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BRIEF REPORT

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Anthrax protective antigen is a calcium-dependent serine protease

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ABSTRACT

Bacillus anthracis secretes a three component exotoxin-complex, which contributes to anthrax pathogenesis. Formation of this complex starts with the binding of protective antigen (PA) to its cellular receptor. In this study, we report that PA is a calcium-dependent serine protease and that the protein potentially uses this proteolytic activity for receptor binding. Additionally our findings shed new light on previous research describing the inhibition of anthrax toxins and exotoxin formation. Importantly, inhibition of the proteolytic activity of protective antigen could be a novel therapeutic strategy in fighting *B. anthracis*-related infections.

Introduction

Bacillus anthracis, the causative agent of anthrax, secretes a three component exotoxin, *i.e.* lethal toxin, which consists of the cell binding protective antigen (PA), and the enzymes edema factor (EF) and lethal factor (LF). This lethal toxin penetrates host cells and is known to contribute to anthrax pathogenesis, making it a commonly used target in the development of vaccines and anti-infectives against this bacterial pathogen [1,2]. In order to penetrate the host cell, anthrax lethal toxin utilizes a multi-step mechanism. First, PA (PA_{83}, 83 kDa) interacts with the host cell surface via at least two receptors, tumor endothelial marker 8 (TEM8/ANTXR1) and capillary morphogenesis gene 2 (CMG2/ANTXR2) [3,4]. Upon receptor binding PA_{83} is cleaved into PA_{63} (63 kDa) by furin, a calcium-dependent serine protease, which is produced by the host. PA_{63} subsequently forms a heptameric structure, known as a 'pre-pore', which interacts with the toxins LF and EF. The entire receptor-toxin complex is then endocytosed and transported into low-pH endosomes. Triggered by the acidic environment a pore forms across the endosomal membrane, after which EF and LF are released into the cytosol of the host cell [5]. Once present in the cell cytosol, EF and LF damage the cell in various ways; LF is a zinc-dependent metalloproteinase that cleaves and inactivates the mitogen-activated protein kinase (MAPK) kinases 1–4, 6 and 7 [6]. The cleavage events result in inactivation of the MAPK pathways thereby hampering a variety of cellular responses including cell division, apoptosis, and survival [7]. EF is a

calmodulin-dependent adenylyl cyclase that elevates intracellular cAMP levels by converting ATP to cAMP, the classical second messenger, thereby causing diverse adverse side-effects [8]. These processes allow *B. anthracis* to evade the hosts` immune system and facilitate proliferation, resulting in the characteristic disease symptoms of anthrax that frequently lead to death. In this respect, understanding the first step in toxin formation, *i.e.* the interaction between PA and its receptor, is critical for the development of effective treatment strategies against *B. anthracis* related infections. In previous research, it has been shown that the interaction between PA_{83} and its receptors resembles an pH-dependent α-integrin/ligand binding interaction [9,10]. Additionally, Bradley and co-workers showed that EDTA inhibits the binding of PA_{83} to its receptor, suggesting a role of divalent cations in receptor binding [3]. The binding-associated regions of both PA_{83} and its receptors were identified [11], though the exact mechanism by which PA_{83} binds to its receptors remains to be elucidated.

In this study, we demonstrated that PA_{83} exhibits a hitherto unknown proteolytic activity which potentially plays a crucial role in the binding of PA_{83} to the ANTXR1 receptor.

