Chapter 11
SARS Accessory Proteins ORF3a and 9b and Their Functional Analysis

Wei Lu, Ke Xu, and Bing Sun

Abstract The SARS coronavirus (CoV) positive-stranded RNA viral genome encodes 14 open reading frames (ORFs), eight of which encode proteins termed as "accessory proteins." These proteins help the virus infect the host and promote virulence. In this chapter we describe some of our latest investigations into the structure and function of two such accessory proteins: ORF3a and 9b. The ORF3a accessory protein is the largest accessory protein in SARS-CoV and is a unique membrane protein consisting of three transmembrane domains. It colocalizes on the cell membrane and host Golgi networks and may be involved in ion channel formation during infection. Similarly the ORF9b accessory protein is 98 amino acids, associates with the spike and nucleocapsid proteins and has unusual membrane binding properties. In this chapter we have suggested possible new roles for these two accessory proteins which may in the long run contain answers to many unanswered questions and also give us new ideas for drugs and vaccine design.

11.1 The SARS-CoV ORF3a Protein

The SARS-CoV genome contains more than 14 open reading frames (ORFs). These ORFs encode various viral proteins, which take part in different virus infection steps (Narayanan et al. 2007). These viral proteins can be classified into three groups: structural proteins, nonstructural proteins and accessory proteins. Generally, researchers take Spike protein, Membrane protein, Envelope protein, and Nucleocapsid protein as the four major structure proteins, since they are all located on the mature virus particles and construct the virus framework. Nonstructural proteins generally refer to viral enzymes, for example the virus RNA dependent

B. Sun (✉)
Laboratory of Molecular Virology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China
e-mail: bsun@sibs.ac.cn

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RNA polymerase and other viral proteinases. Besides these structural proteins and nonstructural proteins, there are many other viral proteins encoded by many other ORFs. The existence and functions of these proteins are all still debatable.

Coronavirus structural proteins and nonstructural proteins have been studied for quite a long time, and their biochemical characteristics and biological functions have been clearly demonstrated. However these studies are not enough for us to understand and conquer the coronavirus. One major problem with the SARS-CoV is that it contains a great number of accessory proteins which help the virus infect the host and promote virulence, and contain answers to many unanswered questions and ideas for drugs and vaccine design.

At the beginning of the SARS-CoV genome are two large ORFs, 1a and 1b, the major source of viral nonstructural proteins. After that, four structural proteins genes are located on the genome in the following order: spike, envelope, membrane and nucleocapsid. Interestingly, there are many small and separated ORFs randomly located among these structural protein genes (Narayanan et al. 2007); these are the SARS-CoV accessory proteins. Researchers in previous years have devoted great efforts to these proteins to understand their purpose and biological significance.

ORF3a is located between the spike and envelope genes on the SARS-CoV genome. It has also been named as SNE protein, X1 protein and U274 protein (Zeng et al. 2004; Kanzawa et al. 2006; Tan et al. 2004a). Protein sequence analysis on ORF3a suggests that it is a membrane protein which has three transmembrane domains at the N terminus. Upon analysis of the full-length sequence of ORF3a, we found that it lacks similarities to any known proteins. However, some researchers divided the protein sequence into several parts and then BLAST-searched them separately. Results clearly showed that the N-terminal domain of ORF3a had 29% identity with the putative cytochrome B-561 transmembrane protein from a bacterium (*Ralstonia solanacearum*) and 24% identity with the opsin fragments from a hawkmoth (*Manduca sexta*) and the rhodopsin from a butterfly (*Popilio glaucus*). The C terminus of ORF3a had moderate similarity to the calcium-transporting ATPase (calcium pump) from a parasite (*Plasmodium falciparum*; 41% identity) and the outer-membrane porin from a bacterium (*Shewanella oneidensis*; 27% identity) (Yu et al. 2004).

At nearly the same time, several research groups reported more new findings on ORF3a using different methods. Using confocal methods, they found ORF3a expressed in SARS-CoV-infected Vero-E6 cells, located mainly on the cell membrane and host cell Golgi networks. They also detected ORF3a in lung sections of SARS-CoV-infected patients (Yu et al. 2004; Yuan et al. 2005; Ito et al. 2005).

Zeng et al. reported that when carrying out proteomics analysis on SARS-CoV-infected Vero-E6 cells, two specific peptides were identified which corresponded to the virus ORF3a sequences. They also detected the presence of anti-ORF3a antibody in the SARS-CoV-infected patient sera by using an antigen peptide derived from the ORF3a sequence (Zeng et al. 2004). Some other groups also demonstrated the existence of ORF3a in purified SARS-CoV virus particles – so that some researchers even proposed that ORF3a may be classified as a new member of the

viral structural proteins. However, no further evidence to date supports ORF3a's function as a structural protein. A gold-labeled immuno-electron microscopy test on purified virus particles is still needed to prove the above hypothesis (Shen et al. 2005).

