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Neutrophil Elastase and Proteinase 3 Cleavage Sites Are Adjacent to the Polybasic Sequence within the Proteolytic Sensitive Activation Loop of the SARS-CoV-2 Spike Protein

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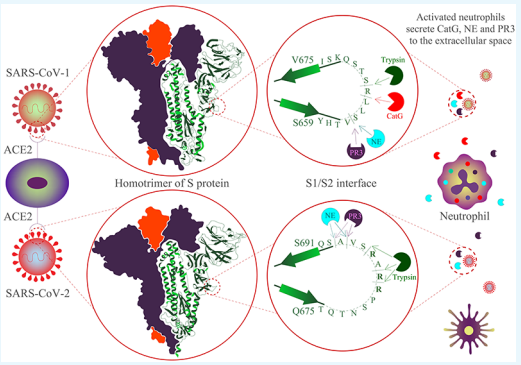
ABSTRACT: Serine proteases neutrophil elastase (NE), protease 3 (PR3), cathepsin G (CatG), and neutrophil serine protease 4 (NSP4) are released by activated neutrophils swarming around the place of pathogen invasion to provoke an immune response. However, uncontrolled proteolytic activity of proteases results in various human diseases, including cardiovascular diseases, thrombosis, and autoimmunity. In addition, proteases can be hijacked by several viruses to prime virus-derived surface proteins and evade immune detection by entering into the host cell. Indeed, porcine elastase increases the suitability of host cells to be infected by SARS-CoV-1. We compared the cleavage sites of human NE, PR3, and CatG as well as porcine-derived trypsin within the amino acid sequence of the proteolytic sensitive activation loop at the interface of S1/S2 of the spike protein (S protein) of SARS-CoV-1 as well as SARS-CoV-2. As a result, NE and PR3, but not CatG, hydrolyze the scissile peptide bond adjacent to the polybasic amino acid sequence of the S1/S2 interface of SARS-CoV-2, which is distinctive from SARS-CoV-1. These findings suggest that neutrophil-derived NE and PR3 participate in priming of the S1/S2 interface during an immune response.



1. INTRODUCTION

The global COVID-19 pandemic is caused by the outbreak of the novel coronavirus SARS-CoV-2. Severe and critical complications of COVID-19 are the result of a dysregulation of the immune and coagulation systems contributing to the formation of systemic inflammation and thrombosis.[1]

Neutrophils are crucial for the first line of defense by migrating from the blood vessel to the site of infection to battle invading pathogens, thereby secreting several mediators to initiate an immune response. These mediators include neutrophil serine proteases (NSPs), such as neutrophil elastase (NE), cathepsin G (CatG), protease 3 (PR3), and neutrophil serine protease 4 (NSP4).[2,3] NSPs are important in host defense against intracellular and extracellular pathogens.[4] The proteolytic activity depends on a catalytic triad composed of aspartate, histidine, and serine residues. Although there is a certain degree of functional redundancy, individual proteases control different cellular processes and are selective for distinct amino acid residues within substrates.[5,6] Additionally, neutrophil extracellular traps (NETs), composed of extracellular webs of chromatin bound to granule proteins and NSPs, are extensively released from neutrophils in response to infections.[7] NETs are also involved in the COVID-19 pathogenesis and inflammation.[8] Whether NSPs are proteolytically active has been challenged since it has been found that the formation of NETs does not require their catalytic activity, and NSPs are released as inactive proteases within NETs.[9]

The S protein is a homotrimer consisting of the S1 subunit embedding the receptor-binding domain (RBD) with the receptor-binding motif (RBM) and the S2 subunit functional as the fusion peptide, important for the cell entry of SARS-CoV-2.[10] The fusion of host cells with the virus is mediated by the sequential proteolytic cleavage of the S protein by host cell proteases at the S1/S2 interface, and at the S2′ site, the cleavage of S2′ generates the fusion peptide, which is inserted into the cell membrane and triggers the membrane fusion process.[11−13] These proteases mainly encompass the cell membrane-associated transmembrane protease serine 2 (TMPRSS2) and furin, which digest at R↑S of the S1/S2 junction as well as between R↑S of S2′ and are important candidates responsible for the entry of SARS-CoV-2 into the host cell.[14−18] Indeed, the broad serine protease inhibitor Camostat Mesylate inhibits the proteolytic activity of

