1 **Biochemical analysis of coronavirus spike glycoprotein conformational intermediates**

2 **during membrane fusion**

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21 **Abstract**

22 A fusion protein expressed on the surface of enveloped viruses mediates fusion of the viral

23 and cellular membranes to facilitate virus infection. Pre- and post-fusion structures of viral

24 fusion proteins have been characterized, but conformational changes between them remain

25 poorly understood. Here, we examined the intermediate conformation of the murine

26 coronavirus fusion protein, called the spike protein, which must be cleaved by a cellular

27 protease following receptor binding. Western blot analysis of protease digestion products

28 revealed that two subunits (67 and 69 kDa) are produced from a single spike protein (180

29 kDa). These two subunits were considered to be by-products derived from conformational

30 changes and were useful for probing the intermediate conformation of the spike protein.

31 Interaction with a heptad repeat (HR) peptide revealed that these subunits adopt packed and

32 unpacked conformations, respectively, and two-dimensional electrophoresis revealed a

33 trimeric assembly. Based on biochemical observations, we propose an asymmetric trimer

34 model for the intermediate structure of the spike protein. Receptor binding induces the

35 membrane-binding potential of the trimer, in which at least one HR motif forms a packed-

36 hairpin structure, while membrane fusion subunits are covered by the receptor-binding

37 subunit, thereby preventing the spike protein from forming the typical homotrimeric pre-

38 hairpin structure predicted by the current model of class I viral fusion protein. Subsequent

39 proteolysis induces simultaneous packing of the remaining unpacked HRs upon assembly of

40 three HRs at the central axis to generate a six-helix bundle. Our model proposes a key

41 mechanism for membrane fusion of enveloped viruses.

42

43 **Importance**

44 Recent studies using single-particle cryo-electron microscopy (cryoEM) revealed the

45 mechanism underlying activation of viral fusion protein at the priming stage. However,

2

46 characterizing the subsequent triggering stage underpinning transition from pre- to post-

47 fusion structures is difficult because single-particle cryoEM excludes unstable structures that

48 appear as heterogeneous shapes. Therefore, population-based biochemical analysis is needed

49 to capture features of unstable proteins. Here, we analyzed protease digestion products of a

50 coronavirus fusion protein during activation; their sizes appear to be affected directly by the

51 conformational state. We propose a model for the viral fusion protein in the intermediate

52 state, which involves a compact structure and conformational changes that overcome steric

53 hindrance within the three fusion protein subunits.

54

Introduction

Class I viral fusion proteins drive the fusion of viral and cellular membranes to facilitate

insertion of the viral genome into the host cytoplasm (1). Structural analyses including cryo-

electron microscopy (cryoEM) and X-ray crystallography revealed pre- and post-fusion

structures, and biochemical analysis led to the proposal of a unified model for conformational

changes of viral fusion proteins; receptor binding primes the formation of a membrane-

embedded homotrimeric pre-hairpin structure that bridges viral and cellular membranes, then

folds back on itself to form a hairpin structure, drawing the viral and cellular membranes into

close proximity, resulting in lipid mixing (1–3). The post-fusion form consists of a central N-

terminal trimeric α-helical coiled-coil (trimer of heptad repeat 1, HR1) surrounded by three

C-terminal helices (HR2), generating a six-helix bundle (6HB) (1–3).

The molecular rearrangements occurring at the early stages of the fusion process

underpinning the receptor-binding step in the fusion protein of human immunodeficiency

virus-1, influenza virus, and coronavirus were revealed by high-resolution cryoEM (4–13).

However, the dynamic rearrangements underpinning the transition from pre- to post-fusion

structures remain largely uncharacterized. During membrane fusion in influenza virus and

herpes simplex virus, V- or Y-shaped density was observed in the intermediate state by

cryoEM (14, 15), and in the retroviral Env trimer, an asymmetric intermediate appeared to

emanate from a single protomer in cryo-electron tomography experiments (16). For the

Moloney murine leukemia virus Env protein, the sequential release of the surface subunit

from the transmembrane subunit induces the formation of an asymmetric trimer (17).

Adoption of an asymmetric conformation presumably overcomes steric hindrance within the

three fusion protein subunits.

The coronavirus spike (S) glycoprotein is a class I viral fusion protein constructed of

S1 and S2 subunits. The N-terminal S1 subunit is responsible for receptor binding, and the C-

80 terminal membrane-anchored S2 subunit is important for virus-cell membrane fusion. Some

81 coronaviruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle

82 East respiratory syndrome coronavirus (MERS-CoV), and mouse hepatitis virus type 2

83 (MHV-2), possess uncleaved 180 kDa S proteins. These viruses utilize cell surface or

84 endosomal proteases (such as TMPRSS2, HAT, trypsin, elastase, or cathepsin L) to cleave S

85 proteins during cell entry (2, 18–31). It remains controversial whether MERS-CoV S protein

86 is processed by the cellular protease furin after internalization via endocytosis (32) because

87 this finding was not supported by a recent study (33).

88   Our previous study showed that the S protein of MHV-2 requires a two-step

89 conformational change process (26). The first step takes place after binding to a soluble form

90 of the MHV receptor (CEACAM1a), upon which the metastable form of S protein is

91 converted to a stable trimer possessing liposome-binding activity. The subsequent step is

92 driven by protease digestion. From a single 180 kDa S protein, trypsin produces a 90 kDa

93 subunit in the absence of receptor, but a 66 kDa subunit in the presence of receptor, and this

94 66 kDa species is thought to involve the formation of a 6HB structure (detected as a

95 proteinase K-resistant 53 kDa subunit) (26). A similar result of the protease digestion pattern

96 was also reported in SARS-CoV and MERS-CoV S proteins (4, 20). Therefore, the sizes of

97 protease digestion products are thought to be directly affected by the conformational state of

98 the coronavirus S protein. Studying the MHV-2 S protein could illuminate the conformational

99 changes occurring in each step, and may provide novel insight into viral class I fusion

100 protein.

101

102 **Materials and methods**

103 **Virus, cells, soluble receptor, and HR2-mimicking peptide**

104   MHV-2 was propagated in DBT cells cultured in Dulbecco's modified Eagle's medium

105   (DMEM) containing 10% tryptose phosphate broth (BD Difco, USA) (34). Viruses were

106   collected at 21 h post-infection and stored at -80°C. The soluble form of the MHV receptor

107   (CEACAM1a) was produced using recombinant baculovirus and purified as previously

108   described (35). HR2-mimicking peptide

109   (DLSLDFEKLNVTLLDLTYEMNRIQDAIKKLNESYINLKE) was provided by B. J. Bosch

110   (36) and dissolved in water at a concentration of 500 μM.

111

112   **Proteases and protease inhibitors**

113   Trypsin (T8802; Sigma, USA), proteinase K (166-21051; Wako, Japan), endoproteinase arg-

114   C (P6056; Sigma), and endoproteinase lys-C (P3428; Sigma) were employed after being

115   dissolved in phosphate-buffered saline (PBS; pH 7.4). Inhibitors camostat (3193; Tocris

116   Bioscience, UK), Pefabloc SC (11429868001; Roche, Switzerland), and E64d (330005;

117   Calbiochem, USA) were dissolved in dimethyl sulfoxide (DMSO). Soybean trypsin inhibitor

118   (STI; T-9128; Sigma) was dissolved in PBS.

119

120   **Liposomes**

121   Lipids l-phosphatidylcholine (PC; egg; Avanti-Polar Lipids, USA), l-

122   phosphatidylethanolamine (PE; egg; Avanti-Polar Lipids), sphingomyelin (Sph; brain;

123   Avanti-Polar Lipids), and cholesterol (Chol; Sigma) were mixed in a 1:1:1:1.5 molar

124   PC:PE:Sph:Chol ratio, dried under N_2 gas in a glass tube, and lyophilized overnight. After

125   addition of 1 ml of PBS (pH 7.2), the lipid suspension was vortexed and extruded 25 times

126   through a 0.4 μm Nucleopore filter (GE Water & Process Technologies, USA) using an

127   Avanti Mini-Extruder. Liposomes were stored at 4°C and used within 1 week.

128

129 **Activation of MHV-2 spike (S) protein and western blot analysis**

130 ***Standard reaction.*** To evaluate conformational changes in the S protein occurring in the first

131 step, a 10 μl solution of MHV-2 (10^7 pfu/100 μl) was mixed with 1.1 μl of soluble receptor

132 (10 μM) and warmed at 37°C for 30 min. For conformational changes in the second step, 1.2

133 μl of trypsin (100 μg/ml) was added and incubated at 37°C for 30 min. A 1/4 volume of

134 sample buffer comprising 30% glycerol, 250 mM TRIS pH 6.8, 2.5% sodium dodecyl sulfate

135 (SDS), a small amount of Bromophenol Blue, 100 mM dithiothreitol (DTT), and 1 mM

136 Pefabloc SC was added to the reaction and boiled at 100°C for 5 min. Samples were

137 separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on

138 3−10% gradient or 7.5% gel (e-PAGEL; ATTO, Japan), transferred to a polyvinylidene

139 difluoride (PVDF) membrane (Immobilon-P; Millipore, USA), and soaked in ImmunoBlock

140 (CTKN001; DS Pharma Biomedical, Japan) for 5 min. Western blot analysis was carried out

141 using anti-S2 antibodies, a mouse monoclonal antibody recognizing the 10G epitope (MAb-

142 10G), and rabbit anti-peptide antibodies recognizing the S2a region, the very highly

143 conserved region (VHCR), and cytoplasmic tail (CT) epitopes (anti-S2a, anti-VHCR, and

144 anti-CT, respectively), followed by horseradish peroxidase-conjugated anti-mouse (32430;

145 Thermo, USA) or anti-rabbit (sc-2054; Santa Cruz Biotech, USA) IgG. Immunoreactive

146 bands were visualized with an enhanced chemiluminescence kit (ECL; RPN2232; GE

147 Healthcare, USA) and a LAS-3000 instrument (Fuji, Japan). All experiments were repeated at

148 least twice.

