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1 **HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated**
2 **by proteolytic processing in the S2' region.**

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14

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25 The GenBank accession number for the France/Lille/2014 spike protein sequence is :
26 MH048989

27

28 **Abstract**

29
30 Human coronavirus 229E (HCoV-229E) is responsible for common colds. As other
31 coronaviruses, HCoV-229E exploits cellular proteases to activate its fusion mediated by the
32 spike protein. We analysed the proteolytic processing of the HCoV-229E spike protein by
33 trypsin-like serine proteases leading to activation of the fusion process. Unlike other
34 coronaviruses, HCoV-229E fusion activation appears to be a one step process. Indeed,
35 cleavage of the S1/S2 interface does not seem to be a pre-requisite, and the fusion activation
36 strongly relies on the S2' region, with arginine residue 683 acting as the recognition site.

37
38 **Main text**

39
40 *Coronavirinae* are enveloped virus with a long positive sense RNA genome. As for other
41 enveloped viruses, they need to fuse their viral envelope with a host cell membrane to deliver
42 their genome to the target cell. Coronavirus spike proteins are major mediators of virus entry
43 and fulfil a dual function by mediating receptor binding and fusion. Spike proteins consist of
44 two distinct domains carrying these functions. The amino-terminal subunit, called S1 domain,
45 is responsible for receptor binding, whereas the C-terminal part, called S2 domain, contains
46 the fusion machinery. Proteolytic priming of coronavirus spike protein is a key feature of the
47 fusion process, and coronaviruses exploit a wide variety of host proteases including
48 cathepsins, furin and members of the transmembrane serine protease (TTSP) family [1] to
49 achieve this requirement. It is likely that the proteolytic processing differs according to the
50 protease used. Depending on the cellular localization of the protease used to activate the
51 fusion, entry can occur either at the cell surface or after internalization of the virus by
52 endocytosis. For SARS-CoV spike protein, two cleavage events mediated by trypsin have
53 been identified. The SARS-CoV virion harbors an uncleaved spike protein at its surface. First,
54 the spike protein is primed by cleavage of the S1 and S2 domain, then to fully activate fusion,
55 a second cleavage occurs at a position called S2' (R797) exposing the fusion peptide [2, 3].
56 Activation of the SARS-CoV spike protein fusion by elastase also involves recognition of a
57 cleavage site in the S2' region [4]. The importance of the S2' cleavage has also been
58 demonstrated for MERS-CoV and MHV [5, 6].
59 HCoV-229E was identified in 1966 in students suffering from common cold [7], but children,
60 elderly and immune-compromised persons can develop a more severe disease [8]. It has been
61 shown that HCoV-229E requires endosomal cathepsin L activity to infect Hela cells [9].
62 However, Shirato et al. have recently shown that clinical isolates of HCoV-229E

63 preferentially uses the transmembrane protease serine 2 (TMPRSS2), a trypsin-like protease,
64 rather than endosomal cathepsin for entry [10]. TMPRSS2 is known to be present at the
65 surface of the human airway epithelia and is likely the main activator of HCoV-229E fusion
66 *in vivo*. In addition, TMPRSS2-mediated entry at the cell surface protects the virus from the
67 inhibitory effects of interferon-induced transmembrane proteins (IFITMs) known to inhibit
68 entry of several enveloped virus [11]. However, the detailed proteolytic processing of HCoV-
69 229E spike protein to activate its fusion remains to elucidate.

