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2015

Liao, Y., Zhang, S. M., Neo, T. L., & Tam, J. P. (2015). Tryptophan-dependent membrane interaction and heteromerization with the internal fusion peptide by the membrane proximal external region of SARS-CoV spike protein. *Biochemistry*, 54(9), 1819-1830.

<https://hdl.handle.net/10356/106972>

<https://doi.org/10.1021/bi501352u>

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1 Tryptophan-dependent membrane interaction and
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3 membrane proximal external region of SARS-CoV
4 spike protein

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10

1 **ABSTRACT**

2
3 The spike (S) protein of severe acute respiratory syndrome-associated CoV (SARS-CoV)
4 mediates membrane fusion and viral entry. These events involve structural rearrangements,
5 including heteromerization between two heptad repeats (HR1 and HR2) to form a trimer-of
6 dimer as a six-helix-bundle (6-HB), a quaternary protein structure which brings two distant
7 clusters of hydrophobic sequences to proximity, the internal fusion peptide (IFP) preceding the
8 HR1 and the highly conserved tryptophan (Trp)-rich membrane proximal external region
9 (MPER) following the HR2. Here, we show that MPER can undergo self-oligomerization and
10 heteromerization with IFP, events that are Trp-dependent. To delineate the roles of Trp residues
11 of MPER in forming these quaternary structures and interacting with membranes, we employed a
12 panel of synthetic peptides: MPER peptide (M-wt) and its alanine (Ala) or phenylalanine (Phe)
13 analogs. Ala or Phe substitutions of Trp, particularly Trp1194Ala, inhibited its association to
14 cellular membranes. Chemical cross-linking experiments showed that M-wt can self-interact to
15 form oligomers and cross-interact with IFP23, a synthetic IFP peptide, to form a hetero-hexamer.
16 In comparison, little high-order oligomer was formed between M-wt and fusion peptide. The
17 specific interaction between M-wt and IFP23 was confirmed by immunofluorescence staining
18 experiments. In aqueous solutions, both M-wt and IFP23 displayed random secondary structures
19 which became helical in hydrophobic solvents. Triple-Ala substitutions of Trp in M-wt, but not
20 the corresponding triple-Phe analog, disrupted oligomerization of M-wt and hetero-
21 oligomerization of M-wt with IFP23. Overall, our results show that Trp residues of MPER play
22 a key role in maintaining the structure and functions of MPER, enabling it to interact with IFP to
23 form a MPER-IFP heteromer, a putative quaternary structure extending from the 6-HB and

function in membrane fusion. Finally, we showed that a MPER peptide could serve as an inhibitor in the entry process.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a contagious atypical pneumonia that caused an epidemic between November 2002 and July 2003 with a 9.6% mortality rate. A previous uncharacterized virus, SARS-associated coronavirus (SARS-CoV), was isolated from patients as the causative agent^{1,2}. The SARS-CoV genome contains a characteristic gene order of 5'-replicase, spike, envelope, membrane, nucleocapsid-3', and short untranslated regions at both termini³.

The spike (S) protein, a non-covalently associated trimer, is a stable quaternary protein structure protruding from the virion surface of coronavirus. It is responsible for viral attachment, fusion and entry into host cells. In SARS-CoV, the S protein becomes fusion competent as it is cleaved into S1 and S2 subunits at a.a.679 by cathepsin L^{4,5}. The S1 subunit mediates receptor binding and determines tropism⁶, whereas the S2 subunit mediates fusion of viral and cellular membranes^{7,8}. Two fusion peptides, FP (fusion peptide) comprising amino acid (aa) 770-778 and IFP (internal fusion peptide) comprising aa 856-888, are located at the N-terminus of S2 subunit, upstream from heptad repeat 1 (HR1) and heptad repeat 2 (HR2)⁹⁻¹². In the pre-fusion state, these fusion peptides are buried in the S protein. Upon receptor binding and cleavage, conformational changes in the S1 subunit and the dissociation of the S1 from the S2 subunit are thought to initiate two major rearrangements in the S2 trimer. Firstly, FP is exposed and then inserted into the target cell membrane, connecting the virus and the target cell. Secondly, HR2 folds back onto HR1 through complementary interaction between aa 916-950 of HR1 and aa 1151-1185 of HR2⁸, resulting in an anti-parallel 6-helix bundle (6-HB)^{7,13,14}. In turn, it facilitates the apposition of

1 virus and target cell membranes to sufficient proximity, allowing the mixing and fusion of lipid
2 bilayers, leading to virus entry.

3 The formation of a 6-HB is characteristic of a class I fusion protein, and the best understood
4 peptide-peptide interaction through helices in forming quaternary protein structures to facilitate
5 the fusion process. Subsequent events following the formation of 6-HB contributing to membrane
6 fusion remain to be elucidated in detail.

7 The FP located at the N-terminus of the S2 protein is known to be responsible for cell
8 membrane insertion ⁹. In contrast, the role of IFP, a hydrophobic segment following FP, is poorly
9 understood. Mutations in IFP have been shown to inhibit 70% of the S protein-mediated cell fusion
10 ¹⁵, but the mechanism of such an inhibition has not been clearly defined. In addition to FP and IFP,
11 the SARS-CoV S protein contains a third hydrophobic and membrane-active region, the membrane
12 proximal external region (MPER) ^{9-12, 16, 17}. The MPER, Y(V/I)KWPW(W/Y)VWL), located
13 between HR2 and the transmembrane (TM) domain of the S2 protein, is rich in aromatic amino
14 acid residues with three to four Trp (W) and two to three Tyr (Y) (Table 1). All coronavirus S
15 proteins share a highly conserved MPER ¹⁸⁻²⁰, and such sequence conservation is also found in
16 other viruses with class I fusion protein, such as human immunodeficiency virus type-1 (HIV-1)
17 ²¹, feline immunodeficiency virus (FIV) ²², influenza virus ²³, and Ebola virus ²⁴.

18 Previously, we have reported that Trp residues in MPER are essential for effective viral
19 infection through a mutational study ¹⁹. Single or multiple Ala substitutions of Trp residues in
20 SARS-CoV MPER abrogates the viral infection, whereas replacing the Trp residues by an aromatic
21 amino acid such as Phe partially restores the infectivity. Biophysical studies suggested that MPER
22 facilitates viral infection by perturbing lipid bilayers and inducing lipid mixing through peptide-

lipid interaction^{18-20, 25}. Synthetic peptides derived from SARS-CoV S protein MPER strongly partitioned into the liposomal membranes, perturbed membrane and caused content leakage^{10, 11, 17}. Similar studies of HIV-1 envelop protein and Ebola virus GP2 using MPER-derived peptides reached similar conclusions, supporting the ability of MPER to interact with lipid membranes^{24, 26}. Furthermore, MPER has been proposed to be structurally flexible. Synthetic MPER peptides derived from HIV envelope protein, Ebola virus GP2, and influenza virus HA protein have been shown to undergo secondary structural changes that include transitions from a predominant β -strand to an α -helix under different pH or when in contact with lipid membranes^{21, 23, 27, 28}. The structural plasticity of MPER together with our mutational study showing the important role played by the Trp residues in viral infectivity raise the possibility that MPER may have an additional partner of interaction, apart from the lipid membranes, in the fusion process. A potential interaction partner of MPER is the IFP, which is placed on the same side of 6-HB and in close proximity to MPER. A putative MPER-IFP complex through a peptide-peptide interaction would provide a quaternary structure that could facilitate the membrane fusion process.

