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Structural and Functional Aspects of the Small Hydrophobic (SH) Protein in the Human Respiratory Syncytial Virus

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1. Introduction

hRSV is the leading cause of respiratory disease in infants, elderly, and immunocompromised populations worldwide (Falsey et al., 2005; Nair et al., 2010). Most individuals are infected at a young age, before 3 years old (Glezen et al., 1986). In fact, RSV infection is the most common cause of hospitalization in children 5 years old and below. When severe infection occurs, respiratory airways and pulmonary development are affected. However, the viral determinants of disease severity are not well defined, as little is known about its molecular mechanism of pathogenesis.

Disease caused by hRSV infection is unique in the sense that repeated infections throughout life can take place even though genetic diversity is not extreme, and antigenic sites are highly conserved between strains (Glezen et al., 1986). It is likely that natural RSV infection only confers imperfect immunity against subsequent infections; the virus probably has evolved to evade the natural immune system so that the durability of antibody response for life-long immunity is poor.

Although the virus was identified half a century ago, there are still no licensed vaccines against infection, and current vaccine-based antiviral therapies are not effective. Reviews describing efforts in the development of antiviral vaccines have been published over the years, e.g. (Collins & Murphy, 2006) and recently (Murata, 2009; Chang, 2011). In the initial trial of RSV vaccine with formalin-inactivated RSV (FI-RSV) during the 1960s, the vaccine proved to be poorly protective and actually enhanced the severity of RSV disease (Kapikian et al., 1969). This failure significantly increased safety concerns surrounding RSV vaccine development. The several hurdles in the development of a pediatric RSV vaccine, and the use of attenuated viruses, subunit particles, peptides, virus-like particles, and live viral vectors as vaccine candidates, which show potential for further development, have been discussed elsewhere, e.g., (Chang, 2011).

In general, currently available prophylactic and therapeutic methods are limited (Murata, 2009; Olszewska & Openshaw, 2009; Weisman, 2009; Chang, 2011). For example, a humanized monoclonal antibody, palivizumab (S. Johnson et al., 1997) targeting hRSV F glycoprotein, a trimeric fusion protein, is licensed for use as prophylactic therapy for the high-risk pediatric population. The drug ribavirin is the only antiviral therapy for patients

with hRSV infection, although not recommended in most cases for its unsatisfactory clinical efficacy and safety concerns (Vujovic & Mills, 2001). In addition to the approved monoclonal antibody palivizumab (Group, 1998), several small molecule inhibitors, e.g., disulfonated stilbenes (Razinkov et al., 2001), benzotriazoles (Cianci et al., 2004), benzimidazoles (Andries et al., 2003), and triphenol compounds (McKimm-Breschkin, 2000) that target F protein are also potent inhibitors of hRSV infectivity.

hRSV is a member of the Paramyxoviridae family of nonsegmented negative strand RNA viruses, and encodes 11 proteins, 9 of which are structural. Amongst these, the genome of hRSV encodes three membrane proteins that are accessible on the surface of the virion: fusion (F), attachment (G), and small hydrophobic (SH) protein. Protein G and F are key factors during virus entry, attachment and fusion (Lamb, 1993; Krusat & Streckert, 1997), and are the only hRSV proteins that induce neutralizing antibodies (Walsh et al., 1987; Connors et al., 1991).

Based on the reactive patterns to monoclonal antibodies, hRSV can be divided into two antigenic subgroups, A and B (P. R. Johnson et al., 1987), which co-circulate in human populations. Although antibodies against both F and G proteins were found in the serum of hRSV infected patients, they only provide temporary protection . Thus, the combination of low immunoprotection and lack of suitable antivirals leads logically towards the search and characterization of new drug targets for the effective treatments of hRSV infection.

2. SH protein

2.1 Topology, polymorphism and localization

In contrast to F and G proteins, little is known about the specific functions played by SH protein in hRSV infection and replication. The SH protein is the smallest transmembrane (TM) surface glycoprotein encoded by hRSV (Murphy et al., 1986; Collins & Mottet, 1993), with 64 to 65 amino acids, depending on the viral strain, A or B, respectively (Collins et al., 1990). Biochemical studies have shown that the SH protein is a type II integral membrane protein with a single TM domain (Fig. 1), where the C-terminus is confirmed to be oriented extracellularly (Collins & Mottet, 1993). The TMHMM algorithm, based on the Hidden Markov Model (Krogh et al., 2001) indicates that the TM domain spans residues 20 to 42 (Fig. 1, red line). This has been confirmed experimentally by us using a synthetic peptide corresponding to the TM domain of SH protein, SH-TM (residue 18-43), which when inserted into supported lipid bilayers was protected from hydrogen/deuterium (H/D) exchange and was α-helical (Gan et al., 2008).

During infection, the majority of the SH protein accumulates at lipid-raft structures of the Golgi complex, the endoplasmic reticulum (ER), and the cell surface (Rixon et al., 2004). Lipid rafts are enriched in cholesterol and sphingolipids and form a platform for various protein-protein interactions necessary during signal transduction events (Dykstra et al., 2003), protein trafficking (Helms & Zurzolo, 2004), and also virus entry, assembly, and budding (Suzuki & Suzuki, 2006). Indeed, hRSV has been shown to utilize lipid rafts, and in particular caveolae, a caveolin-1 enriched subdomain (Werling et al., 1999; Brown et al., 2002), to gain entry and in the assembly of virus particles. Only a very low amount of SH protein is associated with the viral envelope (Rixon et al., 2004).

Several forms of SH protein are present during infection, which vary in their glycosylation status (Olmsted & Collins, 1989): two non-glycosylated forms, a full length 7.5 kDa (SH_0) form, a truncated 4.5 kDa species (SH_t), an N-linked glycosylated form (SH_g), and a polylactosaminoglycan-modified form (SH_p). All these, except the truncated SH_t, are incorporated at the surface of the infected cells, where the non-glycosylated SH_0 appears to be the most abundant form (Collins & Mottet, 1993). In addition to these modifications, the tyrosine residues of SH protein are phosphorylated during infection, and this modification affects cellular distribution (Rixon et al., 2005).

