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Requirements for Anthrax Toxin Entry into Cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

Patricia Lynn Ryan

Committee in charge:

Professor John A.T. Young, Chair
Professor Raffi Aroian
Professor Steve Hedrick
Professor Marc Montminy
Professor Victor Nizet
Professor Kit Pogliano

2010

The dissertation of Patricia Lynn Ryan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

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Chapter 3 contains a reprint of a paper as it appears in PLoSOne. Ryan, P.L., Young, J.A. *Evidence against a human cell specific role for LRP6 in anthrax toxin entry.* PLoS One, 2008. 3(3): p. 1817

VITA

2010	Doctor of Philosophy, University of California, San Diego
2003-2010	Graduate Student and Teaching Assistant Division of Biology, University of California, San Diego
2002-2003	Junior Scientist, University of Minnesota – Twin Cities Laboratory Medicine and Pathology
2002	Bachelor of Science, Zoology The University of Wisconsin – Madison

PUBLICATIONS

Ryan, P.L., Young, J.A. *A role for the recycling pathway in anthrax toxin receptor cell surface expression*. (manuscript in preparation)

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ABSTRACT OF THE DISSERTATION

Requirements for Anthrax Toxin Entry into Cells

by

Patricia Lynn Ryan

Doctor of Philosophy in Biology

University of California, San Diego 2010

John A.T. Young, Chair

Bacillus anthracis secretes a harmful exotoxin called anthrax toxin. Anthrax toxin has deleterious effects on several host cell types and is a significant contributor to anthrax pathogenesis. Toxin-deleted strains of *B. anthracis* are highly attenuated and many of the symptoms of anthrax can be replicated with anthrax toxin alone. Anthrax toxin is an AB-type toxin with two catalytic A moieties. Protective antigen (PA), the B moiety, is responsible for receptor binding, pore formation and translocation of the catalytic moieties, edema factor (EF), an adenylate cyclase, and lethal factor (LF), a zinc metalloprotease that cleaves map kinase kinases. Anthrax toxin binds one of two identified cellular receptors, ANTXR1 or ANTXR2. Previously, it was

assumed that pore formation and toxin translocation were both low-pH dependent and that the requirements for toxin entry mediated by either receptor were the same. Here, I report that while entry mediated by ANTXR2 does require low-pH, when bound to ANTXR1 toxin can form pores in host membranes and translocate under near-neutral pH conditions. Additionally, I report that LRP6, a putative anthrax toxin co-receptor, is not absolutely required for toxin entry into cells. Finally, using a targeted siRNA screen, I identify other cellular factors involved in anthrax toxin entry. Studies characterizing these factors provide evidence in favor of a role for the recycling pathway in anthrax toxin receptor cell surface expression.

Chapter 1.

Introduction

1.1 Brief History of Anthrax

Bacillus anthracis is a Gram-positive, rod-shaped, spore-forming bacterium that causes the disease anthrax. Anthrax is found globally in temperate zones and is one of the oldest documented diseases with recordings of severe outbreaks dating back thousands of years (Bhatnagar and Batra, 2001; Dragon and Rennie, 1995; Glassman, 1958). Typically considered a zoonotic disease, humans can contract anthrax after exposure to contaminated animals or animal products. Thus, human contact with livestock has made *B. anthracis* a pathogen of great historical importance despite no case of human-to-human transmission ever being reported. Considered by some to be the sixth biblical plague of Egypt, anthrax also made appearances in Roman classical literature such as Virgil's third *Georgic* (Ben-Noun, 2003; Dirckx, 1981, 2000a, b). Given its long history and high mortality rates, anthrax has been called many different names throughout recorded time: malignant pustule, Siberian ulcer, malignant edema, black bane, Bradford disease, woolsorter's disease and ragpicker's disease (Bhatnagar and Batra, 2001). The term "anthrax" was coined by the ancient Greek physician, Hippocrates, and is derived from the Greek word for coal, *anthrakis*, which refers to the black lesions that develop in the cutaneous form of the disease (Kyriacou et al., 2006).

Anthrax also holds a significant place in the history of science for its central role in the development of modern germ theory. In 1876, Robert Koch established that isolated anthrax spores caused disease in experimentally infected animals, work that was used to develop Koch's postulates, the criteria required to establish a causal relationship between a microbial organism and disease (Bhatnagar and Batra, 2001; Carter, 1988). Soon after this discovery, in 1881, Louis Pasteur developed the first live attenuated bacterial vaccine by using artificially weakened anthrax bacteria as an inoculum in farm animals (Bhatnagar and Batra, 2001; Carter, 1988).

With the introduction of livestock vaccines and greater regulation and control of animal products, prevalence of anthrax fell and it was briefly considered a minor health concern despite continuing outbreaks in less developed parts of the world (Kyriacou et al., 2006; Schwartz, 2009). Upon induction of government bioweapons programs during the middle of the 20th century, however, anthrax once again became a threat to public health, renewing interest in basic and biomedical anthrax research. Its significance as a biological weapon was underlined when approximately 1 milligram of aerosolized *B. anthracis* spores, the weaponized form of anthrax, was mistakenly released from a Soviet military installation in 1979 sickening hundreds of people and resulting in 80 deaths (Meselson et al., 1994). The Centers for Disease Control classifies *B. anthracis* as one of six Category A

bioterrorism agents, those that pose the greatest threat to national security and public health. In the U.S. anthrax attacks of fall 2001, anthrax spores were disseminated through the mail sickening 22 people and resulting in five deaths (McCarthy, 2001). The spore clean-up costs alone were estimated to be at least 120 million dollars, though it is likely the total economic damage from this event was much greater (Webb, 2003).

1.2 Anthrax Pathogenesis

B. anthracis exists in two forms: vegetative and spore. The spore form is dormant and is usually the infectious agent. Spores are generated by vegetative bacteria during a multi-stage process called sporulation, whereby the bacillus undergoes asymmetric cell division and the larger “mother cell” compartment engulfs the smaller forespore compartment. This forespore compartment then develops into a mature spore after several steps that include the synthesis of a protective protein coat and the production of an outer shell called the exosporium (Donovan et al., 1987; Steichen et al., 2003; Sylvestre et al., 2002). Sporulation is initiated in response to nutrient deprivation and is considered a bacterial survival mechanism. Anthrax spores can persist in the environment for decades and are resistant to many adverse conditions, including temperature and pH extremes, desiccation, and ultraviolet and ionizing radiation (Dragon and Rennie, 1995; Gould, 1977).

Anthrax spores can enter the host via three routes: cutaneous, gastrointestinal, and pulmonary (Fig 1.1). Regardless of host entry route, local macrophages or dendritic cells engulf anthrax spores and it is within these cells that germination into vegetative bacilli occurs (Brittingham et al., 2005; Guidi-Rontani et al., 1999; Ross., 1957). These vegetative bacilli produce two critical virulence factors, an anti-phagocytic poly-D-glutamic acid capsule and a harmful exotoxin, termed anthrax toxin.

Cutaneous anthrax is the most common naturally occurring form of the disease. The mortality rate of cutaneous anthrax in humans is reported to be 20%, however, intervention with antibiotics makes death due to cutaneous anthrax unlikely (Inglesby et al., 1999). Of the 11 cases of cutaneous anthrax contracted during the 2001 anthrax attacks, 100% of the victims survived (Kyriacou et al., 2006; McCarthy, 2001).

Gastrointestinal anthrax occurs after ingestion of either spores or bacteria in improperly cooked meat (Kyriacou et al., 2006). Gastrointestinal anthrax is more severe than cutaneous anthrax with estimated mortality rates of up to 60%. This form is comparatively rare and accounts for only about 1% of all human cases of anthrax (Babamahmoodi et al., 2006; Beatty et al., 2003; Kyriacou et al., 2006).

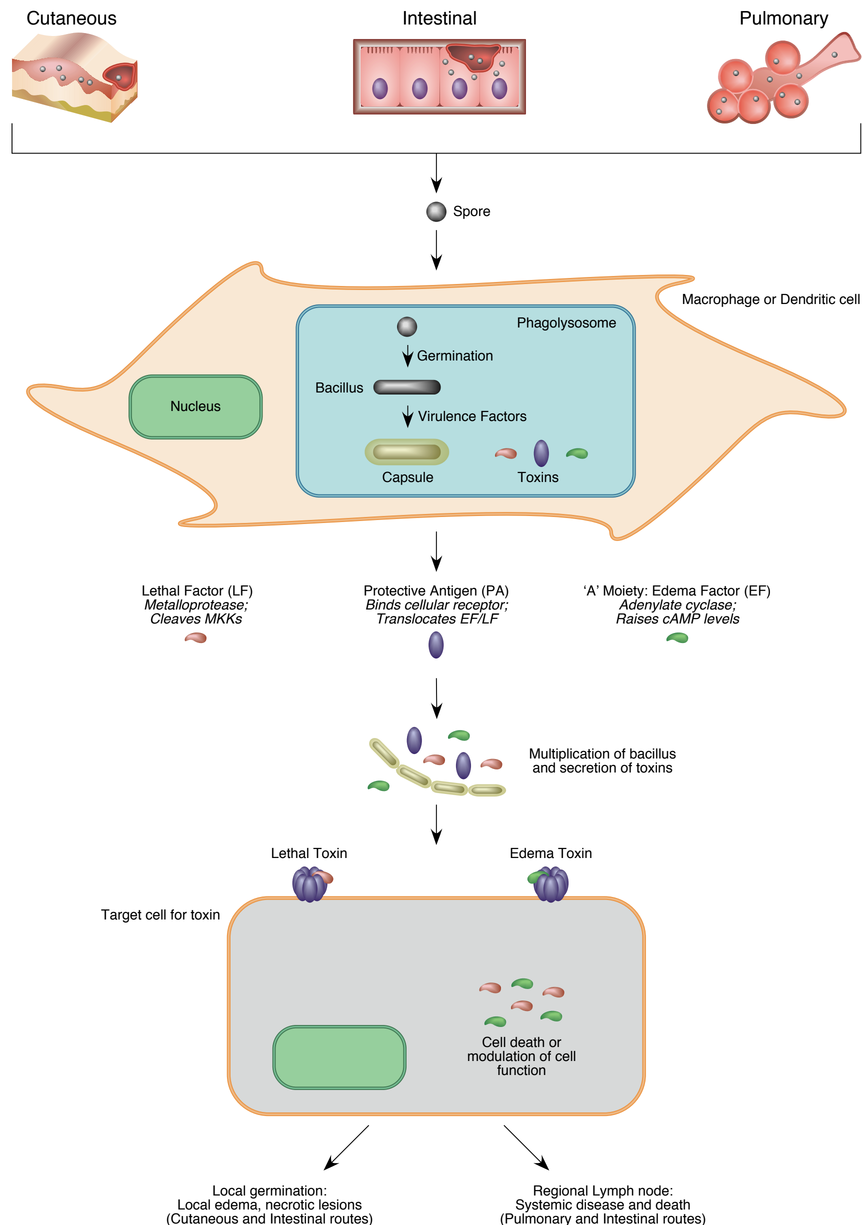

Figure 1.1. *Bacillus anthracis* pathogenesis. The anthrax spore enters the host through three routes: the skin, the intestine and the lung. Spores are engulfed by local macrophages or dendritic cells where they germinate into vegetative bacilli. The vegetative bacillus produces several virulence factors, including an anti-phagocytic capsule and a harmful exotoxin called anthrax toxin. Anthrax toxin has deleterious effects on many host cell types. The infection can remain localized as is common in cutaneous anthrax or, in the case of inhalational anthrax, the infection often becomes systemic. In inhalational anthrax, the spore is taken up by alveolar macrophages and trafficked to regional lymph nodes. The bacillus escapes the macrophage and diffuses into the bloodstream where it replicates freely due in part to its anti-phagocytic capsule. Toxin deleted strains of *B. anthracis* are highly attenuated.

Pulmonary/inhalational anthrax is the most deadly form of the disease, likely because the infection quickly becomes systemic. In inhalational anthrax, spores are taken up by pulmonary macrophages and carried to regional lymph nodes (Ross., 1957). The vegetative bacteria escape the macrophage and diffuse into the bloodstream where they replicate freely due in part to their anti-phagocytic capsule (Makino et al., 1989). Systemic pulmonary anthrax mortality rates approach 100% if left untreated (Shafazand et al., 1999). All 11 patients that contracted inhalational anthrax during the anthrax attacks of 2001 required hospitalization and medical intervention despite which five patients died (Guarner et al., 2003). Systemic anthrax results in high titers of bacteria in the blood and high levels of anthrax toxin that persist after antibiotic treatment (Smith et al., 1954). In small animal models, many symptoms of systemic anthrax can be replicated by anthrax toxin alone, indicating its importance as an anthrax virulence factor (Firoved et al., 2005; Moayeri et al., 2003).

1.3 Anthrax Toxin

1.3.1 Toxin overview

Anthrax toxin was discovered in the 1950s when scientists observed that filtered serum from anthrax-infected guinea pigs was lethal when injected into small animal models (Smith and Keppie, 1954; Smith et al., 1954, 1955). Anthrax toxin is composed of three separate proteins, protective antigen (**PA**;

83 kDa), edema factor (**EF**; 89 kDa) and lethal factor (**LF**; 90 kDa). Though each protein is secreted individually, they self assemble in the bloodstream of host animals or on the surface of receptor-expressing cells to form ternary complexes. PA is responsible for receptor binding, cell entry, and translocation of the catalytic moieties, LF and EF. LF is a zinc metalloprotease that cleaves MAP kinase kinases (MEKs) and EF is an adenylate cyclase that raises cAMP levels (Chopra et al., 2003; Duesbery and Vande Woude, 1999; Leppla, 1982; Vitale et al., 2000; Vitale et al., 1998). In research studies the components are often separated to form two different binary toxins -- lethal toxin (**LF+PA, LT**) and edema toxin (**EF+PA, ET**) -- in order to measure the effects of LF and EF individually. Both LT and ET can be lethal in small animal models and have deleterious effects on several cell types, ranging from disruption of cellular functions to cytotoxicity. *In vivo* and *in vitro* studies suggest that toxins are produced at a ratio of 20:5:1 of PA:LF:EF (Molin et al., 2008; Sirard et al., 1994). Studies addressing the importance of anthrax toxin as a virulence factor have shown that strains lacking functional anthrax toxin are somewhere between 1,000 and 10,000 times less virulent than wild-type strains (Brossier et al., 2000; Pezard et al., 1991).

1.3.2 Protective Antigen

PA is so named for its immunizing properties against anthrax in small animal models (Auerbach and Wright, 1955). PA oligomerizes to form pores

in host cell membranes capable of translocating LF and EF from extracellular to intracellular compartments. Following proteolytic activation by a furin-like protease into a 63-kD subunit, the PA_{63} monomer oligomerizes into either a heptamer or octamer forming a structure called the prepore (Ezzell and Abshire, 1992; Kintzer et al., 2009; Klimpel et al., 1992). Oligomerization produces up to 3 or 4 binding sites for LF and/or EF on the heptamer or octamer, respectively (Kintzer et al., 2009; Lacy et al., 2002; Mogridge et al., 2002a; Mogridge et al., 2002b). In order for the receptor-bound prepore to convert to the SDS-resistant pore species capable of translocating EF and LF, it must encounter acidic pH found in the endosomes of cells (Friedlander, 1986). Agents that raise the intraendosomal pH have been observed to hinder pore formation and translocation of the toxin catalytic moieties (Milne et al., 1994). The effects of pH on pore formation and toxin translocation will be discussed in more detail in Chapter 2.

