LRP5 and LRP6 Are Not Required for Protective Antigen–Mediated Internalization or Lethality of Anthrax Lethal Toxin

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Introduction

Anthrax toxin (AnTx) plays a key role in the pathogenesis of anthrax. AnTx is composed of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). PA is not toxic but serves to bind cells and translocate the toxic edema factor or LF moieties to the cytosol. Recently, the low-density lipoprotein receptor–related protein LRP6 has been reported to mediate internalization and lethality of AnTx. Based on its similarity to LRP6, we hypothesized that LRP5 may also play a role in cellular uptake of AnTx. We assayed PA-dependent uptake of anthrax LF or a cytotoxic LF fusion protein (FP59) in cells and mice harboring targeted deletions of Lrp5 or Lrp6. Unexpectedly, we observed that uptake was unaltered in the presence or absence of either Lrp5 or Lrp6 expression. Moreover, we observed efficient PA-mediated uptake into anthrax toxin receptor (ANTXR)–deficient Chinese hamster ovary cells (PR230) that had been stably engineered to express either human ANTXR1 or human ANTXR2 in the presence or absence of siRNA specific for LRP5 or LRP6. Our results demonstrate that neither LRP5 nor LRP6 is necessary for PA-mediated internalization or lethality of anthrax lethal toxin.

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Abbreviations: AnTx, anthrax toxin; ANTXR, anthrax toxin receptor; bp, base pair; CHO, Chinese hamster ovary; EF, edema factor; EST, expressed sequence tag; LDL, low-density lipoprotein; LeTx, lethal toxin; LF, lethal factor; MEF, murine embryonic fibroblast; MVD, mitogen-activated protein kinase extracellular signal-regulated kinase kinase; OPPG, osteoporosis pseudoglioma syndrome; PA, protective antigen; RT-PCR, reverse-transcription PCR; siRNA, small interfering RNA

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Author Summary

The effects of many pathogenic bacteria are caused by the toxins they release. The toxin released by bacteria that cause anthrax is particularly fascinating since it is made of three different proteins: edema factor, lethal factor, and protective antigen (PA). On their own, each of these proteins is harmless, but when combined, they are deadly. This is because edema factor and lethal factor can exert their poisonous effects only after they have been moved into cells by PA. Determining exactly how PA does this is seen as a critical step in developing medicines that will fight anthrax. That is why a recent report suggesting that LRP6, an outer cell protein, was needed for PA to move the other toxin proteins into cells, was greeted with such interest. However, we now show that mice or cells lacking LRP6, or a related protein called LRP5, are still susceptible to anthrax toxin. The discovery that PA can move lethal factor and edema factor into cells without the help of LRP6 presents a significant challenge to the previously published model. These findings will help focus the efforts of scientists working on new ways to treat anthrax.