Results

PA_{83} proteolytic activity was characterized by measuring the dose-dependent degradation of the PEK-054 substrate using HEPES-buffered saline as reaction

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buffer (Figure 1(A)). No proteolytic activity could be detected when the experiment was performed in phosphate buffered saline (PBS) buffer (data not shown). In view of the calcium-chelating properties of phosphate ions, it was hypothesized that calcium ions are required for the proteolytic activity of PA_{83}. This was corroborated by the finding that EDTA inhibited the proteolytic activity in a dose-dependent manner (Figure 1(B)). PA_{83} activity could be restored by the addition of Ca^{2+} and, to a lesser extent, Mg^{2+} ions (Figure 1(C)). In contrast, the addition of Zn^{2+} ions inhibited the proteolytic activity of PA_{83} (Figure 1(D)). Furthermore, we examined the effect of several (specific) protease inhibitors on PA_{83} activity. The addition of the cysteine protease inhibitor NEM hardly affected protease activity of PA_{83} (Figure 1(E)). In contrast, the serine protease inhibitor aprotinin at 40 µM virtually completely inhibited PA_{83} proteolytic activity (Figure 1(F)). Additional experiments showed that the furin inhibitor D6R was able to inhibit PA_{83} activity (Figure 2(A)). EGA and MDL281700, inhibitors of lethal toxin formation, showed no significant effect on the proteolytic activity of PA_{83} (Figure 2(B,C)).

Identity and purity studies of the purchased PA_{83} protein using LC MS/MS analysis and SDS-PAGE confirmed the identity of PA_{83}. The intact, 83 kDa,

protective antigen (coverage 62%) was not contaminated with any other proteolytically active proteins (Figure 3(A and B)). Additionally, inhibition of the proteolytic activity by a specific antibody for anthrax protective antigen (α-PA) confirms that the activity originates from the PA_{83} protein. No inhibition was observed upon addition of the antibody control (α-human IgG) (Figure 4).

To map the cleavage site for PA_{83}, five FRET-based peptides, covering the full-length of PEK-054 (Figure 5 (A)), were tested in the FRET assay with PA_{83} and LF, the protease for which the PEK-054 substrate was originally designed. Full-length PEK-054 was hydrolyzed by both proteins with comparable efficiency (LF: 172.1 ± 5.6 ΔF/min and PA_{83}: 132.9 ± 5.4 ΔF/min). None of the PEK-054 derivatives was hydrolyzed by LF, however PA_{83} was able to hydrolyze derivatives #1, #2 and #5. Peptide #2 (FITC-KKKVLP-KDbc) was preferentially hydrolyzed by PA_{83} (14.1 ± 0.1 ΔF/min), followed by peptide #1 and #5 (6.7 ± 0.1 ΔF/min and 5.1 ± 0.3 ΔF/min respectively). No hydrolysis was observed with peptide #3 and #4 (Figure 5(A)). A BLAST search, followed by alignment analysis, revealed that peptide #2 has a homology region with the ANTXR1 receptor (Figure 5(B)). This region is present on the outside of each hexamer of the ANTXR1 receptor protein and is therefore accessible to proteases
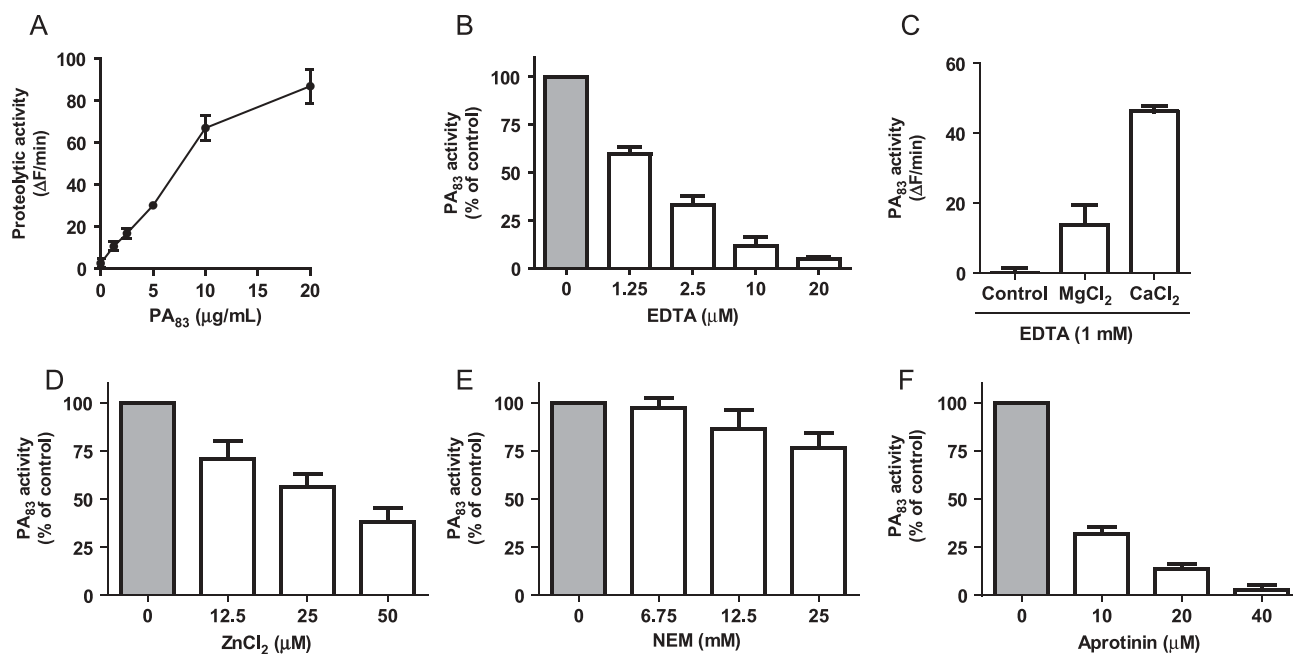

