



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Short communication

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



Rinki Minakshi^{a,b,*}, Kartika Padhan^{a,1}, Safikur Rehman^b, Md. Imtaiyaz Hassan^b,
Faizan Ahmad^b

^a International Centre for Genetic Engineering and Biotechnology, New Delhi, India

^b Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India

ARTICLE INFO

Article history:

Received 28 May 2014

Received in revised form 1 August 2014

Accepted 1 August 2014

Available online 10 August 2014

Keywords:

SARS-CoV

Cyto3a protein

Calcium

Ion channel

Protein conformation

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. ⁴⁵Ca 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.

© 2014 Elsevier B.V. All rights reserved.

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.

* Corresponding author at: Institute of Home Economics, Delhi University, F4, Hauz Khas, New Delhi 110016, India. Tel.: +91 9899785248.

E-mail address: rinki.minakshi@hotmail.com (R. Minakshi).

¹ These authors contributed equally to this work.

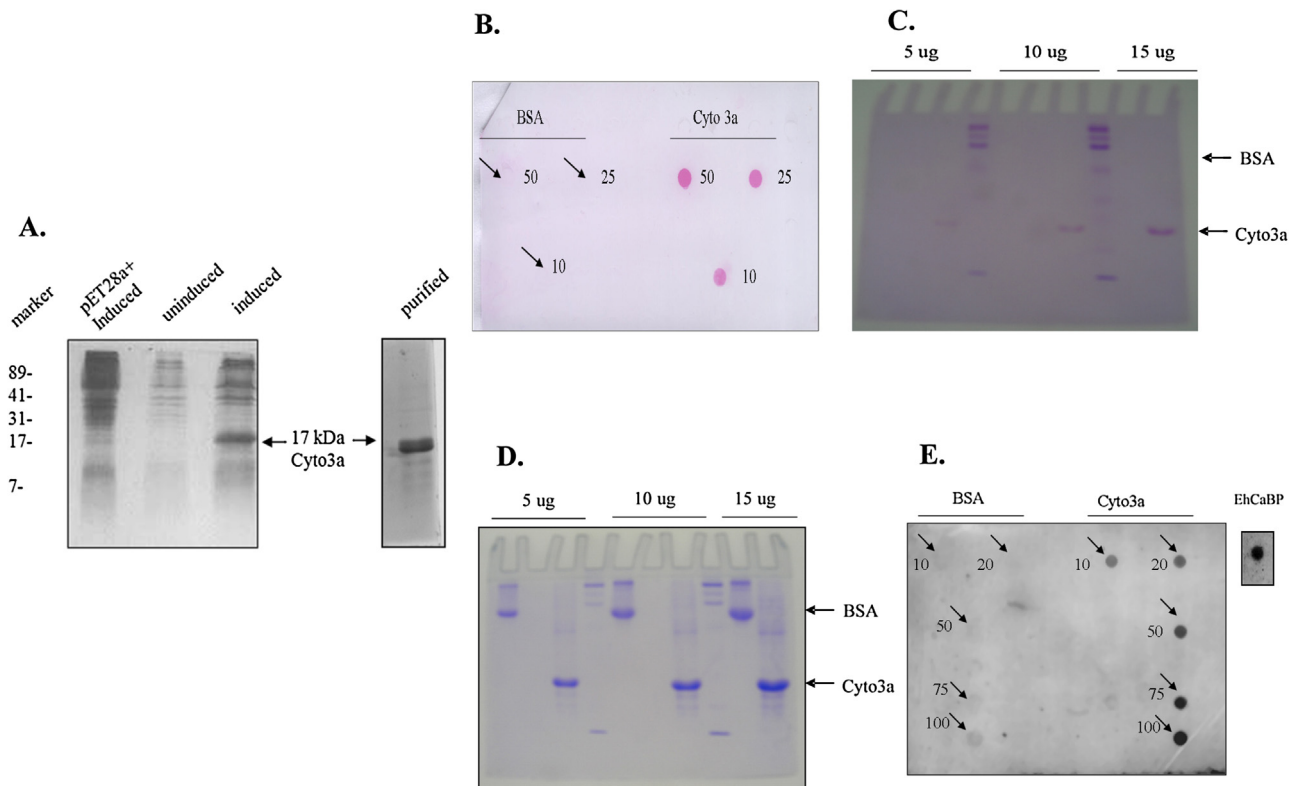


Fig. 1. (A) SDS-PAGE of un-induced and induced sample of *E. coli* BL21 DE3 cells expressing pET28a⁺-Cyto3a. The cytoplasmic domain of 3a was expressed as a protein of approximately 17 kDa. (B) Ruthenium red staining showing red stained blots of Cyto3a while no color with BSA. (C) Stains all staining shows purple band of Cyto3a protein. (D) Coomassie Brilliant blue stains both BSA and Cyto3a. (E) ⁴⁵Ca 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-GCTTTGTGG and X1R1, GTCGACTTACAAGGCACGCTAGTAGT and cloned into pGEMT-Easy, sequence verified, and an *EcoRI-Sall* fragment