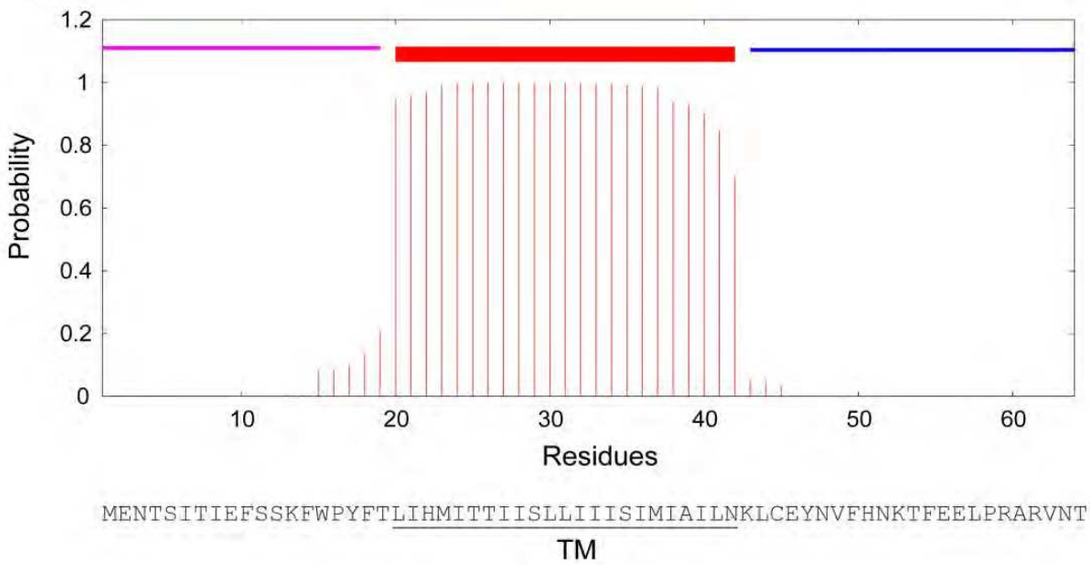


Fig. 1. Prediction of TM helices for SH protein by TMHMM (Krogh et al., 2001) (http://www.cbs.dtu.dk/services/TMHMM/). Only one TM α-helix is predicted (thick red line). The blue line represents residues inside the cell, whereas the magenta line indicates residues outside the cell, although this prediction turns out to be wrong (see text).

2.2 The role of SH protein

The function of SH protein in RSV replication cycle remains unclear. SH has no crucial role in viral survival in *in vitro* cell culture systems (Bukreyev et al., 1997), but it is essential for effective infection in animal models: mouse and chimpanzee (Bukreyev et al., 1997; Whitehead et al., 1999). This suggests a potential role for SH in immune evasion or in immunomodulation. Interestingly, a vaccine based on the use of live attenuated virus carrying a deletion of the SH gene, rA2cpts248/404/1030/ΔSH, showed significant improvement of disease symptoms and protection against re-infection when compared to another version, cpts248/404, which only carried mutations at other two genes, L and M (Karron et al., 2005).

Some studies suggest an ancillary role for SH protein in virus-mediated cell fusion (Heminway et al., 1994; Techaarpornkul et al., 2001). More recently, it has been shown that SH protein from simian virus 5 (SV5) (He et al., 2001), parainfluenza virus 5 (PIV 5) (Fuentes et al., 2007), Mump virus (MuV) (Wilson et al., 2006), and hRSV (Fuentes et al., 2007), all members of the *paramyxoviridae* family, inhibit apoptosis in several mammalian cell lines.

While promotion of apoptosis helps release the virus from the cell, it is possible that inhibition of apoptosis in host cells during infection gives an advantage to the virus in replication. For RSV and PIV 5, SH protein is necessary for the inhibition of tumor necrosis factor alpha (TNF-α)-induced apoptosis (Y. Lin et al., 2003; Fuentes et al., 2007). However, this is also the case in A549 cells, which are insensitive to TNF-α induced cell death (Fuentes et al., 2007). This suggests that this effect is not uniquely mediated by a TNF-α pathway.

In addition to the above, SH increases membrane permeability to low-molecular-weight compounds, as shown when expressed in *Escherichia coli* (Perez et al., 1997). Thus, SH protein has been suggested to belong to the viroporin class, a group of small, highly hydrophobic virus proteins that can oligomerize and form pores (Gonzalez & Carrasco, 2003). Support for this hypothesis was gained when we confirmed that the synthetic peptide corresponding to the predicted TM domain of SH protein (SH-TM) forms pentameric cation-selective ion channels in model planar lipid bilayers (Gan et al., 2008).

Ion leakage may lead to dissipation of membrane potential and disruption of cell homeostasis, but the consequences of these are not clear. Further studies on hRSV infected cells should gain insight into the significance of SH viroporin activity in the hRSV life cycle. One possible indication may be derived from experiments in MDBK and L929 cells, where SH from PIV5 or from RSV A or B subgroups has a protective role against the cytopathic effect (CPE) produced by PIV5 (He et al., 2001; Y. Lin et al., 2003; Wilson et al., 2006). Similarly, the SH protein from PIV5 could be substituted by SH from mumps virus (Wilson et al., 2006), even though these two SH proteins have no sequence homology. These data argue against a mechanism mediated by a specific protein-protein interaction with an unknown protein, and for a possible functional role of a membrane permeabilizing pentameric structure that would be common to all these species.

2.3 Interaction of SH with viral and host proteins

Extensive protein-protein interactions have been observed between the three membrane proteins on the RSV envelope, F, G, and SH (Feldman et al., 2001; Techaarpornkul et al., 2001; Low et al., 2008) and these interactions have an effect on fusion activity of hRSV on the host (Heminway et al., 1994; Techaarpornkul et al., 2001). In cells transiently expressing hRSV membrane proteins, the presence of G and SH proteins enhanced fusion activity mediated by F protein (Heminway et al., 1994). However, using virus-infected cells the presence of G protein alone enhanced F-mediated fusion activity (Techaarpornkul et al., 2001), whereas SH protein in the absence of G protein inhibited it, suggesting a possible interaction between SH and G (Techaarpornkul et al., 2001).

Protein complexes F-G and G-SH have been detected on the surface of infected cells using immunoprecipitation (Low et al., 2008) and heparin agarose affinity chromatography (Feldman et al., 2001). Direct evidence of the existence of an F-SH complex has never been reported. A trimeric complex F-G-SH was not detected on the surface of hRSV infected Hep-2 line cells (Low et al., 2008), but it was present in Vero cell lines co-transfected with F, G, and SH proteins (Feldman et al., 2001), suggesting that this hypothetical ternary interaction may be short lived, or takes place in very specific conditions. These three proteins not only form hetero-oligomers, but also homo-oligomers: F forms trimers (Calder et al., 2000), G forms tetramers (Escribano-Romero et al., 2004), and SH forms pentamers (Collins & Mottet,

1993; Rixon et al., 2005; Gan et al., 2008). Thus, a complicated regulatory network of interactions may exist which probably includes both homo- and hetero-oligomeric forms.

In addition to interactions with viral proteins, the fact that SH proteins of RSV and PIV 5 are necessary for the inhibition of tumor necrosis factor alpha (TNF-α)-induced apoptosis (Y. Lin et al., 2003; Fuentes et al., 2007) also suggests a possible interaction with host proteins, although this has not been confirmed experimentally.