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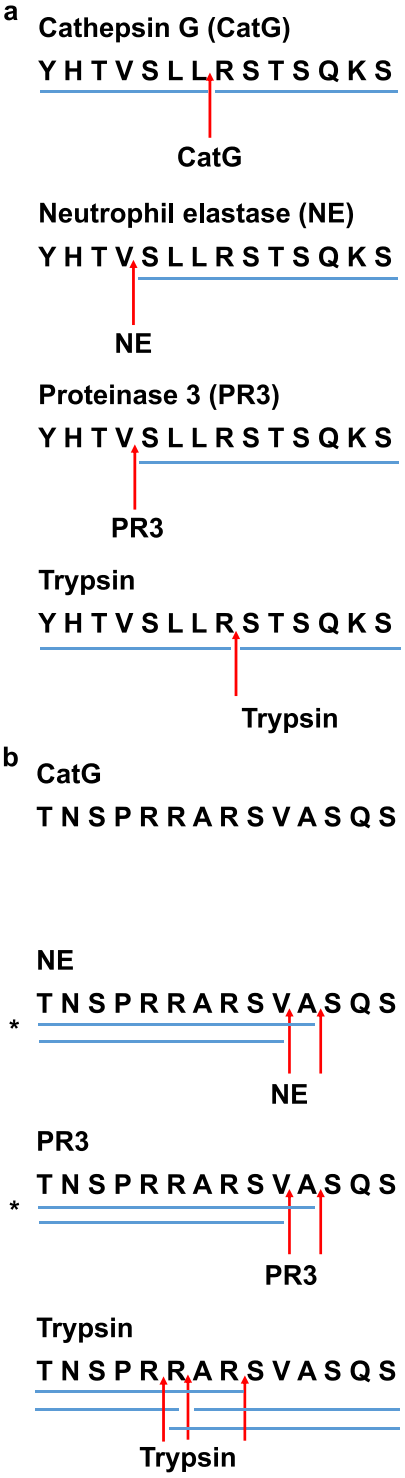

SARS-CoV-1 peptide

Protease	Mass $[M+H]^+$	Expected mass $[M+H]^+$	Peptide
CatG	792.75	793.42	RSTSQKS
	832.16	832.46	YHTVSLL
NE	1106.49	1106.62	SLLRSTSQKS
PR3	1105.85	1106.62	SLLRSTSQKS
Trypsin	636.44	637.32	STSQKS
	988.67	988.56	YHTVSLLR

SARS-CoV-2 peptide

Protease	Mass $[M+H]^+$	Expected mass $[M+H]^+$	Peptide
CatG	1516.71	1516.79	undigested
NE	1143.48	1143.63	TNSPRRARSV
*	1214.67	1214.67	TNSPRRARSVA
PR3	1143.50	1143.63	TNSPRRARSV
*	1214.49	1214.67	TNSPRRARSVA
Trypsin	730.37	730.40	TNSPRR
	804.52	805.42	ARSVASQS
	957.66	957.53	TNSPRRAR
	960.43	961.52	RARSVASQS

Figure 1. Antigen processing of the proteolytic sensitive activation loop. (a) SARS-CoV-1 or (b) SARS-CoV-2 peptides, covering the activation loop of the S protein, were incubated with CatG, NE, PR3, or trypsin, and the resulting digesting pattern was analyzed by HPLC and mass spectrometry. Red arrows indicate cleavage sites, and bars represent peptides detected by mass spectrometry. Asterisk, less dominant peptide; $n = 3$ independent experiments.