LRP5/6 Role in PA-Mediated Uptake of Anthrax Toxin

Neither Lrp5 nor Lrp6 Is Essential for PA-Mediated Uptake of FP59 or LF In Vitro

Wei et al. [22] observed that antisense expression of an EST corresponding to an intronic sequence between exons 21 and 22 of the Lrp6 gene could 1) reduce expression of Lrp6, and 2) protect M2182 prostate carcinoma cells from PA-mediated uptake of FP59, a cytotoxic fusion protein consisting of the N-terminus of LF genetically fused with the ADP-ribosylating domain of Pseudomonas exotoxin A [43]. These observations formed the basis for their conclusion that LRP6 was essential for PA-dependent uptake into cells. To test whether Lrp5 was essential for PA-mediated internalization, we isolated murine embryonic fibroblasts (MEFs) from Lrp5+/− parental, Lrp5+/− heterozygous, and Lrp5−/− nullizygous mice, and treated them with PA plus FP59, Lrp5+/− parental, Lrp5+/− heterozygous, and Lrp5−/− nullizygous MEFs demonstrated equal sensitivity to treatment with a constant amount of PA plus varying concentrations of FP59 (Figure 2A), or with a varying amount of PA plus constant concentrations of FP59 (Figure 2B). These results indicate that loss of Lrp5 expression alters neither MEF sensitivity to FP59 nor the ability of PA to translocate FP59 into cells. As an independent measure of PA-mediated entry, MEFs were treated with PA plus LF, and lysis was immunoblotted for N-terminal proteolysis of mitogen activated protein kinase/extracellular signal-regulated kinase (MEK) 1. Lrp5+/− parental, Lrp5+/− heterozygous, and Lrp5−/− nullizygous MEFs demonstrated equal cleavage of MEK1 following treatment with PA plus LF (Figure 2C). These observations demonstrate that the loss of expression of LRP5 is not sufficient to prevent PA-mediated uptake of FP59 or LF. Similar tests were performed with MEFs from Lrp6+/− parental, Lrp6+/− heterozygous, and Lrp6−/− nullizygous mice. Each of these MEFs demonstrated equal sensitivity to treatment with combinations of PA plus FP59 (Figure 2A and 2B). Again, as an independent measure of PA-mediated entry, MEFs were treated with PA plus LF, and lysis was immunoblotted for N-terminal proteolysis of MEK1. Lrp6+/− parental, Lrp6+/− heterozygous, and Lrp6−/− nullizygous MEFs demonstrated equal MEK1 cleavage following treatment with PA plus LF (Figure 2C). These observations indicate that the loss of expression of Lrp6 is not sufficient to prevent PA-mediated uptake of FP59 or LF. Collectively, these results demonstrate that neither Lrp5 nor Lrp6 is essential for PA-mediated uptake of FP59 or LF.

Neither Lrp5 nor Lrp6 Functions in a Receptor-Specific Fashion

The results discussed above indicate that PA can mediate entry of toxin into cells in the absence of either Lrp5 or Lrp6 and are at odds with a recent study in which an essential role for LRP6 in internalization and lethality of AnTx was reported [22]. In that study, the authors showed that LRP6-specific small interfering RNA (siRNA) and polyclonal antibodies raised against peptides corresponding to the extracellular domain of LRP6 could protect M2182 human prostate carcinoma cells and RAW264.7 murine macrophages from experiment since they do not survive to birth [28]. Regardless of the status of Lrp5 or Lrp6 expression, mice injected with LeTx died within 6 d of the start of treatment (Figure 1). These results indicate that neither loss of Lrp5 expression nor heterozygous expression of Lrp6 impairs LeTx lethality.

Results

Neither Loss of Lrp5 Expression nor Heterozygous Expression of Lrp6 Impairs LeTx Lethality In Vivo

Wei et al. [22] reported that a polyclonal antibody raised against LRP6 could protect cells from killing by LeTx. Based on this result, the authors suggested that the immunological targeting of LRP6 may prove useful in protecting against the effects of accumulated toxin during the late stages of anthrax disease when antibacterial methods normally are no longer of therapeutic value. To test this hypothesis, we challenged mice having targeted deletions of Lrp5 [42] or Lrp6 [28] with daily intravenous injections of anthrax LeTx (50 ug of PA and 10 ug of LF). Previous work in our lab with athymic nude mice on a BALB/c background has shown that this dose of LeTx is sufficient to cause hypotensive shock leading to death within 6 d (unpublished data). Lrp6−/− mice were not used in this
indeed differentially express the previous study and the MEF cells used in this study do have been previously obtained for undetectable levels of loss of internalization via LRP6 may work preferentially through M2182 expressed ANTXR and Lrp6.