Here we describe a structure-activity relationship study of MPER using a panel of synthetic peptides to define the roles played by its Trp residues in forming a putative new quaternary structure of MPER-IFP in the virus-membrane fusion process. Our results show the oligomerization of MPER and the peptide-peptide interaction with IFP through self and complementary interactions, respectively, and that these interactions are Trp-dependent. The Trp residues, particularly Trp1194, played important roles in maintaining the structural plasticity of MPER and in enabling peptide-lipid interaction with cellular membrane. Thus, the MPER-IFP complex may contribute to a quaternary protein structure serving as an extension to 6-HB to

facilitate the fusion of viral and host cell membranes. Lastly, we show that MPER-derived peptides displayed fusion inhibitory effect against viral entry.

EXPERIMENTAL PROCEDURES

Solid phase peptide synthesis. All amino acids and coupling reagents were purchased from Novabiochem (San Diego, CA, USA). Peptides were synthesized using the microwave peptide synthesizer by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry according to manufacturer's instruction. Non-biotinylated peptides were synthesized with Wang resin and C-terminal biotinylated peptides were synthesized with Fmoc-Lys(biotin)-Rink amide resin. Briefly, 0.1 mmol resin (0.2 mmol/g) (Louisville, KY, USA) was swelled in 5 ml dichloromethane (DCM) and subsequently in 10 ml dimethylformamide (DMF). For each coupling, Fmoc was removed by 20% piperidine in DMF, followed by the addition of 0.5 mmol Fmoc-protected amino acid. For hindered amino acids such as Fmoc-Arg(Pbf) and Fmoc-Pro, a double coupling procedure was performed. The Fmoc-amino acid was activated by pyBop (26 g per 100 ml DMF) and diisopropylethylamine (DIEA, 35 ml in 65 ml DMF). The coupling was performed under microwave condition. Final removal of side-chain protection groups and the cleavage of the peptide from the resin were performed with 10 ml reagent R [90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethylene thiol, and 2% anisole] with agitation for 3 hr. Resin was removed by filtration and the peptide in TFA was precipitated by 100 ml diethyl ether through centrifugation at 4000 rpm, 4°C for 10 min. The peptide was washed in diethyl ether for three times. All peptides were purified by HPLC and their molecular masses were confirmed by MALDI-TOF mass spectrometry.

Circular dichroism (CD) spectroscopy. CD spectroscopy analysis was performed to study the secondary structure of single peptide or combination of two peptides (M-wt/IFP23) in increasing trifluoroethanol (TFE) concentrations. TFE was used as a lipid mimetic. The induced environmental changes mimic the increase of local environmental hydrophobicity as experienced by membrane proximal sequences during the later stages of membrane fusion ²⁹. 1 mM peptide dissolved in water with increasing TFE concentrations was subjected to CD spectroscopy measurement using Chirascan CD spectrometer (Applied Photophysics). Cell of 0.1 mm path length (Hellma Uk Ltd.) were used for all the measurements at 25°C. Samples were measured between 190 nm and 240 nm, with a 0.1 nm step resolution, a measurement speed of 60 nm/min and a 1 nm bandwidth. Baselines were either water or the respective buffer solutions in which the peptides were dissolved in. At least four repeat scans were obtained for each sample and its respective baseline. The average baseline spectrum was subtracted from the average sample spectrum before the net spectrum was smoothed with a Savitsky-Golay filter. Secondary structure analyses were performed using CDNN program with the respective spectrum as input. The secondary structure contents were then plotted against TFE percentage used in the experiments.

Chemical cross-linking, Tricine SDS-PAGE, silver staining, and Western blot analysis. 1 mM peptide was incubated with 0, 1, or 5 mM glutaraldehyde at room temperature for 1 hr and was subjected to Tricine SDS-PAGE and silver staining or Western blot. Tricine SDS PAGE was performed as previously described ³⁰. For silver staining, gels were firstly fixed in fixing buffer (50% methanol, 10% acetic acid and 100 mM ammonium acetate) for 30 min, followed by washing with H₂O (double-distilled) twice for 30 min each. Gels were then sensitized in 0.005% sodium thiosulfate for 30 min, before being stained by 0.1% silver nitrate for 30 min. After washing with H₂O, gels were incubated in developer (0.036% formaldehyde, 2% sodium carbonate), and the

development process was stopped by incubating the gel in 50mM EDTA for 30 min. Western blot was conducted using Avidin-HRP (Cell Signaling, USA). Membranes were developed with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, USA) and exposed to X-ray film (Fuji). All procedures were performed under room temperature.

Immunofluorescence assay. Vero E6 cells were seeded on 4-well chamber slides at 50% confluence one day prior to experiment. 50 μ M peptides were applied to the cells and incubated at 37 °C for 30 min. Peptides were then removed and the cells were washed thrice in PBS (phosphate-buffered saline). The cells were then incubated for 30 min at 4 °C with both Alexa Fluor 594-conjugated CT-B (10 μ g/ml) and DylightTM 488-conjugated NeutravidinTM (vol./vol., 1:200 in PBS supplemented with 0.1% BSA, bovine serum albumin). Subsequently, the cells were incubated with rabbit anti-CT-B antibody (vol./vol., 1:50; 30 min, 4°C), which would initiate raft aggregation, making rafts visible as patches under confocal microscope. The cells were subsequently permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 0.1 μ g/ml DAPI in PBS for 10 min in the dark. The cells were fixed with 4% paraformaldehyde (15 min, 4°C). The specimens were mounted with glass cover slips using fluorescent mounting medium containing 15 mM NaN₃. The cells were washed thrice in PBS between incubations. Fluorescently labeled cells were analyzed with a Zeiss LSM 510 META laser scanning confocal microscope with 40 x objective lenses. The images were process using LSM 510 META software.

