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Anthrax Toxin Receptor 2–Dependent Lethal Toxin Killing In Vivo

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Anthrax toxin receptors 1 and 2 (ANTXR1 and ANTXR2) have a related integrin-like inserted (I) domain which interacts with a metal cation that is coordinated by residue D683 of the protective antigen (PA) subunit of anthrax toxin. The receptor-bound metal ion and PA residue D683 are critical for ANTXR1-PA binding. Since PA can bind to ANTXR2 with reduced affinity in the absence of metal ions, we reasoned that D683 mutant forms of PA might specifically interact with ANTXR2. We show here that this is the case. The differential ability of ANTXR1 and ANTXR2 to bind D683 mutant PA proteins was mapped to nonconserved receptor residues at the binding interface with PA domain 2. Moreover, a D683K mutant form of PA that bound specifically to human and rat ANTXR2 mediated killing of rats by anthrax lethal toxin, providing strong evidence for the physiological importance of ANTXR2 in anthrax disease pathogenesis.

Introduction

The spore-forming bacterium Bacillus anthracis causes anthrax and is classified as one of seven Centers for Disease Control and Prevention category A agents that are considered major threats as bioweapons [1]. B. anthracis secretes a toxin which contributes to bacterial virulence and causes many of the disease symptoms. Anthrax toxin is an AB-type toxin, with a single receptor-binding B-moiety, protective antigen (PA), and two catalytic A-moieties, lethal factor (LF) and edema factor (EF). LF is a zinc-dependent metalloprotease that cleaves members of the mitogen-activated protein kinase kinase family (all MKKs except MEK5) [2–4], whereas EF is a calmodulin and calcium–dependent adenylyl cyclase [5,6]. LF and PA combine to form lethal toxin, and EF and PA combine to form edema toxin. These toxins are responsible for disabling host innate and adaptive immune responses, causing vascular leakage, and leading to the death of animals and cultured cells [7–16].

Following binding of PA to cell surface receptors and internalization of toxin complexes, EF and LF are translocated into the cytoplasm through a heptamerized PA pore that forms at endosomal low pH [17–20]. There are two known cell surface receptors for PA, ANTXR1 (anthrax toxin receptor/tumor endothelial marker 8; ATR/TEM8) and ANTXR2 (capillary morphogenesis gene 2; CMG2) [21,22]. These receptors are expressed in various human tissues [22–24], but there is evidence that ANTXR1 may be preferentially expressed in cancer cells and tumor endothelium [25–29]. The relative importance of either receptor for anthrax disease pathogenesis has not been established.

PA interacts with both receptors through a common von Willebrand factor A/integrin-like inserted (I) domain that contains a metal ion adhesion site (MIDAS) with five metal ion coordinating residues [30]. Similar to binding of ligands to α-integrins, binding of PA to its receptors involves direct coordination of a divalent cation in the MIDAS by a carboxylate-containing side chain from PA (residue D683) [31–34] (Figure 1). Previous studies have shown that PA residue D683 is critical for intoxication of cells via ANTXR1 [34].

The ANTXR2 I domain binds to PA domains 2 and 4, and the surface area of the protein interface is much larger (approximately 2,000 Å2) than that of α-integrin–ligand interactions (approximately 1,300 Å2) [31,32]. The large contact surface correlates with a very tight ANTXR2 I domain–PA binding affinity (KD = 170 or 780 pM in Mg2+ or Ca2+, respectively) [35], compared with the affinity of α-integrin–ligand interactions that are usually in the micromolar to millimolar range [36]. ANTXR2 I domain contact with PA domain 2, the domain that forms pores and translocates EF/LF, has been proposed to act as a molecular clamp that prevents pore formation until the complex is trafficked to a low pH endosomal compartment [20,31,32,37] (Figure 1).
Despite strong amino acid sequence homology between ANTXR1 and ANTXR2, there are striking differences in the PA binding activities of the two receptors. When the PA heptamer is bound to ANTXR2, a pH value of approximately 5.0 is required to allow efficient pore formation, whereas approximately pH 6 is required for PA pore formation when the toxin is bound to ANTXR1 [20,37]. Also, the metal-dependent PA binding affinity of the ANTXR1 I domain is weaker than that of ANTXR2 by approximately 1,000-fold ($K_D = 130 \text{nM or } 1.1 \text{M in } \text{Mg}^{2+} \text{ or } \text{Ca}^{2+}$, respectively) [38]. Indeed, the ANTXR1 I domain–PA binding affinity in the presence of metal ions is similar to the ANTXR2 I domain–PA binding affinity in the absence of metal ions ($K_D = 960 \text{nM in EDTA/EGTA}$) [35].