149 ***Reactions in the presence of liposomes.*** A 6 μl sample of liposomes was added, and the

150 volume of receptor and trypsin was raised to achieve the target concentrations. Standard

151 reactions were then carried out as described above.

152 ***Timing of heptad repeat (HR) packing.*** To stop the reaction at the indicated time points,

153 reactions were quickly frozen in dry ice/methanol, and 1.4 μl of HR2-mimicking peptide (500

7

154   µM) was added. Samples were further incubated at 37°C for 20 min to facilitate the formation

155   of 67 and 69 kDa fragments.

156   ***Generation of the proteinase K-resistant 53 kDa fragment.*** After assessing conformational

157   changes of the S protein as described above, reaction mixtures were chilled on ice for 5 min

158   and 1.5 µl of proteinase K (10 mg/ml) was added and incubated on ice for 30 min.

159   ***Deglycosylation of the S2 subunit.*** After assessing conformational changes of the S protein

160   as described above, deglycosylation was carried out using Protein Deglycosylation Mix

161   (P6039S; NEB, UK) according to the manufacturer's instructions.

162   ***SDS-PAGE of unboiled samples (native PAGE).*** After assessing conformational changes of

163   the S protein as described above, sample buffer excluding DTT was added and unboiled

164   mixtures were separated by SDS-PAGE (3−10% gradient or 7.5% gel; e-PAGEL).

165   ***Protein denaturation on PVDF membranes.*** After SDS-PAGE of unboiled samples and

166   electro transfer to a PVDF membrane, initial detection of native S protein was performed by

167   western blot analysis. The membrane was then soaked in stripping buffer (46428;

168   ThermoFisher, USA) at room temperature for 5 min to denature the S protein, rinsed 10 times

169   with rinse buffer (20845; Millipore), blocked with ImmunoBlock, and re-probed with anti-S2

170   antibody.

171   ***Two-dimensional SDS-PAGE.*** S protein was treated with receptor and trypsin as described

172   above, mixed with sample buffer containing molecular size markers (1610373; BioRad,

173   USA) without DTT, and separated by SDS-PAGE using a 3−10% gradient gel (first gel).

174   After electrophoresis, the gel was wrapped in a heat-seal bag, soaked in sample buffer (0.5%

175   SDS), boiled at 105°C for 5 min in an autoclave, sliced along the protein markers, placed

176   onto a 7.5% gel (second gel), and subjected to electrophoresis and western blot analysis.

177

178   **Generating trypsin-treated MHV-2 harboring cleaved S protein**

179   A solution of MHV-2 (500 µl; 10^7 pfu/100 µl) was mixed with 5 µl of trypsin (100 µg/ml) or

180   PBS (for uncleaved control) and incubated at 37°C for 1 h. Next, 5 µl of STI (10 mg/ml in

181   PBS) and 5 µl of camostat (1 mM in PBS) were added and incubated at room temperature for

182   10 min to inactivate trypsin. About 500 µl of virus solution was applied to a 2 ml bed volume

183   of Sephadex G-75 (17005101; GE Healthcare) equilibrated with PBS (column size, 10 ml)

184   (7311550; BioRad, USA). PBS (1.4 ml) was loaded onto the column, and eluent (200 µl

185   fractions) was collected. Western blot analysis was carried out using MAb-10G to identify

186   fractions containing MHV-2. Fractions 3 and 4 were used for the experiments shown in

187   Figure 2.

188

189   **Virus cell entry assay**

190   DBT cells in a collagen-coated 96-well culture plate (4860-010; Iwaki, Japan) were treated

191   with DMEM containing 10 µM E64d cathepsin inhibitor at 37°C for 30 min to block the

192   endosomal virus entry pathway. Approximately 10^5 pfu of virus was used to infect 10^5 cells

193   on ice. After a 30 min adsorption on ice, virus was removed and cells were treated with

194   various concentrations of trypsin. After a 30 min incubation, viral entry was stopped by

195   adding DMEM containing 10 µM camostat and 10 µM E64d, and incubated at 37°C for 5 h.

196   Cellular RNA was isolated from cells with the addition of 200 µl of Isogen (311-02501;

197   Nippon Gene, Japan). Real-time PCR was performed to estimate the amount of newly

198   synthesized viral mRNA7 as described below.

199

200   **Quantitative estimation of viral mRNA by real-time PCR**

201   Real-time reverse transcription PCR was performed to estimate the amount of MHV-2

202   mRNA7 as described previously. The target sequence was the MHV-2 N gene. Hybridization

203   probes labeled with fluorescent dye, 5'-GCTCCTCTGGAAACCGCGCTGGTAATGG-3'

9

204 (3'-labeled with fluorescein isothiocyanate) and 5'-

205 ATCCTCAAGAAGACCACTTGGGCTGACCAAACC-3' (5'-labeled with LCRed640),

206 were used to detect the amplified fragments. To amplify viral mRNA7, oligonucleotides 5'-

207 GTACGTACCCTTTCTACTC-3' (MHV-2 leader) and 5'-CAAGAGTAATGGGGAACCA-

208 3' (MHV-2 mRNA7 reverse) were employed. PCR analysis involved reverse transcription at

209 61°C for 20 min, followed by PCR with an initial denaturation at 95°C for 30 s, followed by

210 40 cycles at 95°C for 5 s, 55°C for 15 s, and 72°C for 13 s. Reactions were performed using a

211 LightCycler Nano instrument (Roche). The amount of virus in cells was calculated from the

212 calibration curve.

213

214 **Electron microscopy**

215 A 6 μl aliquot of ultraviolet-irradiated virus was absorbed onto glow-discharged 300-mesh

216 heavy-duty carbon-coated Cu grids (Veco grids; Nisshin EM, Tokyo, Japan) for 2 min, and

217 the excess was blotted onto filter paper (Whatman; GE Healthcare, Piscataway, NJ, USA).

218 Grids were then washed twice with Milli-Q water and negatively stained with 2%

219 phosphotungstic acid. Data were collected using an HT7700 transmission electron

220 microscope (Hitachi, Tokyo, Japan) operating at 100 kV electrons and magnification of

221 30,000×.

222

223 **Statistical analysis**

224 Two-tailed Student's t-tests were used to analyze statistical significance. A p-value <0.05

225 was considered statistically significant. n.s. = not significant, * = significant ($p \leq 0.05$), ** =

226 highly significant ($p \leq 0.01$), *** = very highly significant ($p \leq 0.001$). Error bars indicate

227 standard deviation (SD).

228

10

229   **Results**

230   **Trypsin digestion generates three different sizes (90, 69, and 67 kDa) of S2 subunit**

231   A schematic diagram of the MHV-2 S protein and the binding sites of antibodies employed in

232   this study is shown in Figure 1A. Three anti-synthetic peptide antibodies, anti-S2a, anti-

233   VHCR, and anti-CT, and a monoclonal antibody (MAb-10G) were used to detect the S2

234   subunits via western blot analysis. To probe conformational changes of the MHV-2 S protein,

235   an authentic virus rather than recombinant S protein was employed. Virus at 10^6 plaque-

236   forming units (pfu) in 10 µl of culture medium was treated with the soluble form of the MHV

237   receptor (1 µM) at 37°C for 30 min, and 10 µg/ml trypsin was added and incubated for 30

238   min. The main difference between previous (26) and present experiments is the concentration

239   of trypsin (10 µg/ml trypsin was used instead of 1 µg/ml trypsin).