PA consists of four discrete domains (Fig. 1.2) (Petosa et al., 1997). Domain 1 is the site of furin cleavage and also constitutes the site where LF and EF bind the oligomeric form of PA. Mutational and structural analysis demonstrated that Domain 2 is involved in both pore formation and receptor binding (Benson et al., 1998; Lacy et al., 2004; Qa'dan et al., 2005; Santelli et al., 2004). This domain contains the 2β2-2β3 loop that inserts into cellular membranes to form the pore. Each PA monomer contributes 2β strands and

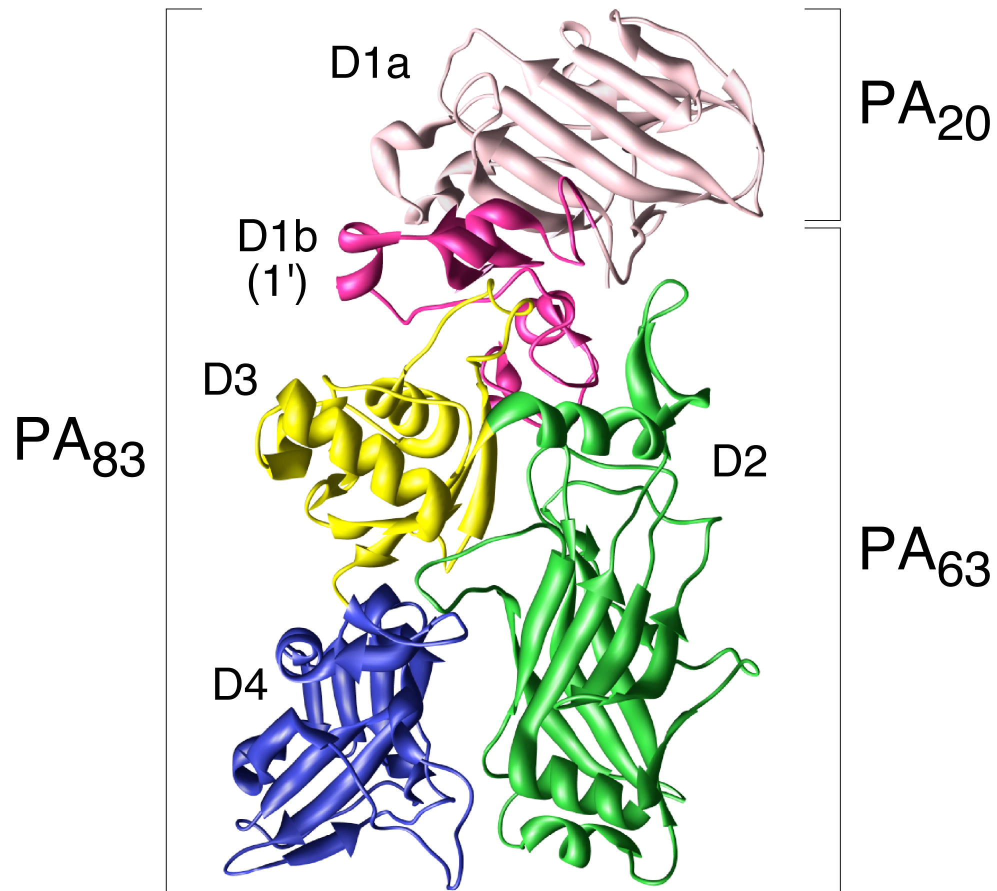

Figure 1.2. Protective antigen (PA) crystal structure. Structural domains of the 83 kD form of PA (PA_{83}). Domain 1 (D1) contains cleavage site for proteolytic activation. After cleavage, PA_{20} (D1a, pink) dissociates from the receptor-bound monomer, and PA_{63} oligomerizes into a either a heptamer or an octamer, also termed the prepore. The binding site for lethal factor (LF) or edema factor (EF) is revealed after proteolytic cleavage and oligomerization, and is on the top of D1' (or D1b, magenta). D2 (green) is involved in receptor binding and pore formation. D3 (yellow) participates in oligomerization. D4 (blue) binds the cellular receptor. Modeled with UCSF chimera program (PDB# IT6B).

one β-loop to form a 14 or 16 β-strand transmembrane β-barrel (heptamer and octamer respectively) through which LF and EF pass. Domain 3 of PA is involved in oligomerization (Mogridge et al., 2001). Domain 4, along with Domain 2, is involved in receptor binding (Lacy et al., 2004; Santelli et al., 2004; Singh et al., 1991).

Since the lumen of the heptameric PA pore is estimated to be ~15Å, it is predicted that EF and LF must unfold in order to pass through the pore, a process that is thought to be facilitated by low pH (Krantz et al., 2004). Central to translocating properties of the PA pore are the 7 or 8 phenylalanines (F427) that line the lumen of the pore and form a “Phe clamp” that transiently interacts with hydrophobic portions of the unfolding catalytic moieties, serving a chaperone-like function and forming a seal that prevents the passage of ions (Krantz et al., 2005). This clamp is thought to facilitate translocation through the cation selective pore in concert with the pH gradient formed between the acidic *cis* compartment of the endosome and the neutral *trans* compartment where EF and LF are delivered (Krantz et al., 2006). Under this model, negatively charged residues are protonated on the acidic *cis* side and allowed to pass through the cation selective channel. On the neutral *trans* side these residues are deprotonated, which prevents diffusion back through the pore and thus translocation occurs in a single direction.

1.3.3 Lethal Factor

LF was given its name due to its lethal effects in small animal models (Beall et al., 1962). LF is a zinc-dependent metalloprotease that inactivates mitogen activated protein kinase kinases (MEK) 1-4, 6, and 7 by cleavage at their N-termini. This cleavage is thought to disrupt many downstream signaling pathways, such as ERK1/2, JNK/SAPK and p38, which are required for various cellular processes and/or cell survival and proliferation. As a result of its catalytic activity, LT disrupts the host vasculature and is capable of inhibiting innate and adaptive immune responses. Despite its name, LT is not cytotoxic in all cell types. The effects of LT have been studied in many different cells but no one cell type has been directly correlated with animal death.

LT targets several cell types that play a role in innate immunity. Treating murine macrophages with LT causes them to undergo either rapid cell lysis or apoptosis. Primary human alveolar macrophages are reportedly resistant to LT entry (Wu et al., 2009), however, human monocytic cell lines can be sensitized to LT-induced programmed cell death following activation (Kassam et al., 2005). In their unactivated state, LT induces monocyte cell cycle arrest. Additionally, human macrophages resistant to LT-induced cell death can reportedly be sensitized after treatment with TNF-α (Kim et al., 2003). Polymorphonuclear cells are also targets of LT with the toxin blocking

chemotaxis of these cells (During et al., 2005). LT reportedly suppresses the function of dendritic cells, but whether this is due to cytotoxicity or a result of inhibition of dendritic cell functions is still a matter of debate (Agrawal et al., 2003; Alileche et al., 2005; Chou et al., 2008).

In addition to disrupting innate immunity, LT also targets cells responsible for development of adaptive immunity. Studies done *in vivo* and *in vitro* have shown that LT suppresses T cell activation and proliferation (Comer et al., 2005; Fang et al., 2005). In addition to disrupting T cell Antigen Receptor (TCR) signaling, LT interferes with T cell chemotaxis (Rossi Paccani et al., 2007). B cells are also likely targets for LT, as *in vivo* and *in vitro* studies demonstrated decreased antibody production in these cells after LT treatment (Fang et al., 2006).

Many small animal models show vascular lesions in response to spore challenge and LT treatment alone. It has been suggested that this vascular assault might be a significant contributor to lethality (Cui et al., 2004; Moayeri et al., 2003). Clinical studies of human anthrax patients show vascular damage in the form of tissue hemorrhage, gastrointestinal bleeding and damage to large and small blood vessels (Friedlander, 2000). Evidence in favor of LT targeting endothelial cells *in vitro* is plentiful. Human umbilical vein endothelial cells (HUVECS) are reportedly sensitive to LT, with reports on their response to intoxication varying from apoptosis to cell cycle arrest to induction

of endothelial barrier dysfunction (Huang et al., 2008; Kirby, 2004; Warfel et al., 2005). Additionally, *in vivo* and *in vitro* studies demonstrated that LT is capable of inhibiting angiogenesis and affects development of tumor vasculature, suggesting the endothelium might be a relevant target in the host (Alfano et al., 2008; Alfano et al., 2009; Duesbery et al., 2001).

1.3.4 Edema Factor

EF is a calcium and calmodulin dependent adenylate cyclase and was first identified based on its ability to induce localized edema in experimental animals (Smith and Keppie, 1954). ET has been reported to be lethal in mice, but it is likely the lethal dose used in these studies is not achieved during an actual infection (Firoved et al., 2005). The murine model of ET intoxication shows extensive tissue lesions and associated hemorrhage with death likely resulting from multi-organ failure. At the cellular level ET is typically not cytotoxic, with the exception of one study on zebrafish embryos (Voth et al., 2005). Instead it appears ET disrupts important cellular functions through production of the second messenger, cAMP (Leppla, 1982). cAMP induction depends on the level of ET present with maximal induction ranging from 200-4,000 fold above resting levels, depending on the study (Gordon et al., 1989; Leppla, 1982; Puhar et al., 2008). Anthrax bacteria lacking EF are attenuated, indicating that EF plays a role in anthrax virulence (Brossier et al., 2000; Pezard et al., 1991).

ET disrupts various processes of immune system cells. In macrophages, ET inhibits chemotaxis and phagocytosis (Rossi Paccani et al., 2007; Yeager et al., 2009). In dendritic cells, ET inhibits pro-inflammatory cytokine secretion, specifically IL-12p70 and TNF-α, while having no effect on anti-inflammatory IL-10 production (Tournier et al., 2005). In neutrophils, ET disrupts phagocytosis and chemotaxis (O'Brien et al., 1985; Szarowicz et al., 2009). ET also inhibits development of innate immunity by disrupting T-cell activation and proliferation (Paccani et al., 2005). My research indicates ET induces cell cycle arrest in the G_1 phase in the RAW 264.7 murine macrophage cell line, which is commonly used as a model to study anthrax intoxication (Appendix Fig. 1). This is consistent with the effects of cAMP in bone marrow derived macrophages where its elevation causes cell cycle arrest at G_1 (Kurokawa and Kato, 1998).

ET is also thought to play a role in the vascular leakage and edema observed in anthrax patients. ET has paradoxically been shown to increase transendothelial barrier resistance *in vitro*. However, it is thought that ET mediates the release of pro-inflammatory mediators, which indirectly results in vascular permeability on a system-wide level (Tessier et al., 2007). Additionally, ET represses platelet aggregation and clotting, possibly contributing to the hemorrhaging associated with ET in mice (Alam et al., 2006).

1.4 Anthrax Toxin Internalization

Upon binding one of two cellular receptors, ANTXR1 or ANTXR2, PA is cleaved by a furin-like protease to yield PA_{63} and a second fragment, PA_{20}, which diffuses into the surrounding medium (Fig. 1.2 and 1.3) (Beauregard et al., 2000). PA_{63} oligomerizes into either a heptamer or an octamer, forming a ring shaped prepore and generating binding sites for LF and EF at the PA_{63}-PA_{63} dimer interface (Cunningham et al., 2002; Mogridge et al., 2002b). LF and EF bind the oligomer competitively through their homologous amino-terminal regions (Bragg and Robertson, 1989; Lacy et al., 2002). The oligomeric form of PA is also capable of binding to receptors after forming in the bloodstream of the host as a result of PA cleavage by serum proteases (Ezzell and Abshire, 1992; Panchal et al., 2005). Indeed, this may be the most physiologically relevant mechanism since the vast majority of PA present in the blood of an infected animal is in the PA_{63} form (Ezzell and Abshire, 1992). The toxin-receptor complex localizes to lipid rafts and is internalized primarily via clathrin-mediated endocytosis (Abrami et al., 2003; Boll et al., 2004). Acidic endosomal pH triggers PA pore formation and subsequent translocation of LF and EF into the cytosol (Abrami et al., 2004). As discussed in Chapter 2, my work in collaboration with Jonah Rainey (a former postdoctoral fellow in the Young lab) revealed that the pH threshold of pore formation depends on which receptor type the toxin binds. If bound to ANTXR1, pore formation occurs at

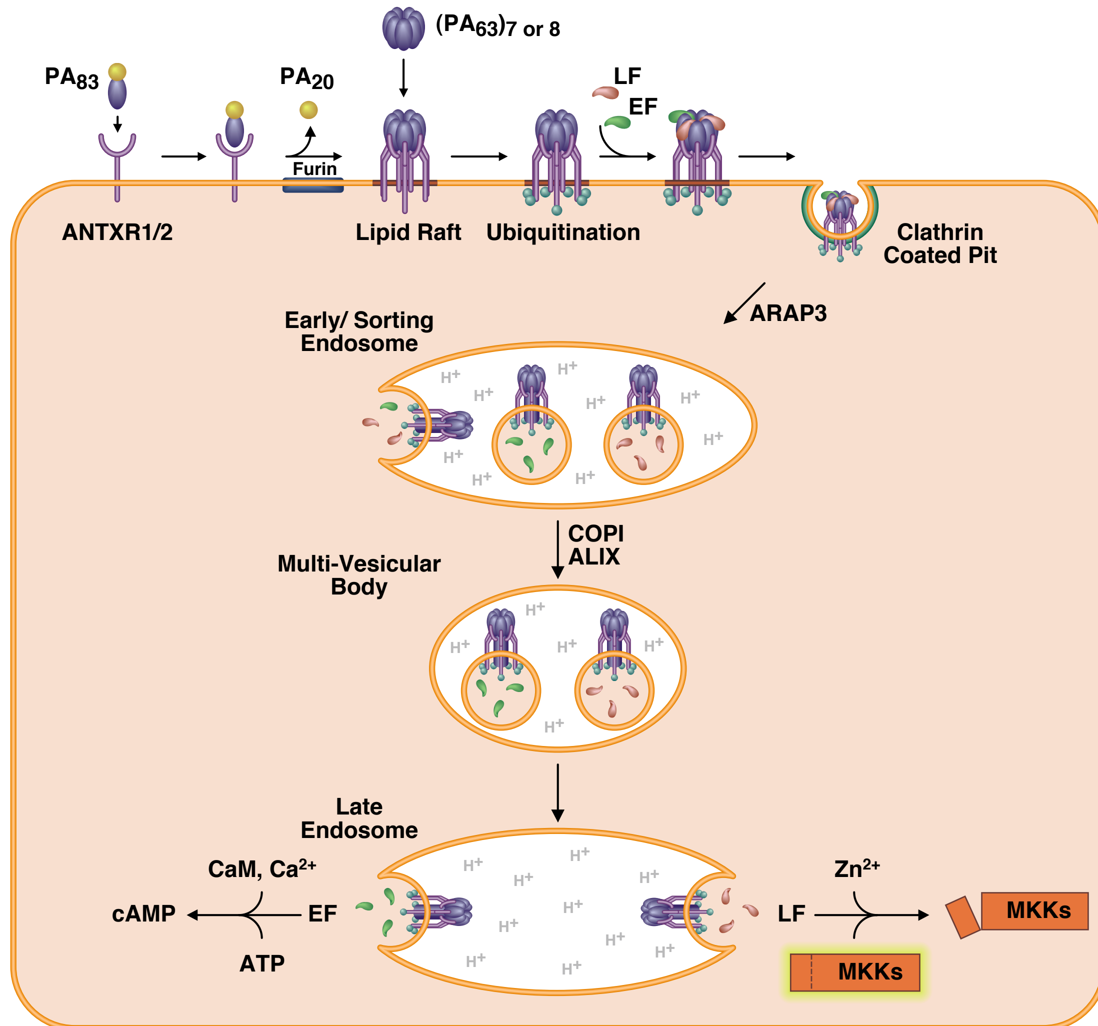

Figure 1.3. Current model of anthrax toxin entry into cells. A full-length 83 kD form of PA (PA_{83}) binds to one of two identified cellular receptors, ANTXR1 or ANTXR2. PA is cleaved by a furin-like protease and the 63 kD form (PA_{63}) remains attached to the receptor. PA_{63} oligomerizes into either a heptamer or an octamer, termed the prepore, producing up to 3 or 4 binding sites for LF and EF. The oligomeric form of PA can also bind to cells after forming in the bloodstream of host animals due to cleavage by serum proteases. The toxin/receptor complex localizes to lipid rafts and the receptor tails are ubiquitinated. The complex is internalized primarily via clathrin-mediated endocytosis in a process that involves cellular protein ARAP3. The acidic pH of the endosome promotes pore formation, which preferentially occurs in the membrane of the intraluminal vesicles within the multivesicular body. EF and LF are translocated into the intraluminal vesicle, the lumen of which is topologically equivalent to the cytosol. The toxin containing vesicles are trafficked to the late endosome and backfusion of the intraluminal vesicle with the limiting endosomal membrane allows for toxin release into the cytosol.

pH 6.0 – equivalent to the pH of early sorting endosomes (Maxfield and McGraw, 2004). If bound exclusively to ANTXR2, pores form around pH 5.2–5.5 which is more characteristic of the pH in late endosomes (Maxfield and McGraw, 2004; Rainey et al., 2005). The nature of the toxin-receptor interactions and receptor-specific pH thresholds for pore formation will be discussed later in the Introduction as well as in Chapter 2.

