Divalent Metal Ion Coordination by Residue T118 of Anthrax Toxin Receptor 2 Is Not Essential for Protective Antigen Binding

Heather M. Scobie, John A. T. Young*

Infectious Disease Laboratory, The Salk Institute for Biological Studies, La Jolla, California, United States of America

The protective antigen (PA) subunit of anthrax toxin interacts with the integrin-like I domains of either of two cellular receptors, ANTXR1 or ANTXR2. These I domains contain a metal ion-dependent adhesion site (MIDAS) made up of five non-consecutive amino acid residues that coordinate a divalent metal ion that is important for PA-binding. The MIDAS residues of integrin I domains shift depending upon whether the domain exists in a closed (ligand-unbound) or open (ligand-bound) conformation. Of relevance to this study, the MIDAS threonine residue coordinates the metal ion only in the open I domain conformation. Previously it was shown that the MIDAS threonine is essential for PA interaction with ANTXR1, a result consistent with the requirement that the I domain of that receptor adopts an open conformation for PA-binding [1]. Here we have tested the requirement for the MIDAS threonine of ANTXR2 for PA-binding. We show that the toxin can bind to a mutant receptor lacking the MIDAS threonine and that it can use that mutant receptor to intoxicate cultured cells. These findings suggest that an open-like configuration of the ANTXR2 MIDAS is not essential for the interaction with PA.

INTRODUCTION

Antigen Binding. PLoS ONE 1(1): e99. doi:10.1371/journal.pone.0000099

Citation: Scobie HM, Young JAT (2006) Divalent Metal Ion Coordination by Residue T118 of Anthrax Toxin Receptor 2 Is Not Essential for Protective Antigen Binding. PLoS ONE 1(1): e99. doi:10.1371/journal.pone.0000099

The crystal structure of the ANTXR2-PA complex has revealed that PA–receptor binding appears to resemble ligand binding to the open conformation of α-integrin I domains. Namely, the ANTXR2-bound divalent cation is directly coordinated by a carboxylate-containing side chain (residue D683) of PA as well as by the MIDAS threonine and serine residues, and water molecules coordinated by the MIDAS aspartate residues (Fig. 1) [7,8]. However, the ANTXR2-PA binding surface which includes residues from the receptor’s I domain MIDAS face and residues from PA domains 2 and 4 (∼2000Å²) is much larger than that of α-integrin-ligand interactions (∼1300 Å²) [7,8].

Previously it was shown that, similar to α-integrin-ligand interactions, the first MIDAS aspartate (D50) and the MIDAS threonine (T118) are critically important for PA binding and intoxication via ANTXR1 [1]. Mutation of the first MIDAS aspartate abolishes metal ion binding whereas mutation of the MIDAS threonine, while permitting metal ion binding, prevents the MIDAS from adopting the configuration associated with the open I domain conformation [6]. Given the importance of residue T118 of ANTXR1 it was proposed that an open-like configuration of the I domain of that receptor is essential for PA-binding [1].

In this report, we have tested whether the same holds true for ANTXR2 and we show that, unlike in the case of ANTXR1, residue T118 is not essential for the ANTXR2-PA interaction, indicating that the I domain of ANTXR2 does not need to adopt a classical open-like conformation to bind PA.

MATERIALS AND METHODS

DNA constructs and cell lines

Quikchange mutagenesis (Stratagene) performed on the previously described ANTXR2-enhanced green fluorescent protein (EGFP) DNA constructs and cell lines

Academic Editor: Debbie Fox, The Research Institute for Children, United States of America

Received October 4, 2006; Accepted November 21, 2006; Published December 20, 2006

Copyright: © 2006 Scobie, Young. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by NIH grant A156013.

Competing Interests: J.A.T.Y. holds equity in PharmAthene, Inc. (Annapolis, MD).

* To whom correspondence should be addressed. E-mail: jyoung@salk.edu
fusion construct (CMG2489-EGFP) and soluble ANTXR2-mycHis fusion constructs (sCMG2) [4,9] to generate D50A and T118A mutations with the oligonucleotide primers 5'CTT CGT CCT GGC AAA GTC TGG GAG TG 3' and 5'CCA GTA GGA GAG GCA TAT ATC CAT G 3', respectively. The open reading frames of all constructs were confirmed by DNA sequencing.

PA receptor-deficient CHO-R1.1 cells, and CHO-R1.1 cells that were engineered to stably express CMG2489-EGFP have been described previously [4]. CHO-R1.1 cells transduced with retroviral vectors encoding the D50A or T118A mutant ANTXR2 proteins were either selected in medium containing G418 (Invitrogen) and/or enriched by flow cytometric sorting on the basis of EGFP expression [4].

