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Anthrax toxin receptor proteins

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Abstract

Anthrax toxin is a key virulence factor for *Bacillus anthracis*, the causative agent of anthrax. Here we discuss what is known about the anthrax toxin receptor (ATR), the cellular receptor for anthrax toxin, and how this information is being used to develop treatments for anthrax as well as to understand aspects of cancer. ATR was identified recently as a type I transmembrane protein with unknown function that contains an extracellular integrin-like inserted (I) domain. The ATR I domain contains the toxin binding site, and a soluble form of this domain was shown to serve as an effective antitoxin to protect cultured cells from toxin action. ATR is encoded by the tumor endothelial marker 8 (*TEM8*) gene, which is selectively up-regulated during blood vessel formation and in tumor vasculature, raising the possibility that this protein normally functions in angiogenesis. Therefore, identification of the cellular receptor for anthrax toxin has made possible new avenues of research in the areas of anthrax pathogenesis, antitoxin development, and cancer biology.

Keywords: Anthrax toxin; ATR; TEM8; Receptor; Antitoxin; Angiogenesis

1. Anthrax pathogenesis

Anthrax is a disease resulting from infection by spores of the Gram-positive bacteria *Bacillus anthracis*. The disease has three clinical manifestations, resulting from three different routes of infection [1]. Cutaneous anthrax, the most common form of the disease, results from infection through a cut or abrasion on the skin. Digestive anthrax involves the consumption of contaminated food, usually meat products, in which case infection occurs through the gut. The final, and most deadly, form of the disease is inhalation or pulmonary anthrax. The *B. anthracis* spore is extremely hardy and resistant to environmental insults, which allows the bacterium to survive for decades in a dormant state. Following contact with an animal host, the spore germinates in response to environmental signals that are not yet fully understood. Germination results in the conversion of the bacterium to the vegetative, or actively dividing, stage of its life cycle. *B. anthracis* then continues to grow, replicating to very high numbers in the blood (up to 10^9/mL in the guinea pig), eventually killing the host [2]. The factors involved in allowing the bacteria to evade the immune system and thus grow to such high numbers, as well as factors involved in the eventual killing of the host, are still being actively studied. However, the current model of pathogenesis implicates the secreted exotoxins from *B. anthracis* as playing pivotal roles in both immune evasion as well as the eventual lethality of anthrax.

B. anthracis expresses two virulence factors, each encoded by a separate extrachromosomal plasmid [3–5]. The first virulence factor, encoded by the plasmid pXO2, is a poly-D-glutamic acid capsule that surrounds the vegetative form of the bacteria and prevents phagocytosis by host immune cells. The second virulence factor, anthrax toxin, is encoded by the pXO1 plasmid. Anthrax toxin is composed of two separate A-B toxins, each with a distinct catalytic A moiety, EF or LF, and a shared binding B moiety, PA. The two toxins are designated as EdTx and LeTx, respectively.

EdTx raises the levels of cyclic AMP (cAMP) in target cells, consistent with EF being an adenylate cyclase [6]. Elevation of cAMP levels in target cells causes disruption of water homeostasis, leading to the swelling or edema commonly seen in anthrax patients. EdTx also inhibits

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Abbreviations: ATR, anthrax toxin receptor; I domain, inserted domain; TEM8, tumor endothelial marker 8; PA, protective antigen; EF, edema factor; LF, lethal factor; EdTx, edema toxin; LeTx, lethal toxin; MEK, mitogen-activated protein/ERK kinase (MAPKK); CHO, Chinese hamster ovary; DTA, diphtheria toxin catalytic (A) domain; cDNA, complementary DNA; MIDAS, metal ion dependent adhesion site; *CMG2*, capillary morphogenesis gene 2.

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phagocytosis of the bacterium by neutrophils, thereby allowing *B. anthracis* to evade the immune system [7].