The current model of anthrax toxin entry suggests that toxin/receptor complex localization to lipid rafts and subsequent internalization is controlled by a series of post-translational modifications of the anthrax toxin receptor cytoplasmic domain. An earlier study using cells that were engineered to express receptor lacking the cytoplasmic tail showed that the tail is not absolutely required for PA binding and toxin entry (Liu and Leppla, 2003). A subsequent study, however, demonstrated that the tail is an important regulator of toxin internalization. The receptor is palmitoylated, which negatively regulates its association with lipid rafts. Upon PA binding, a conformational change occurs which leads to depalmitoylation of the receptor and to proposed receptor association with a hypothetical palmitoylated protein, which in turn leads to lipid raft localization (Abrami et al., 2006). Once localized to the lipid raft the receptor encounters the E3 ubiquitin ligase, Cbl, and ubiquitination of the receptor allows for association with Ub interacting domains such as Eps15, which is involved in the formation of clathrin-coated

pits (Abrami et al., 2006). Additionally, studies using one splice variant of ANTXR1 have shown that the cytoplasmic domain interacts with the actin cytoskeleton and can influence the amount of PA-binding to this receptor (Go et al., 2009).

Interestingly, an electron microscopy study indicated that PA channel formation occurs preferentially in the membrane of intraluminal vesicles within multi-vesicular bodies (MVBs) (Abrami et al., 2004). This is thought to result in EF and LF translocation into the lumen of the intraluminal vesicle, which is then trafficked to late endosomes. It has been further proposed that backfusion of the intraluminal vesicle to the limiting endosomal membrane allows for EF and LF release into the cytosol, as agents that inhibit trafficking to late endosomal compartments also inhibit LF-induced MEK cleavage (Abrami et al., 2004). It is thought that by translocating into the lumen of the intraluminal vesicle, which is topologically equivalent to the cytosol, EF and LF are protected from the hostile, protease-rich environment of the lysosome until they can be delivered into the cytosol. In contrast to this mechanism of entry, diphtheria toxin is internalized via clathrin-mediated endocytosis and trafficked to early endosomes where it preferentially inserts into the limiting endosomal membrane, allowing for the catalytic moiety's release directly into the cytosol (Lemichez et al., 1997).

Nearly all pathogens and bacterial toxins gain access to the cell through one or more cellular entry pathways – phagocytosis, macropinocytosis, or endocytosis (Gruenberg and van der Goot, 2006). Much of the molecular machinery that coordinates MVB-dependent anthrax toxin entry is undefined and, though generally accepted, this entry model requires further validation. Vesicular stomatitis virus (VSV) is the only other type of extracellular cargo known to date that is proposed to enter cells through a pathway similar to that of anthrax toxin (Fig. 1.4). In order to enter cells, VSV-glycoprotein (VSV-G) binds to an unidentified cellular receptor. VSV is then internalized via clathrin-mediated endocytosis and trafficked to early endosomes where viral envelope fusion preferentially occurs with the intraluminal vesicle, resulting in capsid release into the lumen of the intraluminal vesicle. In addition to being required for receptor binding, VSV-G undergoes a pH dependent conformational change that promotes membrane fusion (Blumenthal et al., 1987). The capsid is transported to the late endosome and backfusion of the intraluminal vesicle membrane with the limiting endosomal membrane allows for entry into the cytosol (Fig. 1.4) (Le Blanc et al., 2005). It is possible that other enveloped viruses in addition to VSV follow this route of entry and understanding this shared pathway in detail could have broad implications in understanding and combating microbial

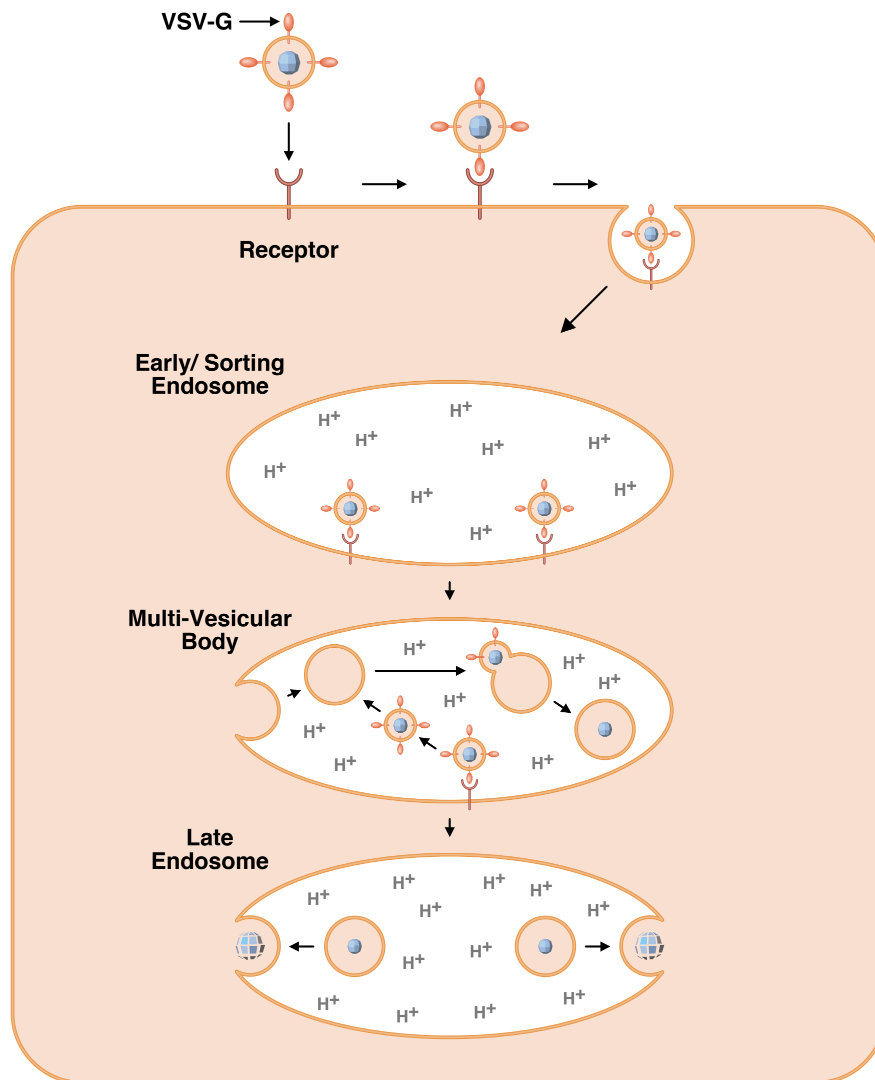

Figure 1.4. Current model of Vesicular Stomatitis Virus-glycoprotein (VSV-G) mediated entry into cells. VSV-G binds an unidentified cellular receptor and the virus/receptor complex is taken into the cell by clathrin mediated endocytosis. Acidic pH of the endosome induces a conformational change in VSV-G that promotes fusion of the viral envelope with the intraluminal vesicle within the multivesicular body, resulting in capsid release into the intraluminal vesicle. Intraluminal vesicles are trafficked to the late endosome, where backfusion of the intraluminal vesicle with the limiting endosome membrane allows for capsid release into the cytosol.

diseases. A study involving candidate factors thought to play a role in VSV-G mediated viral entry and anthrax toxin entry will be outlined in Chapter 4.

1.5 Anthrax Toxin Receptors

1.5.1 Receptor Overview

There are two identified anthrax toxin receptors, ANTXR1 and ANTXR2. ANTXR1 was identified as an anthrax toxin receptor by another former graduate student in our laboratory, Ken Bradley, using a genetic complementation screen (Bradley et al., 2001). ANTXR2 was also identified as an anthrax toxin receptor by a former Young lab graduate student, Heather Scobie, based on its similarity to ANTXR1 (Scobie et al., 2003). Receptor expression in human and mouse tissue samples is nearly ubiquitous as measured by mRNA levels (Su et al., 2004). At least one type of anthrax toxin receptor is expressed in most cultured cells studied to date. Analysis of receptor cell-surface expression has demonstrated a wide range among different cell types, varying between 1,200-240,000 receptors per cell (Abi-Habib et al., 2005). Recent studies have shown ANTXR2 is the primary toxin receptor mediating toxin-induced lethality *in vivo* as ANTXR2 KO mice are resistant to LT challenge, whereas ANTXR1 KO mice are still susceptible (Liu et al., 2009). Additionally, a form of PA engineered to bind specifically to ANTXR2 is capable of mediating lethality in rats (Scobie et al., 2006). The

contribution of each receptor to pathogenesis in a spore challenge model has not yet been addressed.

The receptors' natural functions appear to be associated with cell adhesion and their natural ligands are extracellular matrix proteins. ANTXR1 binds to collagen type I and type VI and ANTXR2 binds to collagen IV and laminin (Bell et al., 2001; Nanda et al., 2004; Werner et al., 2006). Evidence suggests that both ANTXR1 and ANTXR2 play a positive role in angiogenesis (Hotchkiss et al., 2005; Reeves et al., 2009).

Each receptor contains a signal peptide, an extracellular von Willebrand factor A (VWA) domain, a single pass transmembrane domain and a cytosolic tail. Although much is known about toxin/receptor complex internalization, few studies have addressed the requirements for receptor expression at the cell surface or the nature of receptor turnover and internalization in the absence of toxin. It is thought that receptor palmitoylation is not essential for cell-surface expression but does affect the half-life of ANTXR1. Preliminary evidence indicates that palmitoylation defective ANTXR1 mutants are prematurely targeted to lysosomes, reducing the half-life from > 5 hours to ~2 hours (Abrami et al., 2006). Evidence in favor of a role for the recycling pathway in anthrax toxin receptor expression will be presented in Chapter 4.

1.5.2 ANTXR1 and ANTXR2

ANTXR1 was first identified in a screen for mRNA transcripts that were upregulated in tumor endothelium and was thus originally given the name Tumor Endothelial Marker 8 (TEM8) (Fig. 1.5A) (St Croix et al., 2000). *ANTXR1* is expressed in the form of three splice variants: sv1, sv2 and sv3 (Bradley et al., 2001; Liu and Leppla, 2003). The sv1 and sv2 forms both act as anthrax toxin receptors, and differ only in their cytoplasmic domains — the cytoplasmic tail of ANTXR1 sv2 is truncated compared to that of ANTXR1 sv1 (Fig 1.5B).

ANTXR2 was first identified in a screen for genes involved in human capillary tube formation and originally named capillary morphogenesis factor 2 (CMG2) (Fig 5A) (Bell et al., 2001). *ANTXR2* is expressed as three splice variants: 488, 489, and 386 (Scobie et al., 2003). The 488 and 489 isoforms both function as anthrax toxin receptors and are identical except for the last 12 amino acids (Scobie et al., 2003). ANTXR1 sv1 shares 40% identity with $ANTXR2^{488}$ and $ANTXR2^{489}$ in their cytoplasmic regions, and ANTXR1 and ANTXR2 share 60% identity within their PA binding regions (Fig 1.5B).

1.5.3 PA-receptor interaction and pH threshold of pore formation.

The PA-binding regions of ANTXR1 and ANTXR2 are related to the von Willebrand Factor type A (VWA) domains and integrin inserted (I) domains (Bradley et al., 2001; Scobie et al., 2003). One key difference between ANTXR1 and ANTXR2 is the respective affinities of their PA binding domains