240   As reported previously (26), trypsin-mediated cleavage of the 180 kDa S protein (Fig.

241   1B, 10G and CT, lane 1) generated a species of 90 kDa (Fig. 1B, 10G and CT, lane 2) in the

242   absence of receptor. However, two different S2 subunit fragments (67 and 69 kDa) were

243   observed following trypsin-mediated cleavage in the presence of receptor (Fig. 1B, 10G and

244   CT, lane 4). The 67 kDa subunit was observed for the first time when a higher concentration

245   of trypsin (10 µg/ml) was used in the present study. The sizes of these S protein subunits are

246   revised from the previous study; the 200 kDa full-length S protein was revised to 180 kDa,

247   the 80 kDa S2 subunit was revised to 90 kDa, and the 66 kDa S2 subunit was revised to 69

248   kDa (26).

249   The appearance of the 90, 67, 69, and 53 kDa species was assessed at different trypsin

250   concentrations. The 90 and 69 kDa species were detected following treatment with 0.25 or

251   0.5 µg/ml trypsin (Fig. 1C). In addition, a 67 kDa S2 subunit was observed when the

252   concentration of trypsin was higher (≥1 µg/ml; Fig. 1C). Interestingly, both 67 and 69 kDa

253   subunits were observed, even at the highest concentration of trypsin (128 µg/ml), which is

254   ~100-fold higher than needed to induce conformational changes of S protein according to a

255   previous report (26), suggesting that a heterogeneous mix of S2 subunits (67 and 69 kDa) was

256   stably produced from a single (180 kDa) S protein species. Treatment with 1 mg/ml

257   proteinase K was carried out to probe the post-fusion conformation that is thought to involve

258   the formation of a 6HB structure (26). As shown in Figure 1C, a proteinase K-resistant 53

259   kDa band was observed when the concentration of trypsin was ≥0.125 μg/ml (lane 6).

260

261   Quantification of virus cell entry triggered by trypsin

262   The trypsin concentration required to induce virus cell entry was assessed. After virus

263   adsorption for 30 min on ice, cells were treated with various concentrations of trypsin at 37°C

264   for 30 min, and trypsin was then inactivated by addition of the serine protease inhibitor

265   camostat mesylate. After a 5 h incubation at 37°C, cellular RNA was isolated and real-time

266   PCR was performed to quantify viral subgenomic mRNA7. The concentration of trypsin

267   required to induce viral cell entry was ≥0.25 μg/ml (Fig. 1D), which corresponds

268   approximately with the appearance of the 69 and 53 kDa fragments (Fig. 1C), but not the 67

269   kDa fragment.

270

271   Comparison of uncleaved and pre-cleaved S proteins

272   As reported previously, the MHV-2f variant of MHV-2 harbors an amino acid substitution at

273   the S1/S2 cleavage site that is cleaved by furin during biogenesis; this variant is sufficient to

274   induce the cell-cell fusion without supplemental trypsin in the culture medium (29). To

275   clarify whether cleavage at the S1/S2 site is sufficient for S protein priming prior to induction

276   of conformational changes, we compared trypsin-treated MHV-2 and MHV-2f with MHV-2.

277   To generate trypsin-treated MHV-2, the MHV-2 was exposed to 1 μg/ml trypsin (or PBS for

278   the uncleaved virus control) for 1 h, followed by addition of trypsin inhibitors (both STI and

279   camostat). Next, the viruses were purified using a Sephadex G-75 column. Figure 2A shows

280   that, as reported previously (29), half of the S protein of MHV-2f was cleaved (lane 9)

281   whereas almost all of the S protein expressed by the trypsin-treated virus was cleaved (lane 5)

282   (compare with PBS-treated MHV-2 S protein (lane 1)). The trypsin-treated virus was able to

283   enter cells as efficiently as the PBS-treated virus (Fig. 2B). Next, the viruses were exposed to

284   both receptor and trypsin to induce conformational changes. A higher concentration of

285   trypsin (50 µg/ml) was needed to cleave the S protein, presumably due to residual trypsin

286   inhibitors. Although background levels of the 67 and 69 kDa products were observed in the

287   absence of receptor (lane 6) (presumably due to the use of pre-incubated viruses), clear

288   production of a 67 kDa fragment was observed in the presence of receptor and trypsin (lane

289   8). Interestingly, induction of the proteinase K-resistant 53 kDa band from the trypsin-treated

290   virus was observed in the presence of receptor, even in the absence of trypsin (lane 7); by

291   contrast, the other two viruses (PBS-treated MHV-2 and MHV-2f) required both receptor and

292   trypsin. This implies that the two types of cleaved S protein have different conformations: the

293   pre-cleaved S protein (cleaved by furin during biogenesis) needs additional cleavage after the

294   receptor-binding step, whereas the trypsin-treated S protein (cleaved on virus particle) needs

295   only receptor binding to trigger the conformational changes. Cleavage at the S2' site (to

296   generate the 69 kDa species) may trigger conformational changes, as reported previously (20,

297   30); however, cleavage at either the S1/S2 site or the S2' site is presumably enough to

298   activate the MHV-2 S protein. The 67 and 69 kDa species can be considered by-products of

299   the S protein following conformational changes; therefore, these products were examined to

300   probe the intermediate conformation(s) of the S protein.

301

302   **Characterization of a 67 kDa S2 subunit**

303   As identified in previous studies using MHV and SARS-CoV, the 90 and 69 kDa subunits are

304   derived from cleavage at the S1/S2 site (R756) and the S2' site (R907) (11, 19, 27, 28, 30,

305   31). To identify the trypsin cleavage site that produces the 67 kDa fragment, we attempted to

306   gain indirect evidence because the 67 kDa subunit has not been successfully purified from the

307   virus particle for amino-terminal sequencing. Both the 67 and 69 kDa subunits were detected

308   by MAb-10G and anti-CT, but not by anti-S2a, even following increased exposure of blots,

309   and only the 69 kDa subunit was detected by anti-VHCR (Fig. 1B). These results indicate

310   that the 69 kDa subunit is a product of cleavage between S2a and VHCR epitopes at the S2'

311   site, as previously reported (11, 30), whereas the 67 kDa subunit is the result of cleavage at

312   the C-terminal side of the VHCR epitope. Using endoproteinases arg-C and lys-C to identify

313   the specific arginine or lysine residue of the cleavage site, the 67 kDa subunit was found to be

314   cleaved at a lysine residue (Fig. 3A).

315   Next, deglycosylation of the S protein subunit was carried out using a commercial

316   deglycosylation mix containing five enzymes that can completely remove *N*- and *O*-linked

317   glycans from almost all glycoproteins except some plant and insect glycoproteins. The 90, 69,

318   and 67 kDa bands were shifted to 65, 47, and 43 kDa, respectively, following treatment with

319   0.32 or 32 μg/ml trypsin (Fig. 3B). The 4 kDa size difference between 43 and 47 kDa

320   deglycosylated bands indicates that the 67 kDa subunit is cleaved at K951 because the

321   calculated molecular weight of the peptide fragment between S2' (R907) and K951 is 4.8

322   kDa, whereas cleavage at the neighboring lysine K917 would yield a 1.3 kDa size difference,

323   and cleavage at K1008 would afford a 10.6 kDa size difference. The K951 cleavage site was

324   therefore designated postS2'. Notably, the postS2' cleavage site is in the 48th β-strand (β48),

325   which forms an anti-parallel β-sheet with β47 in the pre-fusion form but not in the post-fusion

326   form of the S protein (10, 11). Cleavage at postS2' is unlikely to contribute to S protein

327   triggering because it is located at the C-terminal side of the fusion peptide (FP; Fig. 4A and

14

328   4B), and the appearance of the 67 kDa species does not correspond to the formation of the

329   post-fusion structure (53 kDa) or virus entry (Fig. 1C and 1D), as described above.

330   Additional experiments were carried out to characterize the 67 kDa subunit. The

331   receptor concentration was found to affect the total amount of 67 and 69 kDa species, but not

332   the relative ratio between them (Fig. 3C), implying that two different cleavable features of the

333   S2 subunit were induced in the receptor-binding step. Furthermore, inclusion of dithiothreitol

334   (DTT) at concentrations ≥1 mM in the sample buffer shifted the 67 kDa band to a greater

335   extent than the 69 and 90 kDa bands during SDS-PAGE (Fig. 3D). Two cysteine residues at

336   positions 1169 and 1214 (Fig. 4A and 4B), between the HR1 and HR2 motifs (in the

337   connector region), were predicted to affect the conformation of the 67 kDa subunit.

338   Meanwhile, cysteine residues 932 and 943, located upstream of HR1, and residues 1119 and

339   1130 within the invariant motifs may affect the smaller shift observed for the 69 and 90 kDa

340   bands. The connector region, including the disulfide bond, presumably contributes to folding

341   of the 67 kDa subunit, but not to that of the 69 and 90 kDa subunits.