LeTx, as its name implies, has a more detrimental effect on its host and by itself is lethal as shown in several small animal model systems. LF was shown recently to be a Zn^{2+}-dependent metalloproteinase that cleaves the N-termini of MAPKK family members (MEKs 1, 2, 3, 4, 6, and 7), disrupting a protein–protein interaction site [8–10]. Treatment of murine macrophages with LeTx *in vitro* results in a rapid cell lysis with concomitant release of cytokines and reactive oxygen intermediates (ROIs) [11,12]. While the mechanism of this lysis is unclear, recent work has identified two cellular factors involved downstream of known LF actions. These include a kinesin-like microtubule motor protein, Kif1C, which plays a role in the natural resistance to LeTx-induced macrophage cytolysis seen in some inbred mouse strains [13]. Additionally, a role for the proteasome was implicated by experiments that used inhibitors of proteasome activity to block LeTx-induced cytolysis without blocking MEK cleavage [14]. It is not yet clear how Kif1C and the proteasome contribute to lethal toxin action, but there are surely other cellular factors involved.

LF and EF gain access to the cytosol of target cells by first binding to cell-associated PA. This interaction depends upon the proteolytic processing of PA by furin, which allows PA to oligomerize into a seven-member ring (heptamer) that contains the binding sites for three EF or LF moieties [15–17]. The toxin complex is then endocytosed and trafficked to a low pH endosome where the PA heptamer undergoes conformational changes that allow it to insert into the endosomal membrane, forming a channel or pore, and translocating the A moieties into the host cytosol [18,19].

2. A receptor for anthrax toxin

Initial observations about the nature of the receptor for PA, made more than a decade ago, implicated a single class of proteinaceous receptors on the surface of CHO cells [20]. Most cell lines tested express a receptor for PA as determined by their ability to translocate EF and LF in a PA-dependent manner. Attempts to identify the PA receptor by chemical cross-linking approaches revealed two separate candidate polypeptides with approximate masses of 80 and 22 kDa, respectively [20,21]. However, further attempts to biochemically identify these proteins and/or the PA receptor failed. Therefore, in collaboration with our colleagues at the Harvard Medical School, we used a genetic approach to identify the PA receptor [22].

CHO cells were chemically mutagenized and challenged with a highly potent chimeric toxin that combined the PA dependence of anthrax toxin with a recombinant catalytic moiety (LF_N-DTA) consisting of the amino terminal end of LF fused to the catalytic region of diphtheria toxin. This toxin challenge led to the isolation of resistant cells that were shown subsequently to lack PA receptors. A cDNA expression cloning approach was then used to identify the PA receptor. The complementing cDNA, termed ATR, is a product of the *TEM8* gene that was shown previously to be up-regulated in endothelial cells associated with colorectal cancer [23]. Three different, apparently alternatively spliced, versions of the *TEM8* gene have been described. Splice variant 1 (SV1) is the original TEM8 cDNA that encodes a 564 amino acid protein with a long proline-rich cytoplasmic tail. Splice variant 2 (SV2) is the cDNA identified as ATR that encodes a 368 amino acid protein with a short cytoplasmic tail. Splice variant 3 (SV3) encodes a protein that is identical to the other two throughout most of the extracellular domain but diverges just before the transmembrane region such that it does not contain a recognizable membrane anchoring sequence.

The extracellular region of ATR contains a von Willebrand factor type A domain (VWA), also known as an integrin-like inserted or I domain. I domains are conserved, structurally related protein folding domains that consist of approximately 200 amino acids and function as protein–protein interaction modules. They are present in a large number of extracellular proteins including integrins, matrilins, collagens, and complement components and have been shown to function in binding to cell adhesion molecules and extracellular matrix proteins (reviewed in [24]).

The I domain of ATR was shown to be the binding site for PA [22]. In addition, a MIDAS (metal ion dependent adhesion site) motif (defined by the amino acid sequence DXSXS . . . T . . . D: where X is any amino acid) located within the ATR I domain is important for toxin binding. MIDAS motif residues function to coordinate a divalent cation (most often Mg^{2+} or Mn^{2+}), which is usually necessary for ligand binding. Indeed, this feature of the ATR I domain also seems to be important for PA binding since the PA–ATR interaction was abolished in the presence of EDTA [22]. Work is ongoing to define the molecular details of the interaction between PA and ATR.

