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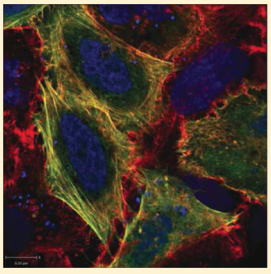
# Binding of Filamentous Actin to Anthrax Toxin Receptor 1 Decreases Its Association with Protective Antigen

Kristopher M. Garlick, Sarah Batty, and Jeremy Mogridge*

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Supporting Information

**ABSTRACT:** ANTXR1 is a type I membrane protein that binds the protective antigen (PA) component of anthrax toxin. The cytosolic domain of ANTXR1 has a novel actin-binding region that influences the interaction of the ectodomain with PA. Here, we have investigated features of the cytosolic domain of ANTXR1 that reduce the association of the receptor with PA. We mutated a stretch of conserved acidic amino acids adjacent to the actin-binding region and found that the mutation increased the affinity for monomeric actin in vitro. ANTXR1 bearing this mutation exhibited increased association with the cytoskeleton and bound less PA compared to the wild-type receptor, confirming the inverse correlation between the two interactions. To determine whether binding of actin is sufficient to regulate the ectodomain, we replaced the actin-binding region of ANTXR1 with that from the yeast protein abp140 and with the WH2 domain of WAVE2. Although both of these domains bound monomeric actin in vitro, only the sequence from abp140 reduced binding of PA to a hybrid receptor. The actin binding regions of ANTXR1 and abp140, but not the WH2 domain, colocalized with actin stress fibers, which suggested that filamentous actin regulates ANTXR1. Consistent with this notion, disruption of actin filaments using latrunculin A increased the amount of PA bound to cells. This work provides evidence that cytoskeletal dynamics regulate ANTXR1 function.



Anthrax toxin has wide-ranging effects on the host that contribute to the pathogenesis of *Bacillus anthracis*.[1,2] These pleiotropic responses to the toxin result from two enzymatic components that exert distinct activities, each of which has a substantial impact on cellular processes. Edema factor is an adenylate cyclase that produces the second messenger cAMP;[3] lethal factor is a protease that disables three mitogen activated protein kinase (MAPK) signaling pathways.[4,5] The ability of anthrax toxin to broadly affect host physiology also results from its third component, protective antigen (PA), targeting two widely expressed receptors, ANTXR1 and ANTXR2, which facilitates delivery of the toxic enzymes into the cytosol of most, if not all, cell types.[6,7]

ANTXR1 and ANTXR2 are type 1 membrane proteins that have similar domain organizations. The ectodomains consist of a von Willebrand factor type A (VWA) domain (also known as an I domain), which binds PA,[8,9] and an immunoglobulin-like domain positioned proximally to the membrane.[10] Both receptors have a single leucine-rich transmembrane domain that contains an oligomerization motif; only the ANTXR1 transmembrane domain has been studied, however, and shown to self-associate in vitro.[11] The cytosolic tails of the receptors appear to lack structured domains, although they do have sequences that influence trafficking and a conserved region that binds actin.[12,13]

Similarities between the receptors suggest related physiological functions. ANTXR1 was originally identified as a gene upregulated in tumor endothelial cells (originally named tumor endothelial marker 8, or TEM8), and ANTXR2 was shown to exhibit increased expression in cells undergoing capillary tube formation (capillary morphogenesis gene 2, or CMG2).[14,15] This implied roles in angiogenic processes, although neither ANTXR1-null nor ANTXR2-null mice appear to have gross vascular defects.[16,17] ANTXR1-null mice accumulate excessive amounts of extracellular matrix (ECM) components in several tissues, which is reminiscent of what is observed in patients with the ANTXR2 related diseases juvenile hyaline fibromatosis and infantile systemic hyalinosis.[18] The receptors may, therefore, be involved in ECM homeostasis: ANTXR1 binds collagen I and VI, while ANTXR2 binds collagen IV and laminin.[15,19,20] Finally, ANTXR1 has been implicated in cell adhesion and spreading by providing a link between collagen I and the actin cytoskeleton.[21]

The ANTXR1−actin interaction also influences the ability of ANTXR1 to bind PA—more PA binds cells that express an actin-binding deficient mutant of ANTXR1 compared to cells that express wild-type ANTXR1.[22] One explanation for this result is that wild-type ANTXR1 adopts both high affinity and low affinity ligand-binding conformations, whereas actin-binding deficient receptors adopt only the high affinity conformation. Consistent with this notion, a recent study used monoclonal antibodies to demonstrate the presence of two structurally distinct populations of ANTXR1 on the cell surface that are regulated by the cytoskeleton: one monoclonal antibody recognized an epitope on one population, and a second antibody recognized an epitope present on both populations.[23] That the epitopes for the monoclonal antibodies map to the VWA domain is perhaps not surprising because these domains are known to undergo conformational changes

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that alter their ligand-binding properties. Ramey and colleagues introduced a mutation into the ANTXR1 VWA domain that is known to lock certain VWA domains into a high affinity conformation and found that it overrode the effect of the cytoskeleton on PA binding.[24] Thus, these studies suggest that the cytoskeleton linkage promotes the low affinity conformation of the VWA domain (Figure 1).