Figure 1. Biochemical characterization of PA_{83} proteolytic activity. PA_{83} proteolytic activity was determined using FRET-peptide PEK-054 with varying PA_{83} concentrations (A). The effect of varying concentrations of (specific) protease inhibitors and metal ions on the hydrolysis of 16 µM FRET-peptide PEK-054 by PA_{83} (20 µg/mL). PA_{83} activity without the addition of inhibitor or metal ions was taken as the 'normalized' (100%) value (B-G). Results are expressed as mean ± SEM ($n = 3$).
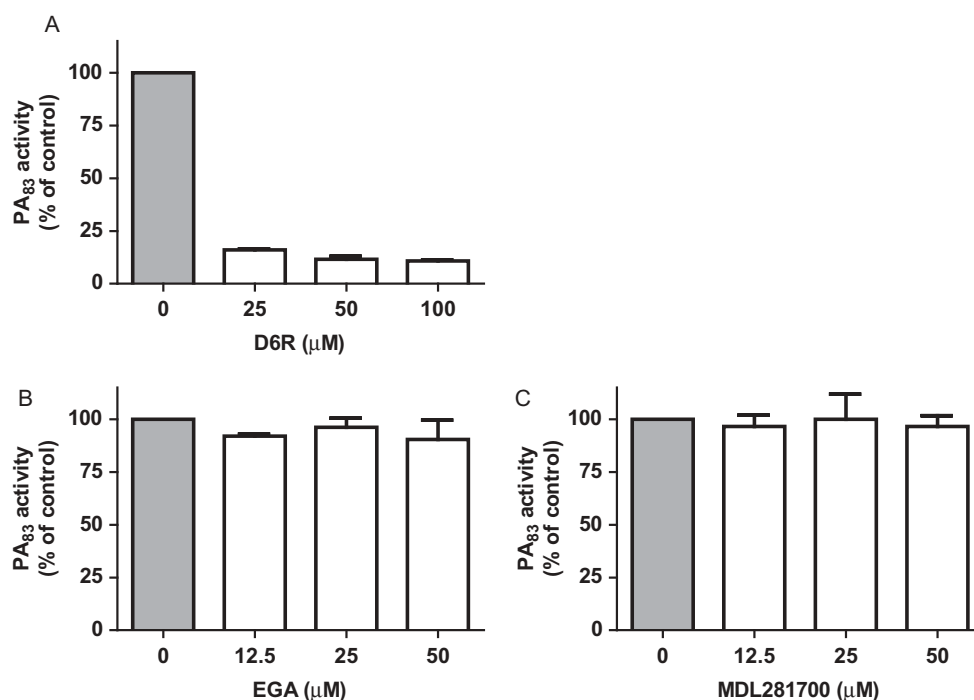
Figure 2. Effect of inhibitors of furin protease activity and lethal toxin formation on PA$_{83}$ proteolytic activity. Varying concentrations of furin inhibitor D6R (A) and lethal toxin formation inhibitors EGA (B) and MDL28170 (C) were incubated with FRET-peptide PEK-054 and PA$_{83}$ (20 µg/mL). PA$_{83}$ activity without the addition of inhibitor was taken as the 'normalized' (100%) value. Results are expressed as mean ± SEM ($n = 3$).

