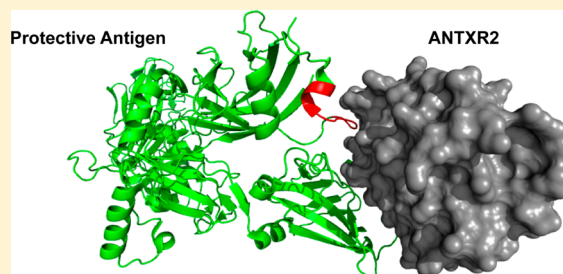


A Protective Antigen Mutation Increases the pH Threshold of Anthrax Toxin Receptor 2-Mediated Pore Formation

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ABSTRACT: Anthrax toxin protective antigen (PA) binds cellular receptors and self-assembles into oligomeric prepores. A prepore converts to a protein translocating pore after it has been transported to an endosome where the low pH triggers formation of a membrane-spanning β -barrel channel. Formation of this channel occurs after some PA–receptor contacts are broken to allow pore formation, while others are retained to preserve receptor association. The interaction between PA and anthrax toxin receptor 1 (ANTXR1) is weaker than its interaction with ANTXR2 such that the pH threshold of ANTXR1-mediated pore formation is higher by 1 pH unit. Here we examine receptor-specific differences in toxin binding and pore formation by mutating PA residue G342 that selectively abuts ANTXR2. Mutation of G342 to valine, leucine, isoleucine, or tryptophan increased the amount of PA bound to ANTXR1-expressing cells and decreased the amount of PA bound to ANTXR2-expressing cells. The more conservative G342A mutation did not affect the level of binding to ANTXR2, but ANTXR2-bound PA-G342A prepores exhibited a pH threshold higher than that of wild-type prepores. Mixtures of wild-type PA and PA-G342A were functional in toxicity assays, and the pH threshold of ANTXR2-mediated pore formation was dictated by the relative amounts of the two proteins in the hetero-oligomers. These results suggest that PA subunits within an oligomer do not have to be triggered simultaneously for a productive membrane insertion event to occur.



The protective antigen (PA) component of anthrax toxin binds receptors on mammalian cells and is proteolytically processed into a 63 kDa fragment that homo-oligomerizes to form ring-shaped heptamers and octamers called prepores.^{1–5} The PA63 prepore recruits the toxin's enzymatic moieties, edema factor (EF) and lethal factor (LF), and upon reaching an intracellular compartment converts into a membrane-spanning pore that translocates the enzymes to the cell cytosol.^{6–9}

The conversion from prepore to pore is a coordinated process that is initiated by acidic conditions and involves large structural rearrangements within the toxin–receptor complex.^{10–14} Studies have revealed that each PA63 monomer of the prepore binds a receptor through two domains. Interaction of PA domain 4 with the receptor is retained throughout the conversion process, whereas domain 2 contacts are lost.^{13,15,16} Loss of the receptor–domain 2 contacts allows a loop to disengage the core of domain 2 and assemble into a β -barrel that projects below the ring-shaped cap of the prepore and embeds in the endosomal membrane.^{12,15,17} The seven loops from a heptamer, or eight loops from an octamer, would have to insert in a concerted manner so that hydrogen bonding requirements of the β -hairpin loops could be satisfied within the hydrophobic membrane environment; the low-pH trigger may facilitate productive insertion events by providing signals simultaneously to each monomer of the prepore.

The pH threshold at which the prepore to pore conversion occurs depends on the receptor to which the prepore is bound. The anthrax toxin receptor 1 (ANTXR1)-bound prepore converts to a pore when it reaches a pH 6 compartment,

whereas the ANTXR2-bound prepore undergoes this transition in a pH 5 compartment.^{18,19} The distinct pH thresholds result from differences in the strengths of the PA63–receptor contacts that must be disrupted for pore formation to occur.^{20,21}