was sub-cloned into the *EcoRI-Sall* digested vector pET28a⁺ in frame with the hexa-histidine tag. The Cyto3a was expressed in *E. coli* BL21 DE3 strain containing pET28 a⁺-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⁻¹) 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

A.

Amino acid sequence of Cyto3a

MRCWLCWKCKSKNPLLYDANYFVCWHTHNYDYCIPYNSVTDITIVVTEGDG
 ISTPKLKEDYQIGGYSEDRHSGVKDYVVVHGYFTEVYYQLESTQITTDGTGIE
 NATFFIFNRLVKDPPNVQIHTIDGSSGVANPAMPDIPICDEPTTTTSVPL

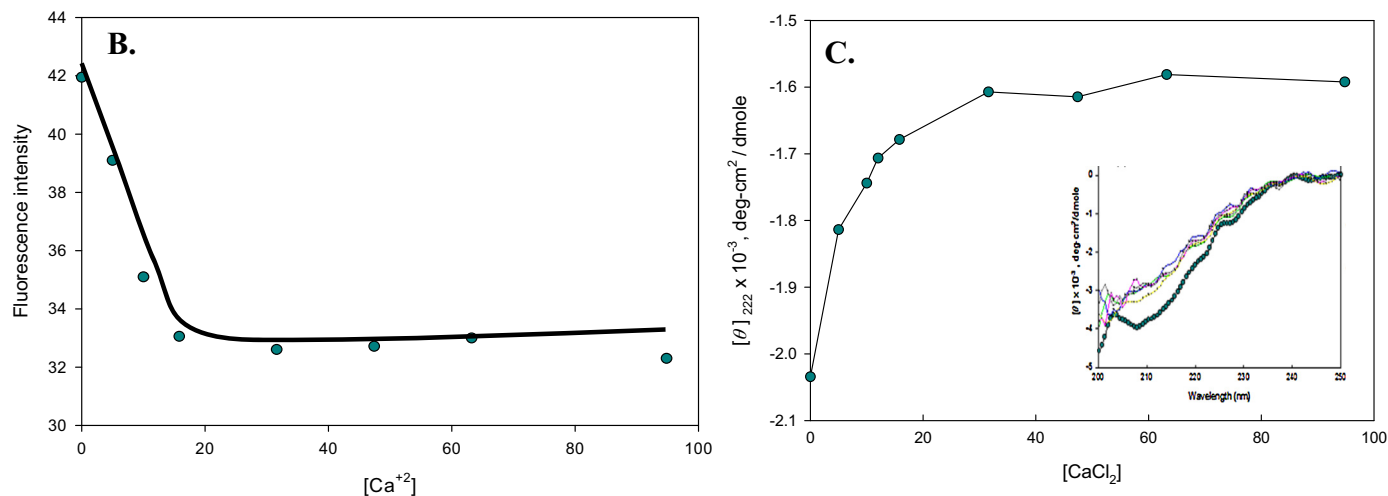


Fig. 2. (A) The amino acid sequence of Cyto3a. (B) The intrinsic fluorescence intensity of Cyto3a at 350 nm as a function of increasing calcium concentration (μM). (C) Plot of the far-UV CD of Cyto3a at 222 nm against increasing calcium concentration (μM). The inset shows the full CD spectra at different [Ca²⁺].

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 μg) 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 ⁴⁵Ca overlay method in which different concentrations (10, 25 and 50 μg) of the purified protein were dot blotted onto the nitrocellulose membrane. Then the membrane was subjected to ⁴⁵CaCl₂ 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 ⁴⁵Ca 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 CaCl₂ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ sensor proteins (Nelson and Chazin, 1998). The fluorescence data showed that binding of Ca²⁺ 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.

Funding

This work was supported by a grant from the Department of Biotechnology (DBT), Government of India. The funding agency had no role in designing and execution of the experiments, data analysis and preparation of the manuscript.

