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Characterization of HCoV-229E fusion core: Implications for structure basis of coronavirus membrane fusion

Cheng Liu [a,b,1], Youjun Feng [b,c,1], Feng Gao [a,b], Qiangmin Zhang [b,c], Ming Wang [a,*]

[a] College of Veterinary Medicine, China Agricultural University, Beijing 100094, PR China
[b] Center for Molecular Virology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, PR China
[c] Graduate School, Chinese Academy of Sciences, Beijing, PR China

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Abstract

Human coronavirus 229E (HCoV-229E), a member of group I coronaviruses, has been identified as one of the major viral agents causing respiratory tract diseases in humans for nearly 40 years. However, the detailed molecular mechanism of the membrane fusion mediated by the spike (S) protein of HCoV-229E remains elusive. Here, we report, for the first time, a rationally designed fusion core of HCoV-229E (HR1-SGGRGG-HR2), which was in vitro produced in GST prokaryotic expression system. Multiple lines of experimental data including gel-filtration, chemical cross-linking, and circular diagram (CD) demonstrated that the HCoV-229E fusion core possesses the typical properties of the trimer of coiled-coil heterodimer (six α-helix bundle). 3D structure modeling presents its most-likely structure, similar to those of coronaviruses that have been well-documented. Collectively, HCoV-229E S protein belongs to the type I fusion protein, which is characterized by the existence of two heptad-repeat regions (HR1 and HR2), furthermore, the available knowledge concerning HCoV-229E fusion core may make it possible to design small molecule or polypeptide drugs targeting the membrane fusion, a crucial step of HCoV-229E infection.

Keywords: Human coronavirus 229E; Virus fusion; Type I membrane fusion; Heptad-repeat regions; Fusion core

The coronaviruses (CoV) are a diverse group of enveloped viruses with positive-stranded RNA genomes of about 30 kb, and cause respiratory and enteric diseases in humans and other animals [1–5]. Human coronaviruses (HCoVs) are notorious for the essence of severe viral pathogens responsible for no less than 30% of upper respiratory tract illnesses worldwide [3]. To date, there are only five different coronaviruses including HCoV-229E, HCoV-OC43, severe acute respiratory syndrome coronavirus (SARS-CoV), HCoV-NL63 [1,6], and HKU1 [7] found in humans. Of being noteworthy, HCoV-229E, a member of group 1 coronaviruses, often leads to coryza, headache, cough, fever, disruption of nasal epithelium, and it can occasionally cause severe lower respiratory tract infection [4,8]. Considering the various severe damages resulting from HCoV-229E infection in humans, different kinds of research have been developed to aim for understanding its pathogenic mechanism and even finding some clues to strategies used for prevention and therapeutics of above illness [4,8–11].

The membrane fusion is the key step during their life cycles for nearly all enveloped viruses, because it is necessary to facilitate the intracellular deposition of the viral genome followed by its replication [1,3,12,13]. It has been known that the envelope protein undergoes a series of conformational changes during the virus fusion process [1,3,12–20]. In general, it is popular that enveloped viruses might adopt a similar molecular apparatus for virus membrane merge in which two types have been proposed [2,5,14–16,19–23]. In type I, human immunodeficiency virus (HIV) [15,17–21,24–26], influenza virus [14,16,27], human respiratory syncytial virus (HRSV) [2,9,10,28,29], and Newcastle virus (NDV) [30–32] are several representa-

* Corresponding author. Fax: +86 01 62732840.
E-mail address: vetdean@cau.edu.cn (M. Wang).
[1] These authors contributed equally to this work.