| Accession no.	Description	Mass	Peptides	Coverage
WP_000746486.1	protective antigen [Bacillus anthracis]	85811	127	62
XP_004016289.1	hemoglobin subunit beta isoform X1 [Ovis aries]	16073	4	39
XP_014959464.1	hemoglobin subunit alpha-1/2 [Ovis aries]	15007	3	26
NP_001070890.2	hemoglobin subunit alpha [Bos taurus]	15184	3	26
XP_003585623.1	hemoglobin subunit alpha [Bos taurus]	15184	3	26
NP_002265.2	keratin type I cytoskeletal 13 isoform b [Homo sapiens]	45837	3	7
NP_705694.2	keratin type I cytoskeletal 13 isoform a [Homo sapiens]	49558	3	7
XP_011539564.1	alpha-amylase 1 isoform X1 [Homo sapiens]	57768	3	6
NP_004029.2	alpha-amylase 1 precursor [Homo sapiens]	57768	3	6
XP_011539565.1	alpha-amylase 1 isoform X1 [Homo sapiens]	57768	3	6
XP_016856547.1	alpha-amylase 1 isoform X1 [Homo sapiens]	57768	3	6
NP_001008222.1	alpha-amylase 1 precursor [Homo sapiens]	57768	3	6
NP_001008220.1	alpha-amylase 1 precursor [Homo sapiens]	57768	3	6
NP_001008219.1	alpha-amylase 1 precursor [Homo sapiens]	57768	3	6
XP_015327143.1	alpha-S2-casein isoform X1 [Bos taurus]	24992	2	10
NP_776953.1	alpha-S2-casein precursor [Bos taurus]	26019	2	10
WP_000808751.1	glycerol phosphate lipoteichoic acid synthase [Bacillus cereus]	74683	2	4
WP_000808758.1	glycerol phosphate lipoteichoic acid synthase [Bacillus anthracis]	74620	2	4
NP_845283.1	lipoteichoic acid synthase [Bacillus anthracis str. Ames]	74578	2	4
WP_000808750.1	glycerol phosphate lipoteichoic acid synthase [Bacillus cereus]	74582	2	4
WP_000808760.1	glycerol phosphate lipoteichoic acid synthase [Bacillus cereus]	74576	2	4
YP_028995.1	lipoteichoic acid synthase [Bacillus anthracis str. Sterne]	74636	2	4
WP_000808762.1	MULTISPECIES: sulfatase [Bacillus]	74636	2	4
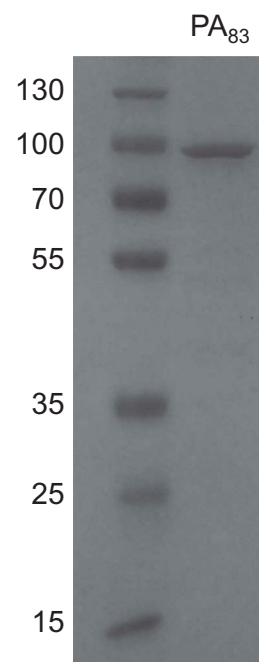
Figure 3. Purity analysis of the PA$_{83}$ protein used within this study. LC MS/MS analysis of the PA$_{83}$ protein. Search results of the Mascot Generic (MGF) files generated against the NCBI database using the Mascot search algorithm (Mascot 2.2.04) are shown. Only the proteins with ≥ 2 peptide hits are listed (A). SDS-PAGE analysis of the PA$_{83}$ protein (2 µg) (B).
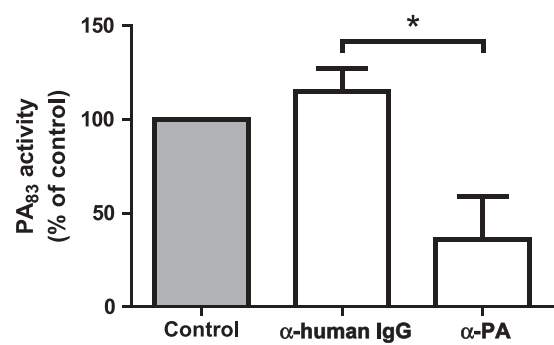
Figure 4. Specificity testing of the PA$_{83}$ proteolytic activity. 20 µg/mL PA$_{83}$ was pre-incubated with 15 µg/mL antibody α-PA/α-human IgG or PBS (control) for 5 hr. PA$_{83}$ was measured using 16 µM FRET-peptide PEK-054. PA$_{83}$ activity without the addition of antibodies was taken as the 'normalized' (100%) value. Results are expressed as mean ± SEM (n = 3). Significance was calculated using an unpaired, two-tailed students t-test (* P-value < 0.05).