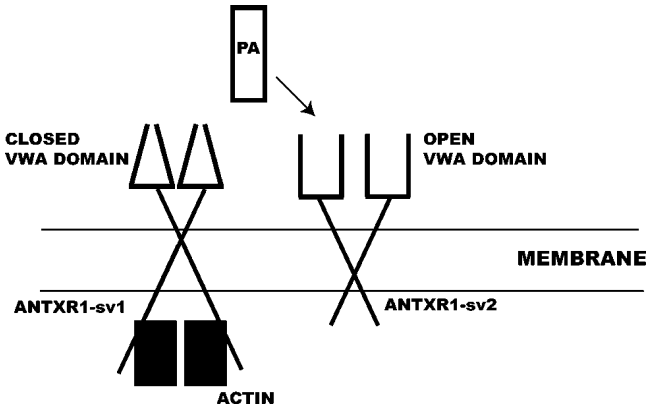


**Figure 1.** Model of how the cytosolic tail of ANTXR1 affects binding of PA. Binding of actin to the tail of ANTXR1-sv1 alters the packing of the transmembrane domains of a receptor dimer causing the VWA/I domains to close. ANTXR1-sv2 lacks an actin-binding region, which allows the transmembrane domains to cross at a different position. This alters how the extracellular domains interact, causing the VWA/I domains to open.

Here, we probe the nature of the intracellular signal that diminishes binding of PA to ANTXR1. Mutation of a stretch of conserved acidic residues adjacent to the actin binding region of ANTXR1 increased its association with monomeric globular actin (G-actin) and decreased its association with PA, confirming the inverse correlation. We next replaced the ANTXR1 cytosolic tail with unrelated sequences that bind actin: the WH2 domain of WAVE2 and a sequence from the yeast protein abp140. Both sequences bound G-actin in vitro, but only the sequence from abp140 diminished PA-binding in a receptor hybrid. Using fluorescence microscopy and an in vitro fractionation assay, we found that the ANTXR1 and abp140 actin binding regions associated with filamentous actin (F-actin), whereas the WH2 domain did not, suggesting that F-actin regulates ANTXR1. Consistent with this idea, the actin filament-destabilizing drug latrunculin A increased the amount of PA bound to cells expressing ANTXR1.

## MATERIALS AND METHODS

**Cell Culture and Transfection.** CHOR1.1 anthrax toxin receptor-negative cells were maintained in F12 medium supplemented with 10% fetal bovine serum (Invitrogen) and 1× penicillin−streptomycin.[6] CHOR1.1 cells stably expressing either pcDNA3-ANTXR1-sv1-HA or pcDNA3-ANTXR1-sv2-HA were maintained in the same medium supplemented with 0.25 mg/mL G418.[22] HeLa cells were maintained in DMEM medium supplemented with 10% FBS and 1× penicillin−streptomycin. CHOR1.1 cells were transfected in 10 cm dishes with polyethylenimine (PEI) at a 4:1 (w:w) PEI:DNA ratio, while HeLa cells were transfected at a 5:1 PEI:DNA ratio.

**Plasmids.** The pcDNA3-ANTXR1-sv1-HA, pcDNA3-ANTXR1-sv2-HA, and pcDNA3-ANTXR1-sv1-Y383C-HA plasmids have been described previously.[22] QuikChange site-directed mutagenesis (Stratagene) was used to introduce a six alanine substitution of amino acids 363−368 into pcDNA3-ANTXR1-sv1 to construct pcDNA3-ANTXR1-sv1-ARM. These mutations were also introduced in the pcDNA3-ANTXR1-sv1-Y383C plasmid. pcDNA3-ANTXR1-sv1$_{1-369}$-HA was constructed by amplifying a PCR product from pcDNA3-ANTXR1-sv1-HA using the forward primer 5′-GCGGGATCCGCCGCCACCATGGCCACGGCGGAGCG-3′ and the reverse primer 5′-GCGGCGGCCGCGCACCATCATCATCTTCTTCCTC-3′ and ligating the product into pcDNA3-HA following restriction digest with *Bam*H1 and *Not*I.

Oligonucleotides encoding the WH2 region of the WAVE2 protein (sequence accession number Q9Y6W5, amino acids 433−464) were synthesized with NotI and XhoI sticky ends: oligonuceotide 5′-GGCCGCGCGTGAGCGATGCCCG-TAGCGACCTGCTTTCAGCCATCCGTCAAGGTTTT-CAGCTGCGCAGGG TTGAGGAGCAGCGGGAACAAGA-GAAGCGGGATGTTC-3′ was annealed to the oligonucleotide 5′-TCGAGAACATCCCGCTTCTCTTGCCGCTGCTCCT-CAACCCTG CGCAG CTGAAAACCTTGACGGATGGCT-GAAAGCAGGTCGCTACGGGCATC GCTCACGCGC-3′. pcDNA3-ANTXR1-sv1$_{1-369}$-HA was digested with NotI and XhoI, and the annealed oligonucleotides were ligated into this plasmid to make pcDNA3-ANTXR1-sv1$_{1-369}$-WH2.