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tives with crystal structure characterization. For type II, flavivirus may be an example with limited knowledge towards its molecular structure basis of membrane fusion [12,33]. Furthermore, it was not very clear how the conformational changes contribute to the fusion, until the crystal structures of dengue virus E protein and Semliki Forest virus, in their post-fusion conformations [12,33–35]. Structural and biochemical studies of fusion (F) proteins, e.g., influenza hemagglutinin, gp41 protein of HIV, S protein of Ebola virus, F protein of HRSV, etc., revealed the mechanistic details of virus entry specific for the class I envelope protein [13,14,16,25,27,28]. In details, once the virus binds to the receptor of host cell, its F or S protein undergoes a series of conformational changes to initiate the membrane fusion [8,12,23,26,30]. Briefly, there are at least three conformational states of the envelope fusion protein, which include pre-fusion native state, pre-hairpin intermediate state, and post-hairpin state [12,22,23,29,36]. Two highly conserved heptad-repeat regions (HR1 and HR2) of F or S protein function as important modules/domains in this process [9,12,18,21–23,25,28,30]. During these state transitions, the HR1 and HR2 are exposed to in an intermediate conformational state but bind to each other to form the coiled-coil structure in an anti-parallel pattern in the post-fusion stage [10,12,13,24,32]. This coiled-coil bundle conformation is proposed to be important for bringing two lipid layers into proximity allowing the membrane merge for viral entry into host cells [9,12,18,31].

The stable HR1/HR2 construct with structure of coiled-coil bundle is so-called the virus fusion core, representing the core structure of the post-fusion fusion protein [2,12,22,23]. In this structure, three HR2 helices pack against the hydrophobic grooves on the surface of central coiled-coil formed by three parallel HR1 helices in an oblique anti-parallel manner [2,12,22,23]. Many studies of fusion cores, including HIV [21,37], influenza virus A [16,27], Ebola virus [13], Newcastle virus (NDV) [30–32], HRSV [2,9,10,28,29], and transmissible gastroenteritis coronavirus (TGEV) [12], confirmed that they all follow the rule of type I virus membrane fusion mechanism. As both HR1 and HR2 are structural α-helical in the fusion core, this structure is also referred to 6-helix bundle or trimer of hairpins [15,23,28,29]. Recently, Xu et al. and Ma et al. separately added another members into type I by depicting the fusion cores of SARS-CoV, murine hepatitis coronavirus (MHV), and TGEV [5,11,12,29,36,38]. More intriguingly, the soluble HR2 derived from SARS-CoV and MHV shows the inhibitory activities for viral fusion, extremely similar to the peptide inhibitor for HIV, Enfuvirtide or T20 [15,17–21,25,26]. As we know, HCoV-229E belongs to group 1 which is same as that of TGEV, but different from those of SARS-CoV and MHV [12,29,36], we are prompted to define the details of HCoV-229E fusion core and even to test whether it is common to the Family *coronaviridae* using the rational design and biochemical characterization of HCoV-229E fusion core.

So far, little of any experimental evidences have been presented to propose that the HCoV-229E fusion core shares the same features as those of well-known enveloped viruses [12,29]. In this study, we tried to unravel the structure basis of HCoV-229E membrane fusion through providing the biochemical and biophysical properties of its fusion core, even to examine the possibility of the HCoV-229E fusion apparatus applied in fusion inhibitor design for the treatment of HCoV-229E-related illness. Thus, we applied bio-engineering technique to produce the soluble protein of the HCoV-229E fusion core (called 2-Helix) in a large scale. The results of gel-filtration combined with circular dichroism (CD), chemical cross-linking, demonstrated that it is of trimer of heterodimer, coiled-coil bundle, implying that HCoV-229E may adopt type I membrane fusion mechanism and even extending our knowledge of membrane fusion apparatus common to the Family *coronaviridae*. Moreover, the 3D structure modeling of HCoV-229E fusion core lively represented its most-likely stereo configuration which is extremely similar to those of coronaviruses, such as TGEV, SARS-CoV, etc. [11,12,36]. In summary, the available knowledge about the HCoV-229E fusion core will help to design the small molecules or polypeptide drugs targeting the crucial step of HCoV-229E membrane fusion, similar to T20 against HIV infection [15,17–21,25,26].

Materials and methods

Phylogenetic analysis of coronaviruses' spiker proteins. Spiker (S) proteins of 15 relevant enveloped viruses, which consisted of 13 closely related coronaviruses in addition to HIV and Influenza virus as references, were collected to perform the multiple alignment in the level of amino acids by the program of Vector NTI Suite 8.0 (Invitrogen), and the available data were processed by TreeView software to generate the phylogenetic tree shown in Fig. 1.