70 To investigate this proteolytic maturation, we first cloned the spike protein sequence from a
71 patient infected by HCoV-229E hospitalized in Lille, France in 2014 (Spike
72 France/Lille/2014, accession number MH048989). The codon-optimized sequence of the
73 France/Lille/2014 spike protein was inserted into the pCDNA3.1(+) vector and fused with a
74 sequence encoding a C-terminal VSVG tag to facilitate the detection of the protein in
75 immunoblot. Then, we analysed the proteolytic processing of the spike protein required for
76 entry mediated by trypsin/trypsin like protease, focusing on the role of the S1/S2 junction and
77 the S2' region. The sequence of the French isolate spike protein shows 99% identity with the
78 spike of the Niigata, 0349 and USA/932-72/1993 isolates and only 94% of identity with the
79 VR-740 laboratory strain. HCoV-229E spike protein contains 3 arginine residues in the S2'
80 region (R679, R683 and R687) which are conserved in different strains of HCoV-229E,
81 except for R679 that is absent in the VR-740 laboratory strain (Fig. 1(a)). First, to confirm the
82 role of the HCoV-229E spike protein S2' region in entry, we inserted a furin cleavage site
83 (Fur687) in the spike protein (Fig.1(a)), at the N-terminal position of the potential fusion
84 peptide. To analyse the effect of the mutation in the context of viral particles, we used a
85 murine leukemia virus (MLV)-based pseudotyping system that produces particles inducing
86 luciferase expression after transduction of permissive cells. Pseudoparticles fully mimic the
87 functionality of the wild-type virus in terms of cell entry and were used because of their
88 technical advantage. Huh-7 cells were inoculated with pseudoparticles harboring the wild type
89 protein or the Fur687 mutant (Fig 1(b)). Neutralization of endosomal pH by ammonium
90 chloride treatment inhibited transduction by HCoV-229E pseudoparticles showing that
91 HCoV-229E infects Huh-7 cells via the endosomal route and uses low-pH active endosomal
92 protease to mediate its fusion. As shown in Figure 1(b), pseudotyped virions with HCoV-
93 229E spike protein containing a furin cleavage site at the S2' position were insensitive to
94 endosomal pH neutralization, but were affected by furin convertase inhibitor. This result
95 shows that the spike protein with the furin cleavage site does not rely on endosomal cathepsin
96 for entry but relies on furin and this confirms the involvement of the S2' region for fusion

97 activation of HCoV-229E, as observed for other coronaviruses [2, 5, 6, 12]. It is interesting to
98 note that the spike proteins incorporated on the pseudotyped virions were not cleaved by furin
99 in producing cells, indicating that the protein is not processed during exit of the producer cell
100 but during entry into the target cell (Fig 1(d)). This suggests that during exit and secretion, the
101 cleavage site is inaccessible to furin and that during entry, conformational change induced by
102 receptor binding may expose the cleavage site. To investigate the role of the S1/S2 junction
103 and the specific role of the 3 arginine residues located in the S2' region (R679, R683 and
104 R687) in the proteolytic activation of HCoV-229E spike protein by trypsin-like serine-
105 proteases, the arginine residues present at S1/S2 and at S2' were mutated individually
106 (R565N, R679N, R683N or R687N). We chose to mutate the arginine residues into
107 asparagine to minimize the effect of the mutations on the conformation/folding of the protein.
108 As seen in Fig 1(c), these arginine residues were not implicated in entry when the virions use
109 the endocytic pathway to invade the cells. The endosomal route of entry of the different
110 mutants was confirmed by inhibition of their entry by ammonium chloride treatment (Fig
111 1(c)). Indeed, when HCoV-229E is internalized, the virus is dependent on the endosomal
112 cathepsin L to mediate its fusion. However, unlike other proteases, cathepsin L has a limited
113 prime-site specificity in terms of recognition sites and the precise sites of proteolysis by
114 cathepsin L remains unclear.

115 In 2005, Simmons et al. showed that SARS-CoV fusion can be induced at the cell surface
116 when virions are bound to the plasma membrane at 4°C and briefly treated by exogenous
117 protease such as trypsin [13]. Particles pseudotyped with the different S1/S2 and S2' arginine
118 spike mutants were bound at the surface of Huh-7 cells treated with ammonium chloride to
119 neutralize the endosomal pH and block the endosomal entry pathway. Then, fusion was
120 induced by a brief trypsin treatment (Fig 2(a)). For the mutant R683N, trypsin treatment was
121 not able to bypass the requirement for cathepsin L activity during the entry process,
122 suggesting that proteolysis by trypsin occurs at R683 residue. Mutations of R679 or R687 had
123 no effect on entry mediated by trypsin. Spike proteins of field strains of HCoV-229E contain
124 an arginine at the position 679 whereas the laboratory strain VR-740 contains a threonine.
125 Field strains were reported to use preferentially the TPPS pathway [10], this result suggest
126 that acquisition of the arginine residue at the position 679 is not involved in this adaptation. It
127 is surprising that mutation of R687 had no effect on entry mediated by trypsin. Indeed, R687
128 is believed to be located at the N-terminus of the fusion peptide and the arginine residue
129 located at the same position in the SARS-CoV spike protein is the major recognition site of
130 trypsin. Unlike SARS-CoV, mutation of the arginine residue located at the S1/S2 junction