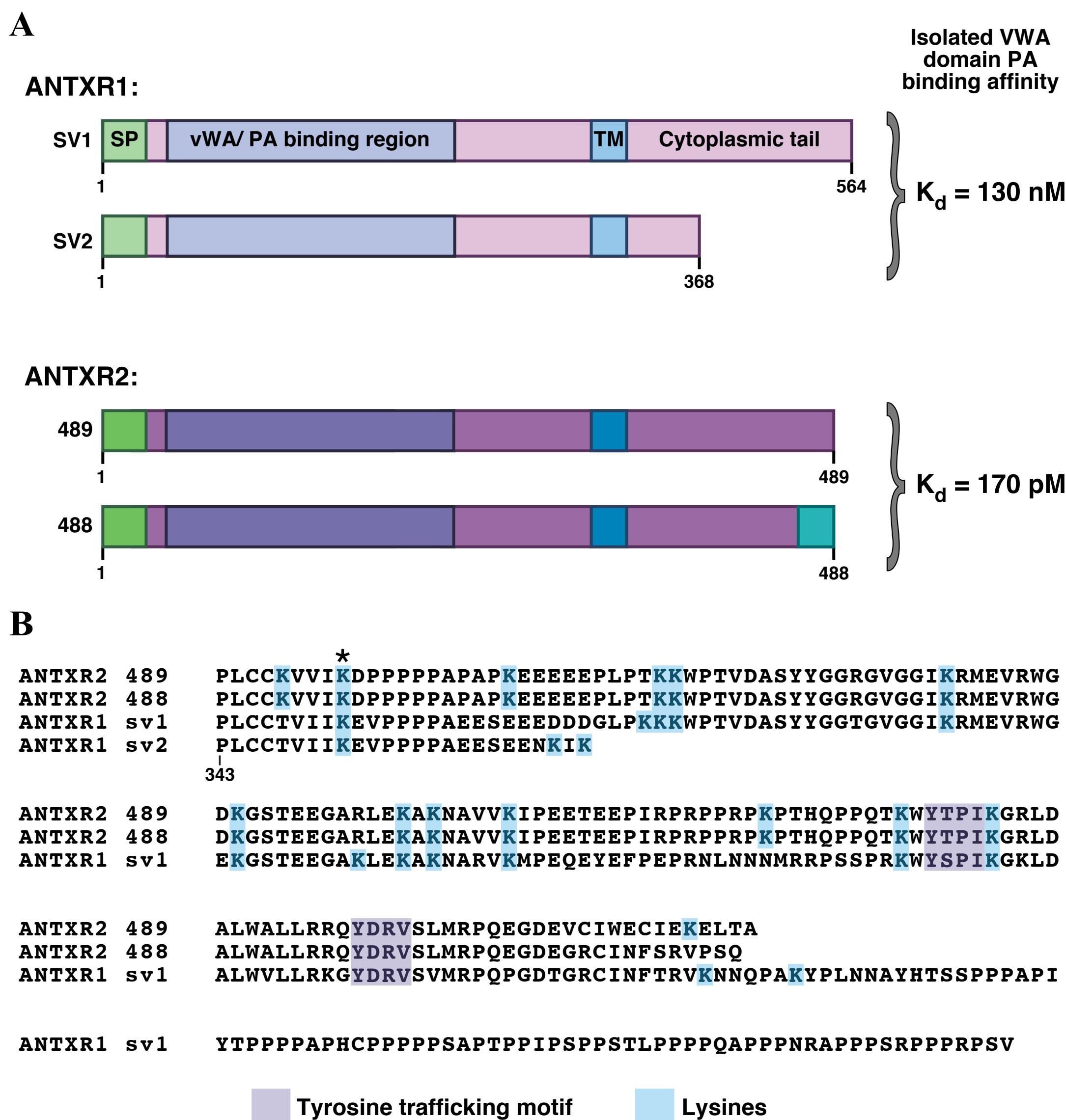

Figure 1.5. Anthrax toxin receptors ANTXR1 and ANTXR2. (**A**) ANTXR1 and ANTXR2 each have two splice variants that function as anthrax toxin receptors. ANTXR1 sv1 and ANTXR1 sv2 are identical except for their cytoplasmic tails, where sv2 is truncated. ANTXR2 488 and 489 splice variants are identical except for their last 12 amino acids. The PA binding affinity of the isolated VWA domain of ANTXR2 is almost 1,000-fold higher than that of ANTXR1. (**B**) The receptor cytoplasmic tails of ANTXR1 sv1 and ANTXR2 488 and 489 share 40% identity. These tails also share common tyrosine based trafficking motifs (purple). There are several conserved lysines (blue), including L351,(*) which is known to be ubiquitinated.

for PA. Despite their sequence similarities, the isolated VWA domain of ANTXR2 has a much higher affinity for PA (K_d = 170 pm) than does that of ANTXR1 (K_d = 130 nM) (Fig. 5A) (Scobie et al., 2007). As noted previously, the two receptors display a striking difference with regard to the pH threshold of pore formation. In the absence of receptor, pores form at neutral pH. However, if bound to ANTXR1 pore formation occurs at pH 6.0 – equivalent to the pH of early sorting endosomes. If bound exclusively to ANTXR2, pores form around pH 5.2–5.5 which is more characteristic of the pH in late endosomes (Rainey et al., 2005). These differences are attributed primarily to receptor interaction with domain 2 of PA (Scobie et al., 2007). Mutational analysis showed that residues G153 and L154 of ANTXR2, which are located on the β4-α4 loop and make contact with PA domain 2, are the major determinants of the lower pH threshold of pore formation when PA is bound to this receptor (Fig 1.6). Mutating these residues to those found on ANTXR1 converts the pH threshold of pore formation to that of ANTXR1. Further residues on ANTXR2 act as determinants for the low pH threshold of pore formation. They are: the additional β4-α4 loop residues 152-157, Q88, Y119, E117, H121, E122 and Y158 (Fig 1.6) (Scobie et al., 2006). In solution and in the absence of receptor, PA forms pores at neutral pH (Miller et al., 1999). It has been proposed that the receptor acts as a molecular clamp

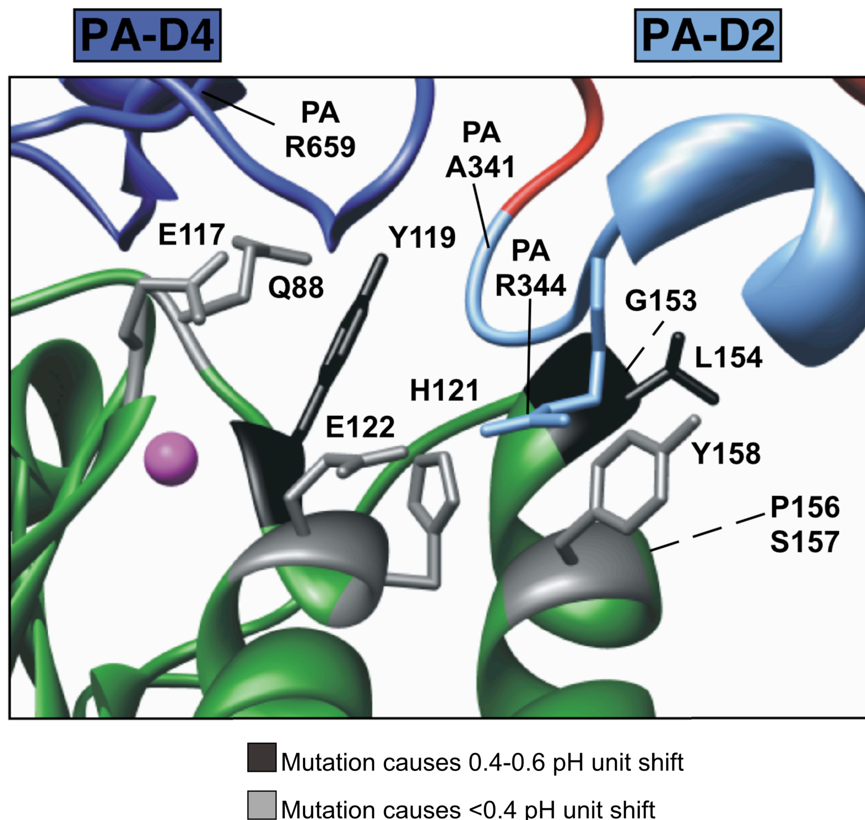

Figure 1.6. Receptor residues involved in molecular clamp function of ANTXR2. Structural diagram of portions of PA domain 4 (PA-D4, dark blue) and PA domain 2 (PA-D2, light blue) with receptor residues involved in molecular clamp function of ANTXR2. PA domain 2 insertion loop is depicted in red. Receptor residues are colored black if mutation of the residue caused a 0.4-0.6 pH unit change in the pH required for pore formation. Receptor residues are colored gray if mutation caused less than a 0.4 unit shift in pH required for pore formation. Residues G153 and L154, which make contact with PA domain 2 and are not conserved on ANTXR1, are the main determinants of the low pH threshold of pore formation associated with toxin bound to ANTXR2. When these residues are mutated to those found on ANTXR1, the pH threshold of pore formation of toxin bound to ANTXR2 is converted to that of ANTXR1 (Scobie et al. 2007). Reprinted with permission from PLoS One.

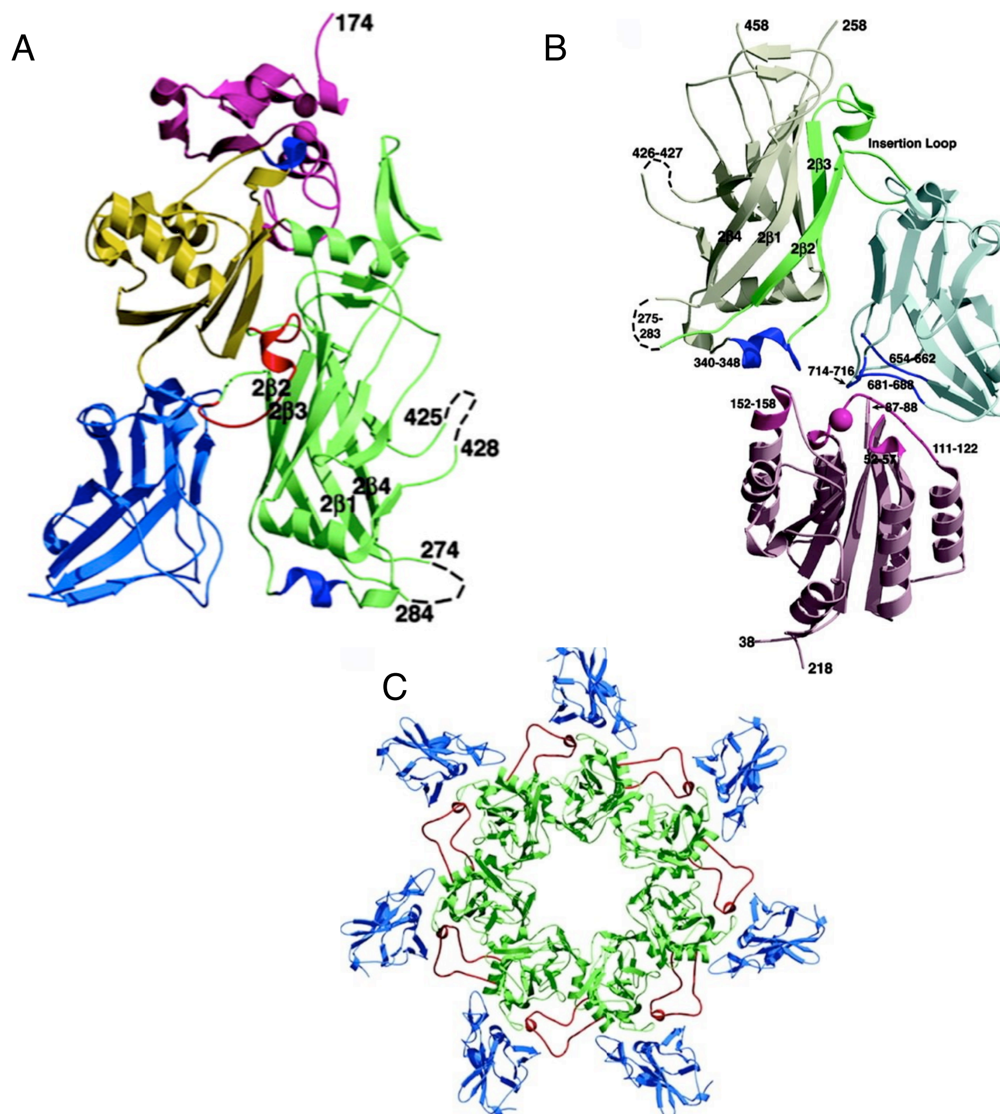

Figure 1.7 Heptameric prepore and receptor - PA interactions. (A) Crystal structure of PA_{63} monomer. Domain 1 is pink, domain 2 is green, domain 3 is yellow and domain 4 is blue. The domain 2 membrane insertion loop is pictured in red. (B) Model of the ANTXR2 VWA domain bound to domain 2 and domain 4 of PA. The ANTXR2 VWA domain (pink) binds both PA domain 2 (white, green, and dark blue) and PA domain 4 (light and dark blue). Direct contacts within the interface are depicted in dark pink (ANTXR2) and dark blue (PA). The PA insertion loop and the 2β2 and 2β3 β-strands (green) are predicted to peel away from the domain 2 β-barrel core to form a pore. Contacts between PA residues 340-348 and the ANTXR2 VWA domain are thought to impede this rearrangement (Lacy et al 2004). (**C**) 3.6-Å heptameric prepore structure as viewed from the bottom. Domains are colored as in (A). The domain 2 insertion loop (red) projects out from the monomer and binds the neighboring monomer in a groove between domains 2 and 4 (Lacy et al. 2004). Reprinted with permission from PNAS.

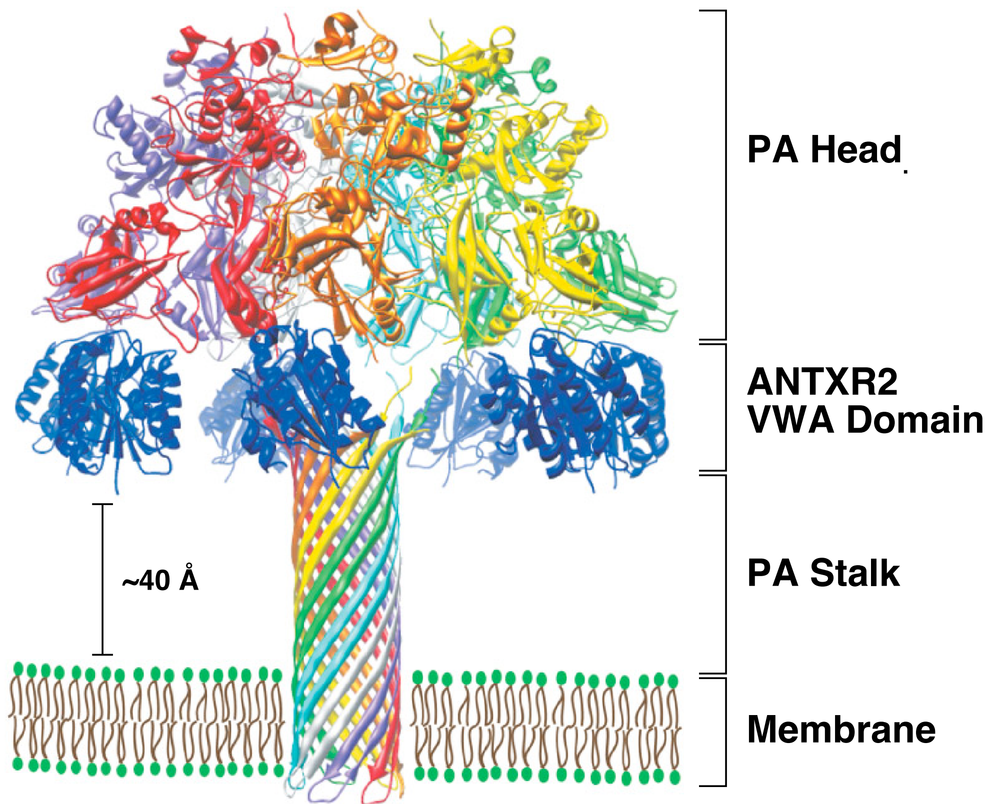

Figure 1.8 Receptor - PA Interactions. Hypothetical PA pore structure based on the prepore crystal structure and the structure of the Staphylococcal alpha-hemolysin pore. Each monomer of PA is shown in a different color and ANTXR2 VWA domain monomers are shown in blue. Each monomer of PA contributes one β-loop from domain 2 to form the β-barrell pore that inserts into the membrane. The barrel is predicted to extend ~50Å above the membrane and it has been suggested the receptor might remain attached to the pore to provide structural support (Santelli et al. 2004). Reprinted with permission from Nature.

that would restrict pore formation until the toxin encounters an acidic intraendosomal environment.

According to the co-crystal structure of the PA heptamer bound to the ANTXR2 I-domain, the receptor likely acts as an impediment to the major conformational rearrangements required for the 2β2-2β3 loop of domain 2 to insert into the membrane during the prepore-to-pore conversion (Fig 1.7). This domain 2 insertion loop packs in between domain 2 and domain 4 of the neighboring monomer (Fig 1.7C), and receptor interaction with domain 2 likely impedes the rearrangement required of this domain in order to form the pore. It is thought that in order for these structural changes to occur, there must be a weakening or complete release of all receptor contacts, at least temporarily (Lacy et al., 2004; Santelli et al., 2004). It has also been proposed that the receptor might remain attached to the pore in order to provide structural support for the stalk region (Fig 1.8) (Nguyen, 2004; Santelli et al., 2004). Evidence consistent with a weakening of receptor-PA interactions coincident with pore formation will be presented in Chapter 2.