Biochemical isolation of lipid rafts. Cells ($2-5 \times 10^7$) were washed thrice with ice-cold PBS and lysed on ice for 30 min in 1 ml of 1% Triton X-100 TNE lysis buffer supplemented with complete protease inhibitor cocktail. The cell lysates were centrifuged for 5 min (4000 $\times$ g, 4 °C) to remove cell debris and nuclei. The supernatant was mixed with 1 ml TNE buffer with 80% sucrose, placed

1 at the bottom of the ultracentrifuge tube, and overlaid with 6 ml of 30% and 3 ml of 5% sucrose in
2 TNE buffer. The lysates were ultracentrifuged at 4 °C in SW41 rotor (Beckman) for 18 hr at 38000
3 rpm. After centrifugation, eleven 1-ml fractions were collected from the top to the bottom of the
4 tube and subjected to 16% Tricine SDS-PAGE or 10% glycine SDS-PAGE, followed by Western
5 blot using Avidin-HRP, anti-S antibody, or anti-caveolin-1 antibody.

6 **IBV-Luc preparation.** The egg-adapted Beaudette strain of avian infectious bronchitis virus (IBV)
7 (ATCC VR-22) adapted to Vero E6 cells was used in this study. IBV-Luc was constructed by
8 replacing 3a3b ORF with the firefly luciferase gene using an *in vitro* ligation protocol. The virus
9 was recovered from Vero E6 cells electroporated with *in vitro* transcripts generated from the full-
10 length IBV-Luc cDNA. Virus stocks were prepared from the infection of Vero cells with 0.1 PFU
11 of virus per cell and incubation at 37 °C for 24 hr. After three rounds of freeze and thaw cycles,
12 cell lysates were spun down at 3000× rpm. Aliquots of the supernatants were stored at -80 °C as
13 virus stock. Virus titers were determined by plaque assay with Vero cells, 1 PFU of virus per cell
14 was used to infect cells in experiment. Vero E6 cells were maintained in Dulbecco modified Eagle
15 medium (DMEM) supplemented with 10% fetal bovine serum and grown at 37°C in 5% CO₂.

16 **Virus inhibition assay and PrestoBlue cell viability assay on Vero E6 cells.** Vero E6 cells were
17 seeded in the 96 well plates one day before infection. Different concentrations of peptides were
18 dissolved in serum free DMEM. IBV-Luc was incubated with peptides for 1 hr at 37 °C, prior to
19 being added to the cells at 1 PFU. Virus and peptides were removed 1 hr post-infection. The cells
20 were incubated for another 20 hr and subsequently subjected to luciferase assay according to the
21 manufacturer's instructions (Promega). Briefly, upon removal of medium, the cells were rinsed in

PBS, lysed with 20 µl lysis buffer per well, and assayed by 100 µl of luciferase assay reagent per well. Percentage of infectivity is determined by luciferase activity.

The cytotoxicity effect of the peptides on Vero E6 cells were determined by PrestoBlue cell viability assay (Invitrogen), according to manufacturer's protocol. Briefly, peptides of different concentrations were added to Vero E6 cells seeded in 96-well plates. Upon 20 hr of incubation, Prestoblue cell viability reagent was added to cells and incubated for 10 min at 37°C. Resulting fluorescence signal was then read and recorded.

RESULTS

Design and synthesis of FP, IFP, MPER peptides and their Ala or Phe analogs. To provide mechanistic insight of our previous work using pseudo-typed SARS-CoV showing that mutating Trp to Ala or Phe in S protein MPER abrogated or diminished infectivity, respectively ¹⁹, we designed a panel of synthetic peptides derived from MPER, FP and IFP, all of which were synthesized by solid phase peptide synthesis (Table 2). The highly hydrophobic nature of the MPER peptide necessitated the inclusion of the upstream charged residues (KYEQ) in the MPER peptide as M-wt (KYEQYIKWPWYVWLGF) to increase its aqueous solubility and to facilitate its purification by reverse-phase HPLC. The Trp, Tyr or Phe residues (W1194, W1196, Y1197, W1199) in the M-wt analogs, M-W1194A, M-W1196A, M-Y1197A, M-W1199A, M-F1202A, M-3W3A, and M-3W3F, and F1202 were substituted by Ala or Phe individually or in combination, respectively. Synthetic peptides FP19, IFP23, and TE20 (see Table 2 for their sequences) were derived from FP¹⁶, IFP^{10, 11} and the region upstream to FP (aa 743-762), respectively. To facilitate product detection in chemical crossing experiments, all peptides were also synthesized with a C-terminal biotin tag and are designated with the letter 'b' following their names (e.g. M-wtb). M-

1 ibv was derived from the MPER of another CoV, avian infectious bronchitis virus (IBV) and
2 designed specifically for virus neutralization study.

3 **MPER peptides interaction with biological membranes and the role of its Trp residues.** To
4 examine the interaction between MPER-derived peptides and biological membrane in details, we
5 employed a live cell system using Vero E6 cells, which are susceptible to SARS-CoV infection.
6 In these experiments, MPER and its analogs were tagged with biotin. They included M-wtb, M-
7 3W3Ab, and a triple-Phe MPER analog, M-3W3Fb with all three Trp in MPER replaced by Phe.

8 Firstly, to study the cellular retention of MPER-derived peptides when applied
9 exogenously, M-wtb, M-3W3Ab, and M-3W3Fb were incubated with Vero E6 cells for 1 hr. Cells
10 and medium were harvested and the retained peptides were detected by immunoblots using avidin-
11 HRP. M-wtb and M-3W3Fb were readily detectable in cell lysate, suggesting cellular retention.
12 Meanwhile, triple Ala substations of M-wtb abrogated the effect (Figure 1). This suggest an
13 important role of aromatic side chains in the association between MPER and the cellular
14 membrane.

15 Immunofluorescence staining was then used to determine the location of the retained
16 MPER peptides. Positive signals were detected in Vero E6 cells treated with M-wtb and M-3W3Fb
17 (Figure 2A). Their signals partially co-localized with that of GM1, a lipid rafts marker. In
18 agreement with the cell-retention experiment, there was no detectable peptide signal in the M-
19 3W3Ab-treated cells.

20 Taken together, these results confirmed that MPER-derived peptide bound to biological
21 membranes, and that its aromatic residues are essential for the MPER-membrane interaction.

1 The immunofluorescence staining showed that M-wtb partially co-localized with the lipid
2 raft marker GM1. The N-terminal region of SARS-CoV MPER, specifically, 1187 KYEQYIK
3 1193, shares sequential homology with the cholesterol recognition amino acid consensus motif,
4 L/V-X(1-5)-Y-X (1-5)-R/K, in which X(1–5) represents one to five residues of any amino acid ³¹.
5 The motif was believed to result in a peptide or protein to preferential associate with cholesterol,
6 a principle component of lipid rafts.