Based on these results, we speculated that PA-mediated internalization via LRP5 or LRP6 does not work preferentially through either ANTXR1 or ANTXR2. Accordingly, we predicted that loss of Lrp5 or Lrp6 expression would disrupt PA-mediated uptake via one ANTXR but not the other. To test this hypothesis, we assayed the effects of siRNA inhibition of Lrp5 and Lrp6 expression upon PA-dependent uptake of FP59 in ANTXR-deficient PR230–Chinese hamster ovary (CHO) cells that were engineered to stably express either human ANTXR1 (T-CHO) or human ANTXR2 (C-CHO). Using RT-PCR, we confirmed previous observations [45,46] that these cell lines express neither ANTXR1 and ANTXR2, ANTXR1, nor ANTXR2, respectively (Figure 3A). By immunoblotting for the NH2-terminus of MEK1, we also confirmed that T-CHO and C-CHO, but not PR230-CHO, were capable of internalizing LF in a PA-dependent fashion (unpublished data; Figure 4B). To knock down Lrp expression in these cells, we tested three siRNA for Lrp5 and three siRNA for Lrp6. Although these siRNAs were designed to inhibit mouse mRNA, we expect that hamster mRNA sequence obtained for regions of Lrp5 and Lrp6 that we used to design our PCR primers were 91%–95% identical to those published for mice (unpublished data). Using real-time PCR, we established that Lrp5-siRNA2 and Lrp5-siRNA3 were most effective in reducing Lrp5 expression, and Lrp6-siRNA1 was most effective for reducing Lrp6 expression (Figure 3B). Notably, the Lrp6-siRNA1 caused a similar level of mRNA inhibition as the siRNA used by Wei et al. [22]. Immunoblots with antibodies specific for Lrp5 showed that its levels were reduced in response to either siRNA-2 or siRNA-3, though the levels of protein did not strictly correlate with the level of mRNA expression (Figure 3C). Antibodies against Lrp6 did not work well in immunoblots (unpublished data), so we indirectly assayed Lrp6 protein expression by assaying the ability of siRNA-treated cells to bind the Lrp6 ligand DKK-1. Whereas treatment of PR230-CHO cells with Lrp6 siRNA-1 caused a clear reduction in DKK-1 binding, treatment with control siRNA did not cause any discernable loss of DKK-1 binding (Figure 3D). These results indicate that siRNA directed against either Lrp5 or Lrp6 can selectively reduce targeted mRNA and protein expression levels.

Regardless of the level of knockdown achieved, none of the siRNA had a demonstrable effect on PA-mediated uptake of FP59 (Figure 4A). In addition, none of the siRNA had any noticeable effect upon PA-mediated uptake of LF, as judged by immunoblotting for NH2-terminal epitopes of MEK1 (Figure 4B). These data indicate that PA-mediated internalization via either LRP5 or LRP6 does not work preferentially through either ANTXR1 or ANTXR2.

Finally, we examined the possibility that Lrp5 or Lrp6 are functionally redundant and that PA-mediated internalization requires expression of either Lrp5 or Lrp6. MEFs isolated from Lrp6 knockout mice were treated with Lrp5-specific siRNA and assayed for sensitivity to PA plus FP59 or LF. Using real-time PCR, we established that Lrp5-siRNA1, Lrp5-siRNA2, and Lrp5-siRNA3 were similarly effective in reducing Lrp5 expression by approximately 66%–80% (Figure 5A). Despite this, reduced expression of Lrp5 in this Lrp6 null background had no effect upon sensitivity of MEFs to PA plus FP59 (Figure 5B) or upon LF cleavage of MEK1 (Figure 5C). Similar results were obtained when we treated Lrp5 null MEFs with siRNA specific for Lrp6, or when we treated T-CHO cells with a combination of Lrp5-siRNA2 and Lrp6-siRNA1 (unpublished data). These results indicate that PA-mediated internalization by MEFs or CHO cells proceeds independently of the expression of Lrp5 and Lrp6.

**Discussion**

Of the three AnTx proteins, PA is the central receptor-binding component, or B moiety, that delivers the catalytic effector molecules, LF or EF, to the cytosol [21]. Before it can do this, PA must bind a cell surface receptor. Cell surface
receptors for PA (ANTXRs) have been identified [15,16]. The first receptor identified (ANTXR1) was a splice variant (sv2) of TEM8, a protein of unknown function that is up-regulated in colorectal cancer endothelium [47]. Young and colleagues now report that of three TEM8 splice variants tested, two (sv1 and sv2) function as an ANTXR, while the third (sv3) does not [16]. sv1 and sv2 differ only in the length of their cytoplasmic tails, but analysis of ANTXR1 deletion mutants indicates that this tail is dispensable for receptor function [45]. More recently, Scobie et al. [16] identified a second receptor (ANTXR2) encoded by capillary morphogenesis gene 2 (CMG2). As this name suggests, ANTXR2 is a protein which is up-regulated during endothelial cell morphogenesis [48]. Both ANTXR1 and ANTXR2 are ubiquitously expressed, making it likely that the participation of each is relevant to the pathology of anthrax.

