The secreted protein toxin produced by *Bacillus anthracis* contributes to virulence of this pathogen and can cause many of the symptoms seen during an anthrax infection, including shock and sudden death. The cell-binding component of anthrax toxin, protective antigen (PA), mediates entry of the toxin into cells by first binding directly to the extracellular integrin-like inserted (I) domain of the cellular anthrax toxin receptor, ATR. Here we report that this interaction requires an intact metal ion-dependent adhesion site (MIDAS) in the receptor as well as the presence of specific divalent cations. Also, we demonstrate that the toxin-receptor interaction is critically dependent on the Asp-683 carboxylate domain of the cellular anthrax toxin receptor, ATR. We propose that this carboxylate group completes the coordination of the MIDAS metal of ATR, mimicking integrin-ligand interactions.

*Bacillus anthracis*, the causative agent of anthrax, is a Gram-positive, spore-forming bacterium that expresses two major virulence factors, a poly-L-glutamate capsule and anthrax toxin. Anthrax toxin is comprised of three secreted proteins: a receptor-binding moiety, protective antigen (PA), and two catalytic moieties, edema factor (EF) and lethal factor (LF). EF is an adenylate cyclase that disrupts water homeostasis and impairs immune function in the host (1, 2). LF is a Zn2+-dependent metalloproteinase that cleaves members of the mitogen-activated protein kinase family of protein kinases and disrupts signal transduction pathways (3–6). PA binds EF and LF to target cells and transports these catalytic subunits into the host cell cytosol where they carry out their enzymatic functions. A cellular receptor that binds PA was identified as anthrax toxin receptor (ATR), a protein encoded by the tumor endothelial marker-8 (TEM8) gene (7). Recently, a second cellular receptor for PA was identified as the human capillary morphogenesis protein 2 (CMG2) (8). PA binds directly to the extracellular integrin-like inserted (I) domains present in ATR and CMG2 (7, 8). Furthermore, the binding of PA to the ATR I domain is dependent on the presence of divalent cations, as EDTA was able to disrupt this interaction (7).

Nine of the 18 integrin α-subunits contain I domains, and when present, these conserved domains constitute major ligand-binding sites in the integrins. In addition, integrin I domains contain a conserved MIDAS motif, defined by the amino acid sequence DXXXS...T.D (where X is any amino acid), which functions to promote ligand binding through coordination of a divalent metal ion, usually Mg2+ or Mn2+ (9, 10). Physiological I domain ligands interact directly with the MIDAS-coordinated metal, explaining the strict metal dependence reported for these integrin-ligand interactions (10). The ATR I domain also contains a conserved MIDAS motif that we hypothesized may play a role in the metal-dependent binding of PA. In this study we identify amino acids in the MIDAS motif of ATR, a carboxylate group in PA, and specific divalent cations that are important for PA-receptor interaction. These data suggest that anthrax toxin binds ATR in a manner that is similar to the binding interactions described for integrin I domains and their natural ligands.

**EXPERIMENTAL PROCEDURES**

Construction and Expression of ATR Variants—QuikChange™ mutagenesis was performed according to manufacturer’s instructions (Stratagene) to introduce the D50A and T118A mutations into the full-length ATR cDNA (TEM8 splice variant 2) (7) using oligonucleotides 5′-GTACTTCATTTTGGCTAAATCAGGAAGTGTG-3′ and 5′-CTGCCAGGAGGAGACGCCTACATGCATGAAGG-3′, and their complements respectively. ATR-, ATR(D50A)-, and ATR(T118A)-EGFP were generated by PCR amplification of the ATR coding region followed by subcloning into the SalI and BamHI sites of pLEGFP-N1 (Clontech).

ATR-mycHis was generated by PCR amplification of the 5′ fragment of ATR corresponding to amino acids 1–229 and subcloning this fragment into the EcoRI and HindIII sites of pcDNA3.1/mycHis(−A)-Clontech. The D50A and T118A mutants of this construct were generated using QuikChange™ according to the manufacturer’s instructions (Stratagene) with oligonucleotides 5′-ACTCTTATTTTGCGCCAATCTCACAGAGCCTACGAGAGG-3′ and 5′-CTGCAAGAGGAGGAGACGCCTACGAGAGGAGG-3′, and their complements, respectively. The resulting constructs were transiently transfected into 293 cells using calcium phosphate precipitation or 293 Freestyle cells using 293fectin (Invitrogen), and
proteins were isolated from the culture media by purification over a nickel-nitrotriacetic acid column (Qagemed and dialysis against Tris-buffered saline (TBS; 50 mM Tris, pH 7.4, 150 mM NaCl). All PCR inserts and genes mutated by QuikChange™ were sequenced and shown to contain no additional mutations.