2.4 Oligomerization of SH protein

Hetero- or homo-dimerization at the TM domain is very common in membrane proteins, e.g. homo- and hetero-dimeric integrins (X. Lin et al., 2006), or trimeric viral fusion proteins (Lamb et al., 1999). Tetramers and above suggest pore or channel formation, e.g., in influenza A M2 (a tetrameric proton channel) (Kovacs & Cross, 1997), CorA (a pentameric divalent cation transporter) (Eshaghi et al., 2006), and MscL (a hexameric mechanosensitive channel) (Sukharev et al., 1997). SH protein can be cross-linked with disuccinimidyl suberate and dithiobis-(succinimidyl)-propionate, to produce higher oligomers, from dimers to pentamers (Collins & Mottet, 1993; Rixon et al., 2005).

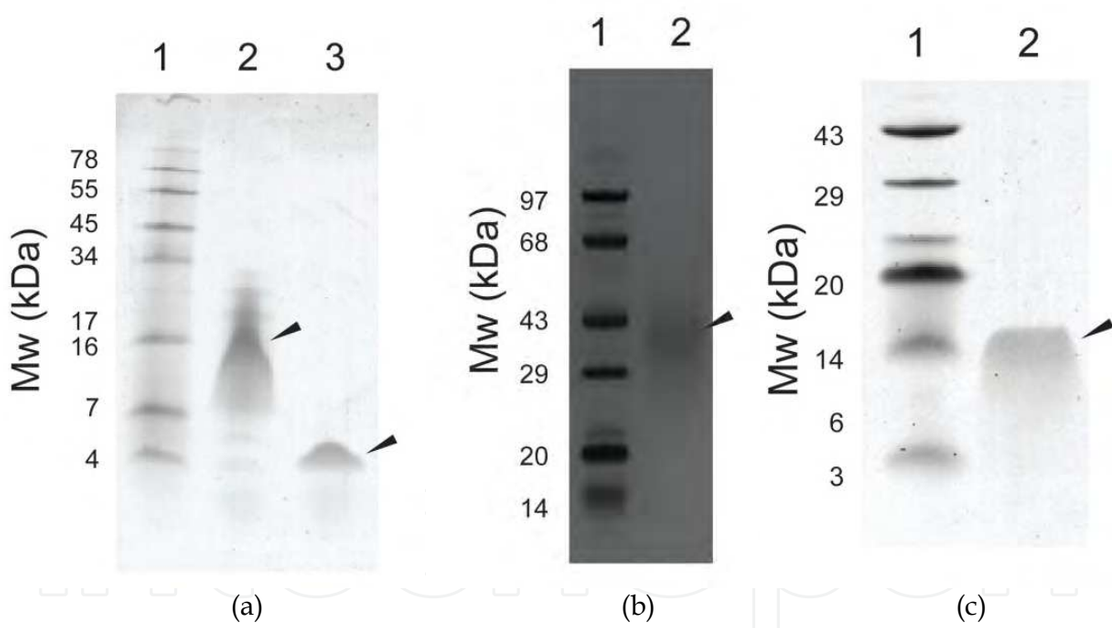
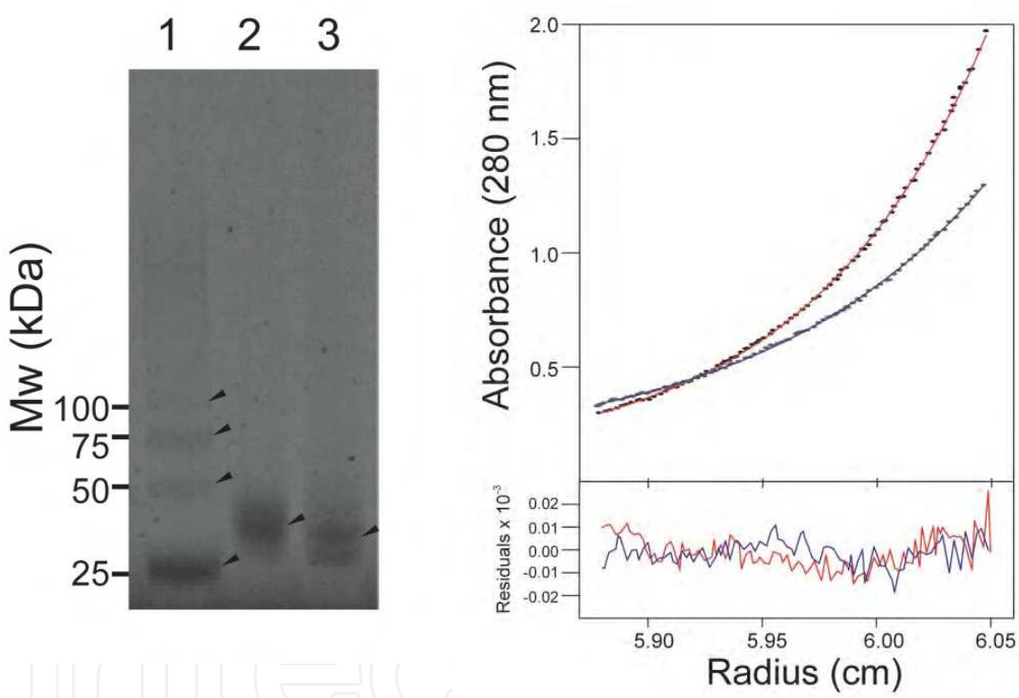


Fig. 2. PAGE analysis of SH protein and SH-TM in SDS ad PFO. (a) SDS-PAGE of SH protein and SH-TM. Lane 1, protein markers; lane 2, SH protein (expected M.W. 7,808 Da); lane 3, SH-TM (expected M.W. 2,983 Da); (b) PFO-PAGE of SH protein. Lane 1, protein markers; lane 2, SH protein; (c) PFO-PAGE of SH-TM. Lane 1 is protein markers and lane 2 is SH-TM.

We have studied SH protein oligomerization using a purified recombinant form corresponding to subgroup A. The protein was successfully over-expressed in *E. coli* and purified by RP-HPLC to high purity. One of the methods that can be used to study oligomerization is SDS-PAGE electrophoresis, which can maintain native oligomeric size in some cases, e.g., glycophorin A and phospholamban (Lemmon et al., 1992; Simmerman et

al., 1996). Usually, however, SDS destabilizes oligomer formation, induces non-specific oligomer formation, or results in anomalous migration (Rath et al., 2009). Thus, in the presence of SDS, SH protein migrated as a diffused band with a molecular weight (~ 17 kDa) consistent with either dimers, or slow monomers (Fig. 2A, lane 2). In contrast, the TM domain of SH protein, SH-TM (residue 18-43), formed only monomers (~3 kDa) in SDS-PAGE (Fig. 2A, lane 3). This indicates that SDS destabilizes possible SH protein oligomers. In contrast to SDS, perfluoro-octanoic acid (PFO) is a milder detergent that protects weak interactions and maintains native oligomeric size (Ramjeesingh et al., 1999). In presence of PFO, SH protein produced a band consistent with a higher molecular weight (~35-40 kDa) compatible with pentamers (Fig. 2B, lane 2). Consistently, the TM domain, SH-TM (residues 18-43), also formed pentamers (~ 15 kDa) in PFO-PAGE (Fig. 2C, lane 2), confirming that the TM domain of SH protein is the main driving force for SH protein pentamerization.