Development of HCoV-229E fusion core. The spiker (S) gene of HCoV-229E was utilized in this work. As shown in the upper panel of Fig. 2, the HCoV-229E S protein belongs to the typical type I membrane protein. The two heptad-repeat regions (HR1 and HR2) were predicted using the computer program of LearnCoil-VMF [39]. The predicted HR1 region covers amino acids 777–916, and the relevant HR2 includes the amino acids 1057–1105 (in the bottom panel of Fig. 2). To achieve the soluble production of the HCoV-229E fusion core, both HR1 and HR2 regions were rationally modified (Fig. 3A), basing multiple lines of considerations including alignments of HCoV-229E with the other related coronaviruses in their corresponding conserved regions (Fig. 3B) [12,29,36]. Subsequently, the HCoV-229E fusion core (2-Helix construct) was designed successfully by connecting the truncated HR1 (789–852) and extended HR2 (1053–1110) with a flexible linker (SGGRGG, single amino acid abbreviation) (Fig. 3C). Finally, the DNA coding sequence with optimized codes in *Escherichia coli* was amplified via the overlapping PCR technique, and then was inserted directionally into the prokaryotic expression vector pGEX-6P-1 (Pharmacia) between the restriction sites of *Bam*HI and *Xho*I (introduced by PCR primers). The resultant recombinant plasmid was confirmed by direct DNA sequencing.

Soluble production of HCoV-229E fusion core. The possible positive clones were transformed into *E. coli* strain BL21 (DE3) competent cells and the single colony was inoculated into Luria–Bertani (LB) medium containing 50 mg/L ampicillin (Sigma, USA) at 37 °C for 8 h. Following, the culture was transferred into the fresh LB medium for large-scale protein production at 37 °C. When the culture density (OD_{600}) added up

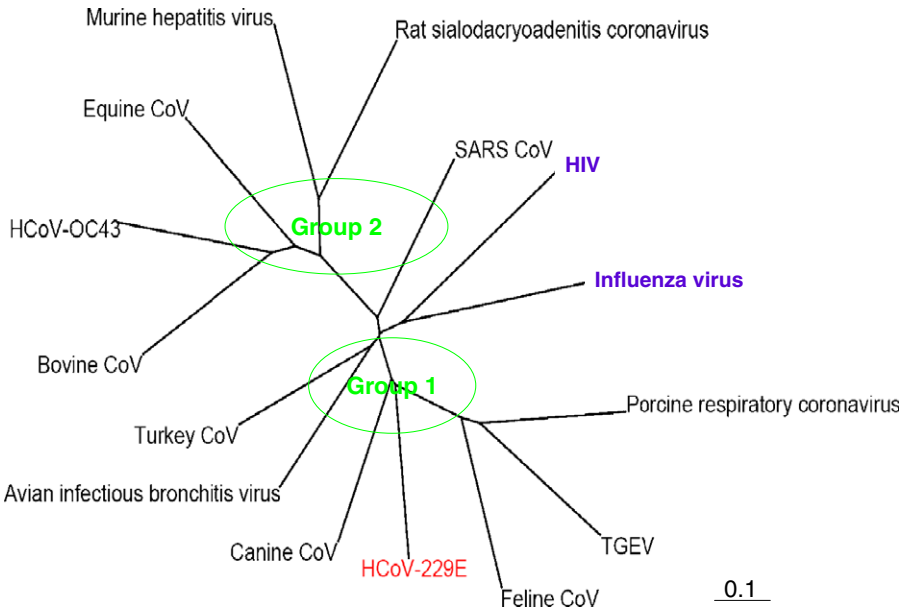

Fig. 1. Phylogenetic tree of fusion proteins from some related coronaviruses. HCoV-229E is highlighted in red; HIV and influenza virus, two of the most classic coronavirues, are indicated in blue; two different groups known are presented in green circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