ANTXR1 and ANTXR2 are type I membrane proteins that bind PA through an extracellular I domain.^{22,23} The sequences of the I domains of the receptors are ~60% identical and contain metal ion-dependent adhesion site (MIDAS) residues that bind a divalent cation.^{24,25} An acidic residue from domain 4 of PA binds the cation directly, which is a type of interaction that also occurs between integrin I domains and their natural ligands, components of the extracellular matrix.²⁶ This interaction is not, however, responsible for the receptor-specific pH thresholds; homology scanning mutagenesis identified receptor residues that contact domain 2 as being primarily responsible for influencing the pH threshold.²⁰ These receptor residues of ANTXR2 form a pocket that makes contact with a PA domain 2 loop (amino acids 340–348) that is thought to shift during pore formation to allow the assembly of the β -barrel.^{15,16} Thus, interactions between the prepore and the receptors restrict pore formation until a suitably acidic compartment has been accessed. A low pH is required to unfold the enzymatic moieties and to provide the force that drives translocation.^{27–29}

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Here we have mutated residue G342 of PA, which is at the tip of the loop that inserts into the ANTXR2 pocket, and have found that an alanine substitution increased the pH threshold of pore formation for the ANTXR2-bound prepore. Mixtures of PA and PA-G342A were toxic to cells, suggesting that hetero-oligomers consisting of PA63 monomers with distinct pH thresholds are able to form pores and that intermonomer interactions may coordinate the assembly of the membrane-inserted β -barrel.

MATERIALS AND METHODS

ANTXR Constructs. The coding sequence of ANTXR1-sv1-HA was subcloned from pcDNA3-ANTXR1-sv1-HA³⁰ into pLJM1³¹ (Addgene plasmid 19319). The existing EcoRI site in ANTXR1-sv1 was silently abolished using QuikChange site-directed mutagenesis (Stratagene). Polymerase chain reaction (PCR) was used to amplify the coding sequence and insert an AgeI site 5' to the Kozak sequence and an EcoRI site 3' to the stop codon after the HA tag DNA sequence. The AgeI- and EcoRI-digested PCR fragment was ligated into the AgeI- and EcoRI-digested pLJM1 vector. The resultant pLJM1-ANTXR1-sv1-HA sequence was confirmed by DNA sequencing.

Similarly, the ANTXR2-sv2-HA coding sequence was subcloned from pcDNA3-ANTXR2-sv2-HA³² into pLJM1. AgeI and EcoRI sites flanking the Kozak sequence followed by the ANTXR2-sv2-HA coding sequences were added via PCR amplification. The AgeI- and EcoRI-digested PCR fragment was ligated into the AgeI- and EcoRI-digested pLJM1 vector, and the resultant pLJM1-ANTXR2-sv2-HA sequence was confirmed by DNA sequencing.

Stable Cell Lines. ANTXR1-HA and ANTXR2-HA were stably introduced into anthrax receptor-negative CHOR1.1 cells²² via transduction with lentiviral particles. Viral particles were produced by transfecting HEK 293T cells with 2.5 μ g of pLJM1-ANTXR1-sv1-HA or pLJM1-ANTXR2-sv2-HA, 1.75 μ g of pSPAX2 (Addgene plasmid 12260), and 0.87 μ g of pMD2.G (Addgene plasmid 12259) with 16 μ g of polyethylenimine. Viral particle-containing medium was collected 20 h post-transfection and filtered through a 0.45 μ m pore, and 2 mL was added to CHOR1.1 cells (80% confluent 10 cm culture dish). Cells were subjected to selective media (10 μ g/mL puromycin) 24 h postinfection, and stable expression of carboxy-terminal HA-tagged ANTXR1 and ANTXR2 was assayed via Western blotting using a polyclonal anti-HA antibody (Santa Cruz Biotechnology). CHOR1.1-ANTXR1-HA and CHOR1.1-ANTXR2-HA were maintained in selective media.

Transient Transfections. Receptor-negative CHOR1.1 cells were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum and kept at 37 °C and 5% CO₂. The cells were plated at a density of 1 \times 10⁶ cells/10 cm dish 20 h prior to transfection. Cells were transiently transfected with 5 μ g of plasmid encoding a C-terminally HA-tagged ANTXR1 or ANTXR2 and 25 μ g of polyethylenimine.