(a) (b)

Fig. 3. BN-PAGE and AUC-SE analysis of SH protein and H22A mutant reconstituted in C14 betaine micelles. (a) BN-PAGE of SH protein and H22A mutant. Lane 1 is AqpZ (monomeric size 25 kDa), which forms oligomers of several sizes in these conditions was used as protein markers; lane 2 is SH protein (expected MW 7808 Da), and lane 3 is H22A mutant (expected MW 7742 Da); (b) Representative traces of a global fit analysis of SH protein to a monomer-pentamer self-association model (red line), and H22A mutant to a monomer-tetramer model (blue line). The data shown were collected with 80 μM protein solubilized in 5 mM C14 betaine micelles, centrifuged at 24,000 rpm. The data is shown as black filled circles. The residuals of the fit are shown below.

As both SDS and PFO are anionic charge detergents, we also studied SH oligomerization in C14 betaine, a zwitterionic detergent. This was assessed in both Blue Native-PAGE (BN-PAGE) and in analytical ultracentrifugation sedimentation equilibrium (AUC-SE) experiments (Fig. 3). In the presence of C14 betaine, SH protein migrates as a single band in BN-PAGE, between monomeric and dimeric AqpZ (i.e., between 25 and 50 kDa), consistent with an SH pentamer (~40 kDa) (Fig. 3A, lane 2). The AUC-SE data for SH protein reconstituted into C14 betaine micelles was also best fitted to a monomer-pentamer model (Fig. 3B, red line). Therefore, the above studies point unequivocally to a pentameric form for SH. Recent electron microscopy studies using a >80 residue long construct containing SH protein have produced ambiguous results that could be assigned to a pentamer or a hexamer (Carter et al., 2010).

The energetics of the interaction between SH monomers was also obtained from AUC-SE data. These studies provide dissociation constant and distribution of oligomeric species over a wide range of concentrations for a reversibly associating system in solution. The calculated standard free energy ($\Delta G°$) was -16.3 kcal/mol, i.e., 78% to 88% of SH protein forms pentamers in these conditions. For comparison, SARS-CoV E protein, also a small membrane protein of 76 amino acids with a single α-helical TM domain (Torres et al., 2006), associates forming pentamers with standard free energy of -9.45 kcal/mol, therefore SH protein has a higher propensity for pentamerization.

2.5 His22 mutation destabilizes the pentameric form of SH protein

The pentameric structure of SH-TM has been modeled by combining evolutionary conservation data in global search molecular dynamics (GSMD) simulations and orientational restrains derived from infrared linear dichroism analysis of an isotopically labeled SH-TM peptide in lipid bilayers (Gan et al., 2008). In this model, His22 was located facing the lumen or inter-helical region of the pentamer. This is reminiscent of a similar residue (His37) found at the TM domain of Influenza A M2 proton channel. In M2, this histidine residue is located in a lumenal orientation, and it has been shown to be important for the tetramerization of M2 (Howard et al., 2002), as well as an essential residue involved in proton transport.

Consistent with this lumenal or interfacial location, a H22A mutant in C14 betaine migrated faster than wild type (WT) SH protein, likely as tetramers (Fig. 3A, lane 3). Also, AUC-SE data in C14 betaine micelles could not be fitted to a monomer-pentamer equilibrium model, but it could be fitted to a monomer-tetramer model (Fig. 3B, blue line). The standard free energy of association was -12.83 kcal/mol. Indeed, histidine is a good candidate to mediate TM α-helix association; the polar Nδ and Nε atoms of the imidazole ring are capable of being both hydrogen bond donor and acceptor.

2.6 SH protein as a viroporin

Viroporin is a general term applied to small hydrophobic proteins encoded by viruses that increase membrane permeability (Gonzalez & Carrasco, 2003). Generally, these proteins are 60-120 amino acids long with one or two α-helical TM domains. They oligomerize in membranes to form pores, allowing passage of ions or small molecules across the lipid bilayer. It has been suggested that viroporins acts as a virulence factor during infection. They are not essential for virus replication but their presence enhances virus growth. Forming an ion

channel may be one of the strategies for viruses to survive in the host system. The channel activity of viroporins leads to the dissipation of the membrane potential and disruption of cells homeostasis, leading to gradually damage of cells as infection progresses. To date, more than ten viroporins have been identified from various viruses, and influenza A virus M2 proton channel is probably the best studied example (Zhou et al., 2001; Schnell & Chou, 2008; Stouffer et al., 2008). Structural and *in vivo* electrophysiological studies of viroporins are lacking, partially due to the difficulty in expression and purification of the hydrophobic membrane proteins. Nonetheless, structures of the SARS-CoV E protein (Pervushin et al., 2009), the HCV p7 protein(Cook & Opella, 2009), and the HIV-1 Vpu proteins (Park et al., 2003; Sharpe et al., 2006) have been studied by NMR methods. Channel activities for these proteins have been confirmed using black lipid membranes (BLM) and can be blocked by hexamethylene amiloride (HMA) (Ewart et al., 2002; Premkumar et al., 2004; Pervushin et al., 2009), whereas we have shown that SARS-CoV E protein also displays channel activity in a mammalian whole-cell patch clamp set-up (Pervushin et al., 2009).

In the case of SH protein, both SH-TM (residues 18-43) (Gan et al., 2008) and full-length SH protein have channel activity when reconstituted in BLMs (Fig. 4A). More direct evidence for channel activity is provided using whole-cell patch clamp experiments of SH protein-transfected HEK293 cell lines. In these experiments, expression of full-length SH gene was monitored by GFP, and the fluorescence intensity was correlated with expression level of SH protein (Marshall et al., 1995). The full-length SH protein displayed channel activity when transiently expressed in HEK293 cells (Fig. 4B). When placed in bath solution with neutral pH, the transfected cells produced significant higher channel activity than the controls, the cells transfected with vector alone (Fig. 4B, left panel). Molecular modeling of the α-helical region of SH protein shows several polar residues lining the lumen of the pore, including charged residues: His22, Lys43, and His51. The pK_a for lysine and histidine are about 11.1 and 6, respectively. Therefore, histidines are most likely contributing to changes in SH channel activity if the pH of the bath solution was changed from neutral to acidic pH.