Acknowledgements

We thank Dr. Shahid Jameel for generously providing us the lab space and reagents for carrying out some experiments. We thank Dr. Narendra Padhan for providing us the purified EhCBP3.

References

- Campbell, K.P., MacLennan, D.H., Jorgensen, A.O., 1983. Staining of the Ca^{2+} -binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye "Stains-all". *J. Biol. Chem.* 258, 11267–11273.
- Chan, C.M., Tsoi, H., Chan, W.M., Zhai, S., Wong, C.O., Yao, X., Chan, W.Y., Tsui, S.K., Chan, H.Y., 2009. The ion channel activity of the SARS-coronavirus 3a protein is linked to its pro-apoptotic function. *Int. J. Biochem. Cell Biol.* 41, 2232–2239.
- Charuk, J.H., Pirraglia, C.A., Reithmeier, R.A., 1990. Interaction of ruthenium red with Ca^{2+} -binding proteins. *Anal. Biochem.* 188, 123–131.
- Ciampor, F., Cmarko, D., Cmarkova, J., Zavodska, E., 1995. Influenza virus M2 protein and haemagglutinin conformation changes during intracellular transport. *Acta Virol.* 39, 171–181.
- Deepa, S., Mishra, A.K., 2005. Fluorescence spectroscopic study of serum albumin–bromadiolone interaction: fluorimetric determination of bromadiolone. *J. Pharm. Biomed. Anal.* 38, 556–563.
- Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., MacKinnon, R., 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 280, 69–77.
- Drosten, C., Preiser, W., Gunther, S., Schmitz, H., Doerr, H.W., 2003. Severe acute respiratory syndrome: identification of the etiological agent. *Trends Mol. Med.* 9, 325–327.
- Ewart, G.D., Sutherland, T., Gage, P.W., Cox, G.B., 1996. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. *J. Virol.* 70, 7108–7115.
- Garbutt, M., Law, L.M., Chan, H., Hobman, T.C., 1999. Role of rubella virus glycoprotein domains in assembly of virus-like particles. *J. Virol.* 73, 3524–3533.
- Gorbalenya, A.E., Koonin, E.V., Lai, M.M., 1991. Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. *FEBS Lett.* 288, 201–205.
- Hirshfeld, K.M., Toptygin, D., Grandhige, G., Kim, H., Packard, B.Z., Brand, L., 1996. Steady-state and time-resolved fluorescence measurements for studying molecular interactions: interaction of a calcium-binding probe with proteins. *Biophys. Chem.* 62, 25–38.
- Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Shi, W., Zhu, J.K., 2000. SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. *Plant Cell* 12, 1667–1678.
- James, P., Vorherr, T., Carafoli, E., 1995. Calmodulin-binding domains: just two faced or multi-faceted? *Trends Biochem. Sci.* 20, 38–42.
- Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., MacKinnon, R.A., 2002a. Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 417, 515–522.
- Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B.T., MacKinnon, R.B., 2002b. The open pore conformation of potassium channels. *Nature* 417, 523–526.
- Khan, M.A., Muzammil, S., Musarrat, J., 2002. Differential binding of tetracyclines with serum albumin and induced structural alterations in drug-bound protein. *Int. J. Biol. Macromol.* 30, 243–249.
- Kumar, S., Padhan, N., Alam, N., Gourinath, S., 2007. Crystal structure of calcium binding protein-1 from *Entamoeba histolytica*: a novel arrangement of EF hand motifs. *Proteins* 68, 990–998.
- Lakowicz, J.R., Maliwal, B.P., Cherek, H., Balter, A., 1983. Rotational freedom of tryptophan residues in proteins and peptides. *Biochemistry* 22, 1741–1752.
- Law, P.T., Wong, C.H., Au, T.C., Chuck, C.P., Kong, S.K., Chan, P.K., To, K.F., Lo, A.W., Chan, J.Y., Suen, Y.K., Chan, H.Y., Fung, K.P., Waye, M.M., Sung, J.J., Lo, Y.M., Tsui, S.K., 2005. The 3a protein of severe acute respiratory syndrome-associated coronavirus induces apoptosis in Vero E6 cells. *J. Gen. Virol.* 86, 1921–1930.