Cytotoxicity Assay. CHOR1.1-ANTXR1-HA and CHOR1.1-ANTXR2-HA stable cell lines were plated at a density of 1 \times 10⁴ cells/well in 96-well plates 24 h prior to treatment. LF_NDTA (a fusion between the PA-binding domain of anthrax lethal factor and the catalytic domain of diphtheria toxin), PA, and PA-G342 mutants were expressed and purified as described previously.³³ Cells were then treated with a mixture of PA or PA-G342 mutants (1 \times 10⁻¹¹ to 1 \times 10⁻⁷ M) and 1 \times 10⁻⁹ M LF_NDTA in Ham's F12 medium (supplemented with 10% fetal bovine serum) and incubated

at 37 °C in 5% CO₂ for 24 h in the presence or absence of 10 mM NH₄Cl. Cell viability was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Following the manufacturer's instructions, 20 μ L of prepared reagent was added to the existing 100 μ L of cell medium, and the cells were incubated for 3 h at 37 °C and 5% CO₂. The absorbance at 490 nm was then recorded. The values were corrected for background (no cells present) and normalized to toxin-untreated controls for each respective stable cell line. For each experiment, the conditions were assayed in triplicate and the mean was determined. The average of the means of three independent experiments \pm the standard error of the mean (SEM) was then plotted using GraphPad Prism version 4.

In experiments in which various molar ratios of PA-G342A and wild-type PA were used to form hetero-oligomers, the concentration of total PA (i.e., PA-G342A + PA) was kept constant at 1 \times 10⁻⁷ M. All other conditions were identical to those described above.

PA Binding Assay. CHOR1.1 cells stably expressing ANTXR1-HA or ANTXR2-HA were grown to ~80% confluency and treated with 1 \times 10⁻⁸ M furin-resistant PA or the PA-G342 mutant for 2 h at 4 °C in Ham's F12 medium supplemented with 1% bovine serum albumin and buffered with 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 8). Cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4) to remove unbound PA and then lysed. Equal amounts of cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using a polyclonal anti-PA antibody raised in rabbits. Blots were stripped and reprobed with an anti- β -actin antibody (Sigma) to ensure equal loading. Bands were visualized using chemiluminescence and a Kodak Image Station 4000MM Pro. The amount of PA and PA-G342 mutant bound to cell surface receptors was determined via densitometry using Carestream Molecular Imaging Software (Carestream Health Inc.). Values were corrected for background and normalized to the amount of PA bound to each cell type. The mean of three independent experiments \pm SEM was determined and plotted using GraphPad Prism version 4.

Cell Surface SDS-Resistant Pore Formation Assay. Receptor-negative CHOR1.1 cells were transiently transfected with a plasmid encoding either ANTXR1-HA or ANTXR2-HA. The transfected cells were then treated with 1 \times 10⁻⁸ M PA in Ham's F12 medium supplemented with 20 mM HEPES (pH 8.0) and 1% bovine serum albumin for 2 h at 4 °C while being gently rocked. Cells were then rinsed three times with PBS (pH 7.4), and cell surface SDS-resistant pore formation was induced by pulsing the cells for 3 min at 37 °C and 5% CO₂ with prewarmed pH-adjusted PBS solutions: 50 mM Tris at pH 6.8, 50 mM Tris at pH 6.5, 50 mM MES at pH 6.0, 50 mM MES at pH 5.6, 50 mM MES at pH 5.4, and 50 mM sodium acetate at pH 5.0. Cell lysates were then analyzed by Western blotting for the presence of SDS-resistant pores using a rabbit polyclonal anti-PA antibody. Blots were reprobed with a mouse monoclonal anti- β -actin antibody (Sigma) to ensure equal loading.

RESULTS

PA Point Mutations Selectively Decrease the Extent of ANTXR2-Mediated Cell Killing. Several substitution mutations of PA residue G342 were made to improve our

understanding of receptor-specific differences in toxin assembly and function. The PA_{340–348} loop fits closely into an ANTXR2 pocket that is in part formed by ANTXR2 residues ¹⁵²DGLVPS¹⁵⁷. These residues are not conserved in ANTXR1 (¹⁵⁴HEDLFF¹⁵⁹),^{15,16,25} and by comparison of the ANTXR1 and ANTXR2 I domain structures, it has been suggested that the PA_{340–348} loop would not make close contact with ANTXR1.²⁵ These data suggest that mutation of G342 to larger amino acids would selectively impair binding of PA to ANTXR2, but not to ANTXR1.

PA residue G342 was mutated to hydrophobic amino acids alanine, valine, leucine, isoleucine, and tryptophan. Each mutant was assessed for the ability to deliver LF_NDTA (the PA-binding domain of LF fused to the catalytic domain of diphtheria toxin) into cells expressing either ANTXR1 or ANTXR2. Each of the mutants intoxicated ANTXR1-expressing cells as effectively as wild-type PA (Figure 1A). In contrast, the mutants were

PA-G342 Point Mutations Result in an Increased Level of Binding to ANTXR1-Expressing Cells.