The pcDNA3-ANTXR1-sv1$_{1-369}$-ABP plasmid was made by inserting DNA encoding a 17 amino acid region of the yeast protein Abp140 (sequence accession number Q08641, amino acids 1−17) into pcDNA3-ANTXR1-sv1$_{1-369}$-HA. We annealed oligonucleotides 5′-GGCCGCGCATGGGTGTCGCA-GATTTGATCAAGAAATTCGAAAGCATCTCAAAGGAA-GAAC-3′ and 5′-TCGAGTTCTTCCTTTGAGATGCTTTC-GAATTTCTTGATCAAATCTGCGACACCCATGCGC-3′ and ligated the annealed product into NotI and XhoI-digested pcDNA3-ANTXR1-sv1$_{1-369}$-HA. QuikChange site-directed mutagenesis was used to introduce the ARM substitutions into pcDNA3-ANTXR1-sv1$_{1-369}$-HA, pcDNA3-ANTXR1-sv1$_{1-369}$-WH2, and pcDNA3-ANTXR1-sv1$_{1-369}$-ABP.

The pEGFP-sv1$_{360-420}$ plasmid has been described.[13] Amplification of this region from the ANTXR1-sv1-ARM plasmid yielded the pEGFP-sv1$_{360-420}$-ARM. Similar cloning techniques were used to generate pEGFP- sv1$_{360-369}$-WH2 and pEGFP-sv1$_{360-369}$-ABP, which were composed of amino acids 360−369 of ANTXR1-sv1, followed by their respective actin-binding regions described above. Amplification of similar regions containing ARM substitutions yielded pEGFP-sv1$_{360-369}$-ARM-WH2 and pEGFP-sv1$_{360-369}$-ARM-ABP.

The pGEX4T1-sv1$_{360-420}$ plasmid was made by amplifying DNA encoding amino acids 360−420 of ANTXR1-sv1 using the forward primer 5′-GCGGGATCCGAGGAGAGTGAG-GAAGAAGATG-3′ and the reverse primer 5′-GCGCTCGAGCTACATCTTGACTCTTGCATTCTTTGC-3′. The PCR products were digested with *Bam*HI and XhoI and ligated into pGEX-4T-1 (GE Healthcare). The pGEX4T1-sv1$_{360-369}$-WH2 and pGEX4T1-sv1$_{360-369}$-ABP plasmids were made using the same forward primer as above, and the reverse primers 5′-GCGCTCGAGCTAAA-CATCCCGCTTCTCTTCTTCCC-3′ and 5′-GCGCTCGAGCT ATTCTTCCTTTGAGATGCTTTCG-3′, respectively.

**PA Binding and Surface Expression Assays.** PA binding assays were performed as described.[22] Briefly, cells in 10 cm dishes were washed with PBS and incubated with 3 mL of $10^{-8}$


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M $PA_{SSSR}$ (a furin-resistant PA mutant) at 4 °C for 2 h in F12 medium supplemented with 20 mM HEPES (pH 8) and 1% BSA. Cells were washed three times in PBS, followed by lysis with 400 μL of EBC buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 0.5% NP-40, 1 mM PMSF). Lysates were cleared by centrifugation, followed by determination of protein concentration by the Bradford assay. Equal amounts of protein (~40 μg) were subjected to SDS-PAGE and Western blotting analysis. $PA_{SSSR}$ was detected using a rabbit polyclonal antibody raised against PA.

For binding assays performed in suspension, 10 cm dishes containing CHOR1.1 cells stably expressing pcDNA3-ANTXR1-sv1-HA or ANTXR1-sv2-HA were washed in PBS, followed by treatment with 1 μM latrunculin A (Calbiochem) in 3 mL of PBS for 2 h at 37 °C. Cells were scraped and resuspended in 1 mL of $PA_{SSSR}$ solution described above, supplemented with either DMSO or latrunculin A. Samples were rotated for 2 h at 4 °C and processed as above.

For determining receptor surface expression, cells were treated with 1 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at 4 °C. Cells were washed three times in PBS supplemented with 100 mM glycine and were harvested as in PA binding assays. Streptavidin agarose resin (80 μL of 50% slurry) (Pierce) was added to equivalent amounts of protein (~40 μg) derived from cell lysates and rotated overnight at 4 °C. Resin was washed three times in EBC buffer, and biotinylated proteins were eluted using SDS sample buffer. SDS-PAGE and Western blot analysis were used to detect precipitated receptors using an anti-HA antibody (Santa Cruz). All blots were visualized using a Kodak Image Station 4000MM Pro.

**F-Actin Association Assays.** HeLa cells seeded in 6-well plates and transiently transfected with pcDNA3-ANTXR1-HA constructs were washed and scraped in PHEM buffer [60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$ (pH 6.9)] as previously described.[13] Cells were lysed in 150 μL of PHEM + 0.15% Triton X-100 for 12 min at 4 °C prior to centrifugation at 16000*g* for 30 min at 4 °C. Soluble (supernatant) and insoluble (pellet) fractions were collected, and the pellet was resuspended in 150 μL of PHEM + 0.15% Triton X-100. SDS sample buffer was added to each fraction and boiled for 5 min, and equal volumes were subjected to SDS-PAGE and Western blotting. Blots were probed with anti-HA antibodies to visualize receptors in each fraction, and intensities of bands were quantified to determine pellet-to-supernatant ratios.