Because PA-ANTXR2 binding appeared to be less dependent on interactions mediated by the metal ion, we reasoned that the PA–ANTXR2 interaction should be less sensitive to mutations of PA residue D683. Here we show that this is the case and that PA proteins with D683 mutations bind specifically to human and rodent ANTXR2 and mediate intoxication via these receptors. We also show that a single region of nonconserved receptor residues that bind PA domain 2 is responsible for the differential abilities of ANTXR1 and ANTXR2 to bind D683 mutant forms of PA. Moreover, we demonstrate that a D683K mutant form of PA mediates lethal toxin killing of rats, implicating ANTXR2 as a physiologically important anthrax toxin receptor.

**Results**

**PA Residue D683 Is Not Essential for Binding or Intoxication via ANTXR2**

Previously, PA residue D683, which contacts the receptor-bound metal ion (Figure 1), was shown to be critically important for the interaction of PA with ANTXR1: mutation of this residue to Asn completely abrogated receptor binding [34]. To test the dependence of ANTXR2-PA binding on PA D683, this residue was replaced with either an asparagine or a lysine. The resultant PA$^{D683N}$ and PA$^{D683K}$ proteins were tested for their abilities to bind to a soluble human ANTXR2 protein (sANTXR2) and a full-length human ANTXR2–enhanced green fluorescent protein (EGFP) fusion protein expressed on PA receptor-deficient Chinese hamster ovary (CHO)-R1.1 cells. Additionally, cells expressing ANTXR2-EGFP were tested for their ability to be intoxicated with PA$^{D683N}$ or PA$^{D683K}$ and LF$_{152}$-DTA, a recombinant protein composed of the N-terminal PA-binding portion of LF fused to the catalytic A chain of diphtheria toxin, which kills CHO cells. For control purposes, these experiments were also
performed with CHO-R1.1 cells engineered to express a human ANTXR1-EGFP fusion protein.

Surface plasmon resonance (SPR) analysis, performed as described previously [35], revealed that PA D683N binds tightly to the sANTXR2 I domain, $K_D = 28 \pm 1.7$ and $17 \pm 0.68$ nM in the presence of Ca$^{2+}$ and Mg$^{2+}$, respectively. This binding was still metal ion dependent because the $K_D$ value was 240 ($\pm 27$) nM in the presence of EDTA, a metal chelator. PA D683K bound the soluble receptor with similar properties, $K_D = 26 \pm 0.81$ and 33 ($\pm 3.1$) nM, in Ca$^{2+}$ and Mg$^{2+}$, respectively, and was 94 ($\pm 8.8$) nM when EDTA was added.

Flow cytometric analysis performed with one of the altered proteins, PAD683K, confirmed that it bound to cells expressing ANTXR2-EGFP but not ANTXR1-EGFP (Figure 2A, 2B, and 2C). Consistently, both PAD683N and PAD683K supported the intoxication of cells expressing human ANTXR2-EGFP (Figure 2D) but not ANTXR1-EGFP (Figure 2E). Indeed, cells expressing ANTXR2-EGFP were just as susceptible to PAD683K-dependent killing as they were to wild-type PA-dependent killing (Figure 2D). We conclude that PA residue D683 is not critical for toxin action mediated by ANTXR2 in the range of toxin concentrations tested.