peptide with an average efficiency of 8.1 ± 1.8 ΔF/min (Figure 5(D)).

Discussion

During a study on LF protease inhibitors we discovered that PA$_{83}$, a protein known to be involved in pore formation during *B. anthracis* infection, possessed proteolytic activity on the FRET-peptide substrate to detect LF activity. This proteolytic activity is a feature of PA$_{83}$ which has not been described before. Characterization experiments revealed that PA$_{83}$ proteolytic activity depends on the presence of divalent cations. Besides, based on its response to specific protease inhibitors, it seems most plausible that PA$_{83}$ belongs to the class of serine proteases. The strong cation dependency of PA$_{83}$ explains why its proteolytic activity was not detected earlier. Besides, the fact that two Ca^{2+} ions are present in the quaternary structure of PA$_{83}$, strengthens the potential role of Ca^{2+} in PA$_{83}$ protease activity [12]. In this respect, Bradley et al showed that binding of PA$_{83}$ to its receptor is cation dependent whereas the binding interaction between the ANTXR1 receptor and PA$_{83}$ is impaired in the presence of EDTA [3]. Additionally, Ramey et al showed that binding of PA$_{83}$ to the ANTXR1 receptor is dependent on divalent

(Figure 5(C)). Based on the 4-amino acid sequence overlap between the ANTXR1 receptor and peptide #2 we designed a 9-amino acid containing FRET-peptide substrate based on the protein sequence of the ANTXR1 receptor protein (FITC-LQKVLPGGD-KDbc (L109-D117)/ANTXR1-peptide). Incubating this peptide with 20 µg/mL PA$_{83}$ showed hydrolysis of the