For F-actin association assays using GST fusion proteins, HeLa cells in 10 cm dishes were washed in PHEM and resuspended in 600 μL of PHEM + 0.15% Triton X-100 at 4 °C. During the 12 min lysis period, 30 μg of total protein of the following GST-containing extracts was added to lysates for incubation: GST, GST-$sv1_{360-420}$, GST-$sv1_{360-369}$-WH2, and GST-$sv1_{360-369}$-ABP. Lysates were processed as described above, and blots were probed with an anti-GST HRP-conjugated antibody (Santa Cruz) to detect levels of GST present in pellet or supernatant fractions.

**Peptides and G-Actin Preparation.** The $sv1_{360-420}$ peptide consisting of amino acids 360−420 of human ANTXR1-sv1 and an amino-terminal biotin tag was synthesized (AnaSpec Inc.). $sv1_{360-420}$-ARM is similar to $sv1_{360-420}$ except for the aforementioned six alanine substitution at positions 363−368. Peptides were bound to streptavidin agarose resin at concentrations of 0.5 mg of peptide/mL of resin or 0.1 mg of peptide/mL of resin for 30 min at room temperature in EBC buffer. Peptide-bound beads were centrifuged at 1500*g* for 5 min and resuspended in equal volumes of EBC. Purified human β-actin (Cytoskeleton Inc.) was diluted and depolymerized in general actin buffer (Cytoskeleton Inc.) according to manufacturer's instructions and as previously described.[13] The absence of actin filaments in the G-actin preparations used for binding assays was confirmed by electron microscopy (data not shown).

**Peptide Binding Assays.** To investigate direct binding between ANTXR1 peptides and G-actin, 50 μL of peptide-bound streptavidin-agarose beads (50% slurry) was rotated with 5 μg of monomeric G-actin for 2 h at 4 °C in 300 μL of EBC buffer. Beads were washed three times with EBC buffer, and proteins were eluted and subjected to SDS PAGE and Western blotting. Blots were probed for β-actin (Sigma) and stripped and reprobed with streptavidin-HRP (Pierce) to detect peptide.

**Confocal Microscopy.** HeLa cells were seeded on coverslips and transfected with the pEGFP plasmids described above. Cells were washed in PBS and fixed with 4% paraformaldehyde for 15 min. Cells were washed with PBS and permeabilized in 0.2% Triton X-100 for 5 min. After blocking with 5% BSA for 1 h, coverslips were stained with 4 units/mL AlexaFluor 555 phalloidin (Molecular Probes) for 1 h, washed three times with PBS, and mounted on slides using ProLong Gold antifade reagent with DAPI (Molecular Probes). Samples were imaged using a Zeiss Axiovert 200 microscope equipped with a spinning disk confocal scan head and a Hamamatsu C9100-13 EM-CCD camera. Z-slices of images were acquired every 0.2 μm and deconvolved using Volocity software (Perkin-Elmer).

**GST Fusion Protein Purification and Binding Assays.** pGEX4T1-$sv1_{360-420}$, pGEX4T1-$sv1_{360-369}$-WH2, and pGEX4T1-$sv1_{360-369}$-ABP were transformed into BL21(DE3) cells and grown at 37 °C to an $OD_{600}$ ~ 0.6. Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was added to cultures that were then shaken for 4 h at 30 °C to induce protein expression. Cells were harvested, resuspended in 20 mM Tris-HCl (pH 8), 1 M NaCl, and then lysed with a French press. Cell lysates were centrifuged to remove cellular debris, and concentrations of resulting extracts were determined using the Bradford assay.

GST fusion proteins were purified through binding extracts to glutathione sepharose, followed by three washes with 20 mM Tris-HCl (pH 8), 1 M NaCl and two washes with PBS. The resulting concentration of the GST fusion proteins was ~2 mg protein/mL glutathione sepharose. For GST pull-down assays, 25 μL of GST-conjugated glutathione beads (50% slurry) was incubated with 5 μg of purified monomeric β-actin for 2 h at 4 °C in EBC buffer. Beads were washed three times with EBC and eluted with SDS sample buffer followed by Western blot analysis. Blots were probed with anti-β-actin antibodies.

## RESULTS

**An Acidic Region in the ANTXR1 Cytosolic Tail Decreases Actin Association.** We demonstrated previously an inverse correlation between the association of ANTXR1 constructs with actin and with extracellular PA: cells that express ANTXR1 splice variant 1 (sv1), which associates with the actin cytoskeleton, bind less PA than cells expressing similar levels of ANTXR1-sv2 or ANTXR1-sv1-Y383C, which do not bind the cytoskeleton.[22] To study this further, we investigated a conserved stretch of acidic amino acids of unknown function situated adjacent to the actin-binding region of ANTXR1-sv1


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(amino acids 373−411). We mutated amino acids 363-EEEDDD-368 to alanines in both ANTXR1-sv1 and ANTXR1-sv1-Y383C to generate acidic region mutants (ARM). Cells that expressed ANTXR1-sv1 bound more PA than cells that expressed ANTXR1-sv1$_{ARM}$ (Figure 2A). In contrast, cells expressing ANTXR1-sv1-Y383C bound similar amounts of PA as those expressing ANTXR1-sv1-Y383C$_{ARM}$.