PA and sANTXR2 protein production

The WT and mutant sANTXR2-mycHis proteins were purified from the extracellular supernatants of transfected human 293Free-style cells (Invitrogen) as described elsewhere [9]. PA was prepared from the periplasm of E. coli BL21 cells that had been transformed with the PA-Pet22b as described previously [9]. The PA protein was purified by FPLC with HiTrap QFF and Superose 12 (Amersham) 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

Cell surface expression of the mutant D50A and T118A ANTXR2 receptors was confirmed using a membrane-impermeable biotinylating reagent (EZ-Link SuLfo-NHS-Biotin; Pierce) and pelleting biotinylated proteins with immobilized streptavidin gel (Immunopure; Pierce) essentially as described elsewhere [1]. The pelleted samples were boiled in reducing SDS sample buffer and subjected to SDS-PAGE using a 10% polyacrylamide gel. The samples were then transferred to a PVDF membrane and immunoblotted with a 1:5000 dilution of anti-GFP antibody (118P; Covance), followed by a 1:2500 dilution of anti-mouse HRP (Dako), in TBST (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 3% non-fat milk. For control purposes, CHO-R1.1 cells transduced with a retroviral vector encoding cytoplasmic EGFP from pLEGFP.C1 (Clontech) were also used to confirm that the biotinylation method used did not label cytoplasmic proteins (data not shown).

PA binding and in vitro intoxication assays

PA binding to cells was monitored by flow cytometric analysis following incubations of cells for 2 hours with 100 nM PA, then with a 1:2000 dilution of an anti-PA rabbit polyclonal serum, and a 1:500 dilution of an APC-conjugated anti-rabbit antibody (Molecular Probes) [4]. Intoxication was monitored by incubating samples of cells with LF/DTA [10] and PA. These experiments were performed either with, or without sANTXR2 inhibitor proteins. Cell viability was measured 25–30 hours later using the WST-1 reagent (Roche), or 45–50 hours later using the CellTiter-glo reagent (Promega) [4,9]. The IC_{50} was determined as the concentration of inhibitor at the midpoint of the soluble receptor titration curve. In all intoxications, % cell viability is the average from three samples, plus or minus the S.D.

RESULTS AND DISCUSSION

The T118A mutant receptor binds PA

To test the importance of ANTXR2 residue T118, it was replaced with an alanine in the context of a human ANTXR2-EGFP fusion
protein [4]. For control purposes, residue D50 of ANTXR2 was also changed to an alanine (a mutation that leads to loss of metal ion binding) in ANTXR2-EGFP. The mutant ANTXR2<sup>D50A</sup>-EGFP and ANTXR2<sup>T118A</sup>-EGFP proteins were then expressed in the PA receptor-deficient cell line, CHO-R1.1 [3]. Flow cytometric analysis performed with PA, an anti-PA antibody and a fluoresceinated secondary antibody revealed that the T118A mutant receptor was expressed at the cell surface and could bind

---

**Figure 3.** Soluble ANTXR2<sup>T118A</sup> inhibits intoxication of cells. WT and mutant sANTXR2 proteins were added in increasing amounts to measure their relative abilities to block CHO-K1 cell intoxication with PA (10<sup>-8</sup> M) and LFN-DTA (10<sup>-10</sup> M). Cell viability was subsequently measured using the WST-1 assay and is represented as the percentage of that seen with cells incubated with LFN-DTA alone (100% viable).

doi:10.1371/journal.pone.0000099.g003

**Figure 4.** The cell surface T118A mutant ANTXR2 supports intoxication. Triplicate samples of the cells shown in Fig. 2 were incubated with 10<sup>-10</sup> M LFN-DTA and varying concentrations of PA and cell viability was measured as described in the Fig. 3 legend.

doi:10.1371/journal.pone.0000099.g004
PA (Fig. 2). By contrast, the D50A mutant receptor did not bind PA (Fig. 2). Cell surface expression of the D50A and T118A mutant receptors was confirmed with a membrane-impermeable biotinylating agent as described elsewhere [1] (data not shown).

As an independent measure of how the D50A and T118A mutations affect PA binding, these mutations were introduced independently into the soluble human ANTXR2 receptor-decoy protein (sANTXR2) which, through binding extracellular PA, can protect cells and animals against intoxication [4,11]. The altered forms of sANTXR2 were tested for their abilities to protect CHO-K1 cells from intoxication by PA and LF_{N-NTA}. Consistent with activity of the full-length T118A mutant and wild-type receptors, the sANTXR2^{T118A} inhibitor was ~13-fold less efficient at blocking intoxication (IC_{50} = 80 nM) as compared to WT sANTXR2 (IC_{50} = 6 nM) (Fig. 3). As expected sANTXR2^{D50A} was not a functional receptor-decoy (Fig. 3). These results confirm that the T118A mutation receptor is still capable of PA binding.