**ANTXR2 Residues That Interact with PA Domain 2 Specify the D683 Mutant PA Interaction**

Based on the co-crystal structures of ANTXR2 bound to PA (Figure 1), there are eight contact residues that would be different at the toxin-binding interface in ANTXR1: A56L, N57H, Q88R, S113L, V115G, D152H, G153E, and L154D [31,32] (Figure S1). We reasoned that the differential abilities of the receptors to bind mutant PA proteins would be due to one or more of these variable amino acids. To test this idea, each of these amino acid substitutions was introduced independently into ANTXR2-EGFP and the altered receptors were expressed in transiently transfected CHO-R1.1 cells. Cell surface expression of the mutant receptors was confirmed by flow cytometry with a polyclonal chicken anti-ANTXR2 serum and AlexaFluor-633-conjugated secondary antibody (Figure 3A). These cells were also subjected to intoxication with PA$^{D683N}$ and LF$_V$-DTA. Cell viability was measured in a flow cytometry-based assay where the percentage of live, EGFP-positive cells remaining after toxin challenge was compared with cells incubated with LF$_V$-DTA alone (no toxin killing). Cells expressing cytoplasmic EGFP or ANTXR1-EGFP were included as negative controls.

The largest defects in PA$^{D683N}$-mediated intoxication were seen with cells expressing G153 or L154 mutant ANTXR2 receptors (Figure 3A). Residues G153 and L154 are located in a surface region of ANTXR2 that contacts PA domain 2 (Figure 1). To further investigate the role of this region in the D683 mutant PA interaction, we made reciprocal exchanges of the residues located between positions 152 and 157 (DGLVPS) in the $\beta_4$-$\alpha_4$ loop region of the ANTXR2 I domain and the corresponding residues of ANTXR1 (154 to 159; HEDLFF) in the context of ANTXR1-EGFP and ANTXR2-EGFP proteins, respectively. These studies revealed that replacement of both G153 and L154 residues in ANTXR2 with the residues from ANTXR1 was sufficient to make cells completely resistant to PAD683N intoxication (Figure 3B). Additionally, replacement of ANTXR1 residues 154 to 159 with the corresponding ANTXR2 residues gave rise to a recombinant receptor that could support PA$^{D683N}$-mediated intoxication (Figure 3B).

**PA$^{D683K}$ Supports LF-Mediated Intoxication in Rats**

To address the possible role of ANTXR2 in lethal toxin killing of Fischer 344 rats, we first confirmed that D683 mutant forms of PA can interact with rat ANTXR2 (rANTXR2) but not rat ANTXR1. To this end, we attempted to isolate a full-length rat ANTXR1 cDNA clone but were not
successful. However, the rat ANTXR1 I domain differs from that of its human counterpart by only two amino acids (residues K72 and R136 of the human protein are, in the rat protein, arginine and serine, respectively). Therefore, we constructed a rat version of the ANTXR1-EGFP protein (designated as ANTXR1 R72,S136-EGFP by replacing these two human-specific residues with those of rat ANTXR1. Also, cDNAs encoding the rANTXR2 open reading frame were isolated from rat liver and brain tissue RNAs; both cDNAs had an identical DNA sequence. Comparison with the human ANTXR2 (hANTXR2) revealed that the I domains of these proteins differ by ten amino acid acids, two of which are PA-contact residues: Arg111 and Ser113 in hANTXR2 are alanine and glutamine in rANTXR2, respectively (Figure S1). CHO-R1.1 cells engineered to express rANTXR2-EGFP were susceptible to PAD683K-dependent killing, albeit slightly less efficiently than the killing observed with wild-type PA (Figure 4A and 4B). By contrast, cells engineered to express either hANTXR1-EGFP or ANTXR1 R72,S136-EGFP were resistant to PAD683K-dependent killing. Taken together, these observations confirm that PA^{D683K} interacts specifically with ANTXR2, both in humans and in rats.