342

343   **Two different sizes of S2 subunit are present in the trimer**

344   We next turned our attention to how two different sizes of S2 subunit (67 and 69 kDa) could

345   be produced by trypsin digestion of a single 180 kDa S protein species, even though a single

346   sized (90 kDa) species is produced in the absence of receptor. We hypothesized that the S

347   protein forms a heterogeneous trimer in the presence of receptor, so that each S protein in a

348   trimer exposes a different cleavage site. In the first native SDS-PAGE step (gels were boiled

349   before blotting so that the trimer could be detected by the antibody, as described in the

350   methods), several different sizes of trimer were detected (Fig. 5A, lanes 2 and 3). In the

351   second denaturing SDS-PAGE step, these trimer bands were separated into monomer bands

352   (Fig. 5A, lanes 5 and 6). To examine the components of the trimer, we performed two-

353   dimensional electrophoresis. Following treatment with 10 μg/ml trypsin, both the 67 and 69

354   kDa bands, or the single 67 kDa band, were separated from three species of trimer at around

355   140 kDa (Fig. 5B and 5C b), whereas only the 69 kDa band was detected following treatment

356   with 0.32 μg/ml trypsin (Fig. 5C a). These results suggest that a subset of S2 subunits can

357   form a heterogeneous trimer comprising the 69 and 67 kDa species at a ratio of 3:0, 2:1, 1:2,

358   or 0:3.

359

360   **Exposed or buried epitope configurations in the S protein globule**

361   Further characterization of S2 subunits was performed to investigate exposed and buried

362   configurations of epitopes, and the results are shown in Figure 6A and 6B. MHV-2 was

363   treated with receptor and trypsin to induce conformational changes as described above. S

364   protein detection was performed using four anti-S2 antibodies recognizing linear epitopes.

365   Following soaking in stripping buffer to denature the S protein on the polyvinylidene

366   difluoride (PVDF) membrane, detection was then repeated with the same antibodies. When

367   antibodies against the VHCR and CT epitopes were used, all bands detected in denatured

368   blots (Fig. 6B) were the same as those observed in native blots (Fig. 6A), indicating that these

369   epitopes are exposed on the S protein in both the native and denatured states. In the post-

370   fusion form of the S protein treated with receptor and trypsin, doublet bands at 140 kDa were

371   observed using anti-CT antibody (Fig. 6A, CT, lane 4), while lower bands were not observed

372   with the anti-VHCR antibody (Fig. 6A, VHCR, lane 4). This observation correlates with the

373   appearance of 67 and 69 kDa bands in SDS-PAGE (Fig. 1B, CT and VHCR, lane 4),

374   suggesting that the upper band of 140 kDa species is composed of 67 and 69 kDa subunits,

375   whereas the lower band comprises three 67 kDa subunits. This is consistent with the results in

376   Figure 5C b showing that the 67 and 69 kDa bands were separated from 140 kDa trimer

377   bands.

378   Using anti-S2a antibody recognizing a linear epitope (Fig. 1B, S2a) (37), a 500 kDa

379   band corresponding to a trimeric assembly was observed only after the receptor-binding step

380   in native blots (Fig. 6A, S2a, lane 3), as observed in a previous study (26), but this was barely

381   detectable in denatured blots (Fig. 6B, S2a, lane 3). We do not currently have an explanation

382   for exposure of the S2a epitope, but this hydrophobic epitope is buried in the globule in the

383   pre-fusion state (10) and is presumably exposed only in the native trimer after the receptor-

384   binding step, before disappearing following cleavage by trypsin. When antibody recognizing

385   the 10G epitope was used, monomeric S2 subunit species were detected in the native blot

386   (Fig. 6A, 10G, lanes 3 and 4), but trimeric species were not observed, although they were

387   detectable in the denatured blot (Fig. 6B, 10G, lanes 3 and 4). This indicates that the 10G

388   epitope adopts a buried configuration in the trimer of pre- and post-fusion forms. These

389   observations provided clues that were used to predict the intermediate structure of the S2

390   subunit.

391

392   **The timing of S protein cleavage**

393   We next analyzed the timing of the appearance of 67, 69, and 53 kDa fragments during

394   conformational changes of the S protein. To stop the reaction after different incubation times

395   at 37°C, reaction tubes were quickly frozen in dry ice/methanol, and immediately boiled in

396   sample buffer containing the trypsin inhibitor. As shown in Figure 7A, trypsin treatment on

397   ice for 5 min following receptor treatment was sufficient to generate the 69 kDa but not the

398   proteinase K-resistant 53 kDa species (lane 2). The 53 kDa band was induced after a 1 min

399   incubation at 37°C (lane 3), and the 67 kDa band appeared and became prominent over 4 min

400   (lane 5), indicating that the conformational changes that induce the 6HB structure are

401   complete at 1 min after treatment with trypsin. In preliminary EM experiments (described

402   below), conformational changes of the S protein were probed in the presence of liposomes.

403 The appearance of S2 fragments (90, 69, and 67 kDa) and the timing of cleavage coinciding

404 with their appearance were the same in the absence and presence of liposomes (Fig. 7B and

405 7C).

406

407 **Unpacked (accessible) and packed (occluded) HR conformations**

408 To characterize the conformational states of 67 and 69 kDa subunits after the receptor-

409 binding step, HR2-mimicking peptide was employed. This 39 amino acid peptide derived

410 from the HR2 region of the S2 subunit is water-soluble and interferes with packing of the

411 HR1/HR2 motif, thereby inhibiting virus infection (36). MHV-2 pre-treated with soluble

412 receptor was treated with HR2-mimicking peptide (50 μM) and various concentrations of

413 trypsin. The 69 kDa but not the 67 kDa subunit disappeared following treatment with HR2-

414 mimicking peptide, and the 55 kDa degradation product appeared after incubation in the

415 presence of 8 μg/ml trypsin (Fig. 8A, lane 13). Presumably, protease-cleavable sites in the S2

416 subunit are vulnerable to scission by trypsin when the HR2-mimicking peptide is present due

417 to the restriction of helical bundle formation by the HR motif; hence the cleavage pattern was

418 altered. This also indicates that the HR1/HR2 motif in the 67 kDa fragment forms a post-

419 fusion species in which the HR1 motif is occluded after the receptor-binding step.

420 Interestingly, HR2-mimicking peptide completely inhibited production of the

421 proteinase K-resistant 53 kDa fragment (Fig. 8C, lanes 11 and 13), even in the presence of

422 the packed 67 kDa species (Fig. 8A, lanes 11 and 13), suggesting that HR2-mimicking

423 peptide interferes with 6HB formation. The trimer in the receptor-binding step is presumably

424 constructed from both packed and unpacked HR1/HR2 motifs, and HR2-mimicking peptide

425 interacts with an unpacked motif, and thereby interferes with 6HB formation. In the presence

426 of 0.5−2 μg/ml trypsin, HR2-mimicking peptide did not appear to affect degradation of the 69

427 kDa fragment due to the low concentration of trypsin (Fig. 8A, lanes 5−10). In addition,

18

428   trypsin degradation products in the presence of HR2-mimicking peptide were detected by

429   MAb-10G antibody (Fig. 8A, lanes 13, 15, and 17) but not by anti-CT antibody (Fig. 8B,

430   lanes 13, 15, and 17), indicating that the 10G epitope region is folded and consequently

431   avoids cleavage by trypsin, whereas the C-terminal side of the 10G epitope including the

432   HR2 region (residues 1253–1302, Fig. 4A) is presumably unfolded and therefore degraded by

433   trypsin. Although the mechanism remains unknown, HR2-mimicking peptide enhanced the

434   production of the 69 kDa subunit following treatment with 0.25 μg/ml trypsin (Fig. 8B, lane

435   3).