1.6 Other host factors involved in anthrax toxin intoxication

In addition to the anthrax toxin receptors, several other host factors involved in anthrax intoxication have been identified. Many of these include proteins involved in toxin uptake and endocytic trafficking. ATP6V0C is a subunit of the vacuolar ATPase/ H+ pump involved in endosome acidification

and was identified as playing a role in LT-mediated cell death in a retroviral insertional mutagenesis screen (Sung O. Kim, 2007). These results are consistent with studies demonstrating that an inhibitor of the vacuolar ATPase/ H+ pump, bafilomycin, protects cells from LT-induced cytotoxicity (Menard et al., 1996). ARAP3 (Arf Gap and Rho GAP with ankyrin repeat and PH domains) is a factor whose absence reduces PA/receptor internalization (Lu et al., 2004) (Fig 1.3). Though the role ARAP3 plays in toxin internalization is unclear, it has been shown to be involved in cell attachment and interacts with CIN85, an adaptor protein that also interacts with Cbl, which, as noted previously, is involved in receptor ubiquitination and toxin/receptor complex internalization (Haglund et al., 2002; Kowanetz et al., 2004). Vesicle-associated proteins such as COP and ALIX are also important for generation of MVBs and thus play a role in trafficking toxin to late endocytic compartments (Abrami et al., 2004; Abrami et al., 2003).

The most controversial host factor is the putative anthrax toxin co-receptor LRP6. LRP6 is a type I transmembrane protein that act as co-receptor for the frizzled receptor in Wnt signaling (Brown et al., 1998; Tamai et al., 2000). It was initially reported that LRP6 is absolutely required for toxin entry and acts as an essential co-receptor for ANTXR1 and ANTXR2. However, several groups, including our own, have challenged these findings. One report showed no involvement of LRP6 in anthrax toxin intoxication of

LRP6 KO mice or MEFs derived from these mice, as measured by intoxication assays and MEK1 cleavage assays (Young et al., 2007). Also, we published a report showing the absence of LRP6 expression has no effect on the kinetics of toxin internalization in HeLa cells (Ryan and Young, 2008), but another group obtained evidence that LRP6 is an efficiency factor that modulates PA pore formation by modestly enhancing toxin-receptor complex endocytosis into another population of HeLa cells. In agreement with our study, these authors concluded that LRP6 is not absolutely required for anthrax toxin entry (Abrami et al., 2008). Further complicating matters, that group showed that over or underexpressing ANTXR1 sv1 and ANTXR2 ablated LRP6 expression. The role of LRP6 in anthrax toxin entry remains controversial and will be discussed in Chapter 3.

1.7 Contributions to the Field

In this dissertation I will outline studies that changed the way we think about anthrax toxin entry. Prior to our work, it was thought that entry characteristics mediated by either toxin receptor were the same. In Chapter 2, I will outline work detailing receptor-specific requirements into cells. In Chapter 3, I describe my work with LRP6, which definitively showed that this factor is not absolutely required for anthrax toxin entry. In the fourth chapter, I will describe the identification and characterization of several other cellular factors that influence anthrax intoxication.

Chapter 2.

Receptor-specific requirements for anthrax toxin entry

2.1 Background

The work presented in this chapter consists of my collaboration with a Young lab postdoctoral fellow, Jonah Rainey, to determine the role receptor type plays in anthrax toxin entry. Individual contributions to the work are outlined below.

Prior to 2005, the prevailing model for anthrax toxin entry required the toxin-receptor complex to pass through an endosomal compartment where acidic pH facilitated pore formation and toxin translocation (Friedlander, 1986; Menard et al., 1996). Evidence in favor of this low-pH dependent entry model came from studies where RAW 264.7 cells were treated with lysosomotropic agents, such as ammonium chloride (NH_4Cl), which raised endosomal pH to near neutral levels and blocked intoxication. One study observed the presence of pores in the membrane of intraluminal vesicles within putative early endosomes of baby hamster kidney cells (BHK). It was suggested that the toxin translocated into intraluminal vesicles and these vesicles were subsequently trafficked to late endosomes where toxin was released into the cytosol (Abrami et al., 2004). The idea that pores formed in early endosomes was controversial, as other studies suggested that pore formation might occur in late endosomes. These studies took advantage of agents that rapidly raise intraendosomal pH and observed that these agents were able to protect RAW

264.7 cells from intoxication when added long after toxin, suggesting pH-dependent entry events occurred in a late endosomal compartment (Dal Molin et al., 2006; Menard et al., 1996). Thus, at the time the work in this chapter was started, several questions remained about the requirements for anthrax toxin entry. Much of the work cited was performed in cells where receptor type was undefined as it was assumed that toxin entered the cells in the same manner regardless of which receptor it bound. In this chapter, I will present evidence that demonstrates receptor-specific requirements for anthrax toxin entry into cells.

In 2004, Rainey showed that receptor-negative cell lines engineered to express different types of anthrax toxin receptor displayed differing sensitivity to PA + LF_NDTA when treated with ammonium chloride (NH_4Cl), a weak base that raises the pH of endosomal compartments to near neutral levels (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981). LF_NDTA is a hybrid toxin consisting of the PA-binding region of LF fused to the catalytic portion of diphtheria toxin A chain. It enters cells in the same way as LF and is a useful tool for studying anthrax toxin entry because it causes cell death in HeLa cells as opposed to LF, which does not. In Rainey's experiments, cells engineered to express either ANTXR1 sv2 or $ANTXR2^{489}$ were challenged with toxin in the presence of NH_4Cl. Cells were incubated with toxin and NH_4Cl for 2 hours at 4°, washed and shifted to 37° in the presence of NH_4Cl for time periods

ranging from 0-8 hours. Viability was assessed 36 hours after toxin addition to cells. Cells engineered to express ANTXR2[489] were nearly completely protected from intoxication. Unexpectedly, cells expressing ANTXR1 sv2 were intoxicated under these very same conditions (Fig 2.1). In order to determine if these findings extended to ANTXR1 sv1, I engineered receptor negative cells to express ANTXR1 sv1 and intoxicated them under the same conditions outlined above. Intoxication mediated by ANTXR1 sv1 was similar to that mediated by ANTXR1 sv2 in that it was not affected by NH_4Cl (Fig 2.2). Given the different sensitivities of these cells to toxin in the presence of NH_4Cl, we came up with two models of to explain these results. In the first model, toxin is differentially stable when bound to ANTXR1 or ANTXR2. In the second, NH_4Cl has no effect on intoxication mediated by ANTXR1.

I then collaborated with Jonah Rainey to show that the difference in NH_4Cl sensitivity was due to distinct receptor-specific pH thresholds for pore formation and toxin translocation. Jonah's work demonstrated that if bound to ANTXR1, pore formation occurs at pH 6.0–6.4 – equivalent to the pH of early sorting endosomes. If bound exclusively to ANTXR2, pores form around pH 5.2–5.5 which is more characteristic of pH in late endosomes. I made the surprising observation that toxin was able to efficiently translocate in the presence of NH_4Cl when entering the cells via ANTXR1, but not ANTXR2 (Rainey et al., 2005). This finding was contrary to the accepted model of toxin

entry, which required acidic pH for toxin unfolding and translocation. Prior to this work it was thought that the characteristics of toxin entry did not vary depending on cell and/or receptor type. The following paper details our work and the new, receptor-specific model for anthrax toxin entry.

2.2 Published Data

Receptor-specific requirements for anthrax toxin delivery into cells

G. Jonah A. Rainey*, Darran J. Wigelsworth†, Patricia L. Ryan*‡, Heather M. Scobie*§, R. John Collier†¶‖, and John A. T. Young*¶‖

*Infectious Disease Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037; †Department of Microbiology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115; ‡Graduate Program, Department of Biology, University of California at San Diego, La Jolla, CA 92093; §Graduate Program in Cell and Molecular Biology, University of Wisconsin–Madison, Madison, WI 53726

Contributed by R. John Collier, July 12, 2005

The three proteins that constitute anthrax toxin self-assemble into toxic complexes after one of these proteins, protective antigen (PA), binds to tumor endothelial marker 8 (TEM8) or capillary morphogenesis protein 2 (CMG2) cellular receptors. The toxin receptor complexes are internalized, and acidic endosomal pH triggers pore formation by PA and translocation of the catalytic subunits into the cytosol. In this study we show that the pH threshold for conversion of the PA prepore to the pore and for translocation differs by approximately a pH unit, depending on whether the TEM8 or CMG2 receptor is used. For TEM8-associated toxin, these events can occur at close to neutral pH values, and they show relatively low sensitivity to ammonium chloride treatment in cells. In contrast, with CMG2-associated toxin, these events require more acidic conditions and are highly sensitive to ammonium chloride. We show, furthermore, that PA dissociates from TEM8 and CMG2 upon pore formation. Our results are consistent with a model in which translocation depends on pore formation and pore formation, in turn, depends on release of PA from its receptor. We propose that because PA binds to CMG2 with much higher affinity than it does to TEM8, a lower pH is needed to attenuate CMG2 binding to allow pore formation. Our results suggest that toxin can form pores at different points in the endocytic pathway, depending on which receptor is used for entry.

capillary morphogenesis protein 2 | tumor endothelial marker 8 | toxin entry

Bacillus anthracis, the causative agent of anthrax, secretes a toxin that is believed to be instrumental in causing anthrax disease symptoms leading to death. Anthrax toxin consists of three proteins, protective antigen (PA), which is a receptor-binding and pore-forming subunit; lethal factor (LF), which is a protease that cleaves mitogen-activated protein kinase kinase family members; and edema factor (EF), which is an adenylate cyclase that raises cAMP levels in cells (1). PA is synthesized as an 83-kDa protein (PA_{83}) for which two cell surface receptors have been identified: tumor endothelial marker 8 (TEM8) (2, 3) and capillary morphogenesis protein 2 (CMG2) (4). Two splice variant mRNAs derived from the TEM8 gene (sv1 and sv2) encode functional anthrax toxin receptors (2, 5). TEM8 expression has been documented in epithelium of the lung, intestine, and skin, the three routes of entry in anthrax infection (6). The CMG2 gene, which has been shown to be broadly expressed in different tissues (4), encodes three protein isoforms, two of which, $CMG2^{488}$ and $CMG2^{489}$, are anthrax toxin receptors (4) (H.M.S., unpublished data).

Receptor-bound PA_{83} is cleaved by a cellular protease to generate a 20-kDa PA_{20} subunit and a 63-kDa subunit (PA_{63}). The larger subunit assembles into the heptameric $(PA_{63})_7$ prepore in lipid rafts (7–9). EF and LF bind to the prepore, and the toxin–receptor complexes are internalized by clathrin-dependent endocytosis and by other endocytic mechanisms (9, 10). These complexes are then exposed to increasingly acidic environments as they are trafficked from early/sorting endosomes to late endosomes. The current model of anthrax toxin entry invokes $(PA_{63})_7$ prepore conversion to a 14-stranded β-barrel-containing pore on the membranes of intraluminal vesicles that are formed within mildly acidic early/sorting endosomes (7, 11–16) (Fig. 1*a*). LF and EF are then believed to translocate into the luminal compartment of these vesicles, which is topologically equivalent to the cytosol. These vesicles then traffic to late endosomes, where they back-fuse with endosomal-limiting membranes to release LF and EF into the cytosol (11).

The PA protein consists of four distinct domains, which have each been assigned different functions (1). Domain 1 is the site of proteolytic processing that gives rise to PA_{20} and PA_{63}. The portion of domain 1 that is associated with PA_{63} binds EF and LF after PA_{63} oligomerization (8). Domain 2 contributes to receptor binding and undergoes low pH-induced structural rearrangements to form the membrane-associated pore (17–19). Domain 3 is involved in PA_{63} oligomerization (20). Domain 4 is primarily involved in receptor binding (21, 22).

X-ray structural studies of complexes consisting of the toxin-binding, integrin-like I domain of CMG2 bound to PA_{83} or to the $(PA_{63})_7$ prepore have revealed how CMG2 binds to PA domains 2 and 4 (17, 18). Based on this model, it has been proposed that low pH causes the release of receptor from domain 2 to facilitate pore formation (17, 18). Consistent with this idea, binding of soluble CMG2 I domain to the $(PA_{63})_7$ prepore shifts the threshold of the conformational prepore-to-pore transition to a more acidic pH range (17). It has further been proposed that the receptor might remain bound, presumably to domain 4 of PA, so that it serves as a structural support for the newly formed pore (18, 23).

There has been speculation that destabilization of the folding of EF and LF by low pH could foster translocation through the newly formed $(PA_{63})_7$ pore (1, 24–26) (Fig. 1*a*). The N-terminal PA-binding regions of EF and LF partially unfold at the pH 5–6 range, generating a molten globule state of these proteins (25), and translocation of LF_N (the N-terminal PA-binding region of LF) through $(PA_{63})_7$ pores that are formed on cell surfaces requires pH values of $\approx$5.5 or less (26).

Before this work, it was believed that characteristics of the toxin entry mechanism were the same regardless of cell type or receptor usage. Here we show that receptor type has a profound impact on the pH threshold required for pore formation and translocation of anthrax toxin into cells. We also provide evidence that receptor dissociation is linked to pore formation and

Abbreviations: TEM8, tumor endothelial marker 8; CMG2, capillary morphogenesis protein 2; PA, protective antigen; LF, lethal factor; EF, edema factor; LF_N-DTA, lethal factor N-terminal diphtheria toxin A chain; MEK, mitogen activated protein kinase; CHO, Chinese hamster ovary.

¶R.J.C. and J.A.T.Y. hold equity in PharmAthene, Inc. (Annapolis, MD).

‖To whom correspondence may be addressed. E-mail: jyoung@salk.edu or jcollier@hms.harvard.edu.