3. Antitoxins

The identification of ATR has opened new avenues for the development of antitoxins. Antitoxins are small molecules or proteins that block toxin action and that may be used in conjunction with antibiotics to prevent disease. There is currently much interest in developing antitoxins for anthrax, as its toxin plays a major role in virulence. In the case of anthrax, infection of guinea pigs with *B. anthracis* leads to bacterial proliferation in the bloodstream. Once a bacterial titer of $>10^6$/mL in the blood is achieved, the animals cannot be rescued with an antibiotic treatment that successfully arrests bacterial growth [25]. This lack of rescue was attributed to the build up of toxin in the bloodstream. In human anthrax cases, antibiotic

treatment is only effective if administered before the onset of symptoms, presumably for the same reasons. Successful vaccination in animal systems involves antibodies against PA, and transfer of anti-PA antibodies provides passive immunity [26,27]. Therefore, blocking PA function is a viable antitoxin approach. The discovery of ATR has led to the development of a soluble receptor-based antitoxin. We predicted that the soluble I domain of ATR (designated as sATR) would compete with cell surface receptor for PA binding and thereby act as a decoy to protect the cells from intoxication. Indeed, we found that sATR could function to block intoxication of CHO cells in culture [22]. The challenge now is to determine the efficacy of sATR as an antitoxin *in vivo*, and to establish whether treatment with this protein leads to any detrimental side-effects.

In addition to sATR, two additional antitoxins have been reported in the last year, each of which provides protection from a purified LeTx challenge in a rat model [28,29]. The first of these is a dominant negative form of PA (DN PA) that can be incorporated into a PA heptamer on the surface of the target cells but that prevents translocation of EF and LF. This mutant PA poisons the entire heptamer such that incorporation of a single DN PA destroys the ability of the heptamer to function. The second antitoxin derives from a peptide that was selected for its ability to block the interaction between LF and the heptameric form of PA. This peptide, while functional, was not an effective antitoxin when used in a monomeric form. Efficiency was increased 7500-fold, however, when it was multimerized using a polyacrylamide backbone [28].

4. Subcellular trafficking of ATR and toxin

Well before the identity of ATR was known, experiments to determine the route of toxin entry were reported. As early as 1986, it was shown that the toxin requires the acidic pH associated with endosomal compartments in order to translocate the catalytic A moieties (LF in this case) into the cytosol [18]. Several groups have since shown that toxin translocation into the cytosol is blocked by treating cells with chemicals that act to neutralize the low pH of endosomes [30–32]. Low pH induces a conformational change in PA that results in membrane insertion and pore formation, effects that are coincident with A-moiety translocation [19,33–35]. These data support a model for receptor-mediated endocytosis of toxin and subsequent trafficking to a low pH endosome.

Recently, it was shown that a mutant form of PA that cannot oligomerize remained on the cell surface for at least 90 min, indicating that the ATR has a very slow rate of endocytosis [36]. However, oligomerization of PA was shown to promote endocytosis of toxin with a half-time of 40–60 min [36,37]. These data are consistent with ATR endocytosis being stimulated by PA-mediated clustering. Interestingly, the rate of uptake of PA is similar to that of diphtheria toxin (DT) bound to its receptor, the heparin binding epidermal growth factor precursor [38]. In the case of the DT receptor, it was shown that the endocytosis of toxin–receptor complexes does not depend upon the presence of a cytoplasmic tail on the receptor [39]. This supports a model in which the receptor–toxin complex is endocytosed non-specifically as a process of bulk turnover of plasma membrane. In the case of anthrax toxin, it seems that PA–ATR complexes are excluded from this turnover until PA heptamerization drives clustering of ATR, at which point the toxin–receptor complex is trafficked to a low pH compartment [36]. In contrast to the slow internalization of PA in primary murine macrophages reported by Beauregard *et al*. [36], it was reported that PA internalization in a murine macrophage cell line (J774) is much more rapid, occurring within 5 min [40]. Beauregard *et al*. suggested that this difference might derive from the different cell types used. Given the fact that there are different isoforms of ATR/TEM8, this difference in toxin uptake may be associated with specific forms of the receptor expressed in the different cell types. Additionally, there may be other cell-type specific differences at the level of ATR/TEM8-interacting proteins that control how a single receptor variant is internalized.