We next measured the binding of the PA-G342 mutants to ANTXR1- and ANTXR2-expressing cells using the PA_{SSR} mutant background, which prevents cleavage by furin to ensure that monovalent interactions were being assessed. Surprisingly, each of the PA-G342 mutations resulted in an increased level of binding of PA to ANTXR1-expressing cells, except for the deletion mutation that did not affect the amount bound (Figure 2A). The increase in the amount of mutant PA bound to these cells ranged from ~2-fold (G342L) to ~6-fold (G342V). In contrast to these results, PA-G342A bound ANTXR2-expressing cells at a level similar to that of wild-type PA, and the remaining mutants exhibited very low levels of binding (Figure 2B). These data are consistent with the notion that binding of PA brings G342 in apposition with ANTXR2, but not with ANTXR1.

The PA-G342A Mutation Increases the pH Required for ANTXR2-Mediated Intoxication.

We next assessed the effects of the G342 mutations on the pH threshold of the prepore to pore conversion because the interaction between the PA_{340–348} loop and the receptors influences this process.^{20,21} NH₄Cl treatment protects ANTXR2-expressing cells, but not ANTXR1-expressing cells, from intoxication because NH₄Cl increases the pH of intracellular compartments above the lower pH threshold of ANTXR2-bound prepore conversion.¹⁸ The G342 substitution mutants were able to intoxicate ANTXR1-expressing cells in the presence or absence of NH₄Cl (Figure 3A). Although the NH₄Cl treatment protected ANTXR2-expressing cells from wild-type PA, it did not confer protection from the G342 substitution mutants (Figure 3B). These results suggest that the increased strength of the interaction between the PA mutants and ANTXR1 was not sufficient to affect the pH threshold, whereas the decreased strength between the PA mutants and ANTXR2 increased the pH threshold.

We investigated PA-G342A further because it is the only mutant that is not defective at binding or intoxicating ANTXR2-expressing cells yet exhibits an increased pH threshold of prepore to pore conversion. Cells expressing either ANTXR1 or ANTXR2 were incubated at 4 °C with wild-type PA or PA-G342A, washed, and then exposed to a buffer at pH values ranging from 6.8 to 5.0 to induce pore formation on the cell surface. Pore formation was monitored by Western blotting because pores remain oligomeric during SDS–PAGE, whereas prepores dissociate to PA63 monomers.³⁴ As expected, PA and PA-G342A formed SDS-resistant pores at pH ≤6.0 when bound to ANTXR1 (Figure 4A). ANTXR2-bound PA formed SDS-resistant pores at pH ~5.0, but PA-G342A pore formation was triggered at pH ~5.6 (Figure 4B). Notably, the 0.6 pH unit increase observed here mimics the pH shift resulting from the mutation of ANTXR2 residues 152–157 to the corresponding residues of ANTXR1.²⁰

Mixtures of PA-G342A and Wild-Type PA Intoxicate Cells.

To investigate PA cooperativity during pore formation, we sought to determine whether mixtures of PA-G342A and wild-type PA could intoxicate cells. ANTXR1-expressing cells were killed by the mutant/wild-type PA mixtures (molar ratios ranging from 1:20 to 10:1) in the presence and absence of NH₄Cl (Figure 5A). This was an expected result because the pH threshold for both wild-type PA and PA-G342A prepores is ~6, so it follows that hetero-oligomers would not be inhibited by NH₄Cl. The PA mixtures were also able to intoxicate ANTXR2-expressing cells, but intoxication was increasingly sensitive to NH₄Cl as the mutant:wild-type PA ratio increased