Wei et al. [22] made a well-reasoned argument that LRP6 is essential for PA-dependent internalization of AnTx. Based on their initial findings, we further tested the requirement for not only Lrp6, but also Lrp5, in PA-dependent uptake of FP59 or LF. However, our results have directed us to the opinion that neither Lrp5 nor Lrp6 is essential for PA-dependent uptake into cells. This position is supported by three independent and compelling lines of evidence. First, mice with targeted deletions of Lrp5 or Lrp6 are as sensitive as wild-type mice to intravenous injections of anthrax LeTx. Second, wild-type MEFs and MEFs lacking expression of Lrp5 or Lrp6 are equally capable of internalizing either FP59 or LF in a PA-dependent fashion. Finally, knockdown of Lrp5 or Lrp6 with siRNA neither alters the sensitivity of CHO cells expressing ANTXR1 or ANTXR2 to FP59 nor prevents NH2-terminal proteolysis of MEK1 by LF. Thus, the available evidence does not support the hypothesis that either LRP5 or LRP6 plays an essential role in PA-mediated uptake.

We propose several alternative explanations for the discrepancy between our results and those of Wei et al. [22].

First, LRP6 may function in a species-specific fashion. Though Wei et al. [22] presented data from both human M2182 prostate carcinoma cells and mouse RAW264.7 macrophages, the protection conferred by siRNA in the latter was only modest (an approximately 2- to 3-fold increase in the IC50) when compared to that in the former (>100-fold increase in the IC50). Perhaps RAW264.7 cells, as well as MEF and CHO cells, express a splice variant of Lrp5 or Lrp6 that is resistant to siRNA treatment? This seems unlikely given the highly conserved nature of LRP6; LRP6 and Lrp6 share 97% protein sequence identity. Moreover, though the Ensembl browser (version 41, October 2006; http://www.

**Figure 2.** In Vitro Treatment of Embryonic Fibroblasts from LRP5- and LRP6-Deficient Mice with PA Plus FP59 or LF

(A) To assess the effects of LRP5 or LRP6 deficiency upon LeTx-sensitivity in vitro, embryonic fibroblasts from LRP5 and LRP6 parental (+/+) and heterozygous (ko/+) and nullizygous (ko/ko) mice were treated with PA (1 ug/ml) and FP59 (20 pg/ml–2 ug/ml). Cell viability (ordinate) at the end of 24 h of incubation is plotted versus the concentration of FP59 (abcissa). Error bars indicate standard deviation between three replicates in a single experiment.

(B) Alternatively, embryonic fibroblasts from the same mice were treated with FP59 (1 ng/ml) and PA (1 pg/ml–10 ug/ml). Cell viability (ordinate) at the end of 24 h of incubation is plotted versus the concentration of PA (abcissa). Error bars indicate standard deviation between three replicates in a single experiment. This plot is representative of three independent experiments.

(C) As an independent indicator of PA entry into embryonic fibroblasts derived from mice with targeted deletions of LRP5 (upper panel) and LRP6 (lower panel), cleavage of MEK1 was assessed by immunoblotting with antibodies that are specific for the NH2-terminus of MEK1. UT, untreated.

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ensembl.org) does predict a novel splice variant of human, but not mouse Lrp5 containing an additional exon (2 base pair [bp]) between exons 13 and 14, splice variants of Lrp6 have not been characterized or predicted based on human or mouse genomic sequence. Alternatively, perhaps human ANTXRs are uniquely dependent upon LRP5 function. However, the CHO cells we used in this study expressed only human ANTXR1 or ANTXR2. Since these cells were equally sensitive to AnTx in the presence or absence of siRNA specific for Lrp5 or Lrp6, this indicates that 1) human ANTXR can bind and internalize AnTx in the absence of (human) LRP5 or LRP6, and 2) human ANTXRs do not have a general requirement for (murine) Lrp5 or Lrp6 in their function.