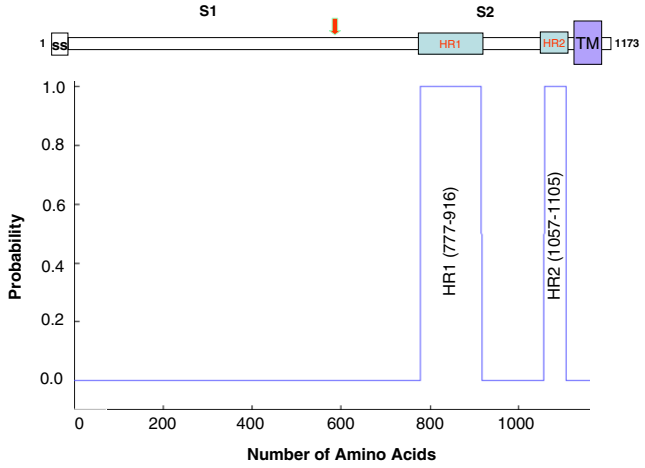

Fig. 2. Cartoon characterization of HCoV-229E fusion protein and prediction of the two heptad repeat regions. In the upper panel, schematic diagram of S protein (amino acids 1–1173) is shown in the upper panel. The cleavage site of S1 and S2 is indicated at a red arrow. SS, signal sequence; S1 and S2, two cleaved fragments of the fusion protein; HR, heptad repeat region; and TM, transmembrane region. In the lower panel, the likelihood of HR1 and HR2 predicted by LearnCoil-VMF program [39] is characterized. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

to 0.8–1.0, the culture was induced with 0.20 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, USA) and grown for another ~12 h at 16 °C untill the bacteria cells were harvested.

The harvested culture was centrifuged to collect the bacterial pellet. The pellet was re-suspended in the iced PBS and homogenized by sonication [12,29,36]. The lysate was centrifuged at 18,600 rpm for 20 min at 4 °C and subsequently filtered through 0.22 μm membrane for clarification [12,29,36]. Then the supernatant was loaded onto a glutathione-Sepharose 4B column (Pharmacia), and the GST-fusion protein was eluted by 20 mM reduced glutathione (Pharmacia). To obtain the GST-removed protein, the GST-3C rhinovirus protease (kept in our laboratory) was added into the resin, and then the mixture was incubated with gentle agitation for about 8 h at 4 °C. The target protein was eluted with 20 ml PBS.

Gel-filtration analysis. The target protein (2-Helix) loaded on a Superdex 75 column (Pharmacia) with an AKTA Purifier System (Pharmacia) after it was concentrated by ultra-filtration (10 kDa cut-off) and exchanged from PBS buffer into the exclusion buffer. The fraction of the peak was collected and analyzed by a 17% SDS–PAGE, and the molecular weight of the interested peak was estimated by comparison with the GST protein run on the same gel [12,29,36].

CD spectroscopic analysis. CD spectra were performed on a Jasco J-715 spectrophotometer in PBS buffer, and wavelength spectra were recorded in a 0.1 cm path-length cuvette at 25 °C.

Chemical cross-linking assay. The purified 2-Helix protein after the gel-filtration was dialyzed against cross-linking buffer (50 mM Herpes, pH 8.3; 100 mM NaCl) and concentrated to approximately 5 mg/L by ultra-filtration (10 kDa cut-off). The resultant proteins were performed to chemical cross-linking reaction with ethylene glycol bis-succinimidylsuccinate (EGS) (Pierce). The reactions were incubated for 1 h on ice at different concentrations of EGS, respectively (0.0, 0.2, 0.5, 1.0, 1.5, 2.0, and 5.0 mM EGS), and quenched with 50 mM glycine. Eventually, the cross-linked samples were analyzed by 17% SDS–PAGE [12].

3D structure modeling. The deduced amino acid sequence of the HCoV-229E fusion core was sent into the CPHmodels 2.0 Server and then was processed. Finally, the coordinates were used to generate the 3D structure of the HCoV-229E fusion core using the program of DeepView/SwissPdb-Viewer 3.7 (SPS) [12].

Results and discussion

Design of HCoV-229E fusion core

Although the similarity of S proteins of coronaviruses seemed to be not high in amino acid level (Fig. 1), the functional domains were extremely conserved (Fig. 2), which may in turn explain their similar membrane fusion mechanisms [8,12,28–30]. In view of the prediction of the LearnCoil-VMF program (Fig. 2) and the multiple

alignments of the putative heptad-repeat regions of HCoV-229E with those of several other coronaviruses (Fig. 3B), the final version of the HCoV-229E fusion core was determined (Fig. 3C). The fusion core consisted of HR1, a truncated type of the LearnCoil-VMF predicted HR1, connected with HR2, an extended version of the LearnCoil-VMF predicted HR2, by a flexible linker (SGGRGG). The fusion core designed here shared the feature of the