As ORF3a is the largest protein in the SARS-CoV accessory protein family, it was considered the most important one, and is postulated to have crucial biological roles. However ORF3a is a very unusual protein as well; compared with other coronavirus proteins, no homologs can be identified with this protein, and hence little functional hint can be obtained using bioinformatics.

11.2 Functional Analysis of ORF3a

More and more evidence has confirmed the presence of ORF3a in SARS-CoV infection. The function of ORF3a becomes the most urgent unanswered question. We know that the viruses in general lead a simple life-style. A limited number of proteins are expressed during their life-cycle and they usually do not carry abundant or unnecessary protein ORFs in their genome.

ORF3a is a membrane-associated viral protein. At first, researchers hypothesized that it must be associated with virus entry into the host cells due to the fact that immunoprecipitation tests with ORF3a showed interaction with the S protein. Thus it is postulated that ORF3a can promote S protein function, and then facilitate virus entry. But little further study has focused on this possibility. One research group proposed that the N-terminal ectodomain of ORF3a can elicit a strong humoral response, which may provide a protective humoral response in SARS patients (Akerström et al. 2006). However, another group tested the neutralization ability of the anti-ORF3a antibody (Qiu et al. 2005), and the results suggested that anti-ORF3a antibody did not show neutralization ability compared with some anti-S antibodies that were tested. We therefore cannot say definitely that ORF3a is a participant in SARS-CoV entry, and many critical experiments still need to be done to answer this question.

Another possible function of ORF3a that researchers have recently uncovered by over-expressing it in several mammalian cell lines is cell apoptosis, using DNA ladder assay and TUNEL assay (Law et al. 2005). This observation is also somewhat debatable because another group compared the apoptosis induction ability of different SARS-CoV proteins, for example N, M, E, ORF7a and ORF3a, and found that only the over-expression of viral ORF7a can induce cell apoptosis (Tan et al. 2004b). However it is noteworthy that both experimental results used two different kinds of cell lines, which may very well be the reason for the differing results.

In 2006, our group found that ORF3a can form an ion channel (Lu et al. 2006). Our previous work demonstrated the presence of ORF3a in virus infection, but we were more interested in its function in the host during SARS-CoV infection. When performing ORF3a expression in mammalian cells, we found that it could interact with itself by disulfide bonds to form homo-dimers and -tetramers. Both

immunoprecipitation and FRET tests confirmed these findings. We also screened the ORF3a sequence and identified that the sixth cystiene is in charge of this homo-polymer formation.

ORF3a is a membrane-associated protein and can form a homo-tetramer. All ion channel proteins form homo- or hetero-polymers and associate with the membrane. Hence we proposed that ORF3a may form an ion channel.

In order to check our hypothesis, we used an electrophysiological method, which combines biology, physiology and physics. There are two fundamental electrophysiological test methods: two-electrode voltage clamp (TEVC), and patch clamp. TEVC is based on the *Xenopus* oocyte cell system. Initially we obtained cDNA from selected samples, transcribed them in vitro into complementary mRNA, and then microinjected these mRNAs into oocyte cells, which are nearly one millimeter in diameter and easily used in microinjection. Several days later, using a TEVC amplifier on these injected oocytes, we analyzed their membrane potential and conductance to test their channel activity. When using ORF3a in this TEVC test, we found a dramatic membrane current through ORF3a-expressing oocytes compared with control oocytes. This result clearly indicated that ORF3a may form an ion channel. We also found this current to be partially inhibited by barium, which is also a specific potassium channel inhibitor. We also tested the channel permeability of ORF3a to potassium ions and found that potassium ions can pass through its channel pore.

What is the possible biological role of the ORF3a channel during the virus infection? To answer this question, we suppressed ORF3a expression by siRNA during SARS-CoV infection. We found that after knocking down ORF3a in the virus-infected cells, virus release into the cell culture is apparently decreased. We proposed that the SARS-CoV ORF3a channel is closely related to virus release. It is interesting to note that some other viral ion channels are also thought to be associated with virus release: for example, HIV encoded ion channel Vpu protein can promote the release of HIV virus particles specifically in human cell lines. p7 protein from HCV has also been proved to form a ion channel, and its absence will cause reduction of HCV virus assembly and release. However, in-depth studies on these viral ion channels have not been reported to date. How could SARS-CoV ORF3a regulate virus release? And is this function dependent on ORF3a ion channel activity?