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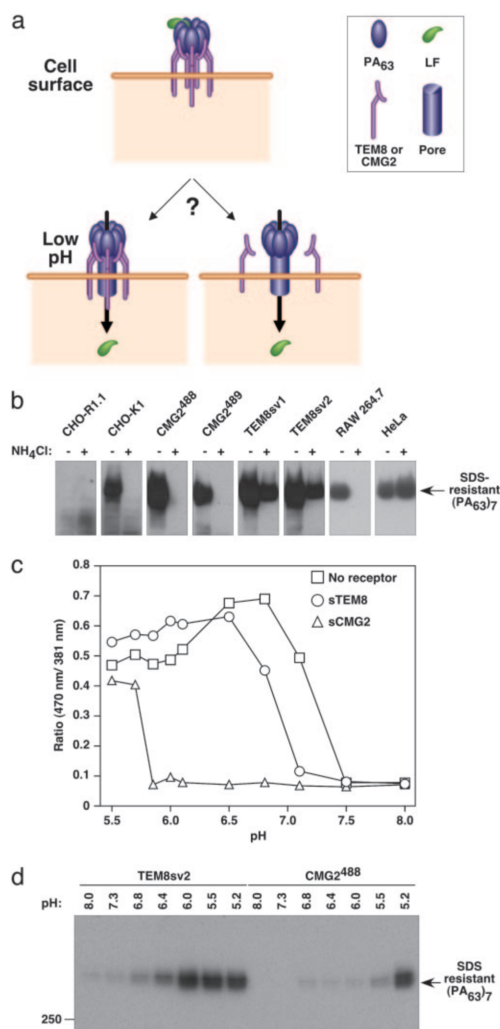

Fig. 1. Pore formation in cells expressing TEM8 does not require low pH. (*a*) Existing model of anthrax lethal toxin internalization and pore formation. The receptor–toxin complex is internalized and trafficked to an acidic endosome. Endosomal low pH triggers $(PA_{63})_7$ pore formation and partial unfolding of the toxin catalytic subunits before translocation through the pore into the cytosolic compartment. (*b*) Different cell types were exposed to lethal toxin (PA plus LF) in the presence or absence of ammonium chloride. The cells were lysed and subjected to SDS/PAGE and immunoblotting with anti-PA antibody. The location of the SDS-resistant $(PA_{63})_7$ heptamer is indicated by an arrow. (*c*) Pyrene-labeled N306C PA was incubated with soluble CMG2 (sCMG2), soluble TEM8 (sTEM8), or without receptors in buffers at different pH values, and the resulting pyrene excimer fluorescence levels were measured. (*d*) PA_{63} was bound to dGAB-treated cells for 2 h at 4°C, unbound PA was removed by washing, and samples were incubated at 37°C for 10 min at the indicated pH in the presence of dGAB. Cells were then lysed, and protein lysates were subjected to SDS/PAGE and immunoblotting with an anti-PA antibody.

show that LF translocation can occur under near-neutral pH conditions when the toxin is bound to TEM8.

Methods

Reagents. PA_{83}, PA_{63}, LF, and anti-PA goat serum were obtained from ListLabs. LF_N-diphtheria toxin A chain (LF_N-DTA) was produced as described in ref. 27. Anti-PA rabbit serum was a gift from Ken Bradley (University of California, Los Angeles). Anti-rabbit-horseradish peroxidase was obtained from Amersham Pharmacia. Anti-MEK1 (MEK, mitogen activated protein kinase) N-terminal was obtained from Upstate Biotechnology (Lake Placid, NY), the C-terminal antibody from Santa Cruz Biotechnology, and anti-EGFP was obtained from Covance (Berkeley, CA). Soluble CMG2 was the S38 protein (28), and soluble TEM8 (residues 1–234) was produced in the 293 FreeStyle expression system according to the manufacturer's instructions (Invitrogen) and purified by Ni-nitrilotriacetic acid and gel filtration.

Cell Culture. Chinese hamster ovary (CHO) cells were maintained in F12 medium with GlutaMAX (Invitrogen) supplemented with 10% calf serum (Invitrogen) and 1× penicillin/streptomycin (Invitrogen). RAW 264.7 and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (HyClone) and 1× penicillin/streptomycin (Invitrogen). For the cells incubated with NH_4Cl and dGAB (see below), these reagents were present throughout each experiment.

SDS-Resistant Pore Formation. Cells were pretreated with or without 30 mM NH_4Cl in supplemented F12 medium for 1 h at 37°C. Cells were incubated with 10^{-8} M PA_{83} and 10^{-9} M LF for 1 h at 37°C. Cells were then washed with PBS, trypsinized, and neutralized with supplemented F12 medium. Cells were then washed twice with PBS and lysed in TBS-containing 1% Nonidet P-40 with Complete EDTA-free protease inhibitor mixture (Roche Applied Science, Indianapolis). Lysates were pelleted, and supernatants were adjusted to 2% SDS in reducing gel sample buffer and subjected to SDS/PAGE on 3–8% acrylamide Tris-acetate gels (Invitrogen) without boiling. Proteins were transferred to polyvinylidene difluoride, and PA was detected by immunoblotting with anti-PA, anti-rabbit-horseradish peroxidase, and SuperSignal femto Western detection reagent (Pierce).

To assay cell-surface SDS-resistant pore formation, cells were pretreated with 50 mM 2-deoxy-glucose (Sigma)/10 mM sodium azide (Sigma)/200 nM bafilomycin A1 (Alexis Biochemicals) (dGAB) in supplemented F12 medium for 45 min at 37°C. Cells were incubated with 10^{-8} M PA_{63} for 2 h at 4°C, washed with PBS, and shifted to 37°C for 10 min in the following buffers diluted into PBS: 50 mM Tris, pH 8; 50 mM HEPES, pH 7.3 or 6.8; and 50 mM MES pH 6.4, 6, 5.5, or 5.2. Cells were then washed in cold PBS, harvested, and lysed, and 20-μg protein samples were subjected to SDS/PAGE and immunoblotting as described above.

Immunoprecipitation. Pores were formed at the cell surface in the presence of dGAB as described above. Cells were washed twice with cold PBS, harvested and lysed on ice as described above except that a 1:500 dilution of an anti-PA rabbit serum was included at all harvest steps. The samples were then subjected to centrifugation at 15,000 × g for 10 min, and the supernatant was incubated at 4°C for 1 h with rabbit anti-PA antibody (1:500 dilution) and then with protein A-Sepharose beads for an additional hour. The beads were then washed and suspended in a gel sample buffer containing 2% SDS. Samples were resolved on 3–8% Tris-acetate or 8% Tris-glycine gels and transferred to polyvinylidene difluoride and probed with anti-PA goat serum or an anti-EGFP mouse monoclonal antibody.

Pyrene Fluorescence. N306C PA_{63} was produced and labeled with pyrene as described in ref. 17. Labeled $(PA_{63})_7$ prepore was mixed with soluble CMG2 or soluble TEM8 as described in ref. 17, except that 1 mM $MgCl_2$ was included in the mixture. pH was adjusted to the indicated values by using 0.1 equivalents of either 1 M Tris, pH 8; 1 M HEPES, pH 7; 1 M BisTris, pH6; or 1 M sodium acetate, pH 5. After 30 min, samples were analyzed in a 1- × 0.5-cm quartz cuvette using an ISS (Champaign, Illinois) fluorometer at an excitation wavelength of 341 nm. Emission was measured from 360 to 600 nm and normalized against the maximal fluorescence at 384 nm.

Intoxication Assays. For LF_N-DTA intoxication and MEK1 cleavage assays, cells were pretreated with or without 30 mM NH_4Cl in supplemented F12 medium for 1 h at 37°C. In the LF_N-DTA experiments, cells were incubated with the indicated concentrations of PA_{83} and 10^{-9} M LF_N-DTA for 2 h at 37°C. The medium was then removed and replaced with fresh medium, and the cells were incubated for an additional 2 h at 37°C. The medium was then replaced with DMEM without glutamine, leucine, and sodium pyruvate (MP Biomedicals, Irvine, CA) supplemented with 2 mM L-glutamine (Invitrogen), 0.45 mM sodium pyruvate (Sigma), and 1 μCi/ml, 173 Ci/mmol [^{3}H]leucine (1 Ci = 37 GBq; PerkinElmer). Cells were incubated at 37°C for a further 2 h, washed three times with cold PBS, resuspended in EcoLume scintillation fluid (MP Biomedicals), and assayed with a Top-Count NXT microplate scintillation counter (Packard).

In the MEK1 cleavage assays cells were incubated with 2.5 × 10^{-8} M PA_{83} and 5 × 10^{-9} M LF for 2 h at 4°C, washed, and shifted to 37°C for the indicated times. In the case of the 4-h time point, the medium was exchanged with fresh NH_4Cl-containing medium after 2 h had elapsed. Cells were lysed in TBS-containing 1% Nonidet P-40 as described above, and 12-mg protein samples were resolved by 8% acrylamide Tris-glycine SDS/PAGE, transferred to a polyvinylidene difluoride membrane, and detected with anti-MEK1 N-terminal and C-terminal antibodies, followed by anti-rabbit-horseradish peroxidase secondary antibodies and SuperSignal femto Western detection reagent (Pierce).

Results

To determine whether the TEM8 and CMG2 receptors influence the triggering mechanism that leads to anthrax toxin pore formation, we investigated the pH thresholds for this process in PA receptor-deficient CHO-R1.1 cells (2) engineered to express either receptor (Fig. 5, which is published as supporting information on the PNAS web site). PA_{83} and LF were added to the cells in the presence of ammonium chloride, a lysosomotropic agent that elevates the pH of acidic endosomes to approximately pH 6.5 or higher (29, 30). Pore formation was monitored by the conversion of PA to an SDS-resistant oligomeric form (7, 16) (Fig. 1 *a* and *b*). Consistent with a low pH requirement, the ammonium chloride treatment blocked $(PA_{63})_7$ pore formation in the CMG2-expressing cells and in CHO-K1 cells and RAW 264.7 mouse macrophage cells that express endogenous anthrax toxin receptors (Fig. 1*b*). By contrast, a significant amount of $(PA_{63})_7$ pore formation occurred with the TEM8-expressing cells and with HeLa cells in the presence of the lysosomotropic agent (Fig. 1*b*). These data indicate that pore formation can occur at near-neutral pH when the toxin is bound to TEM8 but not when it is bound to CMG2. Furthermore, these results revealed an unexpected difference between three cell types (HeLa, CHO-K1, and RAW 264.7 cells) that were previously used to derive a unified model for anthrax toxin entry (9, 11, 15).

To define the pH threshold of TEM8-associated $(PA_{63})_7$ pore formation, we used a pyrene-labeled form of PA (N306C PA) that generates an excimer fluorescent signal upon pore formation (16). Pyrene-labeled N306C $(PA_{63})_7$ prepore was bound to

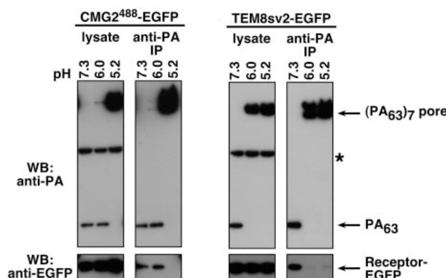

Fig. 2. Toxin dissociates from receptor upon pore formation. PA_{63} was bound to dGAB-treated cells as described in Fig. 1*d*. Cells were treated at the indicated pH values for 10 min and lysed in the presence of an anti-PA rabbit antiserum that does not block PA receptor binding (data not shown). Lysates were resolved directly or immunoprecipitated with anti-PA rabbit serum and immunoblotted with an anti-PA goat serum or an anti-EGFP antibody to detect receptor proteins. IP, immunoprecipitation; WB, Western blot; *, nonspecific cellular protein detected by goat anti-PA.

soluble forms of TEM8 or CMG2 (28) *in vitro* in conditions under which the toxin subunit was fully occupied by soluble receptors, as judged by native PAGE (Fig. 6, which is published as supporting information on the PNAS web site). The pH was adjusted to various values, and the resulting excimer fluorescence was monitored. When bound to TEM8, $(PA_{63})_7$ pore formation occurred at pH values ranging from pH 6.8 to 7.1, but, when bound to CMG2, it occurred at pH values between pH 5.7 and 5.8 (Fig. 1*c*). As noted previously (17), PA formed pores between pH 7.1 and 7.5 when no soluble receptor was present (Fig. 1*c*).

To examine the pH threshold of pore formation when PA is bound to cell membrane-associated receptors, PA_{63} was added to cells in the presence of a mixture of 2-deoxyglucose, sodium azide, and bafilomycin A1 (dGAB) (31). The dGAB treatment prevented toxin-receptor complex internalization (Fig. 7, which is published as supporting information on the PNAS web site). This experiment confirmed that $(PA_{63})_7$ pores formed under mildly acidic pH conditions (pH 6.4) when PA was bound to TEM8 but at more highly acidic conditions (pH 5.2) when it was bound to CMG2 (Fig. 1*d*). The difference between these pH threshold measurements and those obtained with pyrene-labeled N306C PA may be due to the effect of cell membranes on the pH requirements for $(PA_{63})_7$ pore formation.

To determine whether receptors remain bound during $(PA_{63})_7$ pore formation, PA_{63} was bound to dGAB-treated cells that expressed EGFP-tagged versions of CMG2^{488} or TEM8 sv2. The cells were then incubated in buffers at pH 7.3, 6, or 5.2. The cells were then lysed, and PA-associated receptors were detected by immunoprecipitation with an anti-PA antibody followed by immunobloting with an EGFP-specific antibody. The anti-PA antibody used in this study does not disrupt the PA-receptor interaction (data not shown). PA was associated with CMG2^{488}–EGFP in the absence of pore formation (pH 7.3 and pH 6) but not at pH 5.2 when pore formation was observed (Fig. 2 *Left*). Similarly, PA was dissociated from TEM8sv2–EGFP under conditions of pore formation (pH 6 and pH 5.2) (Fig. 2 *Right*). These data indicate that the dissociation of PA from receptor is associated with $(PA_{63})_7$ pore formation.

To determine whether anthrax toxin can be translocated into cells under the mildly acidic conditions that are associated with TEM8-dependent $(PA_{63})_7$ pore formation, the entry of LF_N-DTA and LF into cells was monitored in the presence of ammonium chloride. LF_N-DTA entry was scored as inhibition of

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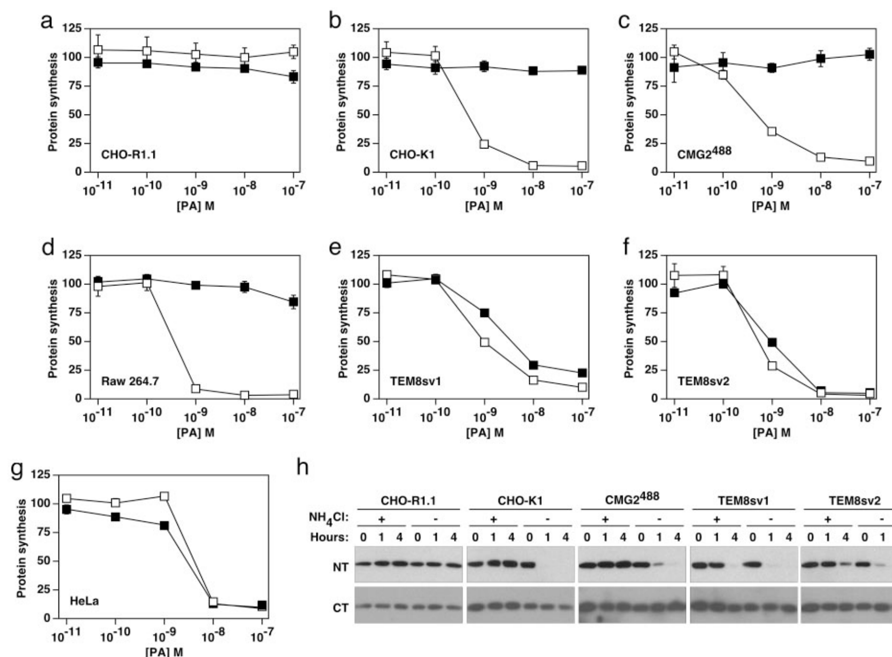

Fig. 3. Ammonium chloride blocks toxin translocation into CMG2-expressing cells but not TEM8-expressing cells. (*a–g*) Cells were incubated with PA plus LF_N-DTA and metabolically labeled with [^{3}H]leucine to monitor protein synthesis in the presence (■) or absence (□) of NH_4Cl. The amount of radiolabeled protein was determined by scintillation counting and graphed from triplicate values ± SEM. (*h*) Cells were incubated with lethal toxin in the presence or absence of NH_4Cl for the indicated times. Cells were lysed, and protein lysates were subjected to SDS/PAGE and to immunoblotting with anti-MEK1 antibodies. NT, N-terminal; CT, C-terminal. Because only eight amino acids are removed from the N terminus of MEK1 by LF cleavage (32, 33), this does not significantly change the molecular weight of the cleaved MEK1 protein that is detected by the C-terminal antibody.

protein synthesis using a [^{3}H]leucine incorporation assay (27), and LF entry was scored by immunoblotting to detect the N-terminal cleavage of the MEK1 substrate (32, 33). Consistent with a requirement for an acidic pH, ammonium chloride treatment inhibited intoxication of the CMG2-expressing CHO-R1.1 cells, CHO-K1 cells, and RAW 264.7 cells (Fig. 3 *b–d* and *h*). By contrast, toxin was able to access the cytosol of TEM8-expressing CHO-R1.1 cells and HeLa cells in the presence of ammonium chloride (Fig. 3 *e–h*), although LF cleavage of the MEK1 substrate was delayed under these conditions (Fig. 3*h*). These data demonstrate that toxin translocation can also occur under the mildly acidic conditions that are associated with TEM8-dependent $(PA_{63})_7$ pore formation.