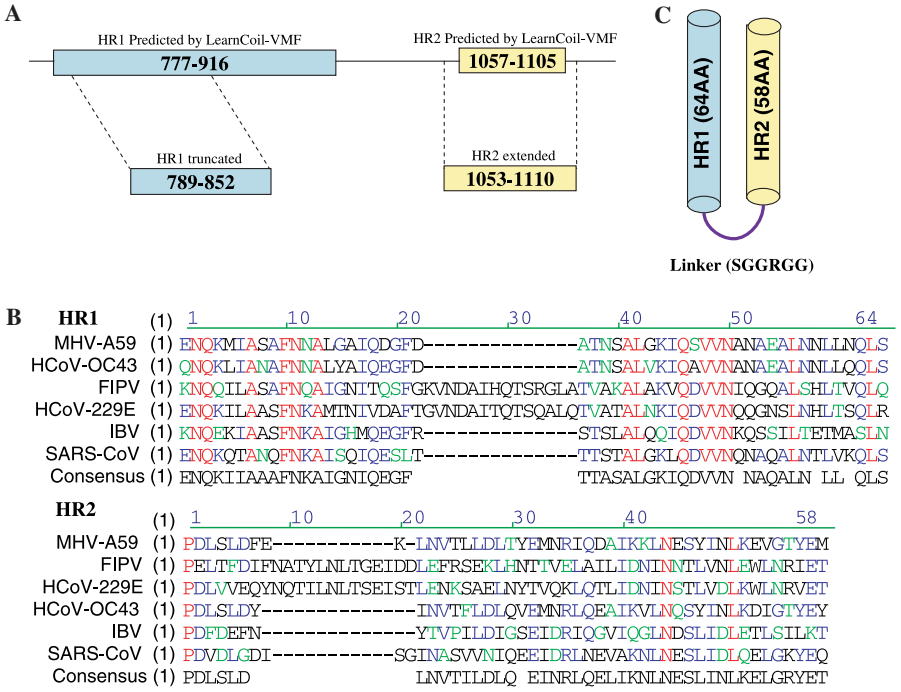

Fig. 3. Developmental strategy for HCoV-229E fusion core. (A) The modification of the final version of the HR1 and HR2 of HCoV-229E used here. (B) Multiple alignments of the final versions of HRs from some closely related coronaviruses selected. (C) Schematic representation of the HCoV-229E fusion core (2-Helix).

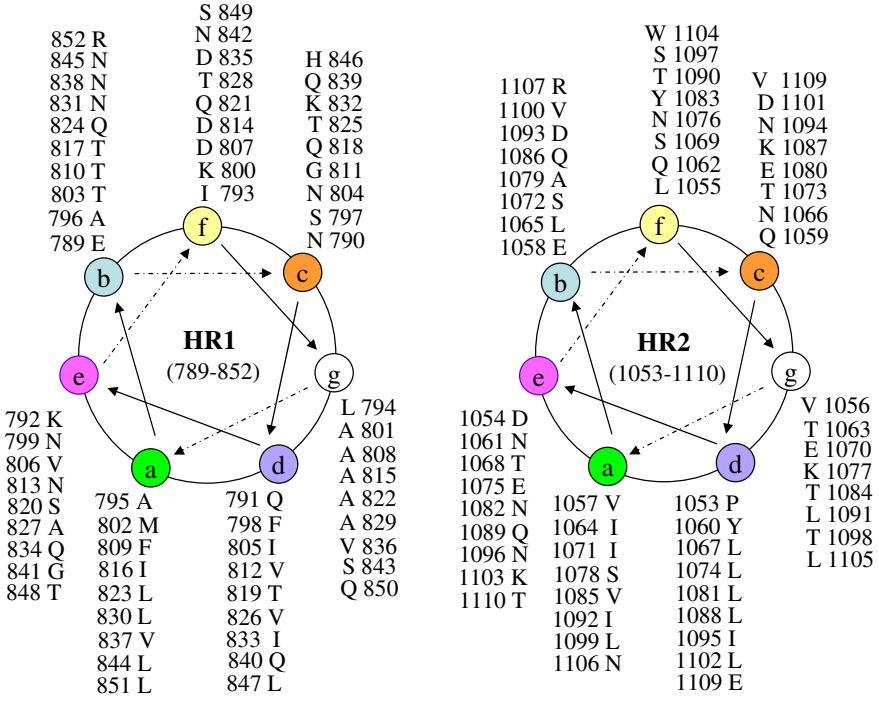

Fig. 4. Helical-wheel representation of the final type of HR regions of HCoV-229E fusion protein.