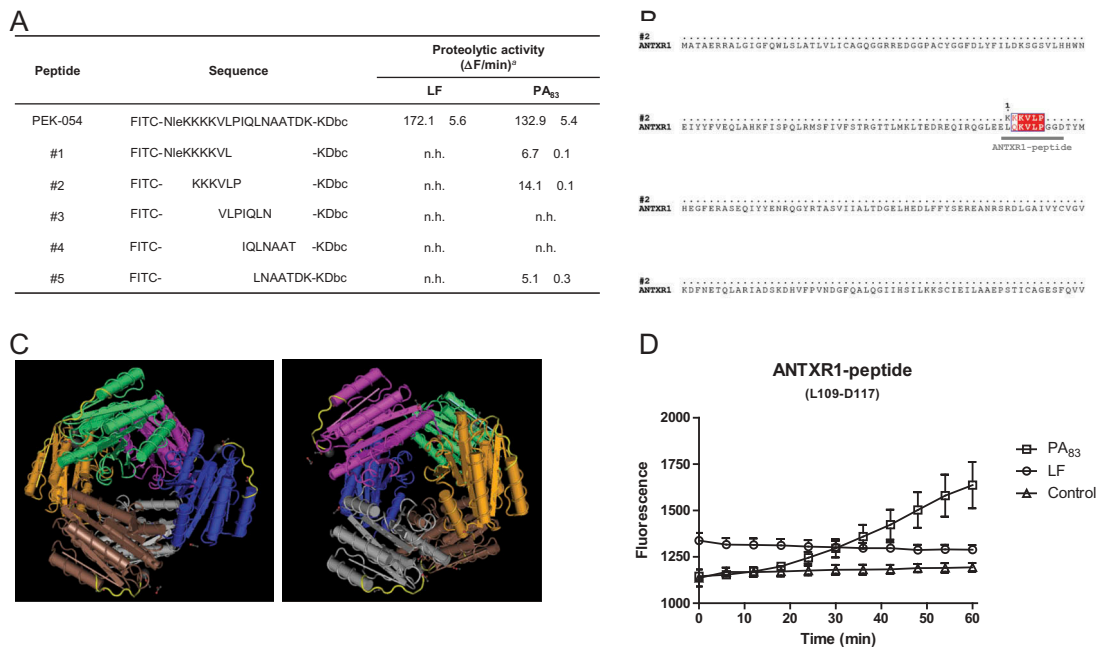| Peptide	Sequence			Proteolytic activity (ΔF/min)[a]	
				LF	PA$_{83}$
PEK-054	FITC-NleKKKKVLPIQLNAATDK-KDbc			172.1 5.6	132.9 5.4
#1	FITC-NleKKKKVL		-KDbc	n.h.	6.7 0.1
#2	FITC-	KKKVLP	-KDbc	n.h.	14.1 0.1
#3	FITC-	VLPIQLN	-KDbc	n.h.	n.h.
#4	FITC-	IQLNAAT	-KDbc	n.h.	n.h.
#5	FITC-	LNAATDK-KDbc		n.h.	5.1 0.3

Figure 5. PA$_{83}$ cleavage specificity and its potential role in ANTXR1 receptor binding. Hydrolysis of FRET-peptide PEK-054 and its derivatives using 20 µg/mL PA$_{83}$ or LF [a] No hydrolysis: n.h. (A). ANTXR1 sequence alignment was performed using software programs ClustalW and ESPript 3.0 (Risler matrix, global score of 0.7). Sequence overlap between ANTXR1 and the peptide is depicted in red (B). Presence of the KVLP-region on the outside of the ANTXR1 protein is marked yellow. The 3D structure of the protein was generated using the Cn3D 4.3 program (C). Hydrolysis of ANTXR1 peptide (L109-D117) using 20 µg/mL PA$_{83}$, 20 µg/mL LF or HEPES buffer (D). Results are expressed as mean ± SEM (n = 3).

cations, such as Mg^{2+} and Ca^{2+} [13]. Based on the above mentioned studies and our observation that not only calcium, but also magnesium, was able to restore PA_{83} proteolytic activity after inhibition with EDTA, we provide preliminary evidence that PA_{83} uses its proteolytic activity in order to facilitate its binding to the ANTXR1 receptor. *In silico* analysis revealed a sequence overlap between the substrate degraded with the highest efficiency, peptide #2 (FITC-KKKVLP-KDbc), and a region in the von Willebrand factor type A domain (vWA domain) of the ANTXR1 protein. A FRET-peptide substrate based on the sequence of this region (ANTXR1-peptide) was successfully degraded by PA_{83}, again suggesting that PA_{83} proteolytic activity plays a role in receptor binding. Further, results published by Fu et al showed that the region of the ANTXR1 receptor that overlaps with the sequence of peptide #2, interacts with the hydrophobic cleft of protective antigen [11].