- Lu, W., Zheng, B.J., Xu, K., Schwarz, W., Du, L., Wong, C.K., Chen, J., Duan, S., Deubel, V., Sun, B., 2006. Severe acute respiratory syndrome-associated coronavirus 3a protein forms an ion channel and modulates virus release. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12540–12545.
- Martina, B.E., Haagmans, B.L., Kuiken, T., Fouchier, R.A., Rimmelzwaan, G.F., Van Amerongen, G., Peiris, J.S., Lim, W., Osterhaus, A.D., 2003. *Virology: SARS virus infection of cats and ferrets.* *Nature* 425, 915.
- Maruyama, K., Mikawa, T., Ebashi, S., 1984. Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* 95, 511–519.
- Nelson, M.R., Chazin, W.J., 1998. An interaction-based analysis of calcium-induced conformational changes in Ca^{2+} sensor proteins. *Protein Sci.* 7, 270–282.
- Pacheco, J.M., Henry, T.M., O'Donnell, V.K., Gregory, J.B., Mason, P.W., 2003. Role of nonstructural proteins 3A and 3B in host range and pathogenicity of foot-and-mouth disease virus. *J. Virol.* 77, 13017–13027.
- Piller, S.C., Ewart, G.D., Premkumar, A., Cox, G.B., Gage, P.W., 1996. Vpr protein of human immunodeficiency virus type 1 forms cation-selective channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 93, 111–115.
- Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. *Cell* 69, 517–528.
- Plugge, B., Gazzarrini, S., Nelson, M., Cerana, R., Van Etten, J.L., Derst, C., DiFrancesco, D., Moroni, A., Thiel, G., 2000. A potassium channel protein encoded by *Chlorella virus PBCV-1*. *Science* 287, 1641–1644.
- Rajini, B., Shridas, P., Sundari, C.S., Muralidhar, D., Chandani, S., Thomas, F., Sharma, Y., 2001. Calcium binding properties of gamma-crystallin: calcium ion binds at the Greek key beta gamma-crystallin fold. *J. Biol. Chem.* 276, 38464–38471.
- Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M.H., Tong, S., Tamin, A., Lowe, L., Frace, M., DeRisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D., Drosten, C., Pallansch, M.A., Anderson, L.J., Bellini, W.J., 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300, 1394–1399.
- Ruiz, M.C., Cohen, J., Michelangeli, F., 2000. Role of Ca^{2+} in the replication and pathogenesis of rotavirus and other viral infections. *Cell Calcium* 28, 137–149.
- Schumacher, M., Adelman, J.P., 2002. Ion channels: an open and shut case. *Nature* 417, 501–502.
- Singh, A.N., Gupta, D., Jameel, S., 2004. Bioinformatic analysis of the SARS virus X1 protein shows it to be a calcium-binding protein. *Curr. Sci.* 86, 842–844.
- Valcarce, C., Selander-Sunnerhagen, M., Tamplitz, A.M., Drakenberg, T., Bjork, I., Stenflo, J., 1993. Calcium affinity of the NH_2 -terminal epidermal growth factor-like module of factor X. Effect of the gamma-carboxyglutamic acid-containing module. *J. Biol. Chem.* 268, 26673–26678.
- Wang, K., Xie, S., Sun, B., 2011. Viral proteins function as ion channels. *Biochim. Biophys. Acta (BBA) Biomembr.* 1808, 510–515.
- Yuan, X., Li, J., Shan, Y., Yang, Z., Zhao, Z., Chen, B., Yao, Z., Dong, B., Wang, S., Chen, J., Cong, Y., 2005. Subcellular localization and membrane association of SARS-CoV 3a protein. *Virus Res.* 109, 191–202.
- Yuan, X., Yao, Z., Wu, J., Zhou, Y., Shan, Y., Dong, B., Zhao, Z., Hua, P., Chen, J., Cong, Y., 2007. G1 phase cell cycle arrest induced by SARS-CoV 3a protein via the cyclin D3/pRb pathway. *Am. J. Respir. Cell Mol. Biol.* 37, 9–19.
- Zhang, G., Wang, A., Jiang, T., Guo, J., 2008. Interaction of the irisfloretoxin with bovine serum albumin: a fluorescence quenching study. *J. Mol. Struct.* 891, 93–97.
- Zhao, H., Su, W., Luo, Y., Ji, Y., Li, Z., Jiu, H., Liang, H., Chen, B., Zhang, Q., 2006. Rectification of excitation with bathochromic shift induced by intense absorption of organic ligands during emission measurement of Eu(III) complex. *Spectrochim. Acta A: Mol. Biomol. Spectrosc.* 65, 846–851.