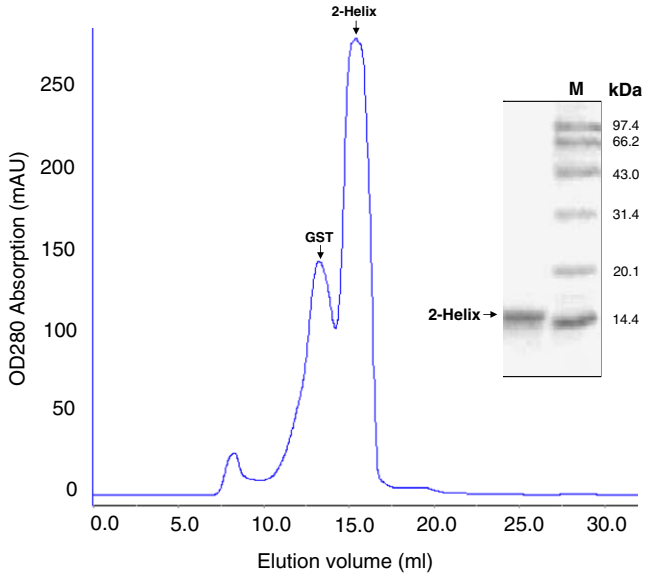

Fig. 5. FPLC profile of the purified HCoV-229E fusion core (2-Helix). M, standard protein marker. The first peak is formed by the GST dimer (52 kDa), and the inset picture is 17% SDS–PAGE analysis of the protein collected from the second peak. The profile of the HCoV-229E fusion core shows clearly it exists in a complex of about 40 kDa, implying it possibly forms a trimer.

helix wheels, a typical characteristic of class I fusion protein (Fig. 4).

Solubility of HCoV-229E fusion core

The HCoV-229E fusion core was synthesized using the overlapped PCR and then cloned into the prokaryotic expression vector, pGEX-6P-1, through the restriction sites (*Bam*HI and *Xho*I) introduced by PCR. Ideally, the fusion core should be 130 Å in length with an ideal molecular mass of about 15.0 kDa. Luckily, the GST-fused virus fusion core protein in the soluble form was observed in the supernatant of the bacteria lysate, and the GST-removed virus fusion core protein run on the 17% SDS–PAGE showed at the position of the expected size (Fig. 5). The availability of much soluble HCoV-229E fusion core protein made it possible to perform the subsequent experiments to classify and characterize HCoV-229E fusion core.

Rule followed by HCoV-229E fusion core

The purified HCoV-229E fusion core protein (2-Helix) was concentrated to10–15 mg/ml in the size exclusion buffer and analyzed by gel-filtration and chemical cross-linking for estimation of the molecular weight. As a result, the 2-Helix protein was eluted at the volume of ~16 ml which followed the position of GST dimmer (52 kDa) presented by the Superdex 200 Column (Fig. 5). Comparatively, the computed molecular mass of the 2-Helix protein was about 14.0 kDa, and then it indicated that the 2-Helix might form oligomers (~40 kDa). Subsequently, the chemical cross-linking experiment demonstrated that the 2-Helix protein oligomer to be a trimer (Fig. 6A) which can be described lively in the model picture (Fig. 6B), and at the same time, the transitional states (monomer and dimer) could be observed clearly on the condition that the concentration of the chemical cross-linker (EGS) was no less than 0.2 mM.

CD spectroscopic profile of the fusion core (2-Helix) presented an absorption curve of the typical alpha-helix