To determine whether the inverse correlation between actin- and PA-binding held with the ARM constructs, we performed an F-actin association assay. Cell lysates were centrifuged to pellet the insoluble actin cytoskeleton (and associated proteins) and then separated into soluble and insoluble fractions. These fractions were subjected to SDS-PAGE and Western blot analysis to determine the pellet:supernatant ratio of the ANTXR1 constructs (Figure 2B). As demonstrated previously, ANTXR1-sv1 was found predominantly in the cytoskeleton pellet, whereas ANTXR1-sv1-Y383C was not. A large majority of ANTXR1-sv1$_{ARM}$ was in the pellet, indicating that the ARM mutation increased association with the cytoskeleton. The acidic region mutation did not markedly increase the amount of the Y383C mutant in the pellet, demonstrating that the acidic region mutation did not override the Y383C mutation. These experiments provide additional support to the notion that the association of the cytoskeleton with ANTXR1 reduces binding of PA.

We next addressed whether mutation of the acidic region of ANTXR1 increased binding of G-actin in vitro. To accomplish this, we synthesized a peptide, sv1$_{360-420}$, composed of amino acids 360−420 of ANTXR1 and a related peptide, sv1$_{360-420ARM}$, containing alanine substitutions at amino acids 363−368. The peptides were attached to streptavidin agarose beads through an amino-terminal biotin group and assayed for binding to purified monomeric human $\beta$-actin. The sv1$_{360-420ARM}$ peptide bound more G-actin than the sv1$_{360-420}$ peptide did (Figure 2C), indicating that the acidic amino acids decrease the affinity of ANTXR1 for actin.

**A Heterologous Actin-Binding Region Reduces the Association of PA with ANTXR1.** To assess whether the association of actin with ANTXR1 is sufficient to reduce the amount of PA bound to the receptor, we replaced the actin-binding region of ANTXR1 with those of two unrelated proteins: WAVE2 and abp140. WAVE2 contains a WH2 domain, which is a well-characterized actin-binding motif found in a variety of proteins.[25] The WH2 domain of WAVE2 binds G-actin in vitro with high affinity.[25] The yeast protein abp140 contains a 17 amino acid motif that binds F-actin (denoted here as the actin binding peptide, ABP).[26] The two motifs were cloned into ANTXR1-sv1$_{1-369}$, which lacks an actin-binding region, and the acidic region mutant ANTXR1-sv1$_{1-369ARM}$ (Figure 3A). As expected, cells expressing ANTXR1-sv1$_{1-369}$ bound more PA than cells expressing ANTXR1-sv1 (Figure 3B). Cells expressing the WH2 domain receptor hybrid bound a high level of PA, whereas those expressing the ABP hybrid bound a low level of PA. Of the receptor constructs tested, the acidic region mutation decreased the amount of PA bound to ANTXR1-sv1 alone. These results correlated with cytoskeleton association: ANTXR1-sv1$_{1-369}$-ABP associated with the F-actin cytoskeleton whereas ANTXR1-sv1$_{1-369}$-WH2 did not; the acidic region mutations did not influence cytoskeleton association of these constructs (Figure 3C).

We next examined the cellular localization of EGFP fusions of the isolated actin-binding regions. EGFP-sv1$_{360-420}$ and EGFP-ABP colocalized with actin filaments, whereas EGFP and EGFP-WH2 did not (Figure 4 and Figure S1). In contrast to EGFP and EGFP-sv1$_{360-420}$, EGFP-sv1$_{360-420ARM}$ was excluded