7 We next examined if the exogenously applied M-wtb was preferentially recruited onto
8 plasma membrane lipid rafts. Lipid rafts were isolated successfully via membrane flotation assay,
9 as highlighted by the raft-associated protein caveolin-1 in fractions 2, 3 and 4 (Figure 2B).
10 Surprisingly, exogenously applied MPER peptide did not co-fractionate with caveolin-1 in
11 fractions 2, 3, and 4, but was detected in fractions 7, 8, 9, 10, and 11. These results suggest two
12 possibilities. First, lipid rafts and their associated proteins are not high-affinity binding targets for
13 the exogenously applied peptide M-wtb, which is a monomer. Second, the association requires
14 MPER in a quaternary structure complex, either as an oligomer or a hetero-oligomer with another
15 domain in the S protein. The second possibility was investigated in the following sections.

16 **MPER-MPER interaction and the role of Trp.** The SARS-CoV S protein interacts through
17 multiple domains, including self-binding of HR1 and HR2 in the prefusion state as a trimer and
18 complementary binding of HR1 and HR2 to form the 6-HB as a trimer-of-dimer in the fusion state.
19 As MPER is the region following HR2, we next investigated the interaction of SARS-CoV MPER
20 to form a quaternary structure similar to HR2, and the role of Trp residues in this process. Synthetic
21 MPER peptides were studied by *in vitro* chemical cross-linking experiments, and glutaraldehyde
22 used as a crosslinker to stabilize transient and weak non-covalent interactions through covalent

bonds. Without glutaraldehyde, M-wt produced a single band with a molecular weight corresponding to the monomer (2,200 Da), as SDS dissociates the noncovalently associated oligomers (Figure 3). With glutaraldehyde, M-wt peptide produced dimer, trimer, and high molecular-weight oligomers, suggesting that the MPER sequence possesses a self-interaction potential. The putative oligomer is termed MPER-MPER interaction in the following sections. Oligomeric formation in M-wt increased with increasing glutaraldehyde concentrations. Similarly, high molecular-weight oligomers were observed for the single-Ala-substitution analogs M-W1194A, M-W1196A, M-Y1197A, M-W1199A, and M-F1202A. However, no trimer or high-order oligomers were detected in the triple-Ala analog M-3W3A after cross-linking by glutaraldehyde. M-3W3F rescued this self-binding ability to form oligomers, suggesting that aromatic side chains are essential for MPER-MPER interaction. However, instead of a discrete order of oligomers, M-3W3F formed a continuous band of oligomers, suggesting an altered MPER-MPER interaction pattern upon triple Trp→Phe mutation.

MPER-IFP hetero-hexamer formation and the role of Trp in MPER-IFP interaction. Upon receptor binding that initiates the N-terminal FP (aa 770-788) inserting into the membrane and HR1/HR2 forming the 6-HB^{9, 16}, FP and IFP (aa 858-886) are positioned adjacent to MPER. We envisioned that their proximity may facilitate heteromerization between the N-terminal hydrophobic sequences of IFP and MPER. This hypothesis was investigated by *in vitro* chemical cross-linking of FP-, IFP-derived peptides, FP19 and IFP29, respectively, with MPER-derived peptides. Peptide TE20 (aa 743-762) was used as a negative control. FP19, IFP23, and TE20 were synthesized with a biotin tag at their C terminus to facilitate detection by Western blot, and are designated as FP19b, IFP29b and TE20b, respectively. Any signal detected by avidin-HRP in the Western blots represented FP19b, IFP23b, or TE20b, but not nonbiotinylated MPER peptides.

Without MPER peptides, FP19b and IFP23b formed mainly dimers with increasing concentrations of glutaraldehyde (Figure 4A), suggesting that the highly hydrophobic FP and IFP sequences could self-interact. TE20 failed to oligomerize even in 2.5 mM glutaraldehyde (Figure 4A). Upon mixing M-wt with IFP23b, predominant hetero-trimer was observed at 1 mM glutaraldehyde concentration. At 2.5 mM glutaraldehyde concentration, hetero-trimers, hetero-tetramers, and hetero-hexamers were observed (Figure 4A). However, FP19b and TE20b produced few high-order hetero-oligomers upon mixing with M-wt. Taken together, these results suggest MPER and IFP contain sequence specificity for complementary interactions, leading to the formation of hetero-hexamers. In contrast, such interaction was not observed between MPER and the other N-terminal hydrophobic region, FP.

To investigate the influence of Trp→Ala and Trp→Phe substitutions in the putative MPER-IFP interaction, M-3W3A and M-3W3F were separately cross-linked with IFP23b, FP19b or TE20b. As shown in Figure 4B, no oligomer was observed in IFP23b/M-3W3A, FP19b/M-3W3A, or TE20b/M-3W3A mixtures, indicating that M-3W3A could not produce high-order oligomers with FP19b, IFP23b, or TE20b peptides. Oligomers were observed in the IFP23b/M-3W3F mixture at 2.5 mM glutaraldehyde. However, they produced weaker bands than those observed for the IFP23b/M-wt mixture. FP19b and TE20b peptides remained in monomeric form in the presence of M-3W3F. These results demonstrate that Trp residues in MPER are important for MPER-IFP interaction. The Trp indole ring may play a role in maintaining the specific interaction between MPER and IFP, as it is not fully replaceable by the phenyl side chain of Phe.

In the previous section, we showed that the exogenously applied M-wtb was partially recruited onto plasma membrane lipid rafts through immunofluorescence staining. We next asked

whether MPER- and IFP-derived peptides could form non-covalent complexes when exogenously applied to Vero E6 cells, under a physiological-relevant condition, to facilitate the recruitment of IFP-derived peptides to cell membrane. IFP23b was applied to cells alone or in combination with M-wt, M-3W3A, or M-3W3F. Signal detected by immunofluorescence staining using DylightTM 488-conjugated NeutravidinTM represents IFP23b. When cells were incubated with IFP23b alone, only a small amount of peptides were detected and retained in Vero cells (Figure 4C). However, in cells treated with IFP23b/M-wt mixture, there was a substantial increase in the signal of IFP23b that was detected in the Vero cells (Figure 4C), with a pattern similar to those cells treated with biotinylated M-wt alone (Figure 2A). This result suggested an interaction between IFP23b and M-wt that enhanced the recruitment and retention of IFP23b to the Vero cells. Consistent with our previous experiments, similar IFP23b signal was observed in cells treated with IFP23b/M-3W3A or IFP23b alone, confirming the inability of IFP23b to bind to M-3W3A. In contrast, an increase IFP23b signal was observed in cells treated with IFP23b/M-3W3F, a level similar to that of those treated with IFP23b/M-wt, highlighting that M-3W3F recovered IFP23b binding ability. These results confirmed a potential interaction between MPER and IFP sequences that require the participation of Trp residues in MPER, both at the presence of cells and *in vitro* chemical crosslinking experiments.