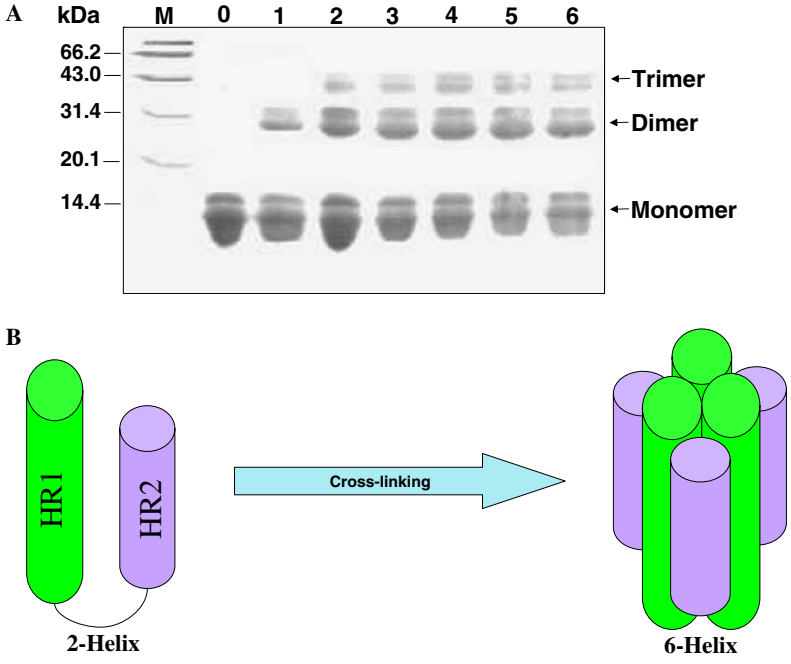

Fig. 6. Chemical cross-linking of HCoV-229E fusion core. (A) Cross- linked products are separated on 17% SDS–PAGE followed by Comassie brilliant blue staining. Protein marker (M) is shown in kDa. Lanes 0–6 indicate the different concentrations of the EGS (0, 0.2, 0.5, 1.0, 1.5, 2.0, and 5.0 mM, respectively). Bands corresponding to monomer, dimer, and trimer are indicated. (B) The cartoon depicting the schematic diagram of the cross-linking.

structure, with double minima at 208 and 222 nm (Fig. 7B), which verified that the result of the protein secondary prediction (Fig. 7A) was in a complete agreement with the previous data of other virus fusion cores [12,22]. Moreover, the thermodynamic experiment of the fusion core protein indicated that it could keep its advanced structure up to above 70 °C (not shown), indicating that the core structure of the post-fusion state of the HCoV-229E in coiled-coil bundle is extremely stable.

Finally, 3D structural modeling of the HCoV-229E fusion core was conducted with the program of Swiss-PdbViewer 3.7 (SPS), and showed lively the typical characteristic of the 2-Helix in molecular level (Fig. 8). The insights into 3D model of HCoV-229E fusion core did enrich the structure biology of coronaviruses and add another hard evidence to draw a conclusion that type I membrane mechanism is somehow common to Family *coronaviridae*.

In one word, the HCoV-229E fusion core actually existed in a 6-helix bundle, a trimer of heterodimer in vitro, which convinced us with the aid of biochemical and biophysical data including gel-filtration, chemical cross-linking, circular diagram (CD), 3D structural modeling, etc. It is convincing that HCoV-229E may adopt the type I

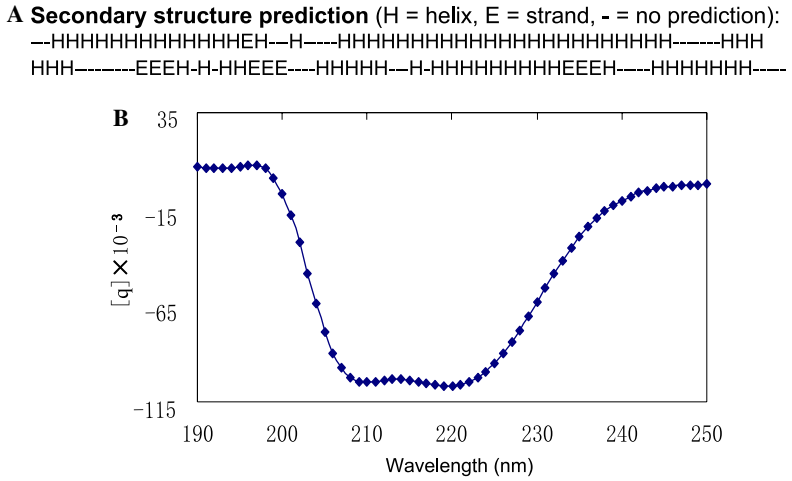

Fig. 7. Secondary structure of HCoV-229E fusion core. (A) Secondary structure prediction of HCoV-229E fusion core, indicating the high percentage of α-helix secondary structure. (B) Typical α-helix secondary structure with double minima at 208 and 222 nm, which is presented by CD experiment and in turn confirms the prediction result above.