Second, LRP6 may function in a cell-specific manner. We cannot exclude this possibility. However, Lrp6 function is compromised even in heterozygous null mice since heterozygous expression genetically enhances a Wnt mutant phenotype [28]. Therefore, we argue that if LRP6 does function in a cell-specific manner, it is unlikely that sensitive cell types will play a significant role in the pathology of anthrax LeTx since heterozygous knockout mice are as sensitive to LeTx as their wild-type counterparts are. Thus, targeting of LRP6 will not likely prove useful in protecting

Figure 3. ANTXR Expression and siRNA Knockdown of LRP5 and LRP6
Reverse-transcription PCR was used to assess ANTXR expression in (A) M2182 prostate carcinoma, RAW264.7 macrophage, MEFs, and ANTXR-deficient PR230-CHO cells that were engineered to stably express either ANTXR1 (T-CHO) or ANTXR2 (C-CHO). PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is included as an mRNA control.
(B) The efficacy with which LRP5- and LRP6-specific siRNA knocked down target gene expression was measured by real-time PCR. Results are presented as an average of three independent samples, each of which was run in duplicate. The error bars indicate the standard deviation about the mean.
(C) The effect of Lrp5-specific siRNA and control siRNA (siRNAc) upon protein levels was assessed by immunoblotting with an antibody specific to Lrp5. Levels of α-tubulin are shown as a control for protein levels. Un., untreated.
(D) The effect of Lrp6-specific siRNA and control siRNA upon Lrp6 protein levels was assessed by DKK-1 binding assays. Nomarski and fluorescence (FITC) images of cells are shown.

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against the effects of accumulated toxin during the late stages of anthrax disease when antibacterial methods normally are no longer of therapeutic value.

Third, LRP6 may function in a receptor-specific fashion. In support of this, our PCR analysis of the MEFs used in our study and the M2182 and RAW264.7 cells used by Wei et al. [22] indicate these cell types differentially express ANTXR1 and ANTXR2. Despite this, knockdown of either Lrp5 or Lrp6 by siRNA failed to protect CHO cells expressing one or the other receptor from PA-mediated entry of FP59 or LF. These results are inconsistent with the hypothesis that LRP5 or LRP6 functions in a receptor-specific fashion.

Fourth, it is possible that LRP5 and LRP6 are functionally redundant with regard to PA-mediated uptake. However, we observed that mouse and hamster cells deficient for both Lrp5 and Lrp6 expression are as sensitive to PA-mediated uptake as are control cells expressing both Lrp5 and Lrp6. Further, since real-time PCR analysis of CHO cells (Figure 3) and M2182 cells (unpublished data) indicates that both cell types express Lsp5 and Lsp6, the insensitivity of M2182 cells to PA-mediated toxicity following Lsp6 knockdown is not likely explained by a deficiency in Lsp5 expression. Finally, though the organization of the extracellular domains of LRP5 and LRP6 are similar to each other, they are markedly different from that of other LDL receptors [49]. So while we cannot exclude the possibility that LRP6 is functionally redundant with another LDL receptor, this possibility is remote.

Finally, the antisense screen used by Wei et al. [22] identified a human EST (image clone 285207) corresponding to an intronic region between exons 21 and 22 of Lrp6. Although the clone harboring this EST showed decreased levels of Lrp6 protein, the lack of a direct relationship between the EST and its apparent target Lrp6 mRNA raises concerns regarding its specificity. Indeed, Wei and colleagues also reported that the same EST matches a sequence of the non-coding strand of an intron of a Bcl-2-like gene (Bcl2L14).

In contrast, for the Lrp5 and Lrp6 knockout mice used in this study, we can be reasonably assured that only Lrp5 or Lrp6 are targeted for inactivation, based on the original Southern blotting of mouse embryonic stem cell (ES) clones. Moreover, we may be reasonably assured that functional Lrp5 or Lrp6 is not expressed in these mice or cells derived from these mice.