The discovery of PA_{83} proteolytic activity sheds new light on previous research involving inhibitors of furin activity. Within lethal toxin formation this human protease cleaves PA_{83} into PA_{63} and PA_{20} after which PA_{63} forms a heptameric pre-pore. Like PA_{83}, furin is a calcium dependent serine protease whose activity is inhibited in the presence of Zn^{2+} ions [14]. Further, the potential benefits of the use of furin inhibitors in the treatment of *B. anthracis* infection has already been proven *in vivo* [15–17]. However, the observed therapeutic effect of furin inhibitors may not only originate from furin inhibition but can also be related to inhibition of PA_{83} proteolytic activity. Here we show that there is indeed cross-inhibition of furin inhibitor D6R with PA_{83} proteolytic activity. Additionally, inhibitors of anthrax lethal toxin formation might work via another mechanism than suggested. For this reason we examined the effect of two compounds, EGA and calpain-inhibitor MDL281700, utilized in studies investigating the inhibition of lethal toxin formation, on the proteolytic activity of PA_{83} [18,19]. These compounds however did not affect PA_{83} proteolytic activity.

The results described in this study indicate that PA_{83} possesses proteolytic activity that potentially contributes to the binding of PA_{83} to its host cellular receptor. Additionally, PA_{83} is able to hydrolyze a protease substrate of which the sequence is based on MAPKK [20], suggesting that PA_{83} might possess LF-like activity. Thus it is hypothesized that by inhibiting the proteolytic activity of PA_{83} it may be possible to decrease the pathogenic potential of *B. anthracis*. In this light, the substrates described in this study could potentially help in future studies aiming to develop PA_{83} protease inhibitors. For example, the sequences of the peptide substrates utilized

in this study could possibly be used to develop competitive inhibitors of PA_{83} protease activity, thereby reducing the efficiency of PA_{83} receptor binding or MAPKK degradation. However additional research is needed to precisely unravel the function of the PA_{83} proteolytic activity.

In conclusion, to the best of our knowledge, we are the first who describe proteolytic activity of PA_{83}. This finding may lead to the development of novel therapeutic strategies against *B. anthracis* infection and virulence.

Materials and methods

Peptide synthesis

The fluorescence resonance energy transfer (FRET-) peptides used in this study were synthesized by solid-phase peptide synthesis using Fmoc chemistry and a MilliGen 9050 peptide synthesizer (Milligen-Biosearch). The peptides were flanked at their C-termini with a fluorescent probe (FITC) and flanked at their N-termini with a lysine coupled quencher (Dabcyl (Dbc)). FRET-peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Purity of the FRET-peptides was confirmed using mass spectrometry, as described previously [21]. Stock solutions (800 µM) of the quenched FRET-peptides were prepared in DMSO and stored at – 20°C.

Protease assay

Proteolytic activity was determined, as described earlier [22], by incubating 16 µM FRET-peptide with varying concentrations of PA_{83} or LF (both List Laboratories) in HEPES buffer (20 mM HEPES, 0.05% Tween-20, pH 8.2) using blackwell clear bottom 96-well plates (Corning). Plates were incubated at 37°C for 60 min, with fluorescence levels being recorded at 2 min intervals using a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Fluorescence values were obtained after correction against the buffer control. Protease activity was defined in ΔF per minute (ΔF/min) [23]. An ΔF/min < 5 was considered as baseline, *e.g.* no hydrolysis (n.h.).