Localization of ATR to the basolateral surface of polarized epithelial cells was also reported prior to the identification of ATR [41]. EdTx entered these cells through the basolateral, but not the apical, surface. This property of ATR is of interest because the cytoplasmic tail regions of the SV1 and SV2 forms of ATR/TEM8 contain an acidic cluster motif (EESEE) that is similar to the basolateral sorting signal of furin [42]. Since PA processing by furin is necessary for PA oligomerization and subsequent EF and LF binding and translocation, it is tempting to speculate that the acidic cluster of ATR/TEM8 causes the receptor to colocalize with furin, although this hypothesis remains to be tested [15,43,44].

5. Toxin–receptor interactions—models and future prospectives

Five other bacterial toxins show homology to PA in all but their receptor binding domains. These include the vegetative insecticidal protein from *Bacillus cereus*, and four clostridial toxins (reviewed in [45]). The conservation of all but the receptor binding domains implies that these toxins derive from a single progenitor and have evolved to interact with their hosts in distinct ways. Understanding how these toxins target their hosts, first by identifying the receptors that they bind, may lead to a better understanding of how they function in the virulence of the respective pathogens. To this end, anthrax toxin is the only member of this group of toxins that has an identified receptor. A chemically mutagenized CHO cell derivative that fails to bind to *Clostridium botulinum* C2 toxin was isolated

and shown to be defective in carbohydrate modifications, but a direct binding receptor has yet to be reported [46,47]. Once identified, comparative analysis of the different receptors may give insight into the characteristics of what constitutes a functional receptor for these related toxins.

Comparative analysis can also be used to determine how anthrax toxin interacts with ATR. In this case, the structure of PA can be compared with the structures of known I domain ligands. The receptor binding domain in PA, domain 4, shows very little structural interaction with the rest of the PA molecule and adopts an autonomous immunoglobulin (Ig)-like fold [35]. Integrins bind to several different types of proteins, including extracellular matrix proteins such as collagen and fibronectin, as well as members of the Ig superfamily such as ICAMs 1–3, MadCAM, and VCAM-1. Structural studies of the binding domain of VCAM-1 and the tenth fibronectin type-III repeat have been reported, and both adopt a similar Ig-like topology even though they share no amino acid similarity [48,49]. In fact, VCAM-1, the fibronectin type-III repeat, and PA domain 4 all share a barrel-shape structure composed of two facing β sheets, each containing three or four anti-parallel β strands. Given this structural similarity, it is tempting to hypothesize that domain 4 of PA either evolved to mimic a natural ligand of ATR or was acquired during the evolution of PA as a binding module for the toxin. In any case, these comparisons allow for the creation and testing of models to explain how PA and ATR interact.