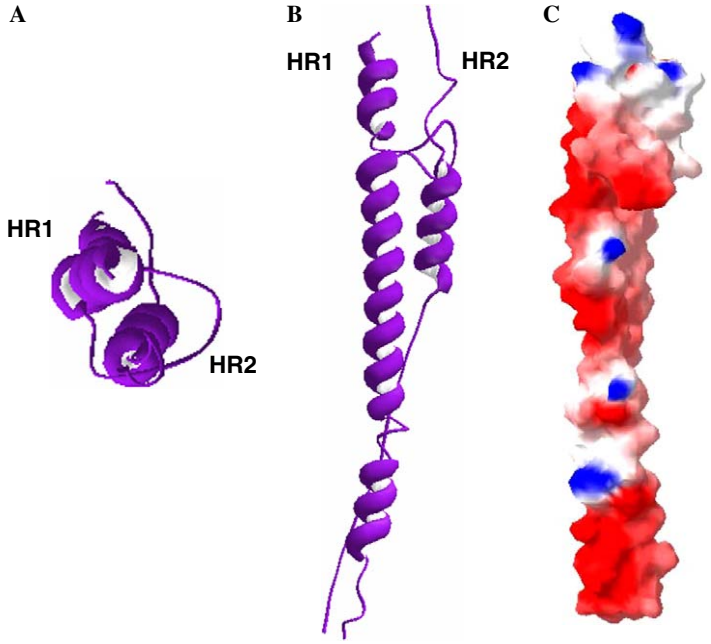

Fig. 8. Structure modeling of HCoV-229E fusion core. (A) Top view of the core structure in ribbon. (B) Front view of the core structure in ribbon. (C) Surface view of the core structure (blue, +; orange, −; others, low charge). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

membrane fusion mechanism to initiate the crucial step toward its successful infection into the host cells.

Feasibility in antiviral therapies

Like porcine transmissible gastroenteritis virus (TGEV), HCoV-229E is also a member of group 1 in Family *coronaviridae*, but different from both murine hepatitis virus (MHV), a representative of group 2 (Fig. 1). Importantly, HCoV-229E, a human pathogen, often leads to coryza, headache, cough, fever, disruption of nasal epithelium, and even causes severe lower respiratory tract infection, resulting in lots of agonies and becoming a big threat to human health worldwide [4,8]. Therefore, many virologists in the world have been prompted to develop the relevant investigations, such as the infection pathway, the pathogenic mechanism, the interaction between HCoV-229E and its host cell, etc. So far, nearly none of very nice ways can be available to effectively prevent or cure the HCoV-229E-related diseases. Thus, it seems to be extremely urgent and important to rationally design and eventually develop some bioengineered vaccines or drugs specific for the HCoV-229E-related diseases [1,2].

Just its following the membrane fusion rule of class I envelope viruses, common to those of both *retrovirus* and *paramyxovirus* [14–21,24–27], the therapeutics of HCoV-229E related diseases should also possess the potential likelihood of applying soluble HR1 and/or HR2. As we know that HR1 or its derivatives of NDV [23,32], HR2 or its derivative coming from HR2 (HIV, SARS-CoV, MHV, etc.) [18,19,24–26,29], and even both HR1 and HR2 of HRSV [8–10] can inhibit the membrane fusion during the virus entry, perturbing the successful infection of virus. Most excitingly, two small molecule drugs against HRSV fusion have been well-documented [33]. Thereby, it will be of interest to test whether HR1 or HR2 (including its equivalent derivative) has the inhibitory activity and to screen some small molecule drugs targeting for inhibition of membrane fusion mediated by HCoV-229E fusion protein in the near future. To our knowledge, it for the first time, provided the biochemical and biophysical basis of the HCoV-229E fusion core, indicating the essence of type I membrane fusion mechanism utilized by HCoV-229E, and also suggesting another novel direction to design the polypeptide or small molecule drugs, which perturbed HCoV-229E membrane fusion with its host cells, for the prevention and therapeutics of HCoV-229E related illness.

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