JOURNAL OF GENERAL VIROLOGY

SHORT COMMUNICATION

Bonnin *et al.*, *Journal of General Virology* 2018;99:908–912

DOI 10.1099/jgv.0.001074

MICROBIOLOGY SOCIETY

HCoV-229E spike protein fusion activation by trypsin-like serine proteases is mediated by proteolytic processing in the S2′ region

Ariane Bonnin, Adeline Danneels, Jean Dubuisson, Anne Goffard† and Sandrine Belouzard*†

Abstract

Human coronavirus 229E (HCoV-229E) is responsible for common colds. Like other coronaviruses, HCoV-229E exploits cellular proteases to activate fusion mediated by the spike protein. We analysed the proteolytic processing of the HCoV-229E spike protein by trypsin-like serine proteases leading to activation of the fusion process. Unlike in other coronaviruses, HCoV-229E fusion activation appears to be a one-step process. Indeed, cleavage of the S1/S2 interface does not seem to be a prerequisite, and the fusion activation is highly reliant on the S2′ region, with arginine residue 683 acting as the recognition site.

Coronavirinae are enveloped viruses with a long positive-sense RNA genome. As for other enveloped viruses, they need to fuse their viral envelope with a host cell membrane to deliver their genome to the target cell. Coronavirus spike proteins are major mediators of virus entry and fulfil a dual function by mediating receptor binding and fusion. Spike proteins consist of two distinct domains carrying these functions. The amino-terminal subunit, called the S1 domain, is responsible for receptor binding, whereas the C-terminal part, called the S2 domain, contains the fusion machinery. Proteolytic priming of coronavirus spike protein is a key feature of the fusion process, and coronaviruses exploit a wide variety of host proteases, including cathepsins, furin and members of the transmembrane serine protease (TTSP) family [1], to achieve this requirement. It is likely that the proteolytic processing differs according to the protease used. Depending on the cellular localization of the protease used to activate the fusion, entry can occur either at the cell surface or after internalization of the virus by endocytosis. For SARS-CoV spike protein, two cleavage events mediated by trypsin have been identified. The SARS-CoV virion harbours an uncleaved spike protein at its surface. First, the spike protein is primed by cleavage of the S1 and S2 domains, and then, to fully activate fusion, a second cleavage occurs at a position called S2′ (R797), exposing the fusion peptide [2, 3]. Activation of the SARS-CoV spike protein fusion by elastase also involves recognition of a cleavage site in the S2′ region [4]. The importance of S2′ cleavage has also been demonstrated for MERS-CoV and MHV [5, 6].

HCoV-229E was identified in 1966 in students suffering from the common cold [7], but children, the elderly and immune-compromised persons can develop a more severe disease [8]. It has been shown that HCoV-229E requires endosomal cathepsin L activity to infect Hela cells [9]. However, Shirato *et al.* recently showed that clinical isolates of HCoV-229E preferentially use the transmembrane protease serine 2 (TMPRSS2), a trypsin-like protease, rather than endosomal cathepsin, for entry [10]. TMPRSS2 is known to be present at the surface of the human airway epithelia and is likely the main activator of HCoV-229E fusion *in vivo*. In addition, TMPRSS2-mediated entry at the cell surface protects the virus from the inhibitory effects of interferon-induced transmembrane proteins (IFITMs) that are known to inhibit the entry of several enveloped virus [11]. However, the detailed proteolytic processing of HCoV-229E spike protein to activate its fusion remains to be elucidated.

To investigate this proteolytic maturation, we first cloned the spike protein sequence from a patient infected by HCoV-229E who was hospitalized in Lille, France in 2014 (Spike France/Lille/2014, accession number MH048989).

Received 14 March 2018; Accepted 20 April 2018

Author affiliation: Université Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 – CIIL U1019 – CIIL-Centre d'Infection et d'Immunité de Lille Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France.

***Correspondence:** Sandrine Belouzard, sandrine.belouzard@ibl.cnrs.fr

Keywords: HCoV-229E; fusion; entry; spike protein.

Abbreviations: HCoV-229E, human coronavirus 229E; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; TMPRSS2, transmembrane protease serine 2; TTSP, transmembrane serine protease.

†These authors contributed equally to this work.

The GenBank accession number for the France/Lille/2014 spike protein sequence is MH048989.