6. ATR/TEM8 in cancer

The natural function of ATR/TEM8 is still unknown, but two lines of evidence indicate a role in cancer. First, TEM8 was identified originally as a mRNA that is up-regulated in tumor endothelium [23]. Subsequently, it was shown in mice that ATR/TEM8 is up-regulated in endothelium associated with both normal and tumor-associated blood vessel formation [50]. Second, an ATR/TEM8-related protein encoded by *CMG2* has been described [51]. CMG2 is highly similar to ATR/TEM8, sharing 51% amino acid identity in the I domain, and was identified originally in a screen for genes differentially regulated during capillary induction *in vitro*. The *CMG2* transcript was found to be up-regulated early during the process of capillary formation, and the CMG2 protein was shown to bind to at least two extracellular matrix (ECM) components, collagen IV and laminin. Both of these ECM components are also up-regulated during capillary morphogenesis and deposited around developing endothelial cell tubes. The subcellular localization of CMG2 was determined to be predominantly to the endoplasmic reticulum. This finding led the authors to suggest that CMG2 may act in assembly of the basement membrane matrix that is produced during new blood vessel formation. In summary, the mRNA expression profile of ATR/TEM8 as well as mRNA expression and binding data of the highly similar protein, CMG2, suggest that one of the physiological roles of ATR/TEM8s may be in angiogenesis. However, the presence of ATR/TEM8 on the cell surface of most cell types tested in culture, as well as its presence on macrophages, indicates that there may be additional roles for this protein, perhaps in cellular adhesion.

One interesting consequence of anthrax toxin using ATR as a binding receptor is that tumor-associated blood vessels would be expected to contain more toxin receptors and would therefore be predicted to bind to and translocate more toxin than other cell types. Consistent with this model, Duesbery *et al*. [52] have reported the use of anthrax LeTx to treat experimental tumors in mice. The authors report that LeTx treatment of V-12 H-ras transformed NIH 3T3 mouse fibroblasts in culture caused the cells to revert to an untransformed phenotype. This finding is consistent with LF cleavage of MEKs interfering with transformation-inducing signaling in these cells. When these transformed cells were injected into mice to induce tumors, followed by *in vivo* LeTx challenge, the authors found that the toxin-treated mice had smaller tumors with a higher degree of necrotic cells and less vascularization. The authors conclude that LeTx inhibits tumor angiogenesis in the V-12 H-ras model system. The discoveries of ATR as the anthrax toxin receptor and its up-regulation during tumor angiogenesis strengthen these conclusions and support the model presented above that tumor vasculature preferentially binds and translocates anthrax toxin. Indeed, the mice used in this study showed no signs of toxic side-effects, suggesting that the tumor, and not the macrophages, may have been the primary target of the toxin. How different tumor types will respond to the LF-mediated cleavage of MEKs remains to be seen.

A related approach to target tumors with anthrax toxin was reported from the laboratory of Stephen H. Leppla and involves the retargeting of anthrax toxin-based fusion toxins [53,54]. In this case, the furin cleavage site in PA is altered to conform to a consensus cleavage site for a metalloproteinase that is up-regulated on the surfaces of tumor cells. Cleavage of PA is a necessary step in the processing of anthrax toxin, and PA is unable to bind to either EF or LF unless this cleavage has occurred [15,43,44]. By altering the cleavage site, Leppla and coworkers have shown that anthrax toxin can be engineered to become activated preferentially on the surface of tumor cells. What we now know about ATR expression will allow a more precise retargeting of anthrax toxin or cytotoxic fusion toxins to tumor vasculature, thereby providing a boost to current toxin-based anti-cancer approaches.

7. Conclusion

The identification of ATR was a long-awaited result that is sure to provide interesting, if not unpredicted, insight into the fields of cell adhesion and developmental and

cancer biology as well as obvious contributions to understanding anthrax pathogenesis. Many other pathogens have evolved to use I domain containing proteins as receptors and have proven to be useful tools in understanding the natural function of these host proteins (reviewed in [55]). Anthrax toxin may likewise prove useful in elucidating the interactions between ATR/TEM8 and its as yet unidentified ligand(s). There is a need for new treatments for anthrax that improve the efficacy of current antibiotic-based therapies. A soluble receptor-based antitoxin is being developed along with at least two other antitoxins in response to this need. The development of anthrax toxin-based anti-cancer treatments has also benefited from the realization that a protein previously known to have increased expression in tumor endothelium is the receptor for anthrax toxin. This suggests that tumors may have an increased sensitivity to anthrax toxin as compared to non-tumor tissues and offers new approaches for targeting cancer.

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