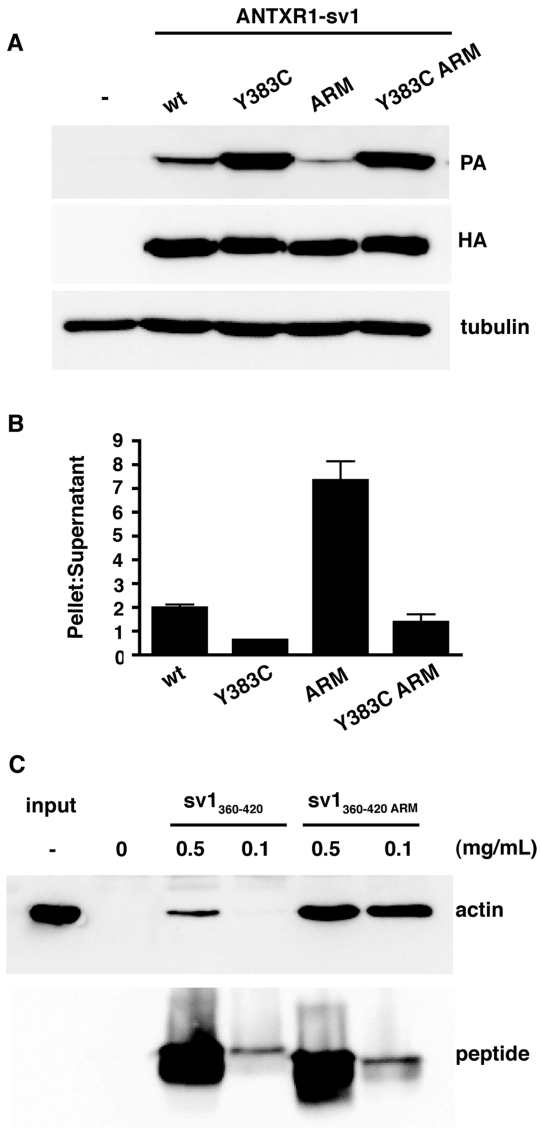


**Figure 2.** Mutation of an acidic region within the cytoplasmic domain of ANTXR1-sv1 decreases PA-binding and increases actin-binding. (A) Cells expressing ANTXR1-sv1-HA, ANTXR1-sv1-Y383C-HA, or the corresponding acidic region mutants (ARM) were exposed to furin-resistant PA for 2 h. Cell lysates were prepared and subjected to SDS-PAGE and Western blot analysis using $\alpha$-PA antibody. Surface expression of the HA-tagged receptors was determined by exposing the cells to a membrane impermeable biotinylation reagent, precipitating biotinylated proteins with streptavidin−agarose, and performing a Western blot assay using anti-HA antibody. Blots are representative of three independent experiments. (B) F-actin association assays of ANTXR1-sv1-HA and ANTXR1-sv1-Y383C-HA receptors expressed in HeLa cells. Cells were lysed and centrifuged for 30 min, and the ratio of the amount of receptors that sedimented with the cytoskeleton to the amount that remained in the supernatant fraction was quantified. Error bars indicate the standard error of the mean of three independent experiments. (C) A biotinylated peptide corresponding to amino acids 360−420 of ANTXR1-sv1 (sv1$_{360-420}$) and a similar peptide containing the acidic region mutation were bound to streptavidin agarose beads and incubated with purified monomeric $\beta$-actin. Proteins were eluted from beads and subjected to SDS-PAGE and Western blot analysis for $\beta$-actin (top) or biotin (bottom). Blots are representative of three independent experiments.


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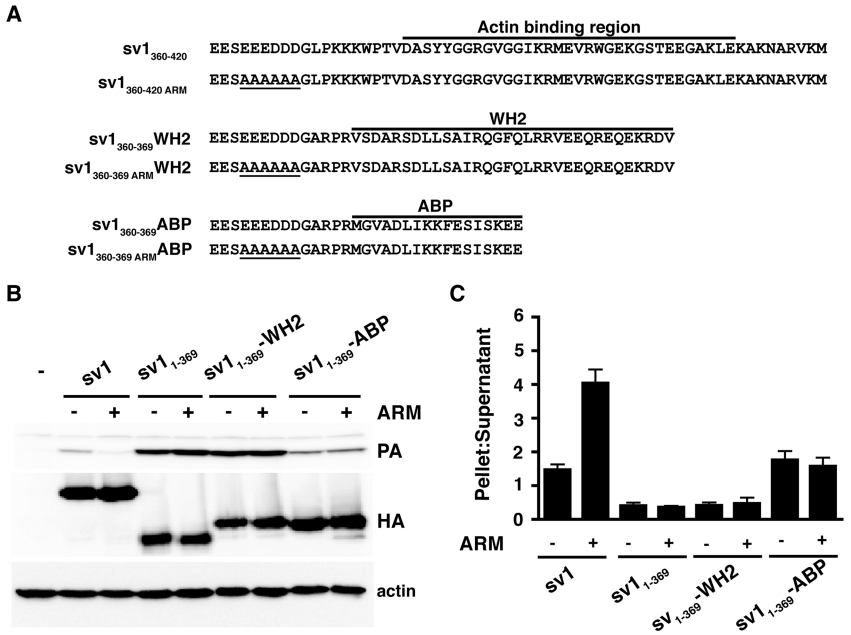


**Figure 3.** Cytosolic tails influence binding of PA to ANTXR1 hybrid receptors. (A) Comparison of sequences of hybrid receptor tails with the region of ANTXR1-sv1 encompassing the actin-binding region and adjacent acidic region. The acidic region mutation (ARM) is underlined. The cytosolic domain of ANTXR1 consists of amino acids 348−564 (not shown). The actin-binding region of ANTXR1-sv1 was replaced with the WH2 domain of WAVE2 or the actin-binding region of abp140 (ABP). (B) Cells expressing indicated receptors were incubated at 4 °C with a furin-resistant form of PA and the amount of bound PA was assessed by Western blotting (top). Actin was used as a loading control (bottom). Surface expression of the HA-tagged receptors was determined by exposing the cells to a membrane impermeable biotinylation reagent, precipitating biotinylated proteins with streptavidin−agarose, and performing a Western blot assay using anti-HA antibody (middle). Blots are representative of three independent experiments. (C) Association of indicated receptors with the F-actin cytoskeleton was determined by lysing cells and sedimenting the cytoskeleton. The amount of each receptor found in the cytoskeleton pellet, and the supernatant was determined by Western blot assays. The ratio of the amount of each receptor localized in pellet fractions to the amount that remained in supernatant was determined. Error bars indicate the standard error of the mean of three independent experiments.

from the nucleus, possibly because the higher affinity of the acidic region mutant for F-actin sequestered this construct from the nucleus. The acidic region mutations did not affect localization of EGFP-ABP, which was excluded from the nucleus, or of EGFP-WH2, which showed diffuse cytoplasmic and nuclear staining similar to the EGFP control.