436   Next, the concentration dependence of HR2-mimicking peptide was assessed. In the

437   presence of ≥0.5 μM HR2-mimicking peptide, the 69 and 53 kDa bands disappeared (Fig. 8D

438   and 8E, lanes 5–7), and the 55 kDa degradation products appeared (Fig. 8D, lanes 5–7). Pre-

439   treatment of HR2-mimicking peptide with 10 μg/ml trypsin did not affect the ability to cause

440   the disappearance of the 69 and 53 kDa fragments (Fig. 8D and 8E, lanes 11–13), indicating

441   that trypsin does not directly affect HR2-mimicking peptide. To determine whether the HR2-

442   peptide actually blocks MHV-2 infection, real-time PCR-based virus entry assays were

443   performed, as previously reported for SARS-CoV in which HR2-mimicking peptide blocks

444   trypsin-mediated direct viral entry from the cell surface (38). In the presence of ≥0.5 μM

445   HR2-mimicking peptide, virus entry was clearly blocked (Fig. 8F).

446

447   **The timing of HR packing**

448   Next, the timing of HR1/HR2 motif packing in the S protein was analyzed. HR2-mimicking

449   peptide was added to the S protein conformational change reaction at the indicated time

450   points after stopping the reaction using dry ice/methanol, and mixtures were re-incubated at

451   37°C for 20 min to facilitate production of the 67 and 69 kDa fragments (re-incubation is

452   required to visualize bands that reflect the structure present upon HR2-mimicking peptide

19

453   addition). At each HR2-mimicking peptide addition time point, if the HR1/HR2 motif forms

454   an unpacked structure, the HR2-mimicking peptide would be expected to bind to HR1 and

455   trypsin would degrade the 69 kDa to produce the 55 kDa lacking the CT epitope as described

456   above (Fig. 8A and 8B). As shown in Figure 8G (lane 2), the 67 kDa subunit appeared, the 69

457   kDa subunit disappeared, and the 55 kDa degradation product appeared following treatment

458   with HR2-mimicking peptide after the receptor-binding step, suggesting that both packed and

459   unpacked HR1/HR2 motifs simultaneously occur in the reaction. After a 1 min incubation at

460   37°C, undegraded 69 kDa subunit, which does not interact with HR2-mimicking peptide, was

461   observed (Fig. 8G, lane 3), suggesting that both the 67 and 69 kDa fragments form a packed

462   structure at this time point.

463

464   **Assembly of three S2 subunits at the center of the S protein trimer**

465   As shown in Figures 5A and 6A, interactions between subunits in the S protein trimer were

466   enhanced by receptor and trypsin treatment, and it remained stable in sample buffer

467   containing 0.5% SDS during SDS-PAGE. To compare the strength of intermolecular

468   interactions within the trimer at each step during the conformational changes, reactions of

469   virus treated with receptor and trypsin were frozen at the indicated time points using dry

470   ice/methanol, mixed with sample buffer containing trypsin inhibitor and 0.5% SDS, and

471   incubated at different temperatures between 25°C and 100°C at intervals of 5°C. Both the

472   trimer and the dissociated monomer were detected via western blotting following soaking of

473   the PVDF membrane in stripping buffer used for trimer detection by MAb-10G. As shown in

474   Figure 9A, the non-treated trimer in the pre-fusion state dissociated into the monomeric form

475   at 25°C. After receptor binding, the dissociation temperature was increased to ~60°C. The

476   dissociation temperature of the trimer was further increased to 90°C after trypsin treatment,

477   and finally reached 95°C after 32 min (Fig. 9B).

478   Next, to explore the interactions of the three S2 subunits in more detail after the

479   protease digestion step, experiments were performed between 81°C and 95°C at intervals of

480   1°C, and the results of western blot analysis were cropped and aligned to compare the

481   dissociated monomers (Fig. 9C). After trypsin treatment on ice, the dissociation temperature

482   was ~85°C, and this gradually increased to 94°C during incubation, corresponding with the

483   appearance of the 67 kDa fragment. These results indicate that the three S2 subunits in the

484   trimer partially assemble at the receptor-binding step, and their interaction is dramatically

485   enhanced by trypsin treatment. The postS2' site is finally cleaved after assembly.

486

487   **Negative-stain EM**

488   Each virus activation step described above was visualized by negative-stain EM (Fig. 10A),

489   and enlarged views of images are shown in Figure 10B. For non-treated virus, intact S protein

490   globules were observed as uniform alignments on the viral membrane (Fig. 10B i). The

491   height (distance) from the viral membrane to the top of the S protein was measured and is

492   presented as a histogram (Fig. 10C). The height of uniform non-treated S protein was ~240

493   Å, but after receptor treatment, the shapes of S proteins became more variable, gaps between

494   S protein globules and the viral membrane were reduced (Fig. 10B ii), and the height of half

495   of the S protein population was decreased to <200 Å (Fig. 10C ii). After trypsin treatment for

496   5 min on ice, the S1 subunits appeared to remain on the S2 subunit, their shapes became

497   obscured, and the gaps were further reduced (Fig. 10B iii and 10C iii). At 1 min after

498   warming at 37°C, a few elongated cone-like structures were observed (Fig. 10B iv), as

499   previously reported using recombinant S2 subunit (11). After additional incubation at 37°C

500   for 30 min, almost all S protein globules disappeared, and many elongated cone-like

501   structures appeared (Fig. 10A v and 10B v).

21

502   Next, the virus activation steps described above were probed in the presence of

503   liposomes. On EM grids, an excessive number of liposomes but very few viruses were

504   observed in the absence of soluble receptor (Fig. 10D i), whereas many virus particles were

505   observed on liposomes in the presence of soluble receptor (Fig. 10D ii). These results

506   correspond to liposome flotation assays in a previous study in which virus binding to

507   liposomes was induced by receptor (26). Virus particles disappeared at 1 min after trypsin

508   treatment (Fig. 10D iv), and fused liposomes were the dominant species observed at 30 min

509   (Fig. 10D v), suggesting that membrane fusion with liposomes was largely complete within 1

510   min, and liposomes then fused with each other using the remaining S protein. An enlarged

511   view of virus-bound liposomes shown in Figure 10D ii and 10D iii is presented in Figure 10E.

512   The gaps between viruses and liposomes that are presumably bridged by S protein were ~200

513   Å after the receptor-binding step (Fig. 10E ii), and this distance was reduced by trypsin

514   treatment on ice (Fig. 10E iii). A hazy density was evident between viruses and liposomes

515   (Fig. 10E).

516

517   **Discussion**

518   The S protein of the MHV-2 virion is uncleaved, similar to the S protein of MERS-CoV and

519   SARS-CoV, which requires cellular protease following receptor binding to induce S1

520   dissociation from S2 and the subsequent conformational changes necessary for membrane

521   fusion (26). By contrast, most MHV variants carry S proteins that are cleaved by cellular

522   furin at the S1/S2 site during biogenesis, which was believed to require only receptor binding

523   to induce membrane fusion (39, 40). However, previous studies of MHV and MERS-CoV

524   suggest that the coronaviruses harboring pre-cleaved S protein (cleaved by furin during

525   biogenesis) at the S1/S2 site require further cellular proteases to facilitate cell entry (20, 30).

526   Our results also suggest that the S protein cleaved during biogenesis by furin requires

527 additional cleavage to induce conformational changes required to adopt a proteinase K-

528 resistant conformation (Fig. 2A, lane 12). However, the trypsin-treated S protein cleaved on

529 virus particles at the S1/S2 site does not require additional cleavage; it needs only receptor

530 binding (Fig. 2A, lane 7). Therefore, these two types of S protein cleaved at the S1/S2 site are

531 predicted to have different conformations. In the present study using MHV-2, the 67 and 69

532 kDa products were considered to be by-products that reflect intermediate conformations of

533 the S protein, although importance of cleavage at the S2' site was reported for coronavirus S

534 proteins (20, 30).

535   Previous studies used cryoEM to examine conformational changes within the

536 coronavirus S protein during the priming stage, which is induced by receptor binding; the

537 results observed after receptor binding indicated an asymmetric trimer with opened and

538 closed domains within the S1 subunit and a tightly assembled central helix in the S2 subunits

539 (4, 6). However, no major differences in the HR region were observed. Although structural

540 analysis using cryoEM is excellent for detecting stable protein structures, unstable structures

541 such as the HR2 motifs in the S2 subunit cannot be analyzed using this technique, even if a

542 large number of protein particles are captured; population-based biochemical analysis is

543 needed to capture features of unstable proteins. Herein, we predicted the dynamic

544 rearrangements of S2 subunits underpinning the transition from pre- to post-fusion structures

545 based on western blot analysis of the 67 and 69 kDa by-products that are induced following

546 trypsin digestion of the S protein.

547   Our model of the S protein conformational changes was constructed based on

548 previously reported pre- and post-fusion structures (protein data bank codes 3JCL and 6B3O,

549 respectively). The S2 subunit of coronavirus features a topology similar to that of the

550 paramyxovirus F protein, comprising a core β-sheet, an upstream helix, and a central helix,

551 and these motifs are essentially identical in pre- and post-fusion F protein structures (10, 41).

23

552   These invariant MHV-2 S protein motifs are assumed not to change conformation, and are

553   displayed as a fixed globule represented by a gray column in Figure 11. The flexible regions

554   in the S2 subunit that undergo conformational changes are positioned in the FP region

555   (residues 867−949) and the HR1 region (residues 954−1072), as shown in Figure 4A and 4B.