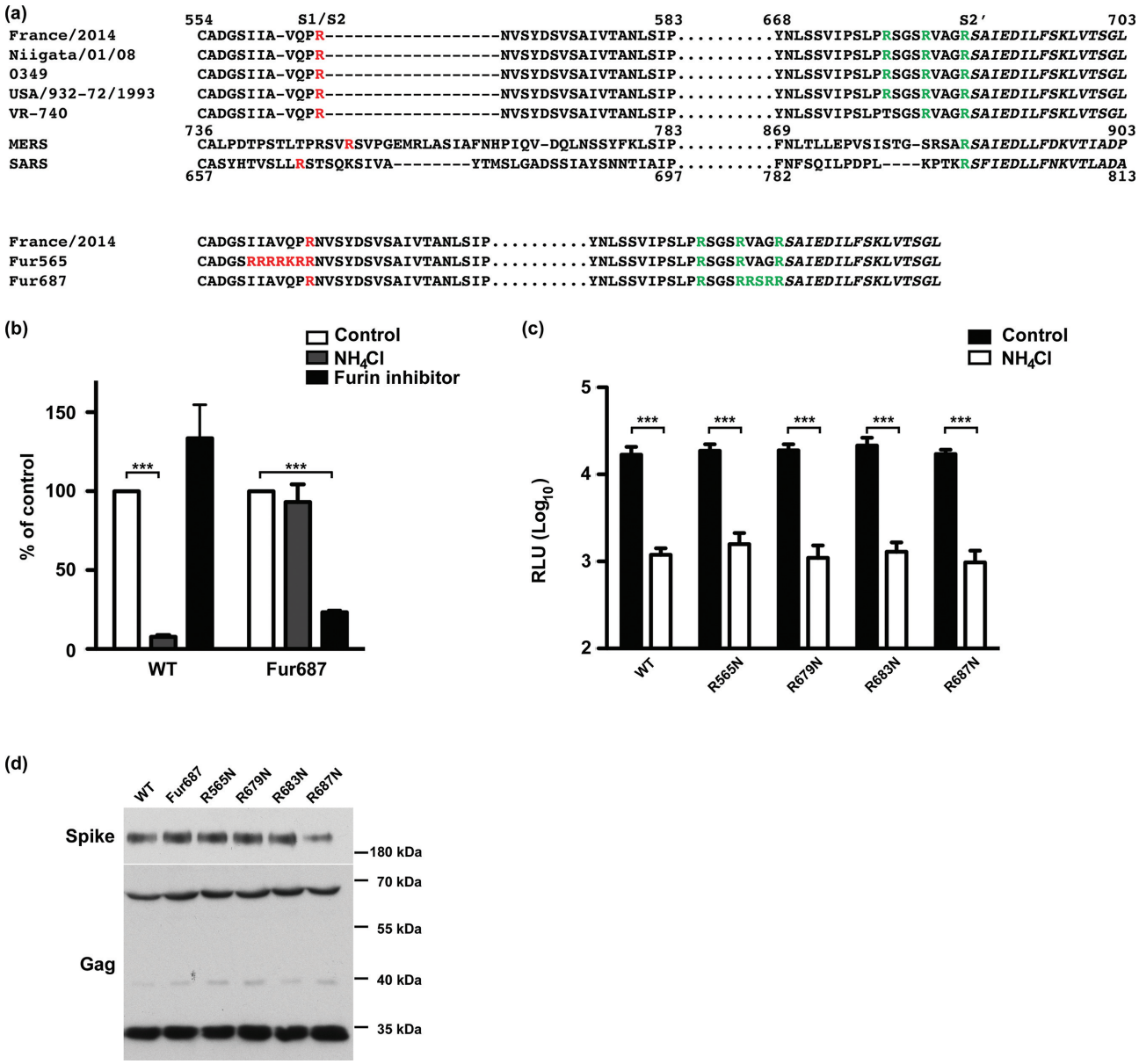

Fig. 1. (a) Alignment of the S1/S2 and S2′ regions of different coronavirus spike proteins (HCoV-229E isolated in France, 2014; HCoV-229E Niigata/01/08; HCoV-229E 0349; HCoV-229E USA/932-72/1993; HCoV-229E laboratory strain VR-740; MERS-CoV; and SARS-CoV). R565 at the S1/S2 boundary is in red and R679, R683 and R687 are presented in green. For SARS-CoV and MERS-CoV the arginine at the S2′ position is also presented in green. The amino sequence between the S1/S2 boundary and the S2′ region is not presented in its entirety, and the omitted sequence is depicted by a dotted line. (b) MLV-based particles pseudotyped with the wild-type spike protein (WT) or with a spike protein in which a furin cleavage site was introduced in the S2′ region (Fur687) were produced and used to inoculate Huh-7 cells in the presence or absence of 25 mM NH_4Cl or 5 µM of furin inhibitor. The results are presented as the percentage of transduction in the absence of drug (DMSO) and are expressed as the mean of three independent experiments. The error bars represent the standard error of the means (SEM). The data were analysed by using an analysis of variance (ANOVA) test (***P<0.001). (c) Endosomal entry of particles pseudotyped with the mutated spike proteins. Pseudoparticles were produced and used to inoculate Huh-7 cells at 37 °C in the presence or absence of 25 mM NH_4Cl. Forty-eight hours later, the cells were lysed and luciferase activity was measured. The results are presented as relative light units (RLU) and are expressed as the mean of five independent experiments. The error bars represent the SEM. The data were analysed by using an ANOVA test (***P<0.001). (d) Analysis of pseudoparticle formation. Pseudoparticles were produced with the different mutated spike proteins and concentrated on a sucrose cushion, before the pseudoparticles were lysed and the production was analysed by Western blotting with anti-VSVG antibody to detect the spike protein and anti-gag antibody.