Based on the sequence of the ATR I domain, the wild type and mutant ATR-mycHis proteins are predicted to contain a single N-linked glycosylation site and have a protein mass of ~29 kDa. To verify that the ATR-mycHis proteins were of the expected size, 3 μg of purified wild type or mutant ATR-mycHis was incubated with peptide N-glycosidase F (500 units), 1% Nonidet P-40, and 50 mM NaPO₄ (pH 7.5) in 30 μl for 4 h at 25 °C. In addition, protein folding was probed by adding trypsin (3194 units/ml) to final concentrations of 0.1, 1, and 5 μg/ml. Reactions were stopped at 80 °C for 60 min and stopped by adding 10 μl reducing SDS sample buffer and heating to 100 °C for 10 min followed by separation on 12% SDS-PAGE. Gels were stained with Coomassie blue and images collected using a Multisimage system (Alpha Innotech).

PA Mutant Proteins—The plasmid pET22b-PA was mutated by QuikChange™ mutagenesis according to manufacturer’s instructions (Stratagene). Oligonucleotides 5’-GAAATTTGAGATCTACAAAGGCCTCTAAAAGAC-3’, 5’-CTGAAGGGCTAAAAACATTTATTAAATGACAG, 5’-CTTAAAGAGGGTAAAATACAGAGATAGTATGTGC-3’, and 5’-GATTTTCATATATATATATATATATATATATATATAG-3’ were used to construct PA-E643Q, PA-E654Q, PA-D658N, and PA-D683N, respectively. The PA genes in these constructs were sequenced and confirmed by automated sequencing.

Infections were performed by adding retroviruses to CHO-R1.1 cells. The resulting cell lines were tested for their ability to support PA binding in a previously described flow cytometric analysis involving integrin-ligand interactions (10, 12). Ca²⁺ nor Mg²⁺ supported toxin binding nor inhibited Mn²⁺-dependent toxin binding at concentrations ranging from 0.01 to 1 mM (data not shown).

To directly test the role of the ATR MIDAS in toxin binding, the first conserved residue of this motif, an aspartate residue at amino acid 50, was changed to alanine (D50A). Similar amino acid substitutions were previously shown to disrupt metal coordination and ligand binding in integrins with MIDAS-containing I domains (13–16). The D50A mutation was introduced into ATR-mycHis, and this change was sufficient to abrogate binding to PA in an ELISA (Fig. 1B). Consistent with mutational analysis of a integrin I domains, the D50A mutation did not cause gross misfolding of the ATR I domain, as determined by limited proteolysis experiments (Fig. 1C).

Integrin I domains can adopt multiple conformations that differ in their affinities for ligands (17). The conserved MIDAS threonine makes a direct contact to the coordinated metal in the “open” or high affinity conformation but not in the “closed” or low affinity conformation. Whereas the ATR(D50A) mutation is predicted to disrupt metal coordination and block all metal-dependent ATR interactions, mutation of the MIDAS threonine (Thr-118 in ATR) is predicted to disrupt metal coordination and ligand binding in integrins with MIDAS-containing I domains (18, 19). Consistent with a direct role for the ATR MIDAS in PA binding, the T118A mutation into the ATR-mycHis protein disrupts toxin-receptor interaction as measured by ELISA (Fig. 1B). As with the D50A mutant form of ATR-mycHis, the ATR(T118A)-mycHis protein displayed a tryptic digest pattern similar to wild type ATR-mycHis, indicating that this mutation does not cause gross misfolding (Fig. 1C).

To test the ability of MIDAS-mutant forms of ATR to serve as a functional PA-binding receptors on cells, the D50A and T118A mutations were engineered into an ATR-enhanced green fluorescent protein (ATR-EGFP) gene fusion, and the resulting proteins were expressed in mutant Chinese hamster ovary cells (CHO-R1.1) that lack anthrax toxin receptors (7). The resulting cell lines were tested for their ability to support toxin binding in a flow cytometry based assay. Wild type ATR-EGFP-expressing cells bound PA and displayed a linear relationship between the amount of receptor expressed and the amount of PA bound (Fig. 2A). In contrast, ATR(D50A)-EGFP-expressing cells were unable to support detectable PA binding, whereas ATR(T118A)-EGFP-expressing cells displayed an impaired ability to support PA binding in this assay (Fig. 2A), indicating that an intact MIDAS is necessary for efficient toxin binding. Cell surface biotinylation demonstrated that all forms of ATR-EGFP were expressed at similar levels on the cell surface (Fig. 2B), excluding the possibility that gross misfold-
ing or improper subcellular localization account for the decreased toxin binding to ATR(D50A)-EGFP or ATR(T118A)-EGFP-expressing cells (20, 21).

Finally, we tested whether the D50A and T118A mutations disrupt the ability of ATR to support intoxication. CHO-R1.1 cells expressing wild type (WT) ATR-mycHis in a range of concentrations either without added divalent cation (boxes) or with 1 mM MnCl₂ (circles), MgCl₂ (inverted triangles), or CaCl₂ (triangles). B, the D50A (triangles) and T118A (inverted triangles) mutant forms of ATR-mycHis were assayed for PA binding in the presence of 1 mM MnCl₂ as described above. RLU, relative light units. C, wild type and mutant ATR-mycHis proteins were treated with peptide N-glycosidase F to remove N-linked carbohydrate, followed by limited proteolytic digest with various concentrations of trypsin as indicated. Following trypsin treatment, samples were analyzed by SDS-PAGE and Coomassie Blue staining. Intact ATR-mycHis proteins are shown by closed arrows, and degradation products are marked by asterisks. A major contaminant of ~70 kDa was seen in some protein preparations (open arrow). Peptide N-glycosidase F is also detected by Coomassie Blue staining (open circle).