Secondary structure of MPER peptides and the contribution of aromatic residues to their structural plasticity. MPER peptides in other Class-1 viruses (e.g. HIV-1) have been known to adopt either β -strand or α -helical secondary structure under different biophysical conditions (e.g. solvent hydrophobicity)^{32, 33}. Their structural plasticity raises the possibility of the existence of more than one structure relevant to viral entry. To investigate the structural plasticity and its determining residues in SARS-CoV MPER, CD spectroscopy was used to analyze the secondary

1 structures of synthetic M-wt and its Ala- or Phe-substituted analogs. The MPER is in close
2 proximity to membrane, and the formation of 6-HB in membrane fusion. Indeed, MPER-derived
3 peptides were capable of binding to cellular membranes, as demonstrated in the previous sections.
4 To include the effect of lipidic environment on MPER secondary structure, increasing
5 concentrations of TFE, a lipid mimetic, was added to the solvent in this study. As shown in Figure
6 5, M-wt predominantly adopted a β -sheet structure in aqueous conditions, and an increase in TFE
7 concentration to 40% prompted the peptide to adopt a more helical-like structure. M-3W3F bearing
8 triple W $\rightarrow$ F substitutions exhibited a similar secondary structural profile of M-wt, under both
9 aqueous and hydrophobic conditions (Figure 5).

10 Ala is a strong helix-former, and the single-Ala substituted analogs of Trp residues yielded
11 contrasting results. Compared to M-wt, the two mutant peptides M-W1196A and M-W1199A
12 exhibited similar structural changes in their CD profiles as solvent became increasingly
13 hydrophobic. However, M-W1194A underwent a more abrupt structural change from a random
14 coil conformation in H₂O to typical α -helix (double minima at 208 nm and 222 nm) in 20% TFE,
15 a conformation change persisted in 80% TFE. M-3W3A, containing triple Ala substitutions at all
16 three Trp positions, adopted a similar structural change profile as M-W1194A (Figure 5).

17 **Secondary structure of FP, IFP, and MPER-IFP mixture.** To study the conformational change
18 of IFP and FP during fusion, the secondary structures of FP19b and IFP23b were assessed by CD
19 spectroscopy in increasing concentrations of TFE. The addition of 20% TFE which increases
20 solvent hydrophobicity triggered IFP23b to adopt a primarily α -helical structure (Figure 6). In
21 contrast, no substantial change was observed in FP19b with increasing TFE concentrations. The
22 CD profile of the M-wt and IFP23b as an equimolar mixture (0.5mM/0.5mM) differed from the

theoretical CD profile averaged from the individual profiles of M-wtb and IFP23b, as the entire profile shifted 4nm to the left, suggesting that there is a weak and transient interaction between M-wt and IFP23b in solution.

Inhibition of virus infectivity by synthetic MPER peptide and the role of Trp. The ability of MPER in both complementary interaction (with IFP) and self-interaction raised the possibility that MPER-derived peptides could serve as viral entry inhibitors. In principle, both MPER and IFP are exposed during viral entry, and their association with a MPER-derived peptide could inhibit the progression of viral entry and infection. For safety reasons, the inhibitory activity of M-wt peptide was tested in neutralization assays using avian infectious bronchitis virus (IBV), instead of SARS-CoV. IBV contains the same MPER sequence and undergoes a synonymous fusion process as SARS-CoV. Specifically, the assay was conducted on recombinant IBV-Luc, which contains a firefly luciferase gene integrated at its ORF3a3b. Successful viral entry and subsequent replication of IBV-Luc was measured by intracellular luciferase activity. MPER peptide derived from IBV, M-ibv (LKTYIKWPWYVWLAIAF) was tested in parallel. Increasing concentrations of synthetic MPER peptides (0, 12, 25, 50 μ M) were mixed with IBV-Luc for 1 hr at 37°C and applied to Vero E6 cells seeded in 96-well plate at 1 PFU. Peptide/virus mixture was removed 1 hr post-infection. Cells were lysed 20 hr post-infection and luciferase activity was measured. As shown in Figure 7, M-ibv and M-wt inhibited IBV-Luc infection in a dose-dependent manner, with IC₅₀ values at 29 μ M and 26 μ M, respectively. M-3W3A was also tested in parallel. M-3W3A completely lost the viral neutralization ability. The observed differential effect of M-wt (inhibition) and M-3W3A (no inhibition) on virus infectivity suggests the specific binding of synthetic M-wt to S protein is a key factor in its anti-viral mechanisms.

To show that the MPER inhibition was not due to its toxicity effect, we determined its cytotoxicity on Vero E6 cells by the PrestoBlue assay. Fifty μ M of the peptides were incubated with Vero E6 cells for 24 hr, before the cell viability being assayed. Viability of cells treated with 50 μ M peptides were shown in Figure 7 B. No statistical difference between viability of control (untreated) cells and treated cells was observed.

DISCUSSION

MPER, a membrane-active region linking the external and transmembrane domains in S2 protein, is important for viral infection as shown in previous mutational studies (17). MPER sequences are highly conserved in *coronaviridae*, and highly enriched with aromatic residues, particularly Trp. How do the Trp residues in MPER contribute to the viral entry and membrane fusion process and, in turn, infectivity? Using a panel of synthetic MPER peptides, this study showed that the Trp residues are important for MPER to interact with cellular membranes. More importantly, they played essential roles in maintaining structural plasticity, self-interaction of MPER-derived peptides and inducing hetero-hexamer formation between MPER- and IFP-derived peptides during the viral entry process. In particular, the formation of MPER-IFP complex provides insights after the formation of 6-HB in the membrane fusion event.

Trp residues in MPER peptides mediate their interaction with membranes. Our study using Trp $\rightarrow$ Ala and Trp $\rightarrow$ Phe MPER analogs showed that Trp residues were essential in lipid mixing . This Trp-dependent effect was pronounced in the Trp $\rightarrow$ Ala analogs when the side-chain indole moiety of Trp was replaced by an alkyl moiety of Ala. In biological membranes, M-wt was found to be retained on Vero E6 cell membranes. In contrast, M-3W3A did not exhibit any binding to cell membranes. The phenotype was restored in the case of M-3W3F, where aromatic phenyl side

1 chains were introduced back to the peptide. These data suggest that the aromatic side chains in
2 MPER peptides are essential for maintaining their interaction in respect to attachment to biological
3 membranes. Structural analysis by CD spectroscopy further demonstrated that single Ala
4 substitution at W1194 as W1194A or triple Ala substitutions as M-3W3A resulted in these MPER
5 analogs losing its structural plasticity and prematurely adopting a helical structure in lipidic
6 environment. Coupling with the previous mutational and biophysical studies, our data suggested
7 that Trp residues in the S protein MPER are essential for membrane lipid mixing during membrane
8 fusion and viral entry, possibly through maintaining a flexible MPER ^{17, 19}.