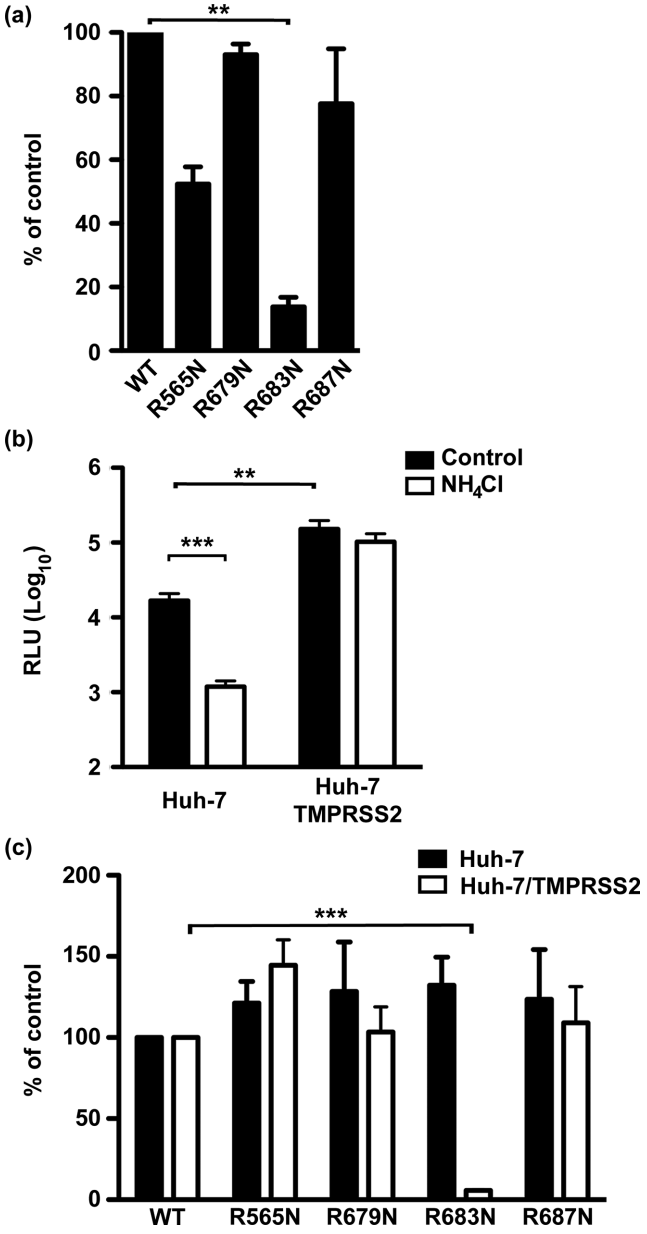

Fig. 2. (a) Trypsin-mediated entry at the cell surface. Particles pseudotyped with the different mutated spike proteins were bound at the cell surface at 4 °C in the presence of 25 mM NH_4Cl and treated with a 5 min incubation with 2 µg ml^{-1} of trypsin to induce fusion. Forty-eight hours later, the cells were lysed and luciferase activity was measured. The results are presented as the percentage of transduction observed with particles pseudotyped with the wild-type protein and are expressed as the mean of three independent experiments. The error bars represent the standard error of the means (SEM). The data were analysed by using an ANOVA test (**P<0.01). (b) Huh-7 cells were transduced or not with a lentivirus expressing human TMPRSS2. Then cells were infected with particles pseudotyped with the wildtype spike protein in the presence or absence of 25 mM NH_4Cl. The results are presented as relative light units (RLU) and are expressed as the mean of three independent experiments. The data were analysed by using an ANOVA test (***P<0.001). (c) Huh-7 cells transduced or not with a lentivirus expressing TMPRSS2 were inoculated with particles pseudotyped with the different mutated spike proteins. The results are presented as the percentage of infection observed with particles pseudotyped with the wild-type protein and are expressed as the mean of three independent experiments. The error bars represent the SEM. The data were analysed by using an ANOVA test (***P<0.001).

The codon-optimized sequence of the France/Lille/2014 spike protein was inserted into the pCDNA3.1(+) vector and fused with a sequence encoding a C-terminal VSVG tag to facilitate the detection of the protein in immunoblot. Then, we analysed the proteolytic processing of the spike protein required for entry mediated by trypsin/trypsin-like protease, focusing on the role of the S1/S2 junction and the S2′ region. The sequence of the French isolate spike protein shows 99 % identity with the spike of the Niigata, 0349 and USA/932-72/1993 isolates, and only 94 % of identity with the VR-740 laboratory strain. HCoV-229E spike protein contains three arginine residues in the S2′ region (R679, R683 and R687), which are conserved