The T118A mutant receptor supports intoxication

The T118A and D50A mutant receptors were also tested for ability to support intoxication, when expressed in CHO-R1.1 cells, with PA and LF_{N-NTA}, a fusion protein with the N-terminal portion of LF fused to the catalytic A chain of diphtheria toxin [10]. For control purposes, these experiments were also performed with the parental CHO-R1.1 cells, and CHO-R1.1 cells expressing a wild-type form (WT) of human ANTXR2-EGFP. These studies revealed that ANTXR2^{T118A} -EGFP supported intoxication albeit less efficiently than the wild-type version of the ANTXR2 protein (Fig. 4A). By contrast, ANTXR2^{D50A} -EGFP could not support intoxication (Fig. 4B), an expected result given that this mutation completely disrupts PA binding (Fig. 2).

This report has investigated the roles played by specific metal ion binding residues in the PA-ANTXR2 interaction. As in the case of ANTXR1 [1], the ANTXR2 interaction with wild-type PA is absolutely dependent upon the first receptor MIDAS aspartate (D50). However, unlike ANTXR1, the MIDAS threonine residue (T118) was not essential for PA interaction as judged by PA-binding either to the mutant cell surface receptor (or to the mutant soluble receptor). Moreover the mutant ANTXR2 protein lacking residue T118 was capable of supporting intoxication mediated by wild-type PA.

Taken together, these results demonstrate that there is no absolute requirement for ANTXR2 to adopt an open-like configuration for binding PA. Consistently, unlike ANTXR1 [1,12] ANTXR2 can bind to, and support intoxication by, mutant PA proteins in which metal-coordinating residue D683 is replaced by either an asparagine or a lysine [13].

The difference in the absolute requirement for an open-like configuration of the receptor MIDAS between ANTXR1 and ANTXR2 is presumably due, at least in part, to the fact that PA binds the ANTXR2 I domain with an approximately 1000-fold tighter binding affinity than it does the ANTXR1 I domain [11,14]. We propose that it is this higher binding affinity associated with ANTXR2 which overcomes an absolute requirement for an open-like configuration of the receptor I domain.

ACKNOWLEDGMENTS

We thank Jeremy Mogridge for providing reagents, D.Wigelsworth, R.J. Collier, D. Borden Lacy, Kenneth A. Bradley, G. Jonah A. Rainey, Richard Barnard, Jared D. Evans and Patricia L. Ryan for their helpful discussions, and John A. Naughton and D. Borden Lacy for help with manuscript preparation.

Author Contributions

Conceived and designed the experiments: JY HS. Performed the experiments: HS. Analyzed the data: JY HS. Wrote the paper: JY HS.

REFERENCES

1. Bradley KA, Mogridge J, Jonah G, Rainey A, Baty S, et al. (2003) Binding of anthrax toxin to its receptor is similar to alpha integrin-ligand interactions. J Biol Chem 278: 49342–49347.
2. Collier RJ, Young JA (2003) Anthrax toxin. Annu Rev Cell Dev Biol 19: 45–70.
3. Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA (2003) Identification of the cellular receptor for anthrax toxin. Nature 424: 225–229.
4. Scobie HM, Rainey GJ, Bradley KA, Young JA (2003) Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A 100: 5170–5174.
5. Shimao A, Takagi J, Springer TA (2002) Conformational regulation of integrin structure and function. Annu Rev Biophys Biomol Struct 31: 485–516.
6. Li R, Ravi P, Griffith DL, Scott D, Armaou AT (1998) Two functional states of the CD11b A-domain: correlations with key features of two Mn2+ -complexed crystal structures. J Cell Biol 143: 1523–1534.
7. Lacy DB, Wigelsworth DJ, Melnyk RA, Harrison SC, Collier RJ (2004) Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. Proc Natl Acad Sci U S A 101: 13147–13151.
8. Santelli E, Bankston LA, Leppla SH, Liddington RC (2004) Crystal structure of a complex between anthrax toxin and its host cell receptor. Nature 430: 905–908.
9. Wesche J, Elliott JL, Falnes PO, Olsen S, Collier RJ (1998) Characterization of membrane translocation by anthrax protective antigen. Biochemistry 37: 15737–15746.
10. Milne JC, Blanke SR, Hanna PC, Collier RJ (1995) Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. Mol Microbiol 15: 661–666.
11. Scobie HM, Thomas D, Marlett JM, Destino G, Wigelsworth DJ, et al. (2005) A soluble receptor decoy protects rats against anthrax lethal toxin challenge. J Infect Dis 192: 1047–1051.
12. Rosovitz MJ, Schuck P, Varughese M, Chopra AP, Mehra V, et al. (2003) Alanine-scanning mutations in domain 4 of anthrax toxin protective antigen reveal residues important for binding to the cellular receptor and to a neutralizing monoclonal antibody. J Biol Chem 278: 30936–30944.
13. Scobie HM, Wigelsworth DJ, Marlett JM, Thomas D, Rainey GJ, et al. (2006) Anthrax Toxin Receptor 2-Dependent Lethal Toxin Killing In Vivo. PLoS Pathog 2: 949–955.
14. Wigelsworth DJ, Krantz BA, Christensen KA, Lacy DB, Juris SJ, et al. (2004) Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen. J Biol Chem 279: 23349–23356.