We have shown in a BLM experiment that SH-TM which contains His22 was acid sensitive (Gan et al., 2008). To test the effect of acidification on channel activity for the full-length SH protein, the bath solution were changed to pH 5.5 after a stable conductance were recorded in neutral pH. In contrast to the control, in which no changes were observed upon pH changes (Fig. 4B, right panel), the SH channel responded more actively in acidic solution. Larger outward current was detected upon exposure of SH channel to acidic solution, therefore we have shown that SH channel activity is pH dependent. Whether residue His22 or His51 is involved in the pH regulation, and the role of the channel activity in virus life cycle, requires further investigation.

Recently, studies have indicated that SH protein can inhibit apoptosis in several mammalian cell lines by blocking the tumor necrosis factor alpha (TNF-α)-mediated apoptotic signaling pathway (Fuentes et al., 2007). However, ion channels may also control apoptosis in cells (Szabo et al., 2004; Lang et al., 2005; Burg et al., 2006; Madan et al., 2008). Disruption of cells homeostasis is a common sign of apoptosis, leading to plasma membrane depolarization associated with intracellular cation overload and cell volume decreases due to anion and water efflux (Burg et al., 2006). In fact, the viroporin of Sindbis virus 6K, murine hepatitisvirus E protein, Influenza A M2 protein, HCV p7 protein, poliovirus 2b and 3A protein have been reported to manipulate apoptosis of infected cells (Neznanov et al., 2001; Campanella et al., 2004; Madan et al., 2008). While promotion of apoptosis helps to release

the virus, inhibition of apoptosis in host cells during infection gives an advantage to the virus to replicate. In future, drugs that block ion channel of several viroporins, such as amantadine, rimantadine, and HMA could be tested on SH ion channel to obtain further understanding of the channel properties of SH protein.

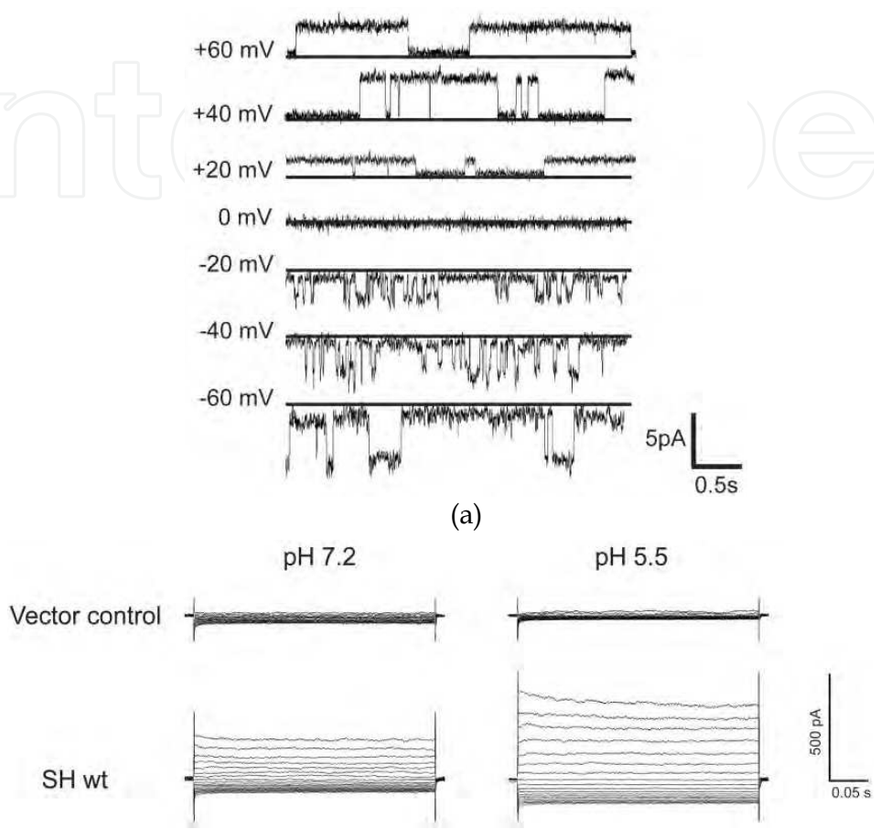


Fig. 4. SH is a viroporin. (a) Single channel elicited by SH protein when inserted into BLMs, recorded in 300 mM KCl, 5 mM Hepes, pH 5.5 buffer solution; (b) Traces of currents evoked in HEK-292 cells transfected with a vector carrying SH protein or vector alone, in neutral or acidic pH bath solutions.

2.7 Secondary structure of SH protein using attenuated total reflection fourier transform infrared (ATR-FTIR) spectroscopy

The amide I region in the infrared spectrum (Fig. 5A) can be assigned to different secondary structure elements (Byler & Susi, 1986). Full length SH protein shows a major peak centered at 1653 cm^{-1} and a shoulder centered at 1632 cm^{-1} indicating a mixture of α-helix and β-strand (Fig. 5A, upper panel). For SH-TM, a narrow band centered at 1654 cm^{-1} indicates a large fraction of α-helix (Fig. 5A, middle panel). For a synthetic peptide that consists of the last 20 C-terminal residues (SH-C20) the spectrum is centered at 1635 cm^{-1}, indicating a majority of β-strand structure (Fig. 5A, bottom panel). A quantification of the α-helix present in full length SH protein produced ~40 residues whereas only ~20 were present in the TM domain alone. This suggests that some α-helix is present in the extramembrane domain.