Figure 4. The Effect of LRP5 or LRP6 Knockdown upon PA-Dependent Uptake of FP59 and Anthrax LF

(A) The effect of Lrp5 or Lrp6 knockdown on cell sensitivity to PA plus FP59 (1 ng/ml) was assessed in ANTXR-deficient PR230-CHO cells that were engineered to stably express either ANTXR1 (T-CHO) or ANTXR2 (C-CHO) using toxicity assays as described in Materials and Methods. Cell viability (ordinate) at the end of 24 h of incubation is plotted versus the concentration of PA (abscissa). Representative data from one of three experiments is presented. Error bars indicate standard deviation between quadruplicate samples, which were run pairwise on two separate plates.

(B) As an independent indicator of PA-mediated entry into the siRNA-treated CHO cells, cleavage of MEK1 following treatment with medium alone (UT), LF alone, or lethal toxin (LT) was assessed by immunoblotting (IB) with antibodies that are specific for the NH2-terminus of MEK1. Only representative data for negative control siRNA (med GC and lo GC siRNA), Lrp5 siRNA2, and Lrp6 siRNA1 are shown, though identical results were obtained for Lrp5 siRNA 1 and 3 as well as Lrp6 siRNA 2 and 3. Immunoblots with an antibody against the carboxy-terminus of MEK1 (MEK1-COOH) are shown as a control for loading and protein degradation.
null mice, a large insertion containing the splice acceptor of the mouse engrailed 2 gene, the transmembrane domain of rat CD4, and the β-galactosidase-neo reporter was inserted between exons 5 and 6. This insertion is therefore expected to generate a fusion construct encoding the first 457 of 1,370 extracellular domain amino acids of Lrp6, corresponding to the first YWTD β-propeller domain and an epidermal growth factor–like repeat of the extracellular domain. Significantly, this protein lacks the intracellular domain that Wei et al. [22] determined was essential for Lrp6 function in AnTx uptake.

In conclusion, using three independent approaches, we have failed to find evidence to support the hypothesis that either LRP5 or LRP6 plays an essential role in PA-mediated uptake. The more likely explanations for the discrepancies between these reports is that either the EST identified by Wei et al. [22] is not specific for LRP6, or the role of LRP6 in PA-mediated uptake is cell-type specific. However, our results should not be interpreted as an indication that an association of ANTXR with PA is sufficient for cellular internalization. Indirect evidence suggests that other membrane-associated proteins may also play a role in AnTx uptake. By chemically cross-linking associated surface proteins, Escuyer and Collier [50] estimated the molecular weight of PA complexed with its receptor at approximately 170 kDa. Since the molecular weights of activated PA, ANTXR1 sv1, ANTXR1 sv2, and ANTXR2 are 63 kDa, 63 kDa, 41 kDa, and 43 kDa, respectively, this indicates that either PA does not bind ANTXR in a 1:1 ratio, or other as yet unidentified proteins are present in this complex.

Materials and Methods

Lrp5- and Lrp6-deficient mice. Lrp5-deficient mice were generated as described in [42]. Lrp6-deficient mice (a gift of W. Skarnes) have been described previously [28]. The knockout mice in this report are maintained on a C57BL background. The genotypes of all mice used in this study were confirmed by PCR analysis of genomic DNA. All experiments were performed in compliance with the guiding principles of the Guide for the Care and Use of Animals by the National Academy of Sciences Institute of Laboratory Animal Resources Commission on Life Sciences. In addition, all procedures were approved before use by the Institutional Animal Care and Use Committee of the Van Andel Research Institute.

Protein expression and purification. PA and LF were expressed in Bacillus anthracis (BH445) and purified essentially as described by Park and Leppla [51]. The concentration of each protein was estimated using the bicinchoninic acid method [52] and by densitometric analyses of Coomassie Blue-stained polyacrylamide gels.

In vivo toxicity assays. To assess the requirement for Lrp5 and Lrp6 in response to LeTx in vivo, 6- to 20-wk-old (male) wild-type, heterozygous, and knockout Lrp5 and Lrp6 mice were injected daily via the tail vein with 50 µl of Hank’s buffered salt solution containing PA (50 µg) and LF (10 µg). The animals were weighed daily and monitored for signs of stress or discomfort.