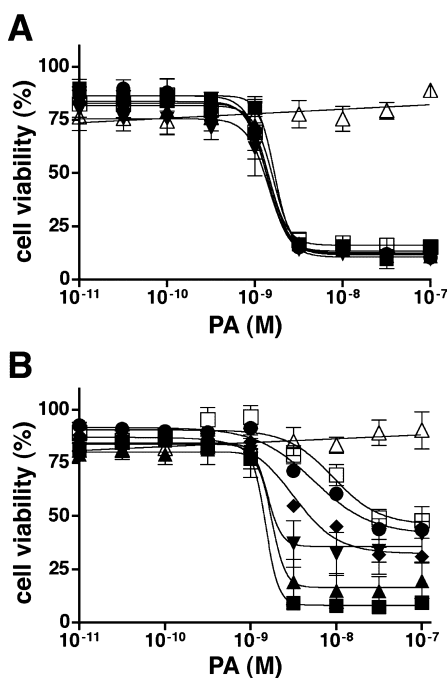


Figure 1. Mutation of PA domain 2 residue G342 results in inefficient killing of ANTXR2-expressing cells. Cells expressing (A) ANTXR1-HA or (B) ANTXR2-HA were exposed to a mixture of PA or PA-G342 mutants (1×10^{-11} to 1×10^{-7} M) and LF_NDTA (1×10^{-9} M) for 24 h. Each treatment condition was assayed in triplicate. The amount of cell death compared to that of the untreated control was then estimated using a metabolic cell proliferation assay. The average of the means of three independent experiments was plotted using GraphPad Prism version 4. The error reported is the standard error of the mean: (■) WT PA, (▲) PA-G342A, (◆) PA-G342V, (▼) PA-G342L, (●) PA-G342I, (□) PA-G342W, and (△) PA-ΔG342.

defective in their abilities to intoxicate ANTXR2-expressing cells, with the exception of PA-G342A, which exhibited activity similar to that of wild-type PA (Figure 1B). An inverse correlation was noted between the size of the side chain and the extent of death of ANTXR2-expressing cells observed at the highest concentration of mutant used.

In an attempt to shorten the PA_{340–348} loop, we constructed PA-ΔG342 and assayed this mutant in cytotoxicity assays. This mutant was completely defective at intoxicating cells that expressed either ANTXR1 or ANTXR2 (Figure 1A,B).

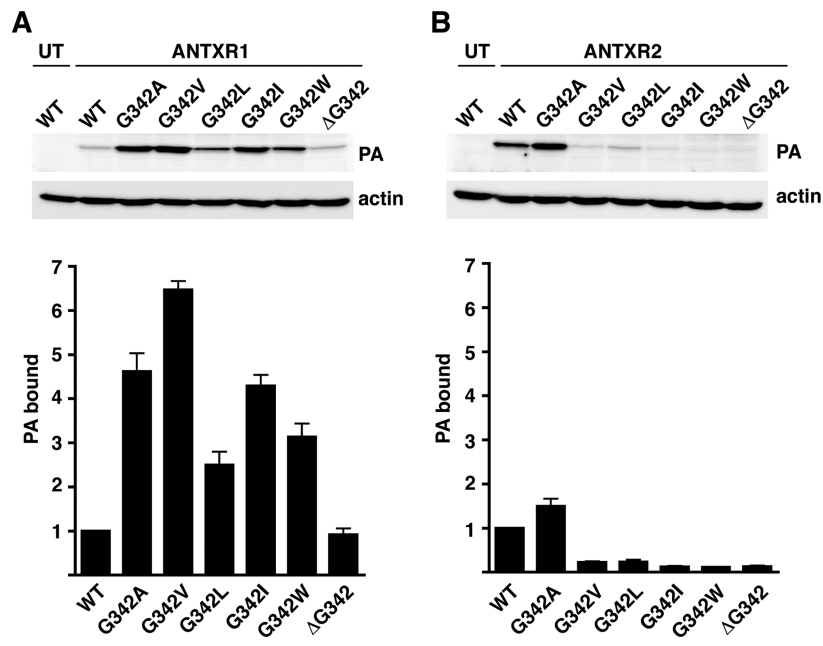


Figure 2. Mutation of PA residue G342 differentially affects binding of PA to ANTXRs. Cells expressing (A) ANTXR1-HA or (B) ANTXR2-HA were exposed to furin-resistant PA or PA-G342 mutants for 2 h. Untransfected cells (UT) served as a negative control. Cell lysates were analyzed by Western blotting using an α -PA antibody (top). Blots were reanalyzed using an anti- β -actin antibody to ensure equal loading. Blots are representative of three independent experiments. The amount of surface-bound PA was determined via densitometry (bottom). The amount of PA-G342 mutant bound to ANTXR1 and ANTXR2 was normalized to that of PA. The mean of three independent experiments and the standard error of the mean are shown.

