. We expressed and purified the recombinant Cyto3a protein with His-tag and subjected it to various staining methods that specifically stain calcium binding proteins to prove its calcium binding nature. Fluorescence emission helps in studying the interaction of a protein with various ligands and presents adequate information about the magnitude and the microenvironment of the protein residues (Hirshfield et al., 1996; Deepa and Mishra, 2005; Zhao et al., 2006; Zhang et al., 2008). Hence, fluorescence spectroscopic study was done to monitor the

changes in the intrinsic fluorescence of the Cyto3a protein upon its interaction with calcium followed by Circular dichroism (CD) study.

The orf3a gene (nucleotides 25,268–26,092) of the SARS-CoV genome (GenBank NC\_004718) was provided by Dr. Vincent Chow (National University of Singapore). From this clone, the Cyto3a was PCR amplified using primers, X1C1, GAATTCATGAGATGTTG-GCTTTGTTGG and X1R1, GTCGACTTACAAAGGCACGCTAGTAGT and cloned into pGEMT-Easy, sequence verified, and an *EcoRI-Sall* fragment was sub-cloned into the *EcoRI-Sall* digested vector pET28a<sup>+</sup> in frame with the hexa-histidine tag. The Cyto3a was expressed in *E. coli* BL21 DE3 strain containing pET28 a<sup>+</sup>-Cyto3a by induction with IPTG; as ~17 kDa protein with His tag (Fig. 1A). We did not remove the His tag because it is not known to bind calcium (Ishitani et al., 2000). The purification was done using Ni-NTA affinity chromatography (Qiagen). All the working buffers like Tris-Cl buffer (10 mM Tris-Cl, pH 8.0) and purified Cyto3a were passed through Chelex 100 resin to remove calcium. The protein samples after passing through Chelex 100 gave calcium free Cyto3a (called here apoCyto3a).

The first hypothesis that the Cyto3a binds calcium was proven by the various staining methods. The purified protein was subjected to stains specific for calcium binding proteins. At first ruthenium red staining was performed in which different concentrations (10, 25 and 50 µg) of the purified recombinant Cyto3a protein were spotted onto the nitrocellulose membrane. The membrane was washed with the Chelex-100 (Sigma-Aldrich) treated water to remove any residual calcium and then subjected to ruthenium red (Sigma-Aldrich) staining (25 µg ml<sup>-1</sup>) for 10 min at room temperature (Charuk et al., 1990). The negative control used here was BSA which is a non-calcium binding protein. Result showed that Cyto3a did stain red whereas BSA did not show any staining (Fig. 1B). However, ruthenium red does not bind exclusively to



**Fig. 2.** (A) The amino acid sequence of Cyto3a. (B) The intrinsic fluorescence intensity of Cyto3a at 350 nm as a function of increasing calcium concentration ( $\mu\text{M}$ ). (C) Plot of the far-UV CD of Cyto3a at 222 nm against increasing calcium concentration ( $\mu\text{M}$ ). The inset shows the full CD spectra at different  $[\text{Ca}^{2+}]$ .

calcium-binding proteins; it can also stain polyanionic substrates (Charuk et al., 1990). So cationic carbocyanine dye “Stains-all” was used which preferentially stains calcium-binding proteins as dark blue or purple (Campbell et al., 1983). Different concentrations of BSA and purified recombinant Cyto3a protein (5, 10 and 15  $\mu\text{g}$ ) were separated by 12% SDS-PAGE. After separation, the gel was fixed and stained with “Stains-all” (Sigma–Aldrich) for 48 h. in the dark. BSA did not show purple staining whereas Cyto3a did (Fig. 1C). After this the same gel was stained with coomassie brilliant blue (Campbell et al., 1983) and both proteins showed blue bands (Fig. 1D). We clearly observed that stains-all stained only Cyto3a while there was no staining of BSA. We finally performed <sup>45</sup>Ca overlay method in which different concentrations (10, 25 and 50  $\mu\text{g}$ ) of the purified protein were dot blotted onto the nitrocellulose membrane. Then the membrane was subjected to <sup>45</sup>CaCl<sub>2</sub> binding by overlay method as described by Maruyama et al. (1984) and Rajini et al. (2001). BSA was the negative control whereas CaBP3 of *E. histolytica*, EhCaBP3 was the positive control (Kumar et al., 2007). The overlay gave strong signals for both Cyto3a and EhCaBP3 whereas for BSA, there was no signal. The <sup>45</sup>Ca signals were proportional to the amounts of the Cyto3a protein blotted on the membrane (Fig. 1E). These results unequivocally demonstrated the presence of calcium binding feature in the Cyto3a protein.

It is established that the intrinsic fluorescence of a protein is well dominated by the contribution of the Trp and Tyr residues (Lakowicz et al., 1983). The emission is highly sensitive to changes in environment of the chromophore, such as binding to a ligand or metal ion, solvent and pH change, etc. The intrinsic fluorescence measurement is more sensitive and features much larger spectral changes during conformational transitions. The interaction of ligand to a protein causes an alteration in the secondary and tertiary structures due to the conformational change in the protein (Khan et al., 2002). The primary protein sequence of Cyto3a shows aromatic amino acids (Fig. 2A) and the fluorescence spectrum of Cyto3a is due to the presence of aromatic residues. So to see if interaction of calcium would confer structural changes in the Cyto3a protein we

monitored changes in fluorescence intensity at 350 nm on the successive addition of different concentrations of CaCl<sub>2</sub> to the purified Cyto3a. The titration of apo Cyto3a protein against increasing calcium concentrations showed sharp inflection in the curve (Fig. 2B). This suggested that the intrinsic fluorescence of Cyto3a protein was quenched by calcium binding indicating a perturbation in the microenvironment around the tryptophan residues in the protein sequence. When a tryptophan residue is buried inside the protein, quenching is not expected to occur, but if the residue is on the protein surface quenching is observed. This leads to exposure of hydrophobic regions of the protein as is reported in many Ca<sup>2+</sup> sensor proteins (Valcarce et al., 1993). The observed result suggests that the interaction of Cyto3a with calcium indeed changes the microenvironment of Trp and Tyr residues in the protein.

Furthermore, the far-UV CD data can also detect large changes in the secondary structure of a protein upon its interaction with the ligand. Here we employed it as a complementary tool to understand the structural changes in the Cyto3a protein upon calcium binding. The binding of Cyto3a to Ca<sup>2+</sup> was associated with very small change in secondary structure of Cyto3a (Fig. 2C). In the cases of calmodulin and troponin C, it has been shown that the conformational change in response to Ca<sup>2+</sup> binding may involve reorientation of pre-existing helices with no changes in the overall content or distribution of regular secondary structures (Nelson and Chazin, 1998). The phenomenon may be caused by rearrangements in the tertiary structure (Nelson and Chazin, 1998). Results herein reported also indicate that the cytoplasmic domain of 3a protein displays some properties that are common in typical Ca<sup>2+</sup> sensor proteins (Nelson and Chazin, 1998). The fluorescence data showed that binding of Ca<sup>2+</sup> would promote the exposure of a hydrophobic cleft due to a conformational change, as observed in regulatory proteins triggered by calcium (James et al., 1995).

The SARS-CoV 3a protein is unique to the virus and till now, there are very few reports for potential homologues in other coronaviruses, which may explain the unexpectedly high virulence of the virus (Law et al., 2005; Wang et al., 2011). The 3a protein has

multitasking behavior. So we emphasize the need of further studies on the calcium binding nature of full length 3a protein which would help in a deeper understanding of the viral pathogenesis.

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