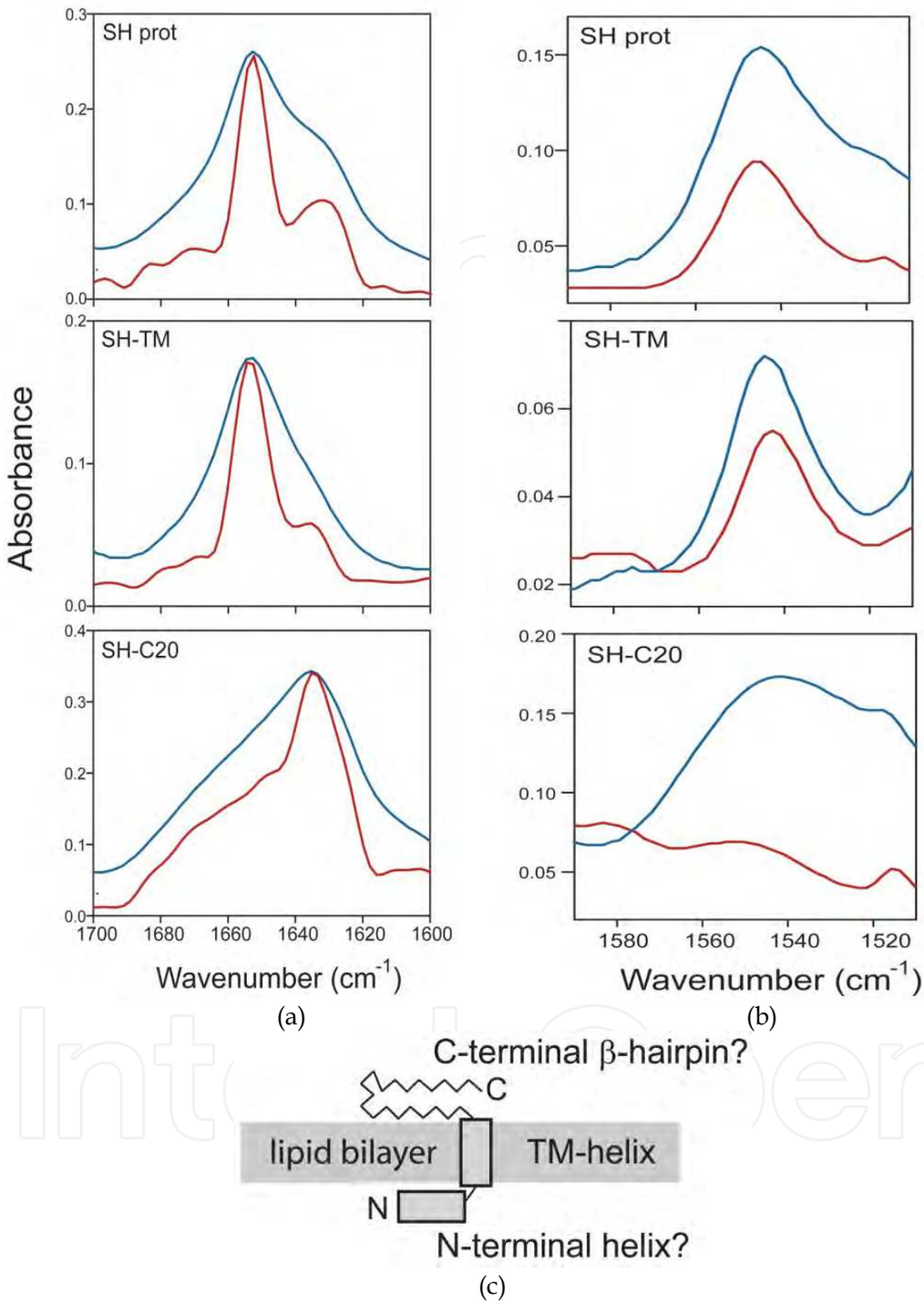


Fig. 5. ATR-FTIR spectra and H/D exchange of SH protein and fragments. (a) Amide I region corresponding to SH protein, SH-TM, and SH-C20. Lines for original spectrum (blue) and Fourier self deconvolved spectrum (red) are shown; (b) Amide II region of the same samples before (blue) and after (red) 1 hour exposure to D_2O (Torres et al., 2006); (c) Sketch representing the main features of SH according to the data in (A-B).

The amide II band in the infrared spectrum, due to peptide backbone N-H bending vibration, is used to monitor protein hydrogen-deuterium (H/D) exchange kinetics. Upon H/D exchange, the frequency of amide II downshifts from ~1545 to ~1450 cm^{-1} (~100 cm^{-1}). Thus, amide exchange can be measured following the decrease in intensity of the unexchanged amide II. H/D exchange can be used to determine the number of residues embedded in the bilayer. The spectra of the amide II band of SH protein, SH-TM, and SH-C20 recorded in H_2O and after one hour exposure to D_2O (Fig. 5B) shows 45%, 80% and 11% of protected residues, respectively. These results suggest that the only embedded fraction corresponds to TM α-helix whereas SH-C20 is not inserted into the membrane. A preliminary sketch of the pentameric model and its secondary structural elements is shown in Fig. 5C.

2.8 Effects of SH, SH-TM and SH-C20 on lipid order

ATR-FTIR is a most suitable tool to study lipid-protein interactions because lipids absorb in many regions of the infrared spectrum. Further, lipid orientational order parameter determination and lipid phase information can be obtained because the frequencies of methylene stretching change upon gel-to-liquid crystal phase transition. The lipid methylene C-H stretching transition dipole is oriented perpendicular to the long axis of an all-*trans* fatty acid chain, therefore measuring linear dichroism of lipid methylene stretching vibrations can be used to probe the orientation of lipid bilayers when deposited on a germanium trapezoid. The order parameter S_L (Tamm & Tatulian, 1997) is calculated for lipid bilayer deposited on the surface of a germanium trapezoid, with electric field components for the evanescent field (Arkin et al., 1997). Thus, a decrease in the dichroic ratio, R_L, corresponds to an increase in the acyl chain order parameter, S_L. Lipid-protein interactions of SH protein, SH-TM, and SH-C20 were investigated in supported DMPC and POPC bilayers (Fig. 6) using both lipid methylene symmetric (~2851 cm^{-1}) and antisymmetric (~2919 cm^{-1}) stretching vibrations to calculate R_L and S_L. The measured values of R_L for DMPC bilayers were 1.14 (S_L = 0.59) for symmetric, and 1.20 (S_L =0.55) for antisymmetric vibrations. These values are in good agreement with published data (Hubner & Mantsch, 1991), indicating well-ordered lipid bilayers.

The frequency of the lipid methylene C-H stretching bands of DMPC (T_m = 23°C) indicated that the membranes were in the gel phase (Tamm & Tatulian, 1997). Therefore, spectra for SH protein and SH-TM were recorded also in POPC (T_m = -2°C), which should form a fluid liquid crystal phase due to the presence of unsaturated bonds in the *sn*-2 chain of the POPC acyl chain. This was evident from the shift in the lipid symmetric stretching vibration, from 2851 to 2853 cm^{-1}, and the anti-symmetric methylene stretching vibration, from 2919 to 2923 cm^{-1} (Tamm & Tatulian, 1997). The values of R_L for POPC bilayers measured in our system were 1.31 (S_L = 0.38) and 1.32 (S_L = 0.37) for symmetric and antisymmetric vibrations, respectively. These values are lower than those of DMPC.

In the presence of protein, for simplicity, only lipid methylene symmetric vibrations were measured. Although no changes in lipid order parameter were observed after SH protein was reconstituted in DMPC (R_L = 1.14 and S_L = 0.59), disorder was observed in POPC (R_L = 1.46 and S_L = 0.23). In contrast, SH-TM increased the order of the acyl chains in both DMPC

(R_L = 1.07 and S_L = 0.69) and POPC (R_L = 1.27 and S_L = 0.43). Interaction of SH-C20 was measured only in DMPC, where a 20% increase in disorder was observed (R_L = 1.27 and S_L = 0.43). Thus, this short β-structure forming peptide (Fig. 5C) is able to destabilize membranes.