in different strains of HCoV-229E, except for R679, which is absent in the VR-740 laboratory strain (Fig. 1a). First, to confirm the role of the HCoV-229E spike protein S2′ region in entry, we inserted a furin cleavage site (Fur687) in the spike protein (Fig. 1a), at the N-terminal position of the potential fusion peptide. To analyse the effect of the mutation in the context of viral particles, we used a murine leukaemia virus (MLV)-based pseudotyping system that produces particles inducing luciferase expression after the transduction of permissive cells. Pseudoparticles fully mimic the functionality of the wild-type virus in terms of cell entry and were used because of their technical advantages. Huh-7 cells were inoculated with pseudoparticles harbouring the wild-type protein or the Fur687 mutant (Fig. 1b). Neutralization of endosomal pH by ammonium chloride treatment inhibited transduction by HCoV-229E pseudoparticles, showing that HCoV-229E infects Huh-7 cells via the endosomal route and uses low-pH active endosomal protease to mediate its fusion. As shown in Fig. 1(b), pseudotyped virions with HCoV-229E spike protein containing a furin cleavage site at the S2′ position were insensitive to endosomal pH neutralization, but were affected by furin convertase inhibitor. This result shows that the spike protein with the furin cleavage site does not rely on endosomal cathepsin for entry, but instead relies on furin, and this confirms the involvement of the S2′ region for fusion activation of HCoV-229E, as observed for other coronaviruses [2, 5, 6, 12]. It is interesting to note that the spike proteins incorporated into the pseudotyped virions were not cleaved by furin in producing cells, indicating that the protein is not processed during the exit of the producer cell, but during entry into the target cell (Fig. 1d). This suggests that during exit and secretion the cleavage site is inaccessible to furin, and that during entry conformational change induced by receptor binding may expose the cleavage site. To investigate the role of the S1/S2 junction and the specific role of the three arginine residues located in the S2′ region (R679, R683 and R687) in the proteolytic activation of HCoV-229E spike protein by trypsin-like serine-