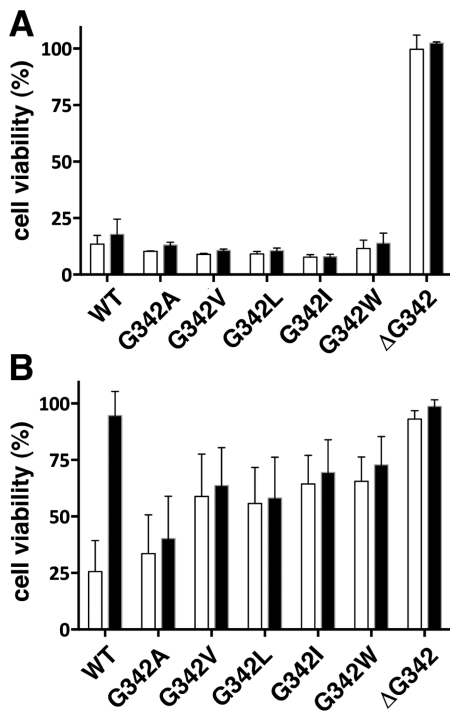


Figure 3. Ammonium chloride does not protect ANTXR2-expressing cells from PA-G342A-mediated intoxication. (A) ANTXR1- or (B) ANTXR2-expressing cells were exposed to PA or PA-G342 mutants (1×10^{-8} M) and LF_NDTA (1×10^{-9} M) for 24 h in the presence (black bars) or absence (white bars) of 10 mM ammonium chloride. Each experimental condition was assayed in triplicate, and the amount of cell death was normalized to the toxin-untreated control. The average of the means of three independent experiments and the standard error of the mean are shown.

(Figure 5B). These data suggest that the pH threshold of membrane insertion of an ANTXR2-bound prepore formed by wild-type PA and PA-G342A is determined by the ratio of the two within a hetero-oligomer: neither wild-type PA nor PA-G342A has complete dominance over the pH threshold.

DISCUSSION

The anthrax toxin pore forms when seven membrane insertion loops from a PA₆₃ heptamer (or eight loops from an octamer) simultaneously embed into the lipid bilayer and assemble into a β -barrel. The coordinated insertion of each loop is required to satisfy the hydrogen bonding requirements of the β -strands within the bilayer and is thought to be accomplished by the simultaneous sensing of an acidic environment by each PA₆₃ monomer–receptor complex. Our findings now suggest that the loop insertions are not completely independent events and that mechanisms for coordinating them exist.

We found one mutation, G342A, that does not adversely affect the association of PA with ANTXR2-expressing cells yet increases the pH threshold of toxin insertion closer to that of an ANTXR1-bound prepore. By allowing hetero-oligomers of wild-type and G342A PA₆₃ monomers to form from an equimolar mixture of the proteins, we then addressed whether each monomer within a PA₆₃ oligomer must reach its pH threshold before a productive insertion event can occur. If the stronger wild-type PA₆₃–ANTXR2 interaction were completely dominant, then only 1% of prepores (i.e., the mutant homo-oligomers) would form pores in the presence of NH₄Cl. What was observed, however, was a level of intoxication that was inconsistent with the notion that a single wild-type PA₆₃ monomer prevents heteropore formation in a pH ~6 compartment.

This result suggests that PA₆₃-G342A monomers induced wild-type PA₆₃ monomers to insert into the membrane at a pH

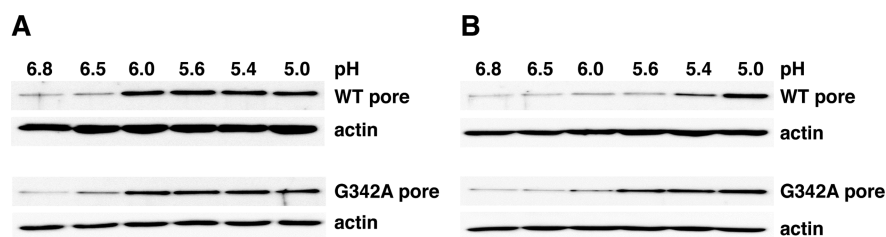


Figure 4. PA-G342A mutation increases the pH required for ANTXR2-mediated SDS-resistant pore formation. Cells expressing either (A) ANTXR1 or (B) ANTXR2 were treated with PA or PA-G342A for 2 h. Unbound PA or PA-G342A was removed, and cells were pulsed in pH-adjusted buffer for 3 min at 37 °C to induce cell surface pore formation. Cell lysates were analyzed by Western blotting with an α -PA antibody and reanalyzed using an anti- β -actin antibody to ensure equal loading. Blots are representative of three independent experiments.