Cleavage characteristics of PA_{83}

20 µg/mL PA_{83} was incubated with 16 µM FRET-peptide PEK-054 (FITC-NleKKKKVLPIQLNAATDK-KDbc) [20] in the presence of varying concentrations of either protease inhibitors or lethal toxin formation inhibitors including 4-bromobenzaldehyde *N*-(2,6-dimethylphenyl) semicarbazone (EGA), calpain-

inhibitor MDL281700, furin inhibitor II (hexa-D-arginine (D6R)), EDTA, $ZnCl_2$, N-ethylmaleimide (NEM) and aprotinin in HEPES buffer (20 mM HEPES, 0.05% Tween-20, pH 8.2). To study the effect of metal ions on PA_{83} proteolytic activity, 20 μg/mL PA_{83} was incubated with 16 μM FRET-peptide PEK-054 in HEPES buffer (20 mM HEPES, 0.05% Tween-20, pH 8.2) supplemented with 1 mM EDTA. Next, 5 mM $CaCl_2$ or 5 mM $MgCl_2$ was added to the reaction to restore potential protease activity. All above mentioned chemicals were purchased from Sigma Aldrich. Protease activity was defined as a percentage of the control (PA_{83} activity without inhibitor or metal ions).

LC MS/MS

Prior to LC MS/MS analysis 10 μg PA_{83} was digested with 0.5 μg trypsin (Difco, BD Biosciences) for 3 hr at 37°C. The trypsinized sample was analyzed by MS/MS using the data dependent LC-auto MS/MS mode. The ten most abundant ions (charge states 2+, 3+ and 4+) in the MS spectrum (300–1300 m/z, 2Hz) of eluted peptides were selected for data dependent MS/MS analysis by collision-induced dissociation using nitrogen as the collision gas. MS/MS scans were acquired over the mass range 100–2000 m/z (2–10 Hz, depending on signal intensities). Data files were exported to mzML format with MSConvert (ProteoWizard 3.0.8764), and searched with Peaks 7.5 (Bioinformatics Solutions Inc.) against a custom database consisting of NCBI RefSeq v77 protein sequences from *Homo sapiens, Bos taurus, Ovis aries* and > 400 selected human pathogenic, commensal and near-neighbor bacterial species. Precursor tolerance was 40 ppm and product ion tolerance was set to 0.04 Da. Semi-tryptic peptides were allowed with up to one missed enzymatic cleavage. Oxidation of methionine was set as variable modification. A PSM score threshold was selected corresponding to a false discovery rate of 0.1%, and protein groups required a minimum of two unique peptides. The resulting FDR at the peptide and protein level were both < 1%.

SDS-PAGE

PA_{83} protein (2 μg) was mixed 1:1 with 2x Laemmli sample buffer (Biorad), incubated for 5 min at 95°C and loaded onto a 10% SDS-PAGE gel. After electrophoresis for 1 hr at 22 mA (max. 160 V) the gel was stained overnight with PageBlue staining solution (Fisher Scientific) and washed two times to visualize bands.

Specificity testing PA_{83}

20 μg/mL PA_{83} was incubated with 15 μg/mL *B. anthracis* protective antigen antibody-[HRP] (α-PA, Novusbio, BAP0102), 15 μg/mL human IgG antibody-[HRP] (α-human IgG, Sigma Aldrich, A0170) or PBS (solvent of the used antibodies) in HEPES buffer for 5 hr at room temperature. Next, 1 μL PEK-054 (800 μM) was added to the reactions and increase in fluorescence was measured at 37°C for 60 min as described above.

Abbreviations

PA	Protective antigen
LF	Lethal factor
EF	Edema factor
TEM8	tumor endothelial marker 8
CMG2	capillary morphogenesis gene 2
MAPK	mitogen-activated protein kinase
vWA	von Willebrand factor type A
D6R	hexa-d-arginine
FRET	fluorescence resonance energy transfer

Disclosure statement

No potential conflict of interest was reported by the authors.

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