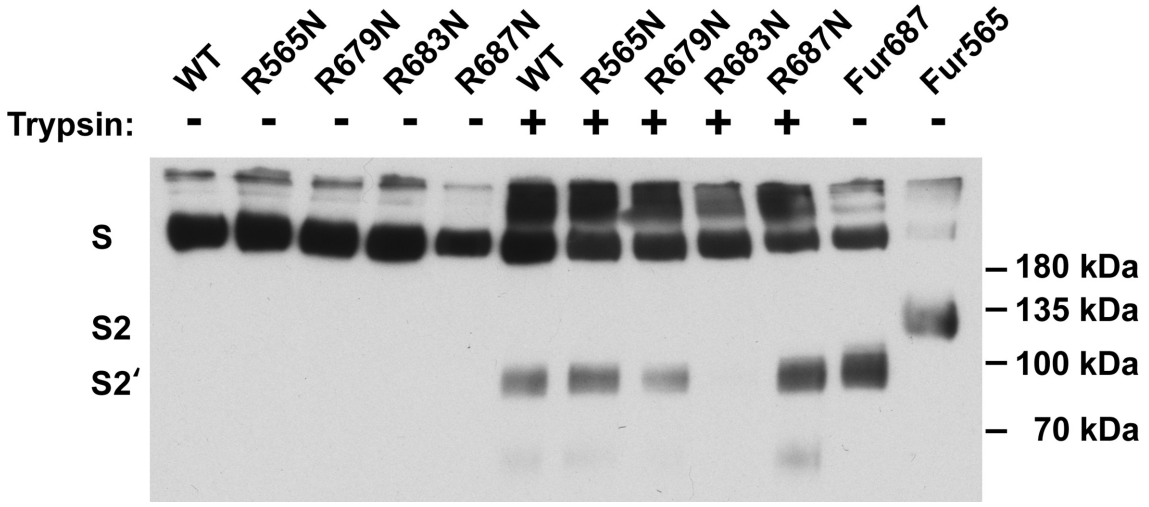

Fig. 3. Spike protein cleavage with trypsin. HEK293T cells were transfected with plasmid encoding the different mutated spike proteins. Forty-eight hours later, cells were treated for 1 h with 2 µg ml^{-1} of trypsin at 37 °C. Then cell surface proteins were biotinylated at 4 °C and the cells were lysed. Biotinylated proteins were precipitated with streptavidin-conjugated beads and analysed by Western blotting with the C-terminal part of the protein being detected with an anti-VSVG antibody.

proteases, the arginine residues present at S1/S2 and at S2′ were mutated individually (R565N, R679N, R683N or R687N). We chose to mutate the arginine residues into asparagine to minimize the effect of the mutations on the conformation/folding of the protein. As seen in Fig. 1(c), these arginine residues were not implicated in entry when the virions used the endocytic pathway to invade the cells. The endosomal route of entry of the different mutants was confirmed by inhibition of their entry by ammonium chloride treatment (Fig. 1c). Indeed, when HCoV-229E is internalized, the virus is dependent on the endosomal cathepsin L to mediate its fusion. However, unlike other proteases, cathepsin L has a limited prime-site specificity in terms of recognition sites and the precise sites of proteolysis by cathepsin L remain unclear.

In 2005, Simmons *et al.* showed that SARS-CoV fusion can be induced at the cell surface when virions are bound to the plasma membrane at 4 °C and briefly treated with an exogenous protease such as trypsin [13]. Particles pseudotyped with the different S1/S2 and S2′ arginine spike mutants were bound at the surface of Huh-7 cells treated with ammonium chloride to neutralize the endosomal pH and block the endosomal entry pathway. Then, fusion was induced by a brief trypsin treatment (Fig. 2a). For the mutant R683N, trypsin treatment was not able to bypass the requirement for cathepsin L activity during the entry process, suggesting that proteolysis by trypsin occurs at the R683 residue. Mutations of R679 or R687 had no effect on entry mediated by trypsin. Spike proteins of field strains of HCoV-229E contain an arginine at position 679, whereas the laboratory strain VR-740 contains a threonine. The field strains were reported to preferentially use the TPPS pathway [10], and this result suggests that acquisition of the arginine residue at position 679 is not involved in this adaptation. It is surprising that mutation of R687 had no effect on entry mediated by trypsin. Indeed, R687 is believed to be located at the N-terminus of the fusion peptide and the arginine residue located at the same position in the SARS-CoV spike protein is the major recognition site of trypsin. Unlike in SARS-CoV, mutation of the arginine residue located at the S1/S2 junction (R565N) has little effect on entry mediated at the cell surface by trypsin. *In vivo,* the major route of entry of respiratory coronavirus is believed to be mediated by the TTPS pathway. To confirm the results we observed with trypsin, we expressed human TMPRSS2 in Huh-7 cells with a lentiviral vector. Transduction of cells with a lentivirus expressing human TMPRSS2 resulted in an increase in transduction by particles pseudotyped with the wild-type S protein that was insensitive to ammonium chloride treatment (Fig. 2b). As previously observed for SARS-CoV [13, 14], HCoV-229E pseudotype entry mediated at the cell surface was more efficient than entry by the endocytic pathway. As seen with infection mediated by trypsin, mutation of the R683 abolished the capacity of the virus to use TMPRSS2 to infect the cells, since its infectivity was sensitive to ammonium chloride treatment of the cells (Fig. 2c). To further confirm that cleavage mediated by trypsin-like serine protease occurs at R683N, HEK293T cells transiently expressing the wild-type or the different mutant proteins were treated with trypsin for 1 h and then proteins at the cell surface were biotinylated at 4 °C. After precipitation of biotinylated protein with streptavidin-conjugated beads, cleavage products were analysed by Western blotting with an antibody directed against a C-terminal tag of the protein. Spike proteins with a furin cleavage site introduced at the S1/S2 boundary (Fur565) or at the S2′ position (Fur687)