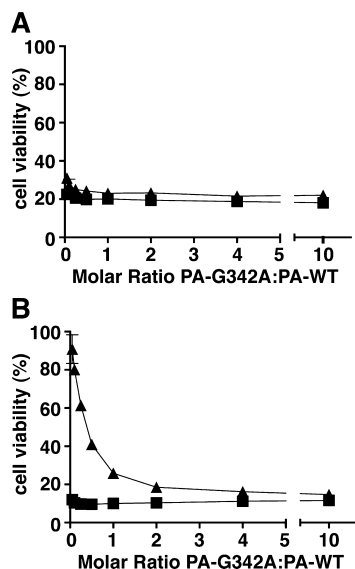


Figure 5. Ammonium chloride protection in the presence of PA-G342A/PA hetero-oligomers. (A) ANTXR1- or (B) ANTXR2-expressing cells were subjected to toxin mixtures containing various molar ratios of PA-G342A and PA (1:20 to 10:1; 1×10^{-7} M combined total) and $\text{LF}_{\text{N}}\text{DTA}$ (1×10^{-9} M) in the presence (\blacktriangle) or absence (\blacksquare) of ammonium chloride. The level of cell death was determined after 24 h and normalized to the that of the toxin-untreated control. Each experimental condition was assayed in triplicate. The average of the means of three independent experiments and the standard error of the mean were plotted using GraphPad Prism version 4.

that is normally too high to trigger the wild-type prepore. Because $\sim 6\%$ of the oligomers would contain one mutant PA63 monomer or no mutant PA63 monomers and NH_4Cl treatment led to an $\sim 15\%$ increase in cell viability, our results further imply that at least two or three molecules of PA63-G342A in a hetero-oligomer are required to induce pore formation at an elevated pH. The disruption of the PA domain 2–receptor interaction in more than one PA63–receptor complex may be needed to induce similar rearrangements in neighboring monomers before a productive insertion event can occur. We note, however, that we have not demonstrated that the assembly of wild-type and mutant PA63 monomers occurs in an even ratio and in a purely random manner, so our estimation of the number of mutant monomers required to exert dominance in a hetero-oligomer may be inexact.

Mutation of G342 to amino acids larger than alanine weakened the binding of PA to ANTXR2, either because the side chains do not fit well into the ANTXR2 pocket or because they interfere with the salt bridge between PA residue R344

and ANTXR2 residue E122.²¹ Interestingly, ANTXR1-expressing cells bound larger amounts of the G342 substitution mutants than wild-type PA. A valine residue at this position is optimal among the amino acids tested, although the increased level of binding of this mutant did not have a significant impact on the LD_{50} and did not shift the pH threshold of pore formation to a degree that caused sensitivity to NH_4Cl treatment. The fact that the larger amino acid side chains did not interfere with translocation of the enzymatic cargo through the channel is not surprising because the side chain of residue 342 projects outward from the β -barrel, rather than inward to the channel.¹²

PA- Δ G342 was the only mutant that showed no activity in the cytotoxicity assays despite exhibiting wild-type levels of binding to ANTXR1-expressing cells, suggesting that the deletion of G342 may inhibit the assembly of the β -barrel. Conductance experiments suggest that the channel interior of the pore is lined with amino acids from a domain 2 β -hairpin; the amino-terminal side of the β -hairpin extends below the cap of the pore toward the membrane from amino acid 276 to the tip of the hairpin (amino acids 313–314) and then back toward the cap to amino acid 351.¹² An alternating pattern of side chain accessibility to the channel interior, consistent with β -strands, was broken between residues 282 and 284 on the amino-terminal strand and at residue 341 on the carboxy-terminal strand. Nassi and colleagues suggest that these β -bulges produce a kink in the β -sheets and may be important for joining the β -barrel to the cap of the pore.¹² Further experiments are required to understand how residue 342 contributes to the assembly of the pore.

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Notes

The authors declare no competing financial interest.

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