11.3 Ion-Channel Activity of the ORF3a Homologue in Other Human Coronavirus

When we initiated our in-depth studies on the relationship between ORF3a channel activity and virus release, several SARS-CoV laboratory infection cases were reported, and regulatory authorities forbid the handling of SARS-CoV in research labs indefinitely. However we found a new way to conduct our research on this

subject. We found that in nearly all the human coronavirus genomes, there was an ORF3a homolog gene located between the viral spike gene and envelope gene. For convenience, we named it *SNE* gene, S-neighbor-E (Zeng et al. 2004).

We used HCoV-229E and OC43 as tools and models to analyze the SNE protein functions. Meanwhile, in order to improve the accuracy of our experiments in electrophysiology, we used another technology, patch clamp analysis, on all these SNE proteins.

Fortunately, we found that all these SNE proteins can form ion channels. Both the TEVC results and patch clamp results confirmed our findings, and we also found that all these ion channels can be permeable to both sodium ions and potassium ions, whereas calcium ions cannot pass through this channel pore. Since SNE channels are nonspecific monovalent cation channels, they result in cell membrane depolarization. Membrane depolarization is an important biological stimulus. There are many cascades downstream of the membrane depolarization, and the most important one is the activation of voltage-dependent calcium channels (VDCC). There are several types of VDCC; L-type is the most common one, found on nearly all types of cells (Lehmann-Horn and Jurkat-Rott 1999). At physiologic or resting membrane potential, L-type calcium channels are normally closed. They are activated at depolarized membrane potentials (Lehmann-Horn and Jurkat-Rott 1999; Friis et al. 2004; Lipscombe et al. 2004; Pringle 2004; Røttingen and Iversen 2000; Catterall et al. 2005).

Using a fluorescence calcium probe, we found that SNE-mediated channel activity causes a dramatic membrane depolarization, which exceeds the threshold of L-type calcium channels, and consequently leads to a calcium influx into the host cells.

Under normal conditions, the intracellular calcium concentration in the cell cytoplasm is very low. There are two pathways to increase cell intracellular calcium after specific stimulation: one is calcium release from the cell organelles' calcium store, such as ER; the other is calcium influx through cell voltage-dependent calcium channels on the cell membrane to allow the calcium ions to be transported from the outside to the inside of the cells (Pringle 2004; Røttingen and Iversen 2000). We found that the latter pathway was responsible for SNE-mediated intracellular increase. When we blocked this calcium influx through L-type calcium channels with the specific inhibitor nimodipine, we found that the virus assembly inside the virus-infected cells was arrested. Virus budding seemed to be inhibited in the absence of calcium influx, and consequently virus release was also interrupted.

It has already been reported that coronavirus assembly begins with the viral genome RNAs interacting with nucleoproteins to form ribonucleoproteins in the host-cell cytoplasm. These ribonucleoproteins move to the budding sites at the ER–Golgi intermediate compartment and the Golgi region (Garoff et al. 1998; Kuo and Masters 2002; Stertz et al. 2007). From the Golgi compartments, infected cells synthesize many vesicles, called multivesicular bodies (MVB). Virus core particles can bud into these vesicles and cause the phospholipid bilayer to form mature virions. Vesicles containing a large amount of mature viruses will then be transported to the cell membrane, where viruses are released after fusion of vesicles with

the cell membrane (Weiss and Navas-Martin 2005a; Qinfen et al. 2004). One or more steps of coronavirus assembly may be influenced by calcium influx through L-type calcium channels. We thus propose that one possibility is that the formation of MVBs is calcium-dependent during coronavirus infection. Calcium influx can lead to an increase in the number or size of MVBs, and subsequently promote efficient virus assembly and release. Viral SNE proteins simply trigger this calcium requirement via activation of L-type calcium channels.

Coronavirus envelope proteins have recently also been shown to have monovalent cation channel activity (Wilson et al. 2004, 2006). It is well documented that coronavirus envelope protein can modulate virus assembly (Ye and Hogue 2007). Expression of coronavirus membrane protein and envelope protein together can result in abundant virus-like particle formation (Vennema et al. 1996). Since envelope protein can form ion channels, perhaps it also employs some pathways to increase intracellular calcium and then influence virus assembly and release.

In conclusion, our findings on ORF3a illustrate the function of human coronavirus SNE proteins and show that they are related to host-cell L-type calcium channels. We also propose that both the viral ion channel and the L-type calcium channel may be appropriate drug targets in therapeutic approaches against coronavirus infection.