556   In addition, the region downstream of the core β-sheet (residues 1155−1302) that includes the

557   HR2 region also appears to be flexible (Fig. 4A and 4B).

558   In the present and previous studies, we observed eight features related to

559   conformational changes that occur in the flexible regions of the S2 subunit. The first four

560   features are induced by receptor binding. **[1]** During this step, the S protein binds to the target

561   membrane, as demonstrated by liposome-binding assays in a previous study (26) and

562   negative-stain EM in this study (Fig. 10D ii). Because the FP region is located close to β48,

563   at least one β48 per trimer must be dissociated from β47 in invariant motifs for the FP region

564   to be free. It is important to remember that the exact location of the FP region of coronavirus

565   S protein remains controversial. The FP region (residues 867−949) immediately follows an

566   S2' cleavage site (42), and another possible FP region (pFP; residues 967−983) is adjacent to

567   the region upstream of HR1 (Fig. 4A and 4B), as reported previously (43, 44). **[2]** The

568   metastable form of the S protein trimer is converted to a stable trimer (Fig. 6A, VHCR, lane

569   3), for which the dissociation temperature was increased from 25°C to 60°C (Fig. 9C).

570   During this stage, the movement of HR1 to form a trimeric α-helical coiled-coil on the distal

571   side of the viral membrane is restricted because the S2 subunit is still covered by the S1

572   subunit in the uncleaved S protein (Fig. 10A ii). Therefore, regions at α29 and α30 (the

573   central helix) are only capable of interacting within the trimer (Fig. 4B). This is consistent

574   with a previous study in which the tightly packed central helix in the S2 subunit was detected

575   by cryoEM after the receptor-binding step (4, 6). **[3]** The 106 amino acids of the connector

576   region (residues 1155−1260, Fig. 4A), including the 10G epitope and disulfide bond at

24

577   positions 1169 and 1214, presumably form a folded structure because the 10G epitope in the

578   trimer was undetectable in the native blot (Fig. 6A, 10G, lane 3), and was resistant to trypsin

579   even in the presence of HR2-mimicking peptide (Fig. 8A). Reduction of the gap between the

580   S protein globule and the viral membrane (~200 Å) in EM images (Fig. 10B ii and 10C ii)

581   may reflect a folded C-terminal side of the S2 subunit. In addition, the 10G region is likely

582   fixed near helix α29 at the top of the S2 trimer to allow 6HB assembly in the following step

583   (11). These observations indicate that, in the S protein structure appearing after the receptor-

584   binding step, the core of the connector leash is packed into the groove of the invariant motif

585   (Fig. 11A ii, purple lines), as seen in the post-fusion structure (11). **[4]** The HR1/HR2 motif

586   forms the packed-hairpin structure observed as the 67 kDa subunit after trypsin treatment that

587   is unable to interact with HR2-mimicking peptide (Fig. 8A, lane 13 and 8G, lane 2). Based on

588   the steric configuration of the post-fusion structure, HR1 of one S2 subunit must be packed

589   with HR2 of another S2 subunit in the trimer to form a hairpin structure (11). Additionally,

590   unpacked HR1, observed as the 69 kDa band that was degraded following treatment with

591   HR2-mimicking peptide and trypsin (Fig. 8G, lane 2), also appeared in this step.

592   Interestingly, both 69 and 67 kDa subunits were present in the trimer, as revealed by two-

593   dimensional electrophoresis (Fig. 5C b), suggesting that a subset of S2 subunits form a

594   heterogeneous trimer constructed from two different conformational states. We predict that

595   the trimer includes at least one packed HR1/HR2 motif and extends at least one unpacked

596   HR1 motif toward the target membrane to expose the FP region, and the S2 subunit is

597   covered by the S1 subunit in the uncleaved S protein (Fig. 11A ii). These features suggest

598   that the S protein cannot form the typical homotrimeric pre-hairpin structure predicted in the

599   current model of class I viral fusion protein assembly (Fig. 11B).

600   The next four features of the conformational changes are induced by trypsin

601   treatment, during which obscure-shaped S proteins were observed by EM (Fig. 10B iii). **[5]**

602   The S1/S2 site is cleaved by trypsin to generate the 90 kDa subunit (Fig. 1C, lane 7 and 7A,

603   lane 2), and the S2' site is subsequently cleaved by trypsin to generate the 69 kDa subunit

604   (Fig. 1C, lanes 7−16). Release of the S1 subunit from the S2 subunit presumably enhances

605   interactions within three S2 subunits and triggers conformational changes in the HR1/HR2

606   motifs. **[6]** All three HR1/HR2 motifs in the trimer appear to form a packed-hairpin structure

607   at 1 min after warming at 37°C because HR2-mimicking peptide cannot interact with them

608   (Fig. 8G, lane 3). **[7]** Simultaneously, the three HR1/HR2 motifs assemble at the center to

609   form a 6HB core at 1 min after trypsin treatment (Fig. 7A, bottom, lane 3). At this stage, the

610   dissociation temperature of the trimer was ~90°C (Fig. 9C). **[8]** The postS2' cleavage site in

611   β48 is finally cleaved by trypsin after assembly of the three HR motifs at the center, resulting

612   in the appearance of the 67 kDa subunit (Fig. 9C bottom). The final 6HB structure is

613   constructed from 67 and 69 kDa subunits or three 67 kDa subunits (Fig. 5C b). Numerous

614   elongated cone-like structures were observed by EM (Fig. 10A v).

615   As described above, at least one HR1/HR2 motif per trimer may form a packed-

616   hairpin structure after the receptor-binding step, and another exposes the FP region, as

617   illustrated in Figure 11A ii. This conformation presumably facilitates formation of an

618   asymmetric trimer. In principle, the class I viral fusion protein trimer must break its rotational

619   symmetry during activation to overcome the steric hindrance within the three fusion protein

620   subunits (45, 46). When the different conformational states of the HR1/HR2 motif assemble

621   in the trimer, the axis of the trimer is presumably tilted against the viral membrane (Fig. 11A

622   vii), resulting in the breaking of rotational symmetry. This is followed by formation of 6HB

623   and membrane fusion. Flexibility at the juxtamembrane region and the tilted orientation

624   against the viral membrane required for membrane fusion were recently reported by a study

625   examining influenza HA in detergent micelles analyzed by cryoEM (47). We anticipate that

626   the mechanism suggested by western blot analysis herein will be confirmed by other

627   approaches such as high-resolution cryoEM and/or *in silico* simulation.

628

629

630

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636

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640

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786

787

788   **Figure Legends**

789   **Fig. 1. Proteolytic activation of the MHV-2 spike (S) protein.** (A) Schematic diagram of

790   MHV-2 S protein organization. Letters A, B, C, and D indicate domains of the S1 subunit.

791   SP, signal peptide; RBD, receptor-binding domain; UH, upstream helix; FP, fusion peptide;

792   HR1/HR2, heptad repeats; CH, central helix; BH, β-hairpin; CR, connector region; TMD,

793   transmembrane domain. The three trypsin cleavage sites are indicated by black arrows, and

794   the four linear epitopes recognized by antibodies are indicated by white arrows. (B) Two-step

795   conformational changes of S protein primed by receptor binding and triggered by trypsin.

796   MHV-2 pre-treated with a soluble form of the receptor (CEACAM1a, receptor) was

797   incubated with trypsin (10 µg/ml). Samples were boiled and subjected to western blot

33

798 analysis using the indicated antibodies. (C) Trypsin concentration-dependent cleavage of S

799 protein. MHV-2 pre-treated with receptor was incubated with various concentrations of

800 trypsin, then with proteinase K. Samples were boiled and subjected to western blot analysis

801 using MAb-10G antibody. (D) Trypsin concentration dependence of virus cell entry. MHV-2

802 was adsorbed onto DBT cells, and various concentrations of trypsin were added. After a 5 h

803 incubation, viral mRNA was quantified by real-time PCR (n = 6). Data were analyzed

804 relative to the no trypsin control using two-tailed Student's t-tests. n.s. = not significant, * =

805 significant ($p \leq 0.05$), ** = highly significant ($p \leq 0.01$), *** = very highly significant

806 ($p \leq 0.001$). Error bars indicate standard deviation (SD).

807

808 **Fig. 2. Proteolytic activation of the pre-cleaved S protein.** (A) Conformational changes in

809 pre-cleaved S proteins. To prepare the virus harboring a cleaved S protein, MHV-2 was

810 treated with 1 µg/ml trypsin or phosphate-buffered saline (PBS) (uncleaved control) for 1 h,

811 treated with trypsin inhibitors, and then purified on a Sephadex G-75 column.