Discussion

The results of this study provide important insights into the role of the receptor in anthrax toxin entry. Specifically, anthrax toxin pore formation and translocation were found to occur under strikingly different acidic conditions when PA was bound to TEM8 instead of CMG2. Complete receptor dissociation was also implicated as being an integral component of the toxin pore-forming mechanism, and the toxin translocated into TEM8-expressing cells at near neutral pH (Fig. 4).

The finding that receptor type dictates different pH thresholds for anthrax toxin pore formation and translocation was unexpected. Previously, it was shown that treatment of peritoneal mouse macrophages and CHO-K1 cells with ammonium chloride, a weak base that neutralizes the pH of endosomes, prevents pore formation and translocation (7, 13). This result holds true for the PA receptor-deficient cells that were engineered to express CMG2, and it holds true for the RAW 264.7 mouse macrophage cells that were tested in this study. However, this finding does not hold true for the cells that were engineered to express TEM8 or for HeLa cells. Presumably, the distinct toxin behavior in RAW 264.7 and HeLa cells is due to differences in CMG2 versus TEM8 expression; however, this remains to be shown directly.

Previously, it was hypothesized that release of PA domain 2 from the receptor by the protonation of key histidine residues that are located at the PA-receptor binding interface triggers pore formation (17, 18). It was further postulated that the receptor remains bound to PA domain 4, perhaps to provide a structural support for the stalk that comprises the pore (18, 23). Because the coimmunoprecipitation experiments that we performed failed to detect receptors associated with the newly formed PA pores, our data instead favor a model in which complete receptor release is required for toxin pore formation. Indeed, this model is compatible with prior observations that demonstrated that functional $(PA_{63})_7$ pores could be formed in membranes lacking anthrax toxin receptors (12).

Previously, various lines of evidence obtained by studying anthrax toxin subunits in solution, on artificial lipid membranes, or on cell surfaces had indicated that the acidic pH characteristic of late endosomal compartments might be required for unfolding

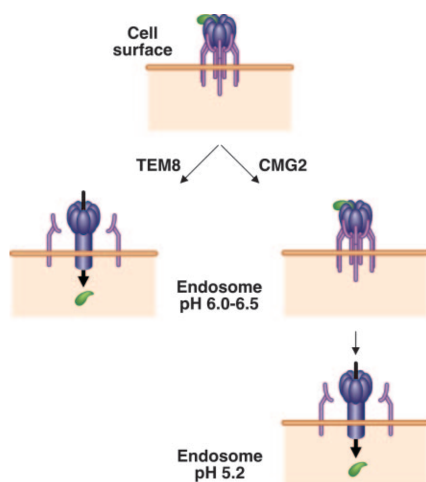

Fig. 4. Model of the distinct receptor-specific pH thresholds for anthrax toxin pore formation and translocation. Receptor–toxin complexes are internalized and trafficked through the endosomal pathway. Pore formation and translocation occur at different pH thresholds for TEM8- versus CMG2-associated toxin, indicative of different endosomal routes of entry. Pore formation is associated with receptor dissociation. See Fig. 1 for a key to the symbols.

EF and LF before translocation through the $(PA_{63})_7$ pore (1, 24–26). By studying the translocation process within cellular endosomes, we show that LF_N-DTA and LF can translocate into the cytosol in TEM8-expressing cells that were incubated with ammonium chloride to raise endosomal pH. However, ammonium chloride treatment did slow the kinetics of LF-mediated cleavage of the MEK1 substrate in these cells (Fig. 3*h*). This effect may be due to less efficient pore formation (Fig. 1*b*) or LF unfolding (25) under more neutral pH conditions; or instead might be due to a reduction in the accessibility of MEK1, which might be concentrated on scaffolds on late endosomes (34), under these conditions. Our results are consistent with different receptor-specific pH requirements for pore formation in the endosomal pathway. Consistent with a previous report that PA forms pores in multivesicular regions of early/sorting endosomes (11), PA bound to TEM8 is predicted to form pores in the early/sorting endosome, where the pH is >6 (35). PA bound to CMG2, on the other hand, requires a much more acidic pH for pore formation (pH 5.2–5.5), one that is more characteristic of late endosomes (35). Based on these properties we propose that the receptor type might dictate the site of toxin pore formation in the endosomal pathway (Fig. 4).

It is likely that the interaction between anthrax toxin and multiple cell types contributes to anthrax pathogenesis. In the initial stages of anthrax infection, toxin plays a role in facilitating spore germination (36–38). Once the vegetative infection is established, many cells are exposed to the toxin. Previous studies have proposed a role for toxin in dendritic cells (39), vascular endothelial cells (40–42), and macrophages (43–47). In most of these cell types, the receptor repertoire is not known. However, because CMG2 and TEM8 appear to be broadly expressed in different cell types (4, 6), it will be important to establish whether heptameric PA-CMG2/TEM8 heterocomplexes can be formed and, if so, to determine the pH values of pore formation that are associated with heterocomplexes containing different ratios of the two receptors. Further investigation is also necessary to directly establish the endosomal sites of toxin entry into cells expressing TEM8 and CMG2 and to determine the effect of substrate subcellular localization on the efficiency of LF and EF action.

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2.3 Conclusions and Discussion

Our work demonstrated that receptor type must be taken into account when determining the characteristics of anthrax toxin entry into cells. If bound to ANTXR1, pore formation occurs at pH 6.0–6.4. If bound exclusively to ANTXR2, pores form around pH 5.2. Interestingly, the pH of the early endosome is thought to be >6.0 whereas the pH of the late endosome is thought to be >5.0 (Maxfield and McGraw, 2004).

This observation of receptor-specific pH thresholds of pore formation carried implications for the accepted model of anthrax toxin entry as the cell lines used to generate this model differed in their expression of the two anthrax toxin receptors. Several early studies on anthrax toxin entry were done on cells where, at the time, receptor type was undefined. One study determined that pores form in early endosomes by isolating early endosomes of BHK cells and monitoring pore formation by western blot. Since the pH of early endosomes is ~6.0, it is unlikely that toxin bound to ANTXR2 could form pores at this stage of the endocytic pathway. It is thus likely that toxin entry was mediated by ANTXR1(Abrami et al., 2004). Other studies suggested pore formation occurred in a late endosomal compartment since agents that blocked endosome acidification were able to protect cells from intoxication when added long after toxin (Menard et al., 1996). These experiments were performed in RAW 264.7 macrophages, which predominantly express

ANTXR2. Thus, pore formation in a late endosomal compartment is consistent with the receptor type expressed in these cells. It is tempting to speculate that anthrax toxin pore formation and toxin translocation occur at various stages of the endocytic pathway and that this is solely dependent on receptor type, though studies isolating early and late endosomes from cells engineered to express different types of receptor and comparing pore formation and the presence of toxin in these compartments remain to be done.

Interestingly, we also found that cells expressing ANTXR1 were capable of translocating toxin under near neutral pH conditions. Prior to this work, it was thought that acid-induced unfolding of LF and EF was necessary for translocation through the narrow pore lumen. These studies used artificial lipid membranes or artificially induced translocation across the plasma membrane (Kochi et al., 1994; Krantz et al., 2004; Wesche et al., 1998). Contrary to this hypothesis, we demonstrated that in the presence of NH_4Cl, an agent that raised intraendosomal pH to near-neutral levels, PA bound to ANTXR1 is still capable of translocating LF as measured by LF-induced MEK1 cleavage. The kinetics of MEK1 cleavage occur at a slower rate, and this may be due to slower kinetics of toxin unfolding and translocation in the presence of near-neutral pH conditions. This evidence is consistent with a model in which acidic pH facilitates unfolding, but is not required. It is possible that the phe clamp present within the PA pore lumen, which transiently interacts with

hydrophobic portions of the unfolding catalytic moieties, is sufficient to reduce the penalty of exposing hydrophobic residues so that unfolding and translocation may still occur (see Introduction). Subsequent studies have demonstrated a transmembrane proton gradient is required for toxin translocation (Krantz et al., 2006). This is consistent with my results, as even in the presence of NH_4Cl a pH gradient likely occurs. Cytosolic pH (which is thought to be equivalent to the lumen of intraluminal vesicles where LF and EF are delivered) is thought to be ~ 7.3, and NH_4Cl is estimated to raise intraendosomal pH to >6.5 (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981).

These studies were subsequently corroborated using a patch-clamp based assay to measure pore formation on the plasma membrane in cells engineered to express ANTXR1 or ANTXR2 (Wolfe et al., 2005). In these studies, pore formation in cellular membranes required receptor expression and the pH thresholds of pore formation depended upon the receptor type expressed. When pore formation was measured in cells engineered to express ANTXR1, efficient pore formation occurred at pH ~6.2. When measured in ANTXR2 expressing cells, pore formation occurred at pH ~5.3.

A second important point addressed in this chapter involves the molecular interaction between PA and the receptors. According to subsequent mutagenesis studies performed by a graduate student in our lab, Heather

Scobie, and research technician, John Marlett, the receptor specific requirements for pore formation can be traced to the receptor interaction with domain 2 of PA (Scobie et al., 2007). The co-crystal structures of PA heptamer and the ANTXR2 I domain show that the receptor makes contact with both domain 4 and domain 2 of PA (Fig 1.6 and 1.7). When residues G153 and L154 of ANTXR2, which make contact with PA domain 2, are mutated to those found on ANTXR1, the threshold pH of pore formation is converted to that of toxin bound to ANTXR1.

Based on studies of the co-crystal structure of ANTXR2 I-domain bound to the heptameric form of PA, it was suggested that receptor might dissociate from the heptamer upon pore formation in order to allow for the structural changes required for the β-loop in PA domain 2 to insert in the membrane and form the pore (Lacy et al., 2004; Santelli et al., 2004). Conversely, it was also suggested that the receptor might remain attached to the pore in order to provide structural support to the β-barrel of the pore, which extends 50Å above the membrane surface (Nguyen, 2004; Santelli et al., 2004). Using co-immunoprecipitation experiments, Jonah showed that cell-surface PA oligomers interact with receptor, but once exposed to low pH co-immunoprecipitation did not bring down detectable levels of receptor, providing support for the idea that receptor/PA interaction is weakened coincident with pore formation. NMR experiments performed since this work was published

have demonstrated that the ANTXR2 VWA-domain dissociates from heptameric PA when exposed to low pH (Williams et al., 2009). Other immunoprecipitation experiments have detected receptor/PA interaction upon pore formation that was allowed to occur within endosomes (Abrami et al., 2006). Future structural studies of PA/receptor interactions at varying pH levels will allow us to address the nature of receptor/PA interaction during the prepore-to-pore conversion.

I am grateful to my co-authors Jonah Rainey, Heather Scobie, John Collier, Darran Wigelsworth, and John A.T. Young for their contributions to the projects documented in this chapter.

Chapter 2 contains a reprint of a paper as it appears in Proceedings of the National Academy of the Sciences. Rainey, G.J., Wigelsworth, D.J., Ryan, P.L., Scobie, H.M., Collier, R.J., Young, J.A. *Receptor-specific requirements for anthrax toxin delivery into cells*. PNAS, 2005. 102(37): p.13278–83.

Chapter 3.

Defining the role of LRP6 in anthrax toxin internalization

3.1 Background

The work in this chapter represents my studies concerning the role of low-density lipoprotein receptor-related protein 6 (LRP6) in anthrax toxin internalization.

LRP6 and the related LRP5 are co-receptors for Wnt in the canonical Wnt/β-catenin signaling pathway (Pinson et al., 2000; Tamai et al., 2000). This signaling pathway controls several development processes, such as cell fate determination, cell proliferation and self-renewal of stem and progenitor cells. LRP5 and LRP6 are single pass transmembrane proteins and are widely expressed during embryogenesis and in adult tissues (Houston and Wylie, 2002).

In an expressed sequence tag (EST) based screen to identify factors involved in anthrax toxin internalization, Cohen and colleagues identified LRP6 (Wei et al., 2006). They demonstrated that LRP6 was essential for intoxication of human M2182 prostate carcinoma cells and played a modest role in intoxication of RAW macrophages. Using siRNAs targeting LRP6 as well as LRP6 deletion mutants, their results demonstrated a critically important role for LRP6 in both PA binding and subsequent toxin internalization into these cells. Additionally, they showed that LRP6 associates with ANTXR1 and ANTXR2. Based on these findings they concluded that LRP6 is an anthrax toxin co-

receptor, essential for intoxication. In favor of this model, they showed that LRP6 antibodies were capable of blocking intoxication, providing preliminary evidence that LRP6 might be a viable target for anthrax therapeutics.

Subsequently, another study performed with mouse embryonic fibroblasts (MEFs) from knockout mice demonstrated no role for either LRP6 or LRP5 in anthrax toxin entry as measured by intoxication and MEK1 cleavage experiments (Young et al., 2007). In this study they also looked at intoxication and MEK1 cleavage in receptor-negative CHO cells engineered to overexpress different isoforms of the human receptor and again found LRP6 did not play a role in these events.

In the initial study identifying LRP6 as a coreceptor, early events in the entry pathway such as toxin binding and pore formation were used to score the importance of LRP6 in anthrax intoxication. Both toxin binding and pore formation are usually completed within 30 minutes of adding toxin to cells. In the subsequent study demonstrating no role for LRP6 in intoxication, toxin internalization was scored using cell viability measurements 24 hours after toxin addition and by measuring MEK1 cleavage 4 hours after toxin addition. Based on these differences in methodology, we hypothesized that LRP6 might modulate the kinetics of toxin entry; an effect that might be missed if anthrax toxin entry is measured using events that occur later in the intoxication process. Additionally, since the most profound effects of LRP6 were observed

in a human cell type, M2182 prostate carcinoma cells, we hypothesized LRP6 might play a human cell-specific role in anthrax toxin internalization. I ruled out both of these options in our report that showed the absence of LRP6 did not affect the kinetics of anthrax toxin entry into HeLa cells, a human epithelial cell line. The results of that study are presented here in section 3.2 (Ryan and Young, 2008).