TMPRSS2 and significantly decreases SARS-CoV-2 infection.[15]

Here, we investigated the processing of the polybasic sequence of SARS-CoV-1 and SARS-CoV-2 by CatG, NE, PR3, and trypsin to mimic the fate of the S protein when NSPs are secreted at the site of infection.

2. RESULTS

A previous study showed that the infection rate of SARS-CoV-1 was augmented when elastase from a porcine pancreas was present in the assay.[19] Moreover, SARS-CoV-2 patients exhibited an upregulation of NE and CatG in nasopharyngeal swabs determined by proteomics analysis,[20] indicating that these proteases could participate in S protein priming. To this

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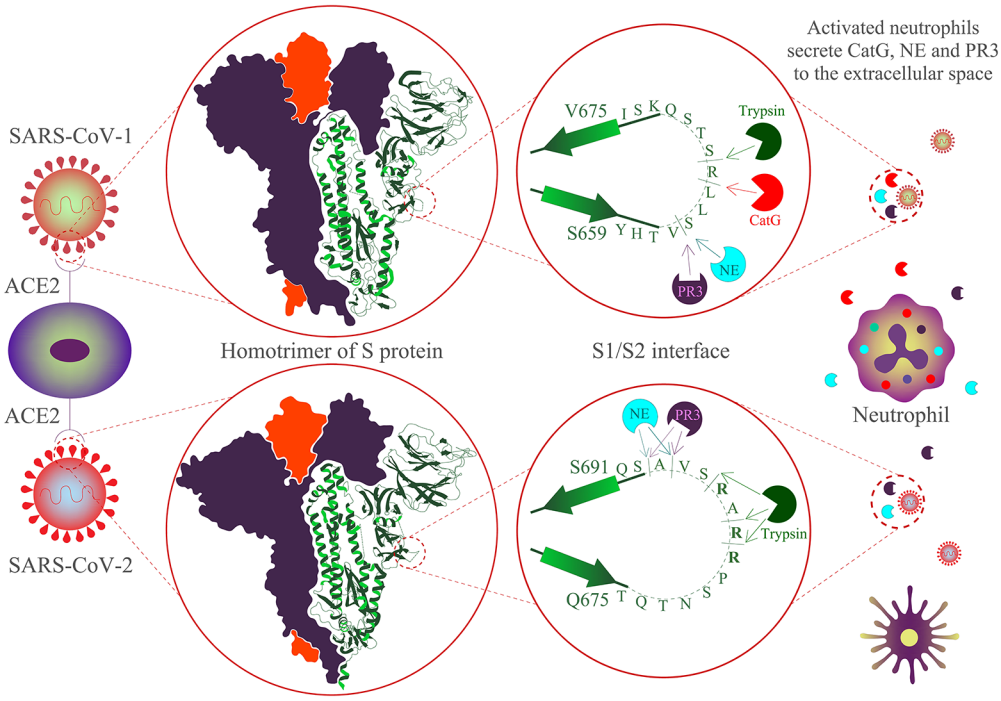

Figure 2. Summary of the proteolytic cleavage sites within the activation loop. The cleavage sites of NE, PR3, CatG, and trypsin are shown for the proteolytic sensitive activation loops of SARS-CoV-1 and SARS-CoV-2. NE, purple; PR3, blue; CatG, red; and trypsin, green. The three-dimensional (3D) structure of S protein (homotrimer) is based on SARS-CoV-1 (PDB ID: 5X5B)[29] as well as SARS-CoV-2 (PDB ID: 7DDN),[30] and the 3D structure was repainted by using AutoCAD software (AutoCAD 2014 for PC, Autodesk, Inc., San Rafael, CA, USA).