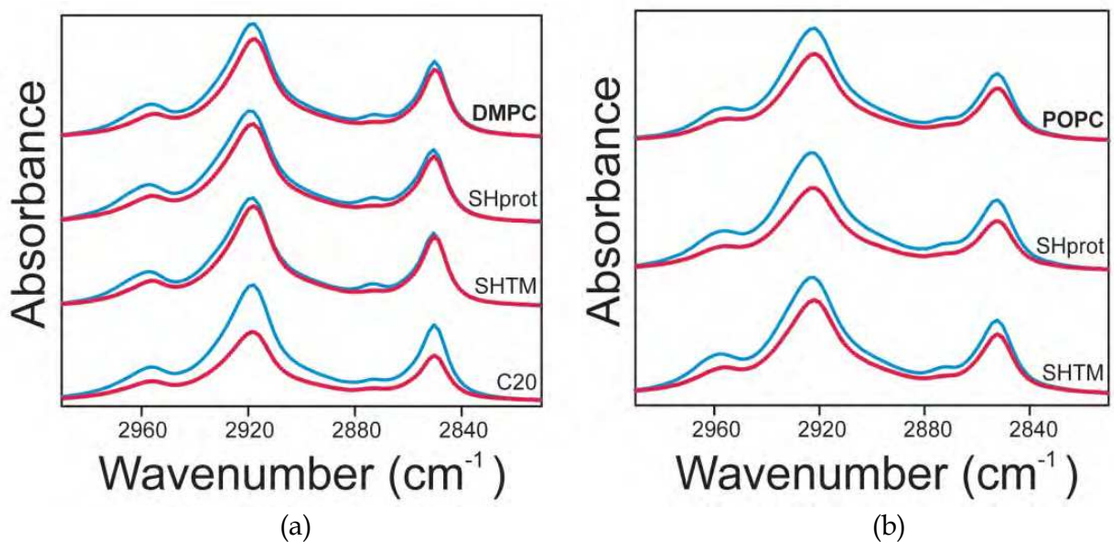
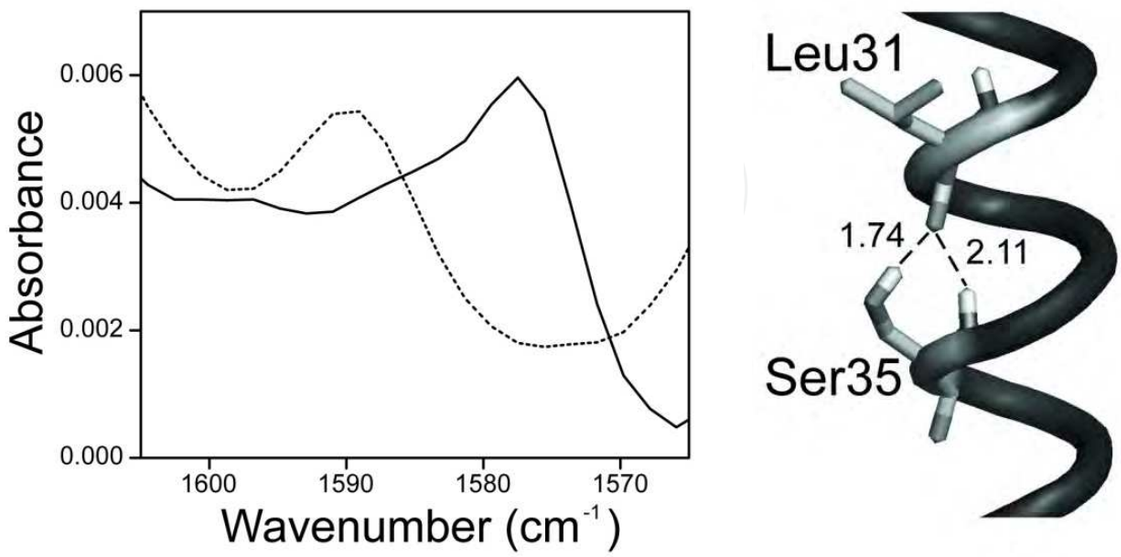


Fig. 6. Polarized ATR-FTIR spectra of the lipid methylene stretching vibrations. Supported DMPC (a) and POPC (b) lipid bilayers in the absence or presence of SH protein, SH-TM, and SH-C20. Blue and red lines correspond to parallel and perpendicular polarizations, respectively.

2.9 Detection of an intra-helical hydrogen bond in SH-TM

During our attempts to measure the dichroism of labeled SH-TM, we observed that the $^{13}C{=}^{18}O$ isotope label at L31 was shifted to a lower frequency, from 1592 cm^{-1} to 1576 cm^{-1} (Gan et al., 2008). According to the harmonic oscillator model, downshift of a vibrational frequency can occur if the reduced mass is increased, or the strength of a bond is weakened. This data is consistent with the presence of an intra-helical hydrogen bond between the hydroxyl side chain of Ser35 and the backbone carbonyl oxygen from Leu31 (Fig. 7B). Indeed, when Ser35 was replaced by alanine, the frequency of the $^{13}C{=}^{18}O$ isotope label at L31 reverted to its expected range, at 1589 cm^{-1} (Fig. 7A). This indicates that an intra-helical hydrogen bond exists in the TM domain of SH protein, which weakens the carbonyl bond, resulting in the downshift observed.

There is a high tendency for serine or threonine residues in α-helices to form intrahelical hydrogen bonds to carbonyl oxygen at position *i*-4 (Baker & Hubbard, 1984), which could induce a kink in the α-helix that may be important for functionality (Ballesteros et al., 2000). Indeed, viral ion channels display certain degree of structural flexibility, as seen in the influenza A virus M2 protein (Li et al., 2007) and SARS-CoV E protein (Parthasarathy et al., 2008).



(a) (b)

Fig. 7. Hydrogen bonding in SH-TM detected by FTIR. (a) Infrared spectra of SH-TM labeled at L31 $^{13}C=^{18}O$ (solid line) and for a S35A mutant (broken line); (b) Schematic representation of the proposed hydrogen bond between Ser35 side chain and Leu31 backbone C=O.

2.10 Structural determination of SH protein in detergent micelles by solution NMR

The HSQC spectrum of ^{15}N labeled SH protein was tested in three detergents: DPC (medium-chain, zwitterionic), DHPC (short-chain, zwitterionic), and SDS (anionic) (Fig. 8). Although SDS is a harsh detergent, well-resolved spectra of membrane proteins have been recorded (Howell et al., 2005; Franzin et al., 2007; Teriete et al., 2007). In contrast, DPC and DHPC have a headgroup that closely mimics that of phosphatidylcholine, the most abundant headgroup in natural membranes, successfully used in KcsA (Yu et al., 2005), human phospholamban (Oxenoid & Chou, 2005), diacylglycerol kinase (Van Horn et al., 2009), Rv1761c from Mycobacterium tuberculosis (Page et al., 2009), influenza A M2 (Schnell & Chou, 2008) or HIV Vpu (Park et al., 2003).