were used as controls (Fig. 3). Cleavage by furin between the S1 and S2 domain gave rise to a band with a size below 135 kDa corresponding to the S2 domain. Cleavage at the S2′ position (Fur687) induces a cleavage product with a smaller size (below 100 kDa). In contrast to other coronaviruses, for which trypsin mainly induces processing at the S1/S2 junction, cleavage of the wild-type HCoV-229E spike protein by trypsin resulted in the appearance of a cleavage product in the Western blot that was of a similar size to the one observed for the mutant Fur687, corresponding to the cleavage of the protein at the S2′ position. Mutation of R683 abolished the cleavage induced by trypsin, confirming the proteolytic processing of the spike protein at this position by trypsin. The absence of any S2 fragment detection suggests that either the S2 fragment is very short-lived, being rapidly processed at the S2′ position, or that the S1/S2 cleavage is not required for the S2′ cleavage. If the S2 fragment was short-lived, then mutation of R683 should lead to the accumulation of the intermediate cleavage product. However, we did not observe any accumulation of cleavage product of larger size when R683 was mutated, again arguing against the requirement for the S1/S2 cleavage to prime the cleavage at the S2′ position, as shown for the SARS-CoV and MERS-CoV spike proteins [2, 6].

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

Funding information
A part of this study was supported by a grant 'Soutien aux thématiques émergentes 2014' from the University Hospital of Lille, France.

Acknowledgements
We thank all the members of the molecular and cellular virology laboratory for helpful discussions and input during the course of this work.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. *Antiviral Res* 2013;100:605–614.
2. Belouzard S, Chu VC, Whittaker GR. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci USA* 2009;106:5871–5876.
3. Madu IG, Roth SL, Belouzard S, Whittaker GR. Characterization of a highly conserved domain within the severe acute respiratory syndrome coronavirus spike protein S2 domain with characteristics of a viral fusion peptide. *J Virol* 2009;83:7411–7421.
4. Belouzard S, Madu I, Whittaker GR. Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain. *J Biol Chem* 2010;285:22758–22763.
5. Burkard C, Verheije MH, Wicht O, van Kasteren SI, van Kuppeveld FJ *et al.* Coronavirus cell entry occurs through the endo-/lysosomal pathway in a proteolysis-dependent manner. *PLoS Pathog* 2014;10:e1004502.
6. Millet JK, Whittaker GR. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. *Proc Natl Acad Sci USA* 2014;111:15214–15219.
7. Hamre D, Procknow JJ. A new virus isolated from the human respiratory tract. *Proc Soc Exp Biol Med* 1966;121:190–193.
8. van der Hoek L. Human coronaviruses: what do they cause? *Antivir Ther* 2007;12:651–658.
9. Kawase M, Shirato K, Matsuyama S, Taguchi F. Protease-mediated entry via the endosome of human coronavirus 229E. *J Virol* 2009;83:712–721.
10. Shirato K, Kanou K, Kawase M, Matsuyama S. Clinical isolates of human coronavirus 229E bypass the endosome for cell entry. *J Virol* 2017;91:JVI.01387–16.
11. Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S *et al.* TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. *J Virol* 2013;87:6150–6160.
12. Yamada Y, Liu DX. Proteolytic activation of the spike protein at a novel RRRR/S motif is implicated in furin-dependent entry, syncytium formation, and infectivity of coronavirus infectious bronchitis virus in cultured cells. *J Virol* 2009;83:8744–8758.
13. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL *et al.* Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proc Natl Acad Sci USA* 2005;102:11876–11881.
14. Matsuyama S, Ujike M, Morikawa S, Tashiro M, Taguchi F. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc Natl Acad Sci USA* 2005;102:12543–12547.