131 (R565N) has little effect on entry mediated at the cell surface by trypsin. *In vivo*, the major
132 route of entry of respiratory coronavirus is believed to be mediated by the TTPS pathway. To
133 confirm our results observed with trypsin, we expressed the human TMPRSS2 in Huh-7 cells
134 with a lentiviral vector. Transduction of cells with a lentivirus expressing the human
135 TMPRSS2 resulted in an increase in transduction by particles pseudotyped with the wild type
136 S protein which was insensitive to ammonium chloride treatment (Fig 2(b)). As previously
137 observed for the SARS-CoV [13, 14], HCoV-229E pseudotype entry mediated at the cell
138 surface was more efficient than entry by the endocytic pathway. As seen with infection
139 mediated by trypsin, mutation of the R683 abolished the capacity of the virus to use
140 TMPRSS2 to infect the cells since its infectivity was sensitive to ammonium chloride
141 treatment of the cells (Fig 2(c)). To further confirm that cleavage mediated by trypsin-like
142 serine protease occurs at R683N, HEK293T cells transiently expressing the wild type or the
143 different mutant proteins were treated by trypsin for 1h and then proteins at the cell surface
144 were biotinylated at 4°C. After precipitation of biotinylated protein with streptavidin-
145 conjugated beads, cleavage products were analysed in western blotting with an antibody
146 directed against a C-terminal tag of the protein. Spike proteins with a furin cleavage site
147 introduced at the S1/S2 boundary (Fur565) or at the S2' position (Fur687) were used as
148 controls (Fig. 3). Cleavage by furin between the S1 and S2 domain gave rise to a band with a
149 size below 135 kDa corresponding to the S2 domain. Cleavage at the S2' position (Fur687)
150 induces a cleavage product with a smaller size below 100 KDa. Contrary to other
151 coronaviruses for which trypsin induces mainly a processing at the S1/S2 junction, cleavage
152 of the wild-type HCoV-229E spike protein by trypsin resulted in the appearance of a cleavage
153 product in western blot with a size similar to the one observed for the mutant Fur687,
154 corresponding to the cleavage of the protein at the S2' position. Mutation of R683 abolished
155 the cleavage induced by trypsin confirming the proteolytic processing of the spike protein at
156 this position by trypsin. The absence of any S2 fragment detection suggests that either the S2
157 fragment is very short-lived, being rapidly processed at the S2' position or that the S1/S2
158 cleavage is not required for the S2' cleavage. If the S2 fragment was short-lived, then
159 mutation of R683 should lead to the accumulation of the intermediate cleavage product.
160 However, we did not observe any accumulation of cleavage product of bigger size when R683
161 was mutated, arguing again against the requirement of the S1/S2 cleavage to prime the
162 cleavage at the S2' position as shown for the SARS-CoV and MERS-CoV spike proteins [2,
163 6].

164 Taken together, our results show that activation of the fusion process by trypsin and trypsin-
165 like serine protease, particularly TMPRSS2 expressed in human airway epithelia, strongly
166 rely on the R683 residue as a cleavage site. It has been shown that the position of the cleavage
167 site in the S2' region of the SARS-CoV spike protein can be shifted from the position relative
168 to the fusion peptide but with some effects on fusion activation and infection. Indeed, for the
169 SARS-CoV spike protein, cleavage at the N-terminus of the fusion peptide results in
170 improved fusion and infection capacities [4]. For HCoV-229E spike protein, the cleavage site
171 by trypsin is not located at the N-terminus of the fusion peptide but is present 3 residues
172 upstream. For the SARS-CoV spike protein, even if the location of the cleavage site presents
173 some flexibility, certain position in the vicinity of the fusion peptide were also not accessible
174 to protease. Indeed, no cleavage product was observed when R683 was mutated (Fig 3),
175 suggesting that both arginine residues 679 and 687 were not accessible to the protease,
176 otherwise, if these residues could be cleaved but without being functional to activate the
177 fusion, the same band would have been observed with the R683N mutant.