9 Both Trp and Phe have aromatic side chains that are able to provide structural stability in
10 hydrophobic lipidic environment. However, the indole ring side chain of Trp potentiates a larger
11 and more intense interaction through their π electrons, as compared to the phenyl side chain of Phe.
12 The indole nitrogen of Trp could function as a weak base and hydrogen bond donor, leading to
13 hydrogen bonds formed with the lipid head groups ³⁴. This could lead to the differential membrane
14 partitioning ability of Trp and Phe, when they are placed at juxtamembrane positions ³⁵. Whereas
15 Trp prefers interfacial position and serves as a ‘buoy’ to adjacent transmembrane domain, due to
16 its balanced attraction towards and repulsion from membrane; Phe is hydrophobic enough to sink
17 into and reside in the hydrophobic core of lipid bilayer with the transmembrane segment ^{18, 34, 36-}
18 ³⁸. The subtle difference between Trp and Phe might have disorganized the orchestrated fusion
19 process and contributed to a substantial loss of viral infectivity when single W→F substitution at
20 critical position (e.g. W1194) was introduced into MPER of the pseudo-typed SARS-CoV ¹⁹,
21 although the M-3W3F and M-wt peptides differed little in structural plasticity and the membrane
22 binding capability.

MPER-derived peptides self-oligomerized and formed hetero-hexamer with IFP-derived peptide. At the pre-fusion state, S proteins exist as trimers through self-interactions between domains on neighboring proteins, such as the HR2. To examine if MPER could exist as a structural extender of trimerized HR2 at the prefusion stage, we investigated the oligomerization ability of MPER-derived peptides. Ala and Phe analogs were examined in parallel to unveil the participation of Trp in this process. Interactions between shorter peptides tend to be weak and unstable, a persistent challenge in the deconstruction approach using peptides to examine protein-protein interactions. The potential interaction between MPERs was stabilized and observed through the chemical crosslinker glutaraldehyde, which captures transient interactions as covalent complexes. M-wt peptide produced dimer, trimer, and high molecular-weight oligomers starting from 1mM glutaraldehyde. No trimer or high order oligomers were detected in the triple-Ala mutant M-3W3A, whereas the single Ala substitution at W1194, W1196, and W1199 did not significantly affect the oligomerization. Together, these data suggested that M-wt oligomerization is maintained by multiple aromatic amino acids, and the loss of aromaticity at a single position is insufficient to disrupt oligomerization.

A possible motif that could explain the requirement of aromatic residues in maintaining MPER-MPER interaction is an aromatic amino acid zipper (AAAZ). The zipper motifs have previously been proposed to exist among aromatic amino acids including Trp, Phe and Tyr, to stabilize peptide or protein structures and induce oligomerization³⁹. Engineered cross-strand Trp-Trp pairs were shown to induce stable β -hairpin peptides, where the indole ring side chains of Trp residues were stacked perpendicularly forming a 'Trp zipper'⁴⁰. Subsequently, 'Trp zipper' motif were either discovered or introduced in proteins to stabilize protein quaternary structure^{41, 42}. Potentially, the oligomerization of MPER peptides could be facilitated by the hydrophobic interaction (e.g. π - π

1 interaction) between aromatic residues on neighboring peptide. The interdigitating stacking of
2 their aromatic ring side chains would generate a zipper-like motif, or AAAZ motif, to facilitate
3 peptide-peptide interaction. Thus, removing aromatic side chains by Ala substitution in W1194,
4 W1196 and W1199 deter the AAAZ formation. In contrast, substitutions of Trp with Phe, another
5 aromatic amino acid could maintain the AAAZ motif, and hence the peptide oligomerization.
6 Furthermore, AAAZ motif is likely sustained by multiple aromatic amino acids, and the loss of
7 one aromatic amino acid at a single position would unlikely disrupt the motif, as shown by our
8 mutational study with single Trp→Ala substitution.

9 As viral entry begins, the HR2 trimer dissociates and folds back onto the central HR1 as a
10 coiled-coil 6HB through complementary binding. Upon the formation of the 6-HB, hydrophobic
11 and membrane-active segments upstream of HR1 (e.g. FP and IFP) and the segments downstream
12 of HR2 (e.g. MPER and TM) come into close proximity, potentiating their interactions. Two
13 groups have used protein engineering to construct a part or the entire ectodomain of gp41 HIV
14 containing HR1 and/or HR2. They showed that the FPPR (fusion peptide proximal region) and
15 MPER come together as continuous helices, extending the trimer-of-trimer structure of the 6-helix
16 bundle ^{43, 44}. The FPPR of gp41 HIV is the equivalent of IFP of SARS-CoV. In our study,
17 crosslinking experiments stabilized and revealed a peptide-peptide interaction between synthetic
18 peptides M-wt and IFP23b, but not between M-wt and FP19b. An advantage of using
19 glutaraldehyde is that the cross-linking reaction is generally mediated by the imine formation with
20 the N-terminal amines. As such, the self-interaction of M-wt or IFP23b is likely through their N-
21 terminal amines and occurs in parallel fashion ⁴³. This weak and transient interaction between M-
22 wt and IFP23b was further observed in the CD spectra as the equimolar mixture adopted a new
23 secondary structural profile (Figure 6).

1 The putative MPER-IFP interaction was further confirmed in a physiologically relevant
2 condition, as the peptides were co-applied to Vero E6 cells and examined through
3 immunofluorescence staining. Indeed, more IFP23b was recruited to and retained on the cell
4 membrane, upon the addition of M-wt. We concluded that the membrane-active M-wt bound to
5 Vero E6 cell membrane and interacted with IFP23b, indirectly enhanced the retention of IFP23b.
6 The ability of MPER to bind and recruit IFP to cell membrane lipid without membrane lysis may
7 be of interest in drug design (see results in using MPER as inhibitor).

8 Amino acids W1194, W1196, and W1199 are also involved in M-wt-IFP23 hetero-
9 hexamer formation. Chemical crosslinking and immunofluorescence staining showed that triple
10 Ala substitution of Trp in M-wt abolishes M-wt-IFP23 interaction, which is partially recovered by
11 Ala→Phe substitution. The putative interaction between MPER and IFP may serve as a continuum
12 of a quaternary protein structure to the 6-HB in providing membrane-interacting surfaces and a
13 low energy barrier path for lipid flow and membrane fusion. Unlike the Leu/Ile zipper of HR1
14 and HR2, the sequence mediating MPER-IFP interaction is short and the interaction is unlikely to
15 be stable.