In an initial experiment, it was found that PA^{D683K} could support lethal toxin-killing of male Fischer 344 rats, albeit several-fold less efficiently than that seen with wild-type PA: the mean time to death (TTD) (±SD) seen with three animals injected intravenously with 8 μg of LF and 10 μg of wild-type PA was 93.3 (±10.2) minutes, similar to that seen with two animals injected with four times as much PA^{D683K} and the same amount of LF, i.e., TTD = 98.5 (±10.6) minutes. This several-fold decrease in the efficacy of PA^{D683K} was also apparent from comparing the mean TTD of two animals
injected with 200 μg of this mutant protein and LF (TTD = 68.5 ± 5.0 minutes compared to those of three animals in each group that received LF with either 20, 30, or 40 μg of wild-type PA [mean TTD = 60.3 ± 2.9; 57.0 ± 2.0; and 54.7 ± 1.5 minutes, respectively]).

To investigate the relative efficacy of PA<sub>D683K</sub> in more detail, a subsequent experiment was performed with rats injected with the same amount of LF and with either a fixed amount of PA<sub>D683K</sub> (100 μg) or varying amounts of wild-type PA (ranging from 10 to 40 μg). Rats injected with LF and 100 μg of PA<sub>D683K</sub> died on average approximately 67 min following toxin administration (Figure 4C). By comparison, rats injected with LF and either 20 μg of PA or 10 μg of PA died an average of approximately 64 and 89 min after toxin injection, respectively (Figure 4C). The difference in TTD between the rats treated with 100 μg of PA<sub>D683K</sub> was statistically significant when compared with those injected with 10 μg of PA (P = 0.0001) but not from those treated with 20 μg of PA (P = 0.104) (Figure 4C). Based on these dosage effects, we conclude that PA<sub>D683K</sub> can mediate anthrax lethal toxin killing of rats, albeit with an approximately 5-fold reduced efficiency compared with wild-type PA.

Discussion

This report demonstrates that D683 mutant forms of PA bind selectively to ANTXR2 and that this selectivity is dependent on amino acid residues of ANTXR2 that contact domain 2 of PA. Moreover, we have shown that a D683K mutant form of PA is capable of mediating lethal toxin killing of rats, implicating an important physiological role for ANTXR2 in this process.

We have demonstrated that the ability of ANTXR2 to bind PA proteins with mutations at the D683 site maps to the receptor surface region containing residues G153 and L154 that engage PA domain 2. Replacing the ANTXR2 G153, which allows a polypeptide backbone turn, and L154, which participates in hydrophobic interactions with PA, with the negatively charged Glu and Asp residues from ANTXR1 resulted in resistance to cellular intoxication with PAD683N. By contrast, exchange of the HEDLFF<sub>154-159</sub> sequence from ANTXR1 for the corresponding DGLVPS<sub>152-157</sub> sequence from ANTXR2 conferred upon ANTXR1 the ability to support PAD683N-mediated intoxication.

Prior to this report it was unclear if ANTXR2 was important for lethal toxin killing in vivo. Our results showing that PA<sub>D683K</sub> supports lethal toxin-mediated killing of rats, implicating an important physiological role for ANTXR2 in this process.

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Prior to this report it was unclear if ANTXR2 was important for lethal toxin killing in vivo. Our results showing that PA<sub>D683K</sub> supports lethal toxin-mediated killing of rats provides direct evidence for the physiological importance of this receptor in this rodent model system. There are several potential explanations for the reduced efficacy of PA<sub>D683K</sub> in vivo, compared to wild-type PA. First, this result could reflect the slightly reduced levels of PA<sub>D683K</sub> intoxication observed in cultured cells expressing rANTXR2 relative to those expressing hANTXR2. This difference may be due to the combined effects of residue differences at positions 111 and 113 of these proteins. The hANTXR2 R111 residue interacts from analysis. Each data point represents a single animal, and the vertical line represents the mean for each group. P-values were determined by Student’s unpaired t-test. Control rats injected with PBS all survived (unpublished data).

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with a negatively charged environment in PA domain 4, and the S113 residue makes H-bond contacts with PA domain 4 in the crystal structure (unpublished data) [31,32]. The rANTXR2 Ala and Gln residues at these respective locations may not be able to participate in these interactions. A second possibility is that cell type–specific differences downstream of initial PA binding might dictate different activities of PA proteins with altered residues at the D683 site. Indeed, we observed a 10-fold difference in intoxication activities of the PA.D683N and PA.D683K proteins despite similar ANTXR2 binding affinities (Figures 2D and 2E). Third, the reduced activity of the PA.D683K protein in vivo might be indicative of a role for ANTXR1 in intoxication of rats by wild-type lethal toxin.