178

179 **Author statements**

180 The authors declare no conflict of interest.

181

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189

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230

231 **FIGURE LEGEND**

232 **Figure 1**

233 (A) Alignment of the S1/S2 and S2' region of different coronavirus spike protein (HCoV-
234 229E isolated in France, 2014, HCoV-229E Niigata/01/08, HCoV-229E 0349, HCoV-229E
235 USA/932-72/1993, HCoV-229E laboratory strain VR-740, MERS-CoV and SARS-CoV).
236 R565 at the S1/S2 boundary is in red and R679, R683 and R687 are presented in green. For

237 SARS-CoV and MERS-CoV the arginine at the S2' position is also presented in green. The
238 amino sequence between the S1/S2 boundary and the S2' region is not totally presented and
239 the omitted sequence is depicted by a dotted line. (B) MLV-based particles pseudotyped with
240 the wild type spike protein (WT) or with a spike protein in which a furin cleavage site was
241 introduced in the S2' region (Fur687) were produced and used to inoculate Huh-7 cells in
242 presence or absence of 25 mM NH₄Cl or 5 μM of furin inhibitor. Results are presented as the
243 percentage of transduction in absence of drug (DMSO) and are expressed as the mean of three
244 independent experiments. Error bars represent the standard error of the means (SEM). Data
245 were analysed by using an ANOVA test (*** P<0.001). (C) Endosomal entry of particles
246 pseudotyped with the mutated spike proteins. Pseudoparticles were produced and used to
247 inoculate Huh-7 cells at 37°C in presence or absence of 25 mM NH₄Cl. 48h later, the cells
248 were lysed and luciferase activity was measured. Results are presented as relative light units
249 (RLU) and are expressed as the mean of five independent experiments. Error bars represent
250 the standard error of the means (SEM). Data were analysed by using an ANOVA test (***
251 P<0.001). (D) Analysis of pseudoparticles formation. Pseudoparticles were produced with the
252 different mutated spike proteins and concentrated on sucrose cushion, then pseudoparticles
253 were lysed and the production was analysed by western blotting with anti-VSVG antibody to
254 detect the spike protein and anti-gag antibody.

255

256 **Figure 2**

257 (A). Trypsin-mediated entry at the cell surface. Particles pseudotyped with the different
258 mutated spike proteins were bound at the cell surface at 4°C in presence of 25 mM NH₄Cl
259 and were treated with a 5 min incubation with 2 μg/ml of trypsin to induce fusion. 48h later,
260 the cells were lysed and luciferase activity was measured. Results are presented as the
261 percentage of transduction observed with particles pseudotyped with the wild type protein and
262 are expressed as the mean of three independent experiments. Error bars represent the standard
263 error of the means (SEM). Data were analysed by using an ANOVA test (** P<0.01) (B)
264 Huh-7 cells were transduced or not with a lentivirus expressing the human TMPRSS2. Then
265 cells were infected with particles pseudotyped with the wild type spike protein in presence or
266 absence of 25 mM NH₄Cl. Results are presented as relative light units (RLU) and are
267 expressed as the mean of three independent experiments. Data were analysed by using an
268 ANOVA test (*** P<0.001). (C) Huh-7 cells transduced or not with a lentivirus expressing
269 TMPRSS2 were inoculated with particles pseudotyped with the different mutated spike
270 proteins. Results are presented as the percentage of infection observed with particles

271 pseudotyped with the wild type protein and are expressed as the mean of three independent
272 experiments. Error bars represent the standard error of the means (SEM). Data were analysed
273 by using an ANOVA test (***) P<0.001).

274

275 **Figure 3**

276 Spike protein cleavage with trypsin. HEK293T cells were transfected with plasmid encoding
277 the different mutated spike protein. 48h later, cells were treated for 1h with 2µg/ml of trypsin
278 at 37°C. Then cell surface protein were biotinylated at 4°C and the cells were lysed.
279 Biotinylated protein were precipitated with streptavidin-conjugated beads and analysed by
280 western blotting with the C-terminal part of the protein detected with an anti-VSVG antibody.