16 **Structural flexibility of MPER and IFP, and the role of three Trp residues.** In agreement with
17 studies by others, we showed that synthetic MPER and IFP derived from SARS-CoV S protein are
18 structurally flexible. They transit from a predominantly β -strand conformation to α -helix in lipidic
19 environmental lipid. MPER may undergo conformational changes, transiting from a β -sheet
20 conformation in its pre-fusion state when it is solvent-exposed, to a helical structure in its post-
21 fusion state which is solvent-shielded and a more hydrophobic environment in the presence of
22 membrane lipids. Similarly, structural flexibility of MPER peptides were also observed in HIV

gp41, Ebola virus GP2, and influenza virus HA protein and is believed to be essential for the conformational transition of fusion proteins^{21, 23, 27, 28}.

A structural basis for the structural flexibility of SARS-CoV S protein in our MPER peptides may be due to their N-terminal sequence KYEQYIK, residues composed of alternating hydrophobic and hydrophilic residues. Such residues constitute a motif favoring β -strand formation. Previous studies have shown that hydrophilic residues (K1187, E1189 and K1193) with a motif of alternating charge distribution (+ - +) can adopt a helix or β -strand arrangement upon environmental changes (e.g. pH or hydrophobicity changes)⁴⁵. The observed hydrophobicity-triggered β -strand to α -helix structural transition for MPER is likely attributed to its flexible N-terminus. The C-terminus (WPWYVWLGF) of MPER is composed of eight hydrophobic residues, five of which are aromatic amino acids (W1194, W1196, Y1197, W1199 and F1202). In aqueous condition, the bulky aromatic residues, especially W1194, of the MPER C-terminus serve as helix-breaker, constraining the peptide into β -sheet conformation.

Of the three Trp in MPER, W1194 appears to play a more important role than W1196 and W1199 in maintaining the structure flexibility. Substituting W1194 with Ala rendered the synthetic MPER analog to prematurely adopt helical structure in lipidic environment. W1194 is the Trp residue adjoining the KYEQYIK segment and Ala is a strong helix former. This combination likely facilitates the α -helical formation of M-W119A. Thus, Trp $\rightarrow$ Ala substitution may render MPER prematurely to a helical structure, hampering proper conformational change and subsequently preventing the putative quaternary intermediate formation. Loss of virus infectivity pseudotyped with S mutants (Ala substitutions of Trp in MPER) may be attributed to this explanation¹⁹.

Synthetic MPER peptide as virus entry inhibitor. MPER has been reported to be an exposed region harboring epitopes for broadly neutralizing antibodies and regarded as desirable target for antiviral therapeutics for its accessibility and sequential conservation⁴⁶⁻⁵¹. Indeed, the inclusion of MPER sequences has been proven essential for both first- and second-generation fusion inhibitors against HIV-1. Similarly, an octapeptide derived from FIV MPER has been shown to act as a mild entry inhibitor^{52, 53}. In virus inhibition assays, both peptides derived from IBV M-ibv and SARS-CoV M-wt inhibited IBV-Luc entry and infection. M-3W3A totally loses the virus neutralization ability. M-wt peptide may play multiple roles when applied to both the virus and human cells. It may bind to S2, interfering with its conformational change, perturbs cell membrane integrity to facilitate fusion or to reduce viral viability.

CONCLUSION

In conclusion, our study on SARS-CoV MPER agrees with our previous work on MPER and those in Class I virus fusion glycoprotein (eg. HIV, EboV). The dominant factor in maintaining MPER diverse roles is its Trp-rich motif, which enables it to self-bind, form a high order complex with IFP, and perform membrane mixing function. Synthetic MPER showed anti-viral activity and has an added advantage in its water solubility compared to fusion peptides, a useful feature for developing a bioactive peptide as a fusion inhibitor.

FIGURE LEDGENDS

Figure 1 Cell binding ability of M-wt and its Ala substitution analogs. 50 μ M M-wtb, M-3W3Ab, or M-3W3Fb was incubated with Vero E6 cells for 30 min. Cells and medium were harvested and subjected to 16% Tricine SDS-PAGE and Western blot using Avidin-HRP.

Figure 2. Cell membrane binding by MPER peptides and the role of Trp residues. A) Subcellular location of M-wt and its analogs. Vero E6 cells were treated with 50 μ M M-wt-b, M-3W3Ab, or M-3W3Fb for 30 min and subjected to immunofluorescence staining with Alex Fluor 594-conjugated CT-B (lipid raft marker GM1 staining, red), DylightTM 488-conjugated NeutravidinTM (biotin staining, green), and DAPI (nucleus staining, purple). M-wt attached onto and translocated into the cells, while its triple Ala-substituted analog M-3W3A lost the cell penetration ability. Triple Phe-substitution in M-wt partially rescued the phenotype. B) Exogenously applied M-wt_b did not show preferential location to lipid rafts. Vero E6 cells were pretreated with 50 μ M M-wt_b for 30 minutes and were lysed on ice in 1% Triton X-100 TNE lysis buffer. Lipid raft fractions were then isolated from post-nuclear cell extracts through membrane flotation assay. 11 fractions were collected from top to bottom after centrifugation and subjected to 16% Tricine SDS-PAGE (for detection of M-wt_b) and 10% Glycine SDS-PAGE (for detection of caveolin-1). Western blot was performed using Avidin-HRP or anti-caveolin-1 antibody.

Figure 3. Importance of Trp residues in self-interaction of MPER peptides. 1 mM M-wt, M-3W3A, M-3W3F, M-W1194A, M-W1196A, M-Y1197A, M-W1199A, or M-F1202A were incubated with 0, 1, or 5 mM glutaraldehyde at room temperature for 1 hr. Samples were subjected to 16% Tricine SDS-PAGE and silver staining.

Figure 4. Formation of M-wt–IFP23 hetero-hexamer and the participation of Trp residues in its interaction. (A) Formation of hetero-hexamer by M-wt and IFP23b. 1 mM M-wt was mixed with FP19b, IFP23b, or control peptide TE20b, at equimolar concentration. Mixtures were incubated with 0, 1, 2.5 mM glutaraldehyde at room temperature for 1 hr and subjected to 16% Tricine SDS-PAGE and Western blot using avidin-HRP. (B) The role of Trp residues in the formation of M-

wt-IFP23 hetero-hexamer. 1 mM peptide M-3W3A or M-3W3F were mixed with FP19b, IFP23b, or control peptide TE20b, at equimolar concentration. The mixtures were incubated with 0, 1, 2.5 mM glutaraldehyde at room temperature for 1 hr and subjected to 16% Tricine SDS-PAGE and Western blot using Avidin-HRP. (C) M-wt enabled the translocation of IFP23b into cell cytoplasm through M-wt-IFP23 interaction. Vero E6 cells were treated with IFP23b, M-wt/IFP23b, M-3W3A/IFP23b, or M-3W3F/IFP23b for 30 min and subjected to immunofluorescence staining with Alex Fluor 594-conjugated CT-B (lipid raft marker GM1 staining, red), Dylight™ 488-conjugated Neutravidin™ (IPF-biotin staining, green), and DAPI (nuclear staining, purple).