**Filamentous Actin Regulates Binding of PA to ANTXR1.** We generated GST fusions of the actin binding regions to compare their abilities to interact with actin in vitro. Binding of the fusion proteins to the F-actin cytoskeleton was assessed by mixing GST, GST-sv1$_{360-420}$, GST-sv1$_{360-369}$-WH2, and GST-sv1$_{360-369}$-ABP with mammalian cell lysates and centrifuging the mixtures. The supernatants and cytoskeleton pellets were subjected to SDS-PAGE and then immunoblotted using an anti-GST antibody. GST and GST-sv1$_{360-369}$-WH2 were found exclusively in the supernatant fraction, whereas GST-sv1$_{360-420}$ and GST-sv1$_{360-369}$-ABP were predominantly found in the pellet (Figure 5A). These results are consistent with how the hybrid receptors fractionated with the cytoskeleton (Figure 3C).

We next measured in vitro binding of the fusion proteins to G-actin using GST pulldown assays. The fusion proteins all bound G-actin, but the GST control did not (Figure 5B). Thus, these experiments indicate that it is the relative affinities for monomeric and filamentous actin that differentiate the actin binding regions of ANTXR1 and abp140 from the WH2 domain. We hypothesized, therefore, that the binding of actin filaments, but not actin monomers, to ANTXR1 diminishes the amount of bound PA. To test this hypothesis, we used latrunculin A to disrupt the cytoskeleton in cells that expressed either ANTXR1-sv1, ANTXR1-sv1-Y383C, or ANTXR1-sv1$_{ARM}$. Latrunculin A treatment increased the amount of PA bound to cells expressing ANTXR1-sv1 and ANTXR1-sv1$_{ARM}$, but not to those expressing ANTXR1-sv1-Y383C, which does not bind actin (Figure 6).

## DISCUSSION

There is mounting evidence that the actin cytoskeleton regulates the ability of ANTXR1-sv1 to bind ligands. Yang and colleagues demonstrated that overexpression of alpha-smooth muscle actin or the actin-binding protein transgelin masks an epitope on the ANTXR1 ectodomain.[23] Their interpretation of these data was that the structure of the ANTXR1 ectodomain is regulated by the cytoskeleton. Our work here using receptor hybrids and the filament disrupting drug latrunculin A suggests that the binding of F-actin, but not G-actin, to ANTXR1 reduces PA binding.

Two possibilities have been proposed for why some receptors do not bind PA.[22−24] The first is that homomeric or heteromeric ANTXR1 ectodomain interactions could sterically occlude PA. A second possibility is that the ectodomain adopts a conformation that does not bind PA. The case for the latter idea is supported by work from Ramey and colleagues, who demonstrated that a mutation in the VWA domain designed to lock it into an open (high-affinity) conformation overrides the negative effect of the cytosolic domain on PA binding.[24] This work suggests that ANTXR1 undergoes a form of regulation similar to integrin inside-out signaling.


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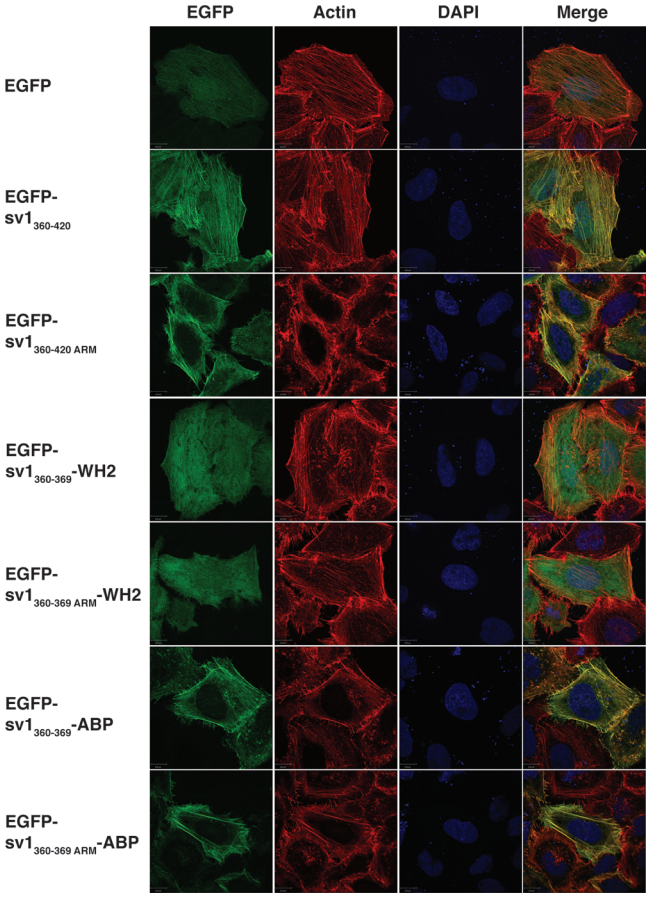


**Figure 4.** Actin-binding regions of ANTXR1-sv1 and abp140 colocalize with actin stress fibers. Confocal micrographs of HeLa cells transfected with EGFP fusion proteins of the isolated actin-binding regions of ANTXR1-sv1, WH2, or abp140 with or without the acidic region mutation (ARM) (green). Cells were stained with AlexaFluor 555 phalloidin (red) to visualize actin and DAPI (blue) to visualize nuclei. Right panels show merged images with colocalization displayed in yellow. Images are representative of three independent experiments.