Cell culture and reagents. J774A.1 cells (obtained from the American Type Culture Collection, http://www.atcc.org) as well as MEFs obtained from wild-type, heterozygous, and knockout Lrp5 and Lrp6 mice were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. M2182 cells were cultured in RPMI1640 supplemented as described previously [53]. All cell lines were maintained at 37 °C in a humidified 5% CO2 incubator. A spontaneous ANTXR-deficient CHO cell mutant (PR290-CHO) as well as PR290-CHO stably transfected with human ANTXR1 (F-CHO) or human ANTXR2 (C-CHO) expression vectors [45] were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cytotoxicity assays. Cells were grown in 96-well plates to 70% confluence. Cells were treated with culture medium containing PA plus F59 at the concentrations indicated and incubated 20 h at 37 °C. At the end of the experiment, cell viability was determined using

Figure 5. The Effect of Lrp5 Knockdown upon PA-Dependent Uptake of FP59 and Anthrax LF in Lrp6 KO MEFs

(A) The efficacy with which LRP5-specific siRNA knocked down target gene expression was measured by qPCR. Results are presented as an average of two independent samples, each of which was run in duplicate. The error bars indicate the standard deviation.

(B) The effect of Lrp5 knockdown on cell sensitivity to PA plus FP59 (1 ng/ml) was assessed in Lrp6 KO MEFs using toxicity assays as described in Materials and Methods. Cell viability (ordinate) at the end of 24 h of incubation is plotted versus the concentration of PA (abcissa). Error bars indicate standard deviation between two independent experiments, each of which was run in quadruplicate.

To generate Lrp5 null mice, part of exon 1, including the signal peptide ATG, was replaced with an IRES-β-galactosidase-dase reporter and MC1-neomycin phosphotransferase selection cassette. Thus, despite the fact that Lrp5−/− mice are viable, functional LRP5 cannot be expressed. To generate Lrp6 null mice, a large insertion containing the splice

\[ \text{LRP5/6 Role in PA-Mediated Uptake of Anthrax Toxin} \]
containing 5 µl of total volume containing 2.5 µl of 10× PCR buffer (Invitrogen, http://www.invitrogen.com), 2.0 µl of DMSO, 1.0 µl of 50 mM MgCl₂, 0.625 mM of each nucleotide, 1 U of Taq polymerase (Invitrogen), a 0.25-jg/ml concentration of primers, LRP5 F or Neo F1, and a 1.25-jg/ml concentration of primer LRP3 5'-targeted (common primer) to detect a 140-bp fragment of the wild-type allele and/or a 1,000-bp fragment of the mutant allele. Samples were amplified for 34 cycles (94 °C for 1 min, 57.8 °C for 1 min, and 72 °C for 1 min). PCR of the wild-type LRP6 allele was carried out as above except with no DMSO and using the LRP6 7757-S primer and the LRP6 8085-AS primer (to detect a 325-bp fragment). Samples were amplified for 30 cycles (94 °C for 45 s, 56.5 °C for 45 s, and 72 °C for 1 min). PCR of the mutant LRP6 allele was carried out as above in 20 µl total volume and no DMSO using the LRP6 7757-S primer and the pGT1.9TM-1388 AS primer (to detect a 586-bp fragment) that was used to amplify 30 cycles (94 °C for 45 s, 56.5 °C for 45 s, and 72 °C for 1 min). PCR products were visualized by ethidium bromide staining in 1.0% agarose gels.

PCR analysis of ANTXR and Lrp 5/6 expression. RNA was isolated using the TRIzol (Invitrogen) method. Briefly, cells were lysed in 10 ml TRIzol and RNA was extracted with chloroform using a 15-ml phase-ethidium bromide staining in 1.0% agarose gels. The identity of the PCR products was confirmed by DNA sequencing. The PCR products were confirmed by DNA sequencing.

Supporting Information

Table S1. List and Sequences of Primers Used in This Study

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