281

282

Figure 1 :

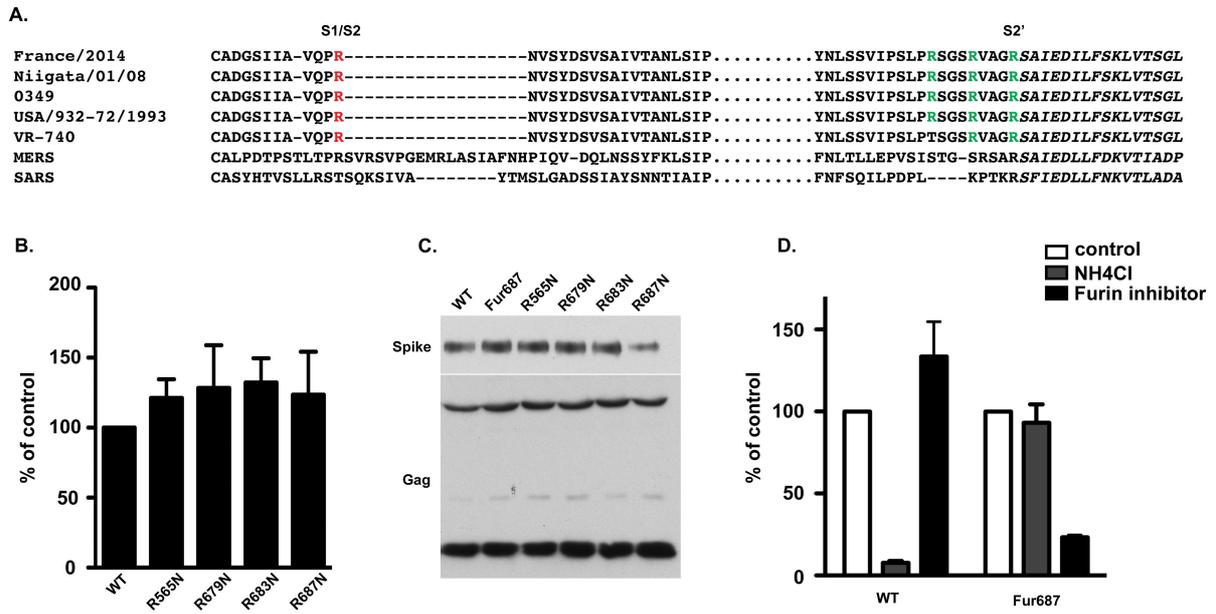


Figure 2 :

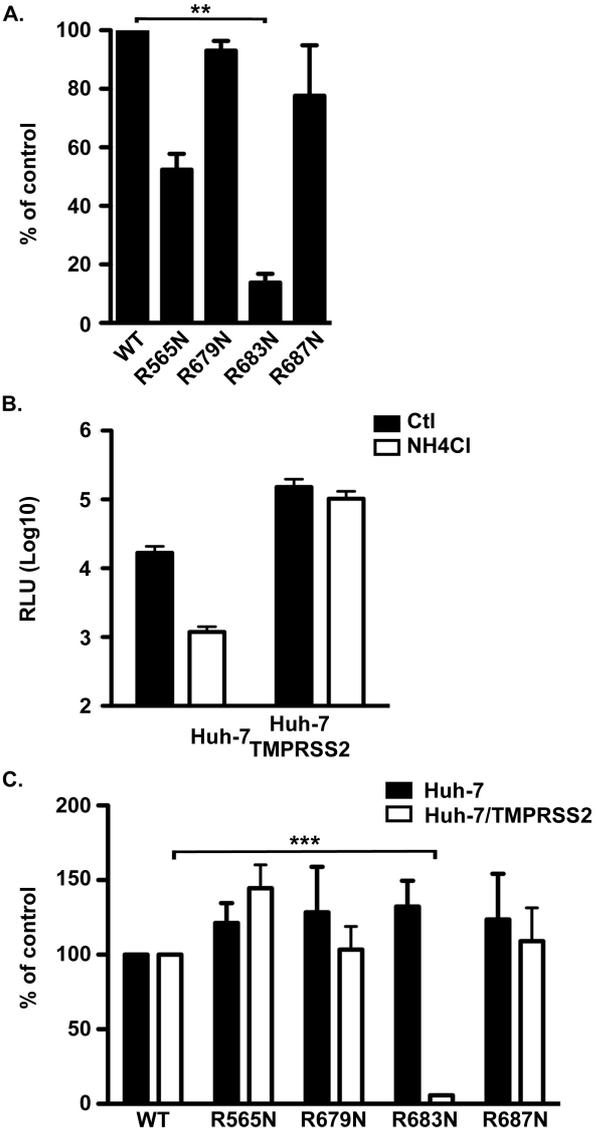


Figure 3 :