Figure 5. CD spectra of synthetic MPER peptides. 1 mM M-wt, M-W1194A, M-W1196A, M-W1199A, or M-3W3A were dissolved in H₂O supplemented with increasing concentrations of lipid mimetic TFE (0%, 20%, 40%, 60%, or 80%), and were subjected to CD spectroscopy measurement at 25°C. Secondary structure analyses were performed using the CDNN program.

Figure 6. CD spectra of FP19b and IFP23b, and M-wt/IFP23b mixture. FP19b adopted β -sheet structure while IFP23b adopted increasingly α -helical structure when dissolved in lipidic solutions. 1 mM FP19b or IFP23b were dissolved in increasing concentrations of TFE (0%, 20%, or 40%) and subjected to CD spectroscopy measurement at 25°C. Secondary structure analyses were performed using the CDNN program. The CD profile of the M-wt and IFP23b equimolar mixture (0.5 mM/0.5 mM) dissolved in ddH₂O was examined with CD spectroscopy and analyzed in a similar fashion.

Figure 7. M-ibv and SARS-CoV M-wt inhibited IBV-Luc infection in Vero E6 cells. **A)** IBV-Luc were mixed with increasing concentrations (0, 12.5, 25, 50 μ M) of M-ibv, M-wt, M-3W3A, or M-3W3F for 1 hr at 37°C and applied to Vero E6 cells at 1 PFU. Virus and peptides were removed 1

hr post-infection. Cells were incubated at 37°C for another 20 hr, before being lysed and subjected to luciferase assay. Percentage of infectivity was calculated based luciferase activity ($n = 3$, mean $\pm$ S.E.). IC₅₀ was calculated using GraphPad Prism software. B) Cytotoxicity effect of MPER-derived peptides was tested on Vero E6 cells with PrestoBlue™ Cell Viability Reagent (Life Technologies), according to manufacturer's protocol. Briefly, 50 μ M M-wt, M-3W3A or M-3W3F was incubated with 10,000 Vero cells for 24 hr. PrestoBlue™ cell viability reagent was subsequently added to the cells, and absorbance at 570 nm and 600 nm was measured.

TABLES

Table 1. Sequential alignment of S protein MPER from members of *coronaviride*

Group I	HCoV-229E	Y	I	K	W	P	W	W	V	W	L
	PEDV	Y	I	K	W	P	W	W	V	W	L
	HCoV-NL63	Y	I	K	W	P	W	W	V	W	L

Group II	TGEV	Y	V	K	W	P	W	Y	V	W	L
	FCoV	Y	V	K	W	P	W	Y	V	W	L
	FIPV	Y	V	K	W	P	W	Y	V	W	L
	PRCoV	Y	V	K	W	P	W	Y	V	W	L
	CCoV	Y	V	K	W	P	W	Y	V	W	L
	MHV	Y	V	K	W	P	W	Y	V	W	L
	HCoV-OC43	Y	V	K	W	P	W	Y	V	W	L
	BCoV	Y	V	K	W	P	W	Y	V	W	L
	RCoV	Y	V	K	W	P	W	Y	V	W	L
	HEV	Y	V	K	W	P	W	Y	V	W	L
	SDAV	Y	V	K	W	P	W	Y	V	W	L
	HKU1	Y	V	K	W	P	W	Y	V	W	L
	CRCoV	Y	V	K	W	P	W	Y	V	W	L
	ECoV	Y	V	K	W	P	W	Y	V	W	L
	MERS-CoV	Y	N	K	W	P	W	Y	V	W	L
	SARS-CoV	Y	I	K	W	P	W	Y	V	W	L
	Bat-SARS-CoV	Y	I	K	W	P	W	Y	V	W	L
Group III	IBV	Y	I	K	W	P	W	Y	V	W	L
	TCoV	Y	I	K	W	P	W	Y	V	W	L
	PCoV	Y	I	K	W	P	W	Y	V	W	L
	DCoV	Y	I	K	W	P	W	Y	V	W	L

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3 **Table 2.** Design and sequences of synthetic peptides derived from CoV S protein

Designation	Virus	Region (a.a. position)	Sequence
M-wt	SARS-CoV	MPER (1187-1202)	KYEQYIKWPWYVWLGF
M-W1194A	SARS-CoV	MPER (1187-1202)	KYEQYIKAPWYVWLGF
M-W1196A	SARS-CoV	MPER (1187-1202)	KYEQYIKWPAYVWLGF
M-Y1197A	SARS-CoV	MPER (1187-1202)	KYEQYIKWPWAVWLGF
M-W1199A	SARS-CoV	MPER (1187-1202)	KYEQYIKWPWYVALGF
M-F1202A	SARS-CoV	MPER (1187-1202)	KYEQYIKWPWYVWLGA
M-3W3A	SARS-CoV	MPER (1187-1202)	KYEQYIKAPAYVALGF
M-3W3F	SARS-CoV	MPER (1187-1202)	KYEQYIKFPFYVFLGF
M-ibv	IBV	MPER (1186 -1202)	LKTYIKWPWYVWLAI AF
FP19	SARS-CoV	FP (770-788)	MYKTPTLKYFGGFNFSQIL
IFP23	SARS-CoV	IFP (866-888)	AGWTFGAGAALQIPFAMQMAYRF
TE20	SARS-CoV	aa 743-762	TQLNRALSGIAAEQDRNTRE

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Funding Sources

This research was supported by the Competitive Research Grant by National Research Foundation in Singapore (NRF-CRP8-2011-05)

ABBREVIATIONS

CoV, coronavirus; SARS, severe acute respiratory syndrome; S protein, spike protein; MPER, membrane proximal external region; FP, fusion peptide; HR, heptad repeat; TM, transmembrane domain; IBV, Avian infectious bronchitis virus; HIV-1, human immunodeficiency virus type-1; FIV, feline immunodeficiency virus; CD, circular dichroism; DMEM, Dulbecco modified Eagle medium; AAAZ, aromatic amino acid zipper.

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Figure 1

Figure 2

Figure 3

A.

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C.

Figure 4

Figure 5

Figure 6

A.

B.

Figure 7

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