812 Conformational changes in PBS-treated MHV-2, trypsin-treated MHV-2, and MHV-2f

813 harboring a pre-cleaved S protein were examined as described in Figure 1. Samples were

814 boiled and subjected to western blot analysis with MAb-10G antibody. (B) Cell entry by

815 viruses. Viruses treated with PBS or trypsin were inoculated onto DBT cells, and cellular

816 RNA was isolated after 0 or 6 h. Viral mRNA was quantified by real-time PCR (n = 4).

817

818 **Fig. 3. Additional characterization of the 67 and 69 kDa subunits.** (A) Specific cleavage

819 of the postS2' site at arginine or lysine residues. Endopeptidase arg-C (20 µg/ml) and/or lys-

820 C (20 µg/ml) were employed instead of trypsin to induce S protein conformational changes.

821 (B) Deglycosylation of S2 subunits. S protein activated by receptor and trypsin was

822 deglycosylated using a deglycosylation enzyme mix. Nonrelevant lanes on the same blot were

823   sliced out in Adobe Photoshop to align the lanes shown. (C) Receptor concentration

824   dependence. MHV-2 was treated with serially diluted soluble receptor, then with trypsin (10

825   µg/ml). (D) Effect of redox potential. The 67 and 69 kDa subunits induced by the treatment

826   of receptor and trypsin (0.32 and 32 µg/ml) were boiled in sample buffer containing the

827   indicated concentration of dithiothreitol (DTT). (A–D) After SDS-PAGE, western blot

828   analysis was carried out using MAb-10G antibody.

829

830   **Fig. 4. Primary structure and topology of the MHV-2 S2 subunit.** (A) Primary sequence

831   of the S2 subunit. Gray areas indicate invariant motifs present in both pre- and post-fusion

832   structures. S-S, disulfide bridge; glyco, N-glycosylation site; scissor mark, trypsin cleavage

833   site. The four antibody-binding sites and the functional motifs are depicted. (B) Topology of

834   the pre-fusion form of S protein based on the cryoEM structure (10). Colors and labels

835   correspond to those in panel A. Magenta dotted lines indicate molecular interactions between

836   motifs.

837

838   **Fig. 5. Components of the S2 trimer after triggering.** (A) Comparison of native and

839   denatured S protein. MHV-2 treated with soluble receptor and trypsin was divided into two

840   aliquots for unboiled (native) and boiled (denatured) treatments. After electrophoresis on a

841   3−10% gel, the gel was boiled in sample buffer at 105°C for 5 min in an autoclave, and

842   transferred to a PVDF membrane. (B) Two-dimensional electrophoresis. The reaction

843   mixture prepared as described for panel A was mixed with sample buffer containing

844   molecular size markers, and two-dimensional electrophoresis was carried out. After the first

845   electrophoresis step, the first gel (3−10%) was boiled in sample buffer, sliced along the

846   markers, and laid onto the second gel (7.5%). After the second electrophoresis step, the gel

847   was transferred to a PVDF membrane. X-marks indicate overlaid molecular size markers. (C)

848 Cropped and enlarged blots. Blots from two-dimensional electrophoresis and western blot

849 analysis corresponding to 0.32 or 10 μg/ml trypsin treatment were cropped at 67 and 69 kDa

850 and enlarged by graphical manipulation software. (A−C) Western blot analysis was carried

851 out using MAb-10G antibody.

852

853 **Fig. 6. Exposed or buried configurations of epitopes in the S protein globule.** (A) Native

854 SDS-PAGE. Unboiled samples were subjected to western blot analysis. (B) Denaturing and

855 re-probing. PVDF membranes from panel A were soaked in stripping buffer for 5 min to

856 denature the bound S protein, and subjected to detection with the same antibodies a second

857 time. (A and B) Western blot analysis was performed using the indicated antibodies.

858

859 **Fig. 7. Timing of the appearance of the 90, 69, 67, and 53 kDa species.** (A) Time course of

860 90, 69, 67, and 53 kDa fragment generation. MHV-2 was treated with receptor and trypsin,

861 and the reaction was stopped by freezing at the indicated time points. (B) Two-step

862 conformational changes in the S protein primed by receptor binding and triggered by trypsin

863 in the presence of liposomes. (C) Time course of the generation of 90, 69, and 67 kDa

864 fragments in the presence of liposomes. MHV-2 was treated with receptor and trypsin in the

865 presence of liposomes, and the reaction was stopped by freezing at the indicated time points.

866 (A−C) Appearance of S2 subunits (90, 69, and 67 kDa) detected by western blot analysis

867 using MAb-10G antibody.

868

869 **Fig. 8. Interaction of HR2-mimicking peptide with the S2 subunit.** (A) Effect of HR2-

870 mimicking peptide (HR2-peptide) during S protein triggering. HR2-peptide (50 µM) was

871 added to MHV-2 after the receptor-binding step, and reaction mixtures were treated with

872 various concentrations of trypsin. (B) The PVDF membrane from panel A was re-probed with

873   anti-CT antibody. (C) Reaction mixtures from panel A were treated with proteinase K to

874   generate the 53 kDa fragment. (D) Effect of trypsin on the HR2-peptide. HR2-peptide non-

875   treated or pre-treated with 10 µg/ml trypsin for 30 min was diluted and added to the reaction

876   containing MHV-2 and receptor, and reaction mixtures were treated with 10 µg/ml trypsin.

877   (E) Reaction mixtures from panel D were treated with proteinase K to generate the 53 kDa

878   fragment. (F) Blocking virus cell entry. MHV-2 was adsorbed onto DBT cells, and 10 µg/ml

879   trypsin was added in the presence or absence of HR2-mimicking peptide. After a 5 h

880   incubation, viral mRNA was quantified by real-time PCR (n = 6). Data were analyzed

881   relative to the no peptide control using two-tailed Student's t-tests, as described in the legend

882   of Figure 1. (G) Time course of HR1/HR2 motif packing. During S protein activation by

883   receptor and trypsin, the reaction was stopped by freezing at the indicated time points, and

884   HR2-mimicking peptide (HR2-peptide) was added and incubated for 20 min to facilitate the

885   formation of 67 and 69 kDa fragments. (A−E and G) Western blot analysis was performed

886   using the indicated antibodies.

887

888   **Fig. 9. Timing of S2 subunit assembly.** (A) Thermostability of the trimer in the receptor-

889   binding step. The reaction analyzing conformational changes of S protein was stopped by

890   freezing at the indicated time points after 30 min treatment with receptor. (B) Thermostability

891   of the trimer in the proteolysis step. Reaction mixtures prepared as described for panel A

892   were treated with trypsin and stopped by freezing at the indicated time points. (C) More

893   detailed analysis of S protein thermostability. The dissociation temperature of mixtures

894   prepared as described for panel B was explored between 81°C and 95°C at intervals of 1°C.

895   (A−C) Reaction mixtures were treated with sample buffer containing protease inhibitor and

896   0.5% SDS on ice, then incubated at the indicated temperature using a Veriti thermal cycler

897   (ThermoFisher). After electrophoresis and electroblotting, the PVDF membrane was soaked

898   in stripping buffer for trimer detection by MAb-10G. Dissociated monomer bands from each

899   step of the conformational changes were cropped and aligned to compare the dissociation

900   temperature in panel C.

901

902   **Fig. 10. Visualization by negative-stain electron microscopy (EM).** (A) Virus particles.

903   MHV-2 treated with or without receptor (1 μM) and trypsin (10 μg/ml) to induce

904   conformational changes in S protein was subjected to negative-stain EM. (B) Enlarged view

905   of S protein on the viral particle. (C) Histogram of S protein height above the membrane. S

906   proteins (n = 32) in five viral particles were measured and represented as a histogram. (D)

907   Visualization of virus binding to liposomes. Liposomes were added to the reaction to induce

908   conformational changes in the viral S protein. Viruses are indicated by arrows. (E) Enlarged

909   view of virus binding to liposomes. Virus particles observed in panel D are enlarged.

910

911   **Fig. 11. Schematic diagram of S protein activation.** (A) Conformational changes of the S

912   protein trimer. (B) Homotrimeric pre-hairpin structure in the unified model of class I viral

913   fusion protein assembly. FP, fusion peptide; HR1/HR2, heptad repeats.