The PA.D683K protein is the first engineered toxin that can discriminate between the two anthrax toxin receptors, binding specifically to ANTXR2. This ANTXR2–specific form of PA can now be used to study the role of ANTXR2 in mediating the effects of lethal toxin in other cell types, and it can also be engineered into the bacterium to probe the importance of this receptor for various aspects of anthrax disease pathogenesis following infection with B. anthracis spores.

Materials and Methods

DNA constructs and cell lines. The ANTXR2-EFGP, ANTXR1-EFGP, and sANTXR2-myHis fusion constructs have already been described (CMG2489–EFGP, ATRTEM8 sv2-EFGP, and sCMG2-myHis, respectively) [22,38]. QuikChange mutagenesis (Stratagene, La Jolla, California, United States) was performed with the oligonucleotide primers in Table S1 to generate various mutant versions of the PA.D683K allele in the PA-Pet22b construct [39] and the ANTXR1.R72,S136-EFGP construct. To isolate rat ANTXR2 (rANTXR2) cDNA, rat liver and brain RNA were prepared by homogenizing tissue from adult male Fischer 344 rats with TriZOL reagent (Invitrogen, Carlsbad, California, United States) according to the manufacturer’s instructions, followed by purification with RNeasy mini spin columns (Qiagen, Valencia, California, United States). The rANTXR2 open reading frame was isolated using a nested, touchdown PCR protocol on 3’ RACE cDNA products (Clontech, Palo Alto, California, United States) with a Universal Primer Mix (Invitrogen) and primer 5’ CCC GAG CCC AAG GGA CTG TGA GC 3’ for the first round, and primers 5’ aat gag gac AAC AGG ATG GTG GCC GGT CCG TCC C and 5’ aat tag aag GCA TCG TGC TGA GCA GGT TAT GC in the second round PCR. Primers included engineered Sall and BamHI restriction sites (underlined), respectively, for cloning rANTXR2 as an EFG fusion protein in the pLEGPFP11 plasmid. The open reading frames of all constructs were confirmed by sequencing. The PA.D683N-Pet22b plasmid [34] was a gift from Jeremy Mogridge.

PA receptor–deficient CHO-R1.1 cells and CHO-R1.1 cells that were engineered to stably express recombinant EFGP–fusion proteins, CMG2489–EFGP or ATRTEM8 sv2-EFGP, have been described previously [22,34]. For transient expression of receptors, approximately 5 × 10⁶ CHO-R1.1 cells was transfected with 1 µg of receptor–EFGP fusion construct and 5 µg of pBSI K (+) or pTR2Shyg plasmids as carrier DNA and LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were split and intoxicated at 24 h post-transfection, and analyzed for cell viability or receptor cell surface expression at 48 h post-transfection.

PA and sANTXR2 protein production. The sANTXR2-myHis protein was expressed from the extraplastidic human 293 Freestyle cells (Invitrogen) stably expressing this protein as described elsewhere [38]. The wild-type and altered forms of PA were prepared from the periplasm of E. coli BL21 cells that had been transformed with the PA-Pet22b, PA.D683K–Pet22b, or PA.D683K–Pet22b plasmids, as described previously [40]. The PA proteins were purified by FPLC with HiTrap QFF and Superose 12 (Amersham, Little Chalfont, United Kingdom) or HiLoad Superdex 200 (Amersham) columns, and the relative protein purity was determined by ImageQuant analysis (Amersham) of Coomassie-stained protein samples following SDS-PAGE.