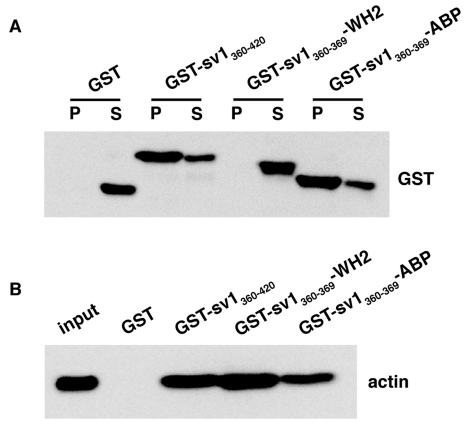


**Figure 5.** Actin-binding regions of ANTXR1-sv1 and abp140 bind F-actin. (A) GST fusion proteins containing the actin-binding region of ANTXR1-sv1, the WH2 domain of WAVE2, or the actin-binding region of abp140 were incubated with cell lysates and the mixtures were centrifuged to pellet the F-actin cytoskeleton. The pellet and supernatant fractions were subjected to SDS-PAGE and Western blot analysis with an $\alpha$-GST antibody. (B) GST fusion proteins were bound to glutathione sepharose and incubated with purified monomeric $\beta$-actin. Bound proteins were eluted and subjected to SDS-PAGE and Western blot analysis to detect monomeric actin. Blots are representative of three independent experiments.

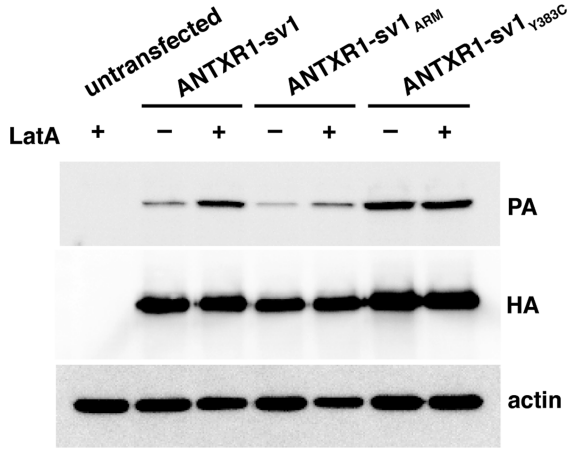


**Figure 6.** Binding of actin filaments to ANTXR1-sv1 diminishes PA-binding. CHOR1.1 cells stably expressing ANTXR1-sv1 or ANTXR1-sv1-Y383C or ANTXR1-sv1$_{ARM}$ were treated with DMSO vehicle or with the actin filament disrupting agent latrunculin A for 2 h. Furin-resistant PA was incubated with cells for 2 h, and the amount of PA that bound cells was assessed by Western blotting. Receptor surface expression was assessed using a biotinylation assay. Blots are representative of three independent experiments.

To understand the nature of the signal that prevents binding of ANTXR1 to PA, we replaced the region of ANTXR1 that binds actin with two unrelated actin-binding motifs. The WH2 domain bound G-actin in vitro, but it did not label actin stress fibers in cells or fractionate with the cytoskeleton in vitro. This could be because it has a higher affinity for G-actin than for F-actin. In contrast, the actin-binding motif from abp140 bound F-actin. That the actin-binding motif from abp140, but not the WH2 domain, decreased PA binding to ANTXR1 suggested that F-actin was responsible for regulation of the receptor. This notion was supported by an experiment demonstrating that latrunculin A increased the amount of PA bound to cells expressing ANTXR1-sv1. Incubation of ANTXR1-sv1 expressing cells with this drug did not, however, increase the level of bound PA to the level observed with ANTXR1-sv1-Y383C expressing cells, which might be because latrunculin A treatment did not lead to the disruption of all of the filamentous actin. We note that filament binding proteins have been shown to stabilize filaments,[27] which could mean that filaments bound to ANTXR1 were long-lived and did not depolymerize during the latrunculin A treatment. This might explain why we previously failed to observe an effect of latrunculin A on the amount of PA bound to cells.[22]

The ability of F-actin, but not G-actin, to regulate ANTXR1 function suggests that force generated by the filaments could be important for this process. Tensional force generated by the cytoskeleton has been shown to induce a conformational change in the integrin adaptor, talin, which exposes a binding site for vinculin during the formation of focal adhesions.[28] It remains to be determined whether mechanotransduction plays a role in the regulation of ANTXR1.

Adjacent to the actin-binding region of ANTXR1 is a conserved segment of acidic amino acids. Mutation of these amino acids increased the association of ANTXR1 with the F-


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actin cytoskeleton and increased binding of a cytosolic tail peptide to G-actin in vitro. The inhibitory effect of the acidic amino acids on actin binding appears to be context-specific because the mutations had no effect on the WH2 and ABP receptor hybrids. Although these acidic amino acids decrease the affinity of ANTXR1 for actin, their primary function might instead be related to a distinct process.

## ■ ASSOCIATED CONTENT

### Supporting Information

Statistical analysis of colocalization of actin with EGFP fusion proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*E-mail: jeremy.mogridge@utoronto.ca; Ph: 416-946-8095; Fax: 416-978-5959.



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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

ANTXR1, anthrax toxin receptor 1; ARM, acidic region mutant; PA, protective antigen; sv1, splice variant 1.

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