An Aspartate Residue in PA Is Critical for Receptor Binding—Having shown a requirement for divalent cations and for the ATR MIDAS motif in toxin binding, we next addressed how PA contributes to the interaction with its receptor. Proteins that bind preferentially to the open conformation of MIDAS-containing I domains have conserved glutamate or aspartate residues that contribute to binding by completing the coordination of the MIDAS metal by virtue of their carboxylate side chains (9, 17, 18, 23, 24). To explore whether PA mimics a natural I domain ligand, we searched the crystal structure of PA for solvent-exposed aspartate and glutamate residues that receptor function. Interestingly, ATR(T118A) retains a low level of receptor activity as seen with cell binding and intoxication assays (Fig. 2, A and C), consistent with this mutation inducing a closed conformation with reduced affinity for PA.

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could contribute to binding (25). Four such amino acids were identified in the receptor-binding domain, domain 4 (D4) (Fig. 3), and each was mutated individually to the corresponding amine-containing residue (Asp to Asn and Glu to Gln). Each PA mutant was expressed in *E. coli*, purified, and tested for its ability to function in a cell killing assay when combined with LFN-DTA. Although three mutants behaved as wild type, a single mutant, D683N, was unable to participate in cell killing (Fig. 4A). Limited tryptic digestion profiles of the wild type and the D683N PA proteins were identical (data not shown), indicating that this PA mutant is properly folded. These results identify Asp-683 as a residue critical for PA function.
To test whether the PA-D683N mutation disrupted toxin function at the level of receptor binding, we engineered this mutation into a previously described glutathione S-transferase tagged receptor-binding domain of PA (GST-D4) (7) and tested the ability of this protein (GST-D4(D683N)) to interact with ATR-mycHis in a GST pull-down assay. GST-D4(D683N) was not able to bind to ATR-mycHis (Fig. 4B), consistent with a role for Asp-683 in receptor binding.

**DISCUSSION**

Successful vaccination against anthrax in animal models is correlated with an antibody response to PA, and the most effective neutralizing antibody responses are those directed against the receptor binding domain of PA (domain 4) (26, 27). Furthermore, transfer of anti-PA antibodies provides passive immunity to anthrax infection (27–29). Therefore, strategies to prevent binding of PA to its cellular receptors may lead to development of new anthrax antitoxins. Development of such antitoxins will be aided by a thorough understanding of how PA interacts with its cellular receptors. The goal of this work was to extend our understanding of anthrax intoxication by defining the molecular determinants of the PA-ATR interaction. PA binds directly to the extracellular region of ATR, which contains an integrin-like inserted or I domain, also known as a von Willebrand factor type A domain (VWA). VWA/I domains are conserved, structurally related protein-folding domains that consist of ~200 amino acids and function as protein-protein interaction modules (30). These domains are found in intracellular proteins expressed in eukaryotes, eubacteria and archaea, but an evolutionary divergence in metazoans gave rise to extracellular proteins with VWA/I domains (30). Proteins with extracellular I domain include integrins, matrilins, collagens, and complement components, and the I domains in these proteins bind directly to cell adhesion molecules and extracellular matrix proteins (30, 31). Although the natural function of ATR/TEM8 is still unknown, it is likely that the ATR I domain serves as a protein-protein interaction domain, the function of which is predicted to rely on an intact MIDAS. The requirement for an intact ATR MIDAS for toxin binding suggests that PA may compete with natural ligands for binding to ATR.

That PA binding may compete with natural ATR ligands is further supported by the absolute requirement for an aspartate residue at position 683 in PA. Such a requirement for carboxylate-containing residues is a common feature of α-integrin I domain ligands. Interestingly, Asp-683 is positioned in an exposed loop in PA, which was previously implicated in receptor binding (25, 32). Indeed, we and others (7, 32) have identified Asn-682 as important for PA-ATR binding. During the preparation of this manuscript, Rosovitz et al. (33) reported that replacing Asp-683 in PA with alanine results in a 1000-fold decrease in toxicity, which is correlated with a lack of mutant PA binding to cell surfaces, consistent with our findings here.

The loop that contains Asp-683 exists between two anti-parallel β-strands and folds such that Asp-683 is presented at the furthest tip, extending into solvent (Fig. 3). Binding of a integrin I domains to their ligands requires divalent cations, an intact MIDAS, and the presence of a conserved aspartate or glutamate in a solvent-accessible loop in the ligand. Given the dependence for toxin-receptor binding on Mn$^{2+}$ or Mg$^{2+}$, the requirement for an intact MIDAS in ATR, and the requirement for an aspartate in an exposed loop in PA (Asp-683), we propose that PA is mimicking a natural I domain ligand and that the receptor binding function of Asp-683 involves coordination of the MIDAS-bound metal ion present in ATR.

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