914

Fig. 1

A

trypsin cleavage site

S1/S2 S2' postS2'

SP RBD A B C D UH FP pFP HR1 CH BH CR HR2 TMD

S2a VHCR 10G CT

antibody-binding site

B

antibody S2a VHCR 10G CT
receptor − − + + − − + + − − + + − − + +
trypsin − + − + − + − + − + − + − + − +

kDa 250 150 100 75 50 37

kDa 180 90 69 67

1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

C

MHV-2 + receptor at 37℃ for 30 min

trypsin (µg/ml) at 37℃ for 20 min

0 0.008 0.016 0.031 0.063 0.125 0.25 0.5 1 2 4 8 16 32 64 128

kDa 250 150 100 75 50 37

kDa 180 90 69 67

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

proteinase K (1 mg/ml) on ice for 30 min

50 53

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

D

MHV-2 / DBT cells

viral mRNA (log10)

5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5

n.s. n.s. n.s. n.s. n.s. *** ** ***

trypsin (µg/ml)

0 0.0625 0.125 0.25 0.5 1 2 4 8 16

Fig. 2

A

PBS-treated MHV-2   trypsin-treated MHV-2   MHV-2f

1 mg/ml proteinase K on ice for 30 min

B

Fig. 3

A

MHV-2 + receptor
at 37°C for 30 min

endopeptidases
at 37°C for 30 min

- / argC / lysC / argC and lysC / argC then lysC / lysC then argC / trypsin

kDa 250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷

kDa
◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5 6 7

B

MHV-2 + receptor
at 37°C for 30 min

marker / 0.32 µg/ml trypsin (- / deglyco) / 32 µg/ml trypsin (- / deglyco)

glycosylated size (kDa)
deglycosylated size (kDa)

kDa 250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷
25 ▷

90 → 65
69 → 47
67 → 43

1 2 3 4

C

receptor (nM): 0 / 3.125 / 6.25 / 12.5 / 25 / 50 / 100 / 200
trypsin: + + + + + + + +

kDa 250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷

◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5 6 7 8

D

0.32 µg/ml trypsin   32 µg/ml trypsin

boiled with DTT (mM): 0 / 0.0001 / 0.001 / 0.01 / 0.1 / 1 / 10 / 100   0 / 0.0001 / 0.001 / 0.01 / 0.1 / 1 / 10 / 100

kDa 250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷

◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8

Fig. 4

Fig. 5

A

MHV-2 + receptor

trypsin
(µg/ml)   0 0.32 10   0 0.32 10

kDa
155 ⎤
    ⎥
140 ⎦

                                        kDa
                                        ◄ 180

                                        ◄ 90

                                        ◄ 69
                                        ◄ 67

        1   2   3   4   5   6
        native   denatured

B

treated with
10 µg/ml trypsin

|← 1st gel →|

boiled
sample ↓

        kDa ▷250 ▷150 ▷100 ▷75 ▷50 ▷37 ▷25

kDa
250 ▷
150 ▷
100 ▷
75 ▷
                                        ◄ 69
                                        ◄ 67
50 ▷

37 ▷
25 ▷

2nd gel

C

(a) treated with
0.32 µg/ml trypsin

1st gel →
150 kDa
▼

                ◄ 69

                ◄ 67

(b) treated with
10 µg/ml trypsin

1st gel →
150 kDa
▼

                ◄ 69   ⎤
                            ⎥ 2nd gel
                ◄ 67   ⎦
                            ↓

Fig. 6

A

Unboiled native S protein

| antibody | S2a | VHCR | 10G | CT |

669 ▷
500 (3 mer S)
450 ▷
250 ▷
180 (1 mer S)
150 ▷
140 (3 mer 67&69 kDa)
(3 mer 67 kDa)
100 ▷
90 (1 mer 90 kDa)
75 ▷
50 ▷
37 ▷

B

Unboiled native S protein (denatured on the PVDF membrane and reprobed)

669 ▷
500 (3 mer S)
450 ▷
250 ▷
180 (1 mer S)
150 ▷
140 (3 mer 67&69 kDa)
(3 mer 67 kDa)
100 ▷
90 (1 mer 90 kDa)
75 ▷
50 ▷
37 ▷

Fig. 7

A

10 µg/ml trypsin
on ice for 5 min
then warmed at 37°C

no trypsin

0 1 2 4 8 16 32 min

kDa
250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷

◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5 6 7 8

proteinase K (1 mg/ml)
on ice for 30 min

◀ 53

1 2 3 4 5 6 7 8

B

liposome   −   +
receptor − − + +   − − + +
trypsin   − + − +   − + − +

kDa
250 ▷
150 ▷
100 ▷
75 ▷
50 ▷

◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5 6 7 8

C

(i) MHV-2 + liposome
(ii) +receptor at 37°C for 30 min
(iii) +trypsin on ice for 5 min
(iv) incubated at 37°C for 1 min
(v) incubated at 37°C for 30 min

kDa
250 ▷
150 ▷
100 ▷
75 ▷
50 ▷

◀ 180
◀ 90
◀ 69
◀ 67

1 2 3 4 5

Fig. 8

A

MHV-2 + receptor at 37℃ for 30 min

HR2-peptide (50 µM)

+ − | + − | + − | + − | + − | + − | + − | + − | + −

trypsin (µg/ml) at 37℃ for 20 min

0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32

MAb-10G

kDa
◄ 180
◄ 90
◄ 69
◄ 67
◄ 55

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B

anti-CT

◄ 180
◄ 90
◄ 69
◄ 67

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

C

1 mg/ml proteinase K on ice for 30 min

MAb-10G

◄ 53

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

D

MHV-2 + receptor at 37℃ for 30 min

HR2-peptide (µM) pretreated with PBS | HR2-peptide (µM) pretreated with 10 µg/ml trypsin

0 | 0.005 | 0.05 | 0.5 | 5 | 50 | 0.005 | 0.05 | 0.5 | 5 | 50

0 | 10 µg/ml trypsin at 37℃ for 20 min

MAb-10G

kDa
◄ 180
◄ 69
◄ 67
◄ 55

1 2 3 4 5 6 7 8 9 10 11 12 13

E

1 mg/ml proteinase K on ice for 30 min

MAb-10G

◄ 53

1 2 3 4 5 6 7 8 9 10 11 12 13

F

viral mRNA (log10)

n.s. n.s. * *** ***

0 | 0.005 | 0.05 | 0.5 | 5 | 50

HR2-peptide (µM) treated with 10 µg/ml trypsin

G

10 µg/ml trypsin on ice for 5 min then warmed at 37℃

add HR2-peptide | −

no trypsin | 0 | 1 | 2 | 8 | 32 | 32 min

kDa
250 ▷
150 ▷
100 ▷
75 ▷
50 ▷
37 ▷

◄ 180
◄ 90
◄ 69
◄ 67
◄ 55

detected using MAb-10G

1 2 3 4 5 6 7

Fig. 9

A

denaturation temperature (°C)

100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25

no treat

◄ 3 mer
◄ 2 mer
◄ 1 mer

receptor
at 37°C

30 min

◄ 3 mer
◄ 2 mer
◄ 1 mer

detected using anti-CT

B

denaturation temperature (°C)

100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25   kDa

trypsin
on ice

5 min

◄ 155
◄ 90
◄ 69

at 37°C

1 min

◄ 155
◄ 90
◄ 69
◄ 67

4 min

◄ 150
◄ 90
◄ 69
◄ 67

32 min

◄ 140
◄ 90
◄ 69
◄ 67

detected using MAb-10G

C

denaturation temperature (°C)

100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25   kDa

no treat

◄ 1 mer

receptor
30 min

◄ 1 mer

trypsin
on ice

5 min

denaturation temperature (°C)

95 94 93 92 91 90 89 88 87 86 85 84 83 82 81

◄ 69

at 37°C
1 min

◄ 69

4 min

◄ 69
◄ 67

32 min

◄ 69
◄ 67

detected using MAb-10G

Fig. 10

A
(i) non-treated
(ii) plus receptor at 37°C for 30 min
(iii) then plus trypsin on ice for 5 min
(iv) then warmed at 37°C for 1 min
(v) additionally incubated at 37°C for 30 min

■ 200 Å

B
(i) non-treated
(ii) plus receptor at 37°C for 30 min
(iii) then plus trypsin on ice for 5 min
(iv) then warmed at 37°C for 1 min
(v) additionally incubated at 37°C for 30 min

■ 200 Å

C
(i) non-treated
(ii) plus receptor at 37°C for 30 min
(iii) then plus trypsin on ice for 5 min
(iv) then warmed at 37°C for 1 min
(v) additionally incubated at 37°C for 30 min

count
spike height (Å)

D
(i) non-treated MHV-2 + liposome
(ii) plus receptor at 37°C for 30 min
(iii) then plus trypsin on ice for 5 min
(iv) then warmed at 37°C for 1 min
(v) additionally incubated at 37°C for 30 min

■ 200 nm

E
(ii) plus receptor at 37°C for 30 min with liposome
(iii) then plus trypsin on ice for 5 min with liposome

200 Å   200 Å

■ 200 Å

Fig. 11

A

(i) pre-fusion

invariant motifs — HR1
FP —
connector region — HR2

trimer of 180 kDa

receptor →

(ii) intermediate
incubated with receptor
at 37°C for 30 min

FP — receptor
unpacked —
leash in
groove —
packed

trimer of 180 kDa

trypsin →

(iii) intermediate
incubated with trypsin
on ice for 5 min

unpacked
packed

trimer of 69 kDa

(iv) intermediate
incubated with trypsin
at 37°C for a few minutes

trimer of 67 and 69 kDa

(v) post-fusion
incubated with trypsin
at 37°C for 30 min

trimer of 67 kDa

B

homotrimeric
pre-hairpin structure

FP
HR1
invariant motifs
connector region — HR2