Cell surface receptor expression analysis. Anti-ANTXR2 antibody–boiled-in chickens against the sANTXR2-myHis protein [22,38] that should include ANTXR2 residues 34 to 232 as a mature protein (Aves Labs, Tigard, Oregon, United States). Antibodies were affinity purified against the same antigen by FPLC with a HiTrap NHS-activated HP column (Amersham). For flow cytometric analysis of ANTXR2 expression on the surfaces of transfected cells, cells were incubated with a 1:100 dilution of the anti-ANTXR2 antibody, followed by a 1:1,000 dilution of an AlexaFluor-633–conjugated anti-chicken IgG (Molecular Probes, Eugene, Oregon, United States). All FACS data were collected on an LSR flow cytometer (BD Biosciences, San Diego, California, United States) and analyzed with a Flojo software package (Tree Star, Ashland, Oregon, United States). The geometric mean of AlexaFluor-633 fluorescence after incubation with primary and secondary antibody was graphed after subtracting background binding of cells incubated with secondary antibody. Cell surface expression of human ANTXR1-EFGP, ANTXR2-EFGP, ANTXR1.R72,S136-EFGP, and rat ANTXR2 proteins shown in Figures 4 was confirmed by flow cytometric analysis using an AlexaFluor-633–conjugated monoclonal form of PA.D683K ( unpublished data).

PA binding and in vitro intoxication assays. PA binding to cells was monitored by flow cytometric analysis following incubations of cells for 2 h on ice with 100 nM PA or PA.D683N, then with a 1:2,000 dilution of an anti-PA rabbit polyclonal serum, and a 1:500 dilution of an APc-conjugated anti-rabbit antibody (Molecular Probes) [22]. Intoxication was monitored by incubating samples of cells with LF₅-DTA [41] and either PA, PA.D683N, or PA.D683K proteins. Cell viability was measured 10 to 50 h later using the CellHet-Glo reagent (Omega, Madison, Wisconsin, United States). In the case of transiently expressed receptors, samples of cells were incubated with LF₅-DTA and either PA, PA.D683K, or PA.D683N proteins and analyzed by FACS 20 to 24 h later. Cell viability was determined by dividing the percentage of live, EFGP-positive cells in the samples with complete toxin by that in the LF₅-DTA only control (100% cell viability). In all intoxications, percent cell viability is the average from three samples, ± SD.

Binding affinity analysis. All experiments were performed at 25 °C using the Biacore 2000 system and sensor chips with immobilized PA proteins as described previously [35]. Concentrations of sANTXR2 proteins used ranged from 24 nM to 4.8 µM in HBS (10 mM HEPES [pH 7.6], 150 mM NaCl) with either 1 mM CaCl₂, 1 mM MgCl₂ or 2 mM EDTA at pH 7.6. Serial injections were made at 10 µl/min, followed by a 40-µl buffer injection to allow for off-rate measurements. All kinetic data were analyzed using Origin Software. The equilibrium dissociation constants were calculated from kinetic measurements of the association and dissociation rate constants according to $K_D = k_{on}/k_{off}$, and the errors were propagated. All results described are the average values of two independent trials.

Rat intoxication challenge. Animal lethal toxin challenges were performed according to protocols approved by the Scripps Institutional Animal Care and Use Committee. Male Fischer 344 rats (180 to 200 g; Harlan) were anesthetized with isofluoranes and inoculated with 500 µl of a toxin mixture through a jugular vein cannula. The toxin mixture was prepared for each group by mixing 8 µg of LF (List Biological Laboratories, Campbell, California, United States) with 10 to 40 µg of purified PA or with 100 µg of PA.D683K in a 500-µl volume per rat. Rats recovered from anesthesia within 5 min and were monitored for symptoms of intoxication and death (defined by cessation of respiration). The Student’s unpaired t-test (Prism) was used for statistical analysis.

Supporting Information

Figure S1. Alignment of the Amino Acid Sequence from Human ANTXR1 (hANTXR1), hANTXR2, and sANTXR2.

Sequences were aligned by the ClustalW method (MacVector). The amino acid sequences of hANTXR1 and hANTXR2 protein and Crystal sequences aligned are indicated above. The dark line above the sequence indicates the I domain region that binds PA. The residues of the MIDAS are indicated with green boxes. The residues implicated in PA contact in the hANTXR2-PA co-crystal structure are indicated with red boxes, and residues implicated in binding by PA.D683K or PA.D683N are indicated with purple boxes. Asterisks (*) and periods (.) below the sequence designate conserved and similar residues, respectively, at that site.
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