end, we synthesized peptides spanning the S1/S2 interface of the S protein of SARS-CoV-1 ($_{660}$YHTVSLLRSTSQKS$_{673}$) and SARS-CoV-2 ($_{678}$TNSPRRARSVASQS$_{691}$), which is also called the polybasic sequence. Both SARS-CoV-1 and SARS-CoV-2 peptides were incubated with CatG, NE, PR3, and trypsin, and the digestion pattern was resolved by high-performance liquid chromatography (HPLC) as well as mass spectrometry (Figure 1). CatG, NE, PR3, and trypsin digest SARS-CoV-1 peptides at distinct sites; of these, CatG hydrolyzed the scissile peptide bond between $_{666}$LR$_{667}$, NE and PR3 mainly between $_{663}$VS$_{664}$, and trypsin strictly at $_{667}$RS$_{668}$ (Figure 1a), which has comparable cleavage sites such as TMPRSS2[21,22] and digests the SARS-CoV-1 S protein precisely at $_{667}$RS$_{668}$.[23]

These findings are in contrast to the processing results obtained from SARS-CoV-2 peptides, where CatG was not able to cleave after any amino acids within the SARS-CoV-2 peptide sequence (Figure 1b). NE and PR3 mainly catalyzed the hydrolysis between $_{687}$VA$_{688}$ residues, whereas trypsin showed several cleavage sites at the polybasic sequence: $_{682}$RR$_{683}$, $_{683}$RA$_{684}$, and $_{685}$RS$_{686}$. Additionally, the cleavage sites for CatG, NE, PR3, and trypsin within the proteolytic sensitive activation loop of SARS-CoV-1 and SARS-CoV-2 are summarized in Figure 2.

3. DISCUSSION

Protease-mediated entry is one of the major determinants of a successful infection by coronaviruses; therefore, it has been speculated that the acquisition of new mutations at the S protein might serve to enhance viral pathogenicity.[19] The polybasic insert at the junction of S1/S2 is one such mutation.[24] It is suggested that this unique cleavage site might promote replication of viruses in humans and has evolved due to a strong selective pressure.[25] Structural analysis of the S protein of SARS-CoV-2 revealed that the four-amino acid insert $_{681}$PRRA$_{684}$ at the S1/S2 interface forms a proteolytically sensitive and flexible activation loop, which is lacking in related SARS-CoV viruses.[26] The activation loop can be easily accessed by host cell proteases to catalyze the cleavage of the polybasic sequence, thereby supporting S protein-mediated membrane fusion.[26] However, the processing of the SARS-CoV-2 S1/S2 activation loop has not been investigated experimentally by neutrophil-derived proteases. Thus, we were interested in monitoring the precise proteolytic cleavage of the polybasic sequence by NSPs, which theoretically support the immune evasion of SARS-CoV-2, and found that CatG is not responsible for priming the S protein of SARS-CoV-2 at this position. In contrast, NE and PR3 cleave adjacent to the polybasic sequence of SARS-CoV-2, indicating novel cleavage sites that are absent in SARS-CoV-1.

It was determined that elastase from a porcine pancreas increases the infection rate of SARS-CoV-1.[19] Notably, the cleavage specificity differs between NE and porcine elastase. Even though both NE and porcine elastase recognize valine and alanine at the P1 position, porcine elastase has a higher preference for methionine and glycine at this position. In addition, glycine and alanine are better recognized by porcine elastase at P2, P3, and P4 subsites,[27] demonstrating the significance of using proteases from human neutrophils in the assay.

The hydrolysis by NE and PR3 of the SARS-CoV-2 peptide are exclusively toward the C-terminal end of the furin cleavage site $_{685}$RS$_{686}$, and CatG does not digest within the proteolytic sensitive activation loop, in contrast to the SARS-CoV-1 peptide (Figure 2). These findings might be important since the "destruction" of the activation loop by CatG (NE and

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PR3) of the SARS-CoV-1 peptide could be assumed as an innate immune response. Additionally, NE was preincubated with Camostat, a serine protease inhibitor with an inhibitor capacity for TMPRSS2, followed by addition of the SARS-CoV-2 peptide; however, only a high concentration (100 μM) of Camostat inhibits the turnover of the SARS-CoV-2 peptide (Supporting Information). Thus, protease inhibitors, such as alpha-1-antitrypsin, which inhibits TMPRSS2, NE, and PR3, are indicated to interfere with SARS-CoV-2 infection.[28] Overall, protease inhibitors might be beneficial for treatment.