For SH protein, $^{1}H/^{15}N$-HSQC spectra showed limited peak dispersion, resonances not well-resolved, and overlapping peaks (Fig. 8). Only about 50% of the peaks could be observed in SDS and DHPC, whereas DPC appeared to be the best detergent with about 75% of peaks observed. Sample heterogeneity was observed in all three detergents, evidenced by the double resonance observed for the tryptophan indole side chain, Nε-Hε, at around 10.0-10.5 ^{1}H ppm (see the inserts of Fig. 8). As only one tryptophan residue is present in SH protein, only one peak should be observed. This indicates the presence of two backbone conformations, or two different rotameric states of the tryptophan indole side chain.

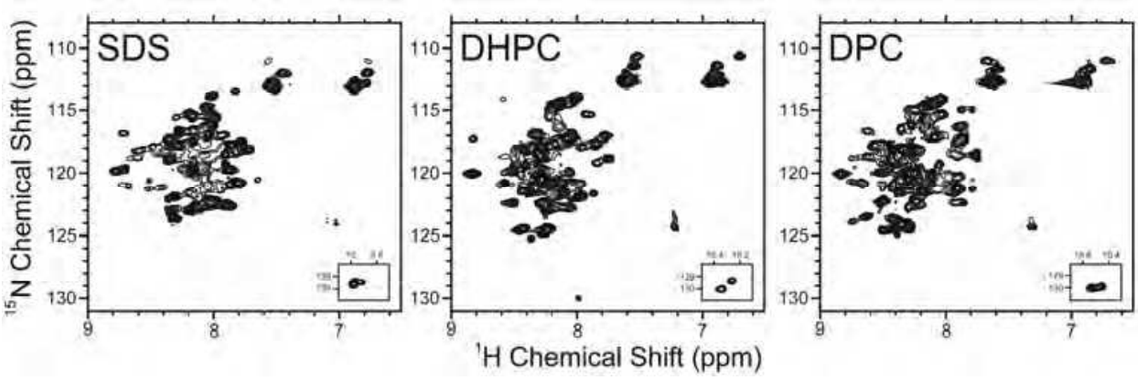


Fig. 8. Effect of different micellar environments on SH protein shown by $^{1}H/^{15}N$-HSQC spectra. The detergents SDS (left), DHPC (middle), and DPC (right) were used to obtain the spectra. In each graph, the resonance of Trp15 side chain is shown in the insert.

3. Summary and conclusions

Viral respiratory diseases pose serious threats to human health and effective antiviral agents are limited. Specifically, hRSV is one of the main agents responsible to widespread infection causing millions of clinical cases each year. Vaccines, antibodies and small drugs are being sought, but effective treatments are not yet available. The discovery of proteins known as 'viroporins' in many viruses has opened a potentially effective field for antiviral therapy. In hRSV, biochemical and biophysical studies have shown that SH protein has the characteristics of a viroporin, and this chapter has described biophysical properties of this purified protein that ultimately may help elucidate its role in RSV infection.

A first step in the characterization of a protein is its successful expression and purification. Both the full length and the TM domain of SH protein assemble as homopentamers, with His22 having a stabilizing role. The study of SH protein by solution NMR methods is in progress, and it shows good potential, especially in DPC micelles and in bicelles, although at present dual conformations are observed. Other lower resolution techniques, such as FTIR, have provided data that allow to obtain a preliminary model of full length SH protein, based on H/D exchange, β-structure in the last 20 amino acids, and the higher than expected helical content in the full length protein, when compared to the TM domain. The C-terminal β-structure has a destabilizing effect on membranes and may fold as a β-hairpin. FTIR of isotopically labeled TM domain has detected an intra-helical hydrogen bond between the backbone carbonyl oxygen of Leu31 and the side chain of Ser35 in the TM domain that could be important for channel activity. Conductance studies of SH protein provide compelling evidence that SH behaves as an ion channel, where histidine side chains may have an important regulatory role. The involvement of histidine as a pH sensor has been observed in other ion channels. In influenza A M2, His 37 is responsible for pH sensitivity (Schnell & Chou, 2008; Stouffer et al., 2008), and other examples of histidine regulated pH sensitive ion channels exist, for example the bacterial potassium channel KscA (His25) (Takeuchi et al., 2007), the potassium channel ROMK1

(four histidine residues are involved) (Chanchevalap et al., 2000), the potassium channel TASK1 (His98) (Yuill et al., 2007), potassium channel TREK-1 (His126) or TREK-2 (His151) (Sandoz et al., 2009). Therefore, the hypothesis that the TM histidine in SH protein (His22) has a regulatory role is worth exploring.

SH protein has been associated with fusion activity during infection, and with membrane permeabilization, and the structural features observed seem to reflect these functions.

Finally, channel activity in other viroporins can be blocked, e.g., the influenza A virus M2 proton channel blocked by amantadine and rimantadine (Wang et al., 1994; Chizhmakov et al., 1996), the amiloride derivative hexamethylene amiloride (HMA) inhibits ion channel activity of HIV-1 Vpu proteins (Ewart et al., 2002), SARS-CoV E protein (Pervushin et al., 2009), and HCV p7 protein (Premkumar et al., 2004). At present, there are no drugs reported that can block SH channel activity. The discovery of such compounds is important both for antiviral therapy and to understand the biology of SH protein.

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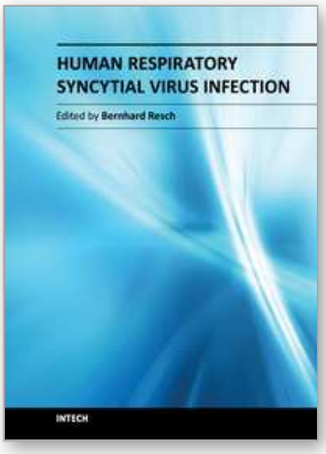

Human Respiratory Syncytial Virus Infection

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In this online Open Access book on "Human RSV Infections", several distinguished authors contribute their experience in respiratory syncytial virology. A major focus lies on the fascinating pathophysiology of RSV and represents recent and actual work on different mechanisms involved in the complex pathogenesis of the virus. The second section elucidates epidemiologic and diagnostic aspects of RSV infection covering a more clinical view of RSV disease. At last, treatment modalities including the search for a vaccine that is still not in sight are discussed and conclude this book, thus building up a circle that runs from experimental models of RSV related lung disease over clinical aspects of disease to the latest news of therapeutic and prophylactic approaches to human RSV infection.

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