11.4 The SARS-CoV ORF9b Protein

There are nine subgenomic mRNAs in SARS-CoV, four of which (mRNA3, 7, 8, 9) are bicistronic producing two ORFs starting at the first or additional downstream start codon separately (Thiel et al. 2003; Weiss and Navas-Martin 2005b). On bicistronic mRNA9, a 98 amino acid viral accessory protein 9b was encoded from a complete internal ORF within the *N* gene.

We expressed ORF9b protein in *E. coli*, and raised specific antibodies against it. In order to test the presence of ORF9b, we produced SARS-CoV infected cell supernatant and by sucrose density gradient centrifugation, the released viral particles in this supernatant were collected. These viral particles were then Western blot analyzed, using anti-ORF9b antibodies. A specific band was detected, which clearly proved the existence of PRF9b in the mature virus particles. We also found that ORF9b was present in the virus-like-particles (VLP), using the same density gradient centrifugation technique.

ORF9b was shown to be associated with viral S and N proteins. S protein is known to be located on the membrane of the virion envelope, and N protein is located inside the particles. The interactions among S, N and ORF9b may thus help to localize ORF9b inside the particles but closer to the envelope. We also investigated the intracellular localization of ORF9b in both transfected and infected cells. Cytoplasmic ORF9b was diffused in the cytoplasm, but it was difficult to determine whether the signal really represented ORF9b localization.

We detected the expression of ORF9b in infected cells and in clinical specimens; however, further details of the properties and function of ORF9b and its role in viral pathogenesis remain a mystery.

11.5 Functional Role of ORF9b

ORF9b is even less studied than ORF3a. The crystal structure was reported recently; it indicated that ORF9b is an unusual membrane binding protein with a long hydrophobic lipid-binding tunnel. It was therefore proposed to be associated with intracellular vesicles and may have some function in SARS-CoV assembly (Meier et al. 2006). However, further analysis of the properties and function of ORF9b are still necessary to understand its contribution to virus pathogenesis.

We found there was a similar protein, called I protein, present in the mouse hepatitis virus (MHV). There are also corresponding proteins similar to SARS-CoV ORF9b, so-called "internal" or "I" proteins, in other group II coronaviruses (Fischer et al. 1997; Lapps et al. 1987; Senanayake and Brian 1997). In MHV, I protein is an accessory viral structural protein which can contribute to plaque morphology (Fischer et al. 1997). In our study, the I protein in SARS-CoV (ORF9b) was also shown to be a structural component of virions. It is reasonable to propose that ORF9b in SARS-CoV may contribute to viral pathogenesis as I protein in MHV does. However, a detailed functional analysis of ORF9b is still needed to understand its role in the viral life-cycle.

In order to investigate the function of ORF9b, we used the *ORF9b* gene as bait in a yeast two-hybrid screening experiment. We found several interesting genes that can strongly interact with ORF9b, some of which are virus infection related host factors. Further work on this is still underway.

11.6 SARS-CoV Accessory Proteins: An Important Part of the Virus Genome

SARS-CoV possesses a long positive-strand RNA as genome. It is a single-stranded positive-sense RNA 29,727 nucleotides in length, excluding the polyadenylation tract at the 3′ end (Rota et al. 2003). Fourteen ORFs have been identified, encoding two replicases (1a and 1b), four structural proteins (S, E, M, N) and eight accessory proteins (ORFs 3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b) (Rota et al. 2003; Marra et al. 2003). All current studies on accessory proteins of coronaviruses including SARS-CoV suggest that they are not essential for virus replication (de Haan et al. 2002; Yount et al. 2005), but do affect viral release, stability, and pathogenesis, and finally contribute to virulence (Weiss and Navas-Martin 2005c).

Our study on ORF3a also shows similar results where it functions as an ion channel in SARS-CoV to facilitate virus release (Lu et al. 2006). In addition, ORF7a has also been characterized as a virion-associated protein of SARS-CoV (Huang et al. 2006), which induces apoptosis (Tan et al. 2004b; Kopecky-Bromberg et al. 2006) and arrests the cell cycle (Yuan et al. 2006) when over-expressed. Most recently, SARS-CoV ORF6 and ORF7b proteins have been identified as being incorporated into virus particles, although the functions of these two proteins are still unknown (Huang et al. 2007; Schaecher et al. 2007).

Understanding the properties and functions of SARS-CoV specific accessory proteins may help explain the differences in pathogenicity between SARS-CoV and other known coronaviruses.

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