3.1. Limitations of the Study. It would be interesting to extend our findings to the processing of the homotrimer protein of SARS-CoV-1 and SARS-CoV-2. Therefore, NSPs or the content of secreted NSPs from activated primary neutrophils could be used followed by analysis by LC−MS/MS. Moreover, substrate recognition and specificity by proteases can be altered by post-translational modifications. In the polybasic sequence, two potential *O*-glycosylation sites are T678 and S686, which might influence the proteolytic activity of proteases.

4. MATERIALS AND METHODS

4.1. *In vitro* Digest, HPLC, and Mass Spectrometry. SARS-CoV-1 and SARS-CoV-2 peptides were synthesized by EMC Microcollections GmbH (Tübingen, Germany). The respective peptides were purified by reversed-phase HPLC using a C18 250 × 8 column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany), and the accurate mass was determined by matrix-assisted laser desorption/ionization−time of flight (MALDI-TOF) mass spectrometry (Reflex IV, Bruker Daltonics, Bremen, Germany). 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix (Reflex IV, Bruker Daltonics). Both peptides were lyophilized, solved in phosphate-buffered saline (PBS) pH 7.4 to a concentration of 10 mg/mL, and stored at −20 °C.

SARS-CoV-1 and SARS-CoV-2 (200 μg/mL) were incubated with 4 μg/mL of NE (Cat. no. SE563, elastase from leukocytes of human sputum, Elastin Products Company Inc., EPC, Owensville, MO, USA), 4 μg/mL of PR3 (Cat. no. ML734, proteinase 3 from leukocytes of human sputum, Elastin Products Company Inc., EPC, Owensville, MO, USA), 4 μg/mL of CatG (Cat. no. E-002, neutrophil-derived human CatG, BioCentrum Ltd., Krakow, Poland), or 4 μg/mL of trypsin (Cat. no. 5455.1, trypsin from a porcine pancreas, Roth, Karlsruhe, Germany) in PBS pH 7.4. The digest was performed for 2 h at 37 °C, and the digestion pattern was resolved by reversed-phase HPLC. A gradient by using an Intelligent Pump L-6200 (Merck-Hitachi, Darmstadt, Germany) connected to a Reprosil 100, 250 × 2 mm, C18 with a 5 μm particle diameter column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was performed. The flow rate was 0.3 mL/min, and the separation of the digest products was done by using the following linear gradient of 0−50% acetonitrile in 0.05% trifluoroacetic acid for 30 min. UV chromatograms were acquired at 214 nm by a UV−vis detector L-4200 (Merck-Hitachi, Darmstadt, Germany) and Chromato-Integrator D-2500 (Merck-Hitachi, Darmstadt, Germany).

The peptides were collected and analyzed by mass spectrometry (MALDI-TOF, Reflex IV, Bruker Daltonics, Bremen, Germany). The acquired mass spectra were manually compared with predicted peptides generated by an Expasy FindPept tool (https://web.expasy.org/findpept/, Swiss Institute of Bioinformatics, Lausanne, Switzerland).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00363.

Supplementary figure showing the inhibition of NE by Camostat. SARS-CoV-2 peptide was preincubated with different concentrations of Camostat. Afterward, NE was added, incubated for 2 h at 37 °C, and the digestion pattern was resolved by HPLC (PDF)

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Author Contributions

H.K. and T.B. performed the experiments. T.B. and A.Z. generated the figures. Z.M., A.Z., and T.B. wrote, reviewed, and edited the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Cat	cathepsin
CatG	cathepsin G
COVID-19	coronavirus disease 2019
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NSPs	neutrophil serine proteases
NSP4	neutrophil serine protease 4
RBD	receptor-binding domain
RBM	receptor-binding motif
PR3	proteinase 3
RAS	renin angiotensin system
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
S protein	spike protein
TMPRSS2	transmembrane protease serine 2

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