3.2 Published Data

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Evidence against a Human Cell-Specific Role for LRP6 in Anthrax Toxin Entry

Patricia L. Ryan[1,2], John A. T. Young[1]*

1 Infectious Disease Laboratory, The Salk Institute for Biological Studies, La Jolla, California, United States of America, 2 Division of Biological Sciences, University of California San Diego, La Jolla, California, United States of America

Abstract

The role of the cellular protein LRP6 in anthrax toxin entry is controversial. Previous studies showed that LRP6 was important for efficient intoxication of human M2182 prostate carcinoma cells but other studies performed with cells from gene-knockout mice demonstrated no role for either LRP6 or the related LRP5 protein in anthrax toxin entry. One possible explanation for this discrepancy is that LRP6 may be important for anthrax toxin entry into human, but not mouse, cells. To test this idea we have investigated the effect of knocking down LRP6 or LRP5 expression with siRNAs in human HeLa cells. We show here that efficient knockdown of either LRP6, LRP5, or both proteins has no influence on the kinetics of anthrax lethal toxin entry or MEK1 substrate cleavage in these cells. These data argue against a human-specific role for LRP6 in anthrax toxin entry and suggest instead that involvement of this protein may be restricted to certain cell types independently of their species of origin.

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* E-mail: jyoung@salk.edu

Introduction

Bacillus anthracis, the etiological agent of anthrax, secretes a tripartite toxin, which is one of two major virulence factors. Anthrax toxin is composed of the receptor binding moiety, protective antigen (PA), and two catalytic moieties: Lethal factor (LF), a zinc –dependent metalloprotease that cleaves MAP kinase kinases (MAPKKs), [1–3] and edema factor (EF), a calcium- and calmodulin-dependent adenylate cyclase that raises cAMP levels [4]. LF and EF combine with PA to form lethal toxin (LeTx) and edema toxin (EdTx), respectively. Both 83 kD and 63 kD forms of PA (PA_{83} and PA_{63}) can bind to either of two cellular receptors, ANTXR1 (anthrax toxin receptor 1/tumor endothelial marker 8; ATR/TEM8) or ANTXR2 (anthrax toxin receptor 2/capillary morphogenesis factor 2;CMG2) [5,6]. Receptor-bound PA_{83} is cleaved by cell-surface furin into the 63 kD form [7]. PA_{63} forms a heptameric ring structure [$PA_{63(7)}$], termed a prepore, at neutral pH [8]. EF and LF bind the prepore and the toxin/receptor complex is endocytosed primarily via clathrin-mediated endocytosis [9] and trafficked to an endocytic compartment where low-pH triggers $PA_{63(7)}$ pore formation and translocation of EF and LF into the cytosol [10–12].

Currently there is a controversy about the role played in anthrax toxin entry by the low-density lipoprotein receptor-related protein LRP6, which interacts with both ANTXR1 and ANTXR2 [13]. Evidence in favor of a specific role was provided by Cohen and colleagues [13]. In that study, LRP6 was identified through a genome-wide antisense RNA screening approach to be important for intoxication of human M2182 prostate carcinoma cells by PA and FP59, a recombinant toxin comprised of the N-terminal portion of LF fused to *Pseudomonas* exotoxin A. Consistently, siRNA-mediated knockdown of LRP6 levels in these cells reduced their toxin sensitivity by several orders of magnitude, an effect that was partially overcome by expression of an siRNA-resistant form of LRP6. LRP6 deficiency in M2182 cells was also associated with reduced levels of PA binding and pore formation, and expression of a dominant-negative form of LRP6, lacking its cytoplasmic domain, rendered these cells resistant to intoxication. This group also showed that LRP6 could be co-precipitated with both ANTXR1 and ANTXR2. Furthermore, they showed that siRNA-mediated knockdown of LRP6 rendered RAW264.7 mouse macrophages resistant to intoxication by PA and LF, although this effect was much more modest (~3-fold) than that seen with M2182 cells. LRP6-specific antibodies also protected RAW264.7 cells from intoxication.

By contrast, Duesbury and colleagues found that $LRP6^{+/-}$ and $LRP5^{-/-}$ mice were just as susceptible to killing after LeTx injection as wild-type mice [14]. LRP5 is a protein that is highly related to LRP6 [15]. In addition, they showed that mouse embryo fibroblasts (MEFs) that were isolated from $LRP6^{-/-}$ or $LRP5^{-/-}$ mice were just as susceptible to intoxication by PA and FP59, and to MEK1 cleavage by LF and PA, as those isolated from wild-type mice [14]. They went on to show that there is no obvious receptor-specific role for LRP6 since knocking down this protein had no effect on the toxin sensitivity of PA receptor-deficient Chinese hamster ovary cells that were engineered to express either ANTXR1 or ANTXR2.

Duesbury and colleagues put forward several possible explanations for the discrepant results. Since their data argue against an anthrax toxin receptor-specific role for LRP6, and there is no evidence for functional redundancy between LRP6 and LRP5 in toxin entry, they suggested that instead LRP6 might function in

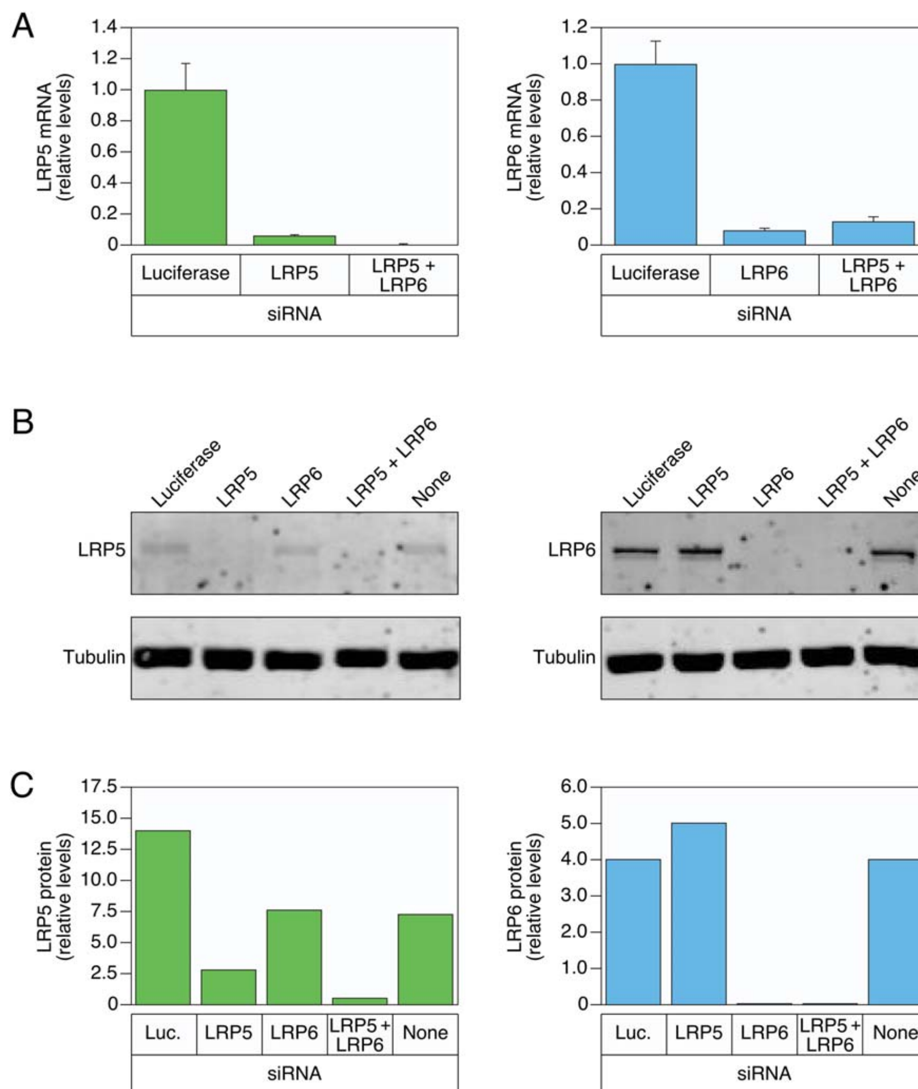

Figure 1. siRNA knockdown of LRP5 and LPR6 mRNA and protein expression in HeLa cells. A) RT-PCR analysis of LRP5 and LRP6 levels in HeLa cells transfected either with cognate pools of siRNAs or with siRNAs directed against luciferase (negative control). These experiments were each performed with triplicate samples and the mean average results are shown along with the standard deviation of the data indicated by error bars. B) Immunoblot analysis of LRP5 and LRP6 protein levels in cells transfected with the pools of siRNAs described in panel A. To control for equivalent cellular protein levels imunoblot analysis was also conducted on tubulin. C) The relative levels of LRP5 and LRP6 proteins in each sample shown in panel B were quantitated relative to the levels of tubulin in each sample using the fluorescence-scanning method described in materials and methods.

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either a human-specific or cell type-specific manner. In this report we show that siRNA-mediated knockdown of LRP6 and/or LRP5 levels has no impact on the kinetics of anthrax toxin entry into human HeLa cells. These data argue against a human-specific role for either LRP6 or LRP5 in anthrax toxin entry and suggest that the requirement for LRP6 might be restricted to certain cell types.

Results and Discussion

Efficient siRNA-mediated knockdown of LRP6 and LRP5 expression in HeLa cells

HeLa cells were chosen as a model human cell type because they have been used extensively for anthrax toxin entry studies and they are efficiently transfected with siRNAs [9,16,17]. To

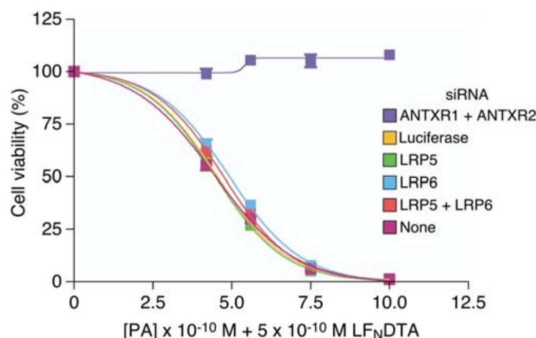

Figure 2. Reduced levels of LRP6 and/or LRP5 do not influence the toxin sensitivity of HeLa cells. siRNA-transfected cells were incubated with varying amounts of PA83 (as shown) and with 5×10^{-10} M LF_NDTA. These experiments were performed with triplicate samples and the results shown are the mean average values with the standard deviation of the data indicated with error bars.
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determine the efficiency of knocking down LRP6 or LRP5 expression, these cells were transfected with cognate siRNAs. These studies revealed an approximately 20-fold decrease in LRP5 and a 10-fold decrease in LRP6 mRNA transcripts in the transfected HeLa cells, relative to the levels observed with cells transfected with a negative control siRNA directed against luciferase (Fig. 1A). Consistently, siRNA transfection significantly reduced LRP5 and LRP6 proteins levels, as judged by immunoblotting (Fig. 1B and 1C). Importantly, LRP6 protein was reduced to undetectable levels in HeLa cells transfected with the corresponding siRNAs (Fig. 1B and C). Taken together these studies show that expression of LRP5 and LRP6 can be markedly reduced in siRNA-transfected HeLa cells. In addition, the level of siRNA-mediated knock-down of LRP5 and LRP6 exceeded those achieved in previous studies concerning the role of LRP5 and LRP6 in anthrax toxin internalization.

LRP6 and LRP5 are not important for intoxication of HeLa cells

To test the importance of LRP6 and/or LRP5 for anthrax toxin entry, HeLa cells transfected with different siRNAs were tested for their susceptibility to intoxication in the presence of varied amounts of PA_{83} and a fixed amount of LF_N-DTA. LF_N-DTA is a hybrid toxin consisting of the PA-binding subunit of LF fused to the catalytic domain of diphtheria toxin A chain [18]. This recombinant toxin uses precisely the same PA-dependent pathway for cellular entry as that used by wild-type LF, and indeed it has been used as a convenient tool to study the anthrax toxin entry mechanism because, unlike wild-type LF, it can cause cell death in many cell types following its translocation into the cytosol [19–21]. For positive control purposes, HeLa cells were also transfected with siRNAs directed against both ANTXR1 and ANTXR2, which results in complete protection of the cells from intoxication because of efficient receptor knockdown (Fig. 2).

These studies revealed that the cells transfected with LRP5- and/or LRP6-directed siRNAs were just as susceptible to intoxication as were untransfected cells or cells transfected with a control firefly luciferase-specific siRNA (Fig. 2). These data argue against a specific requirement for either LRP6 or LRP5 in anthrax toxin entry.

LRP6 and LRP5 do not influence the kinetics of $PA_{63(7)}$ pore formation in HeLa cells.

The toxin sensitivity studies shown in Fig. 2 are conducted over a two day time frame and, as such, they do not provide any information on the kinetics of toxin entry which is usually complete within a 60- to 90-minute time period [2,22,23]. To explore the possibility that LRP6 and/or LRP5 might influence the kinetics of toxin entry we first evaluated the rate of $PA63_{(7)}$ pore formation in siRNA-transfected HeLa cells. Pore formation was evaluated during the first 35 minutes after initiating toxin internalization, using a standard approach, namely by the acid-pH-dependent conversion of $PA63_{(7)}$ to an SDS-resistant oligomeric species [8,11,24]. These studies revealed that the kinetics of pore formation in cells transfected with LRP5 and/or LRP6 siRNAs were precisely the same as those seen in cells transfected with the control luciferase siRNA (Fig. 3A and 3B). As expected, HeLa cells treated with siRNAs targeting ANTXR1 and ANTXR2 showed little quantifiable pore formation (Fig. 3A and 3B). Taken together these studies demonstrate no obvious effect of knocking down LRP5 and/or LRP6 expression on the early kinetics of toxin uptake and acid pH-dependent PA pore formation.

LRP6 and LRP5 do not influence the kinetics of MEK1 substrate cleavage in HeLa cells

To confirm that LRP6 and LRP5 play no role in anthrax toxin entry into HeLa cells, we monitored the effect of knocking down expression of these proteins upon the kinetics of MEK1 cleavage by LF. LF cleavage removes the eight N-terminal amino acids of MEK1, an event that can be scored by immunoblotting using an antibody specific for the N-terminal region of MEK1 [1–3,23]. MEK1 cleavage was monitored by immunoblotting at 0, 30, 45, 60, 75 and 90 minutes after LeTx addition and the signal obtained in each case was compared to that of Ku86, a cellular protein that is not a LF substrate. These studies revealed that siRNA-mediated knockdown of LRP6 or LRP5 levels had no influence on the MEK1 cleavage kinetics in HeLa cells (Compare these samples to the control samples transfected with luciferase siRNA: Fig. 4A and 4B). As expected, HeLa cells transfected with siRNAs targeting ANTXR1 and ANTXR2 were completely resistant to LF-mediated MEK1 cleavage (Fig. 4A and 4B).

In summary, the data obtained in this report argue against a human specific role for LRP6 or for the related LRP5 protein in anthrax intoxication. Efficient siRNA-mediated knockdown of expression of either or both LRP5 and LRP6 in HeLa cells had no impact on the kinetics of anthrax toxin uptake or pore formation in acidic endosomes. Furthermore, these treatments did not alter the kinetics of MEK1 cleavage following LeTx uptake into HeLa cells. These data lead us to conclude that LRP6 may be important for anthrax toxin uptake in different cell types independently of their species of origin, as was suggested by Duesbury and colleagues [14]. However, future studies in this area will be required to clarify the precise context(s) under which anthrax toxin entry is dependent upon LRP6.

Materials and Methods

siRNA transfections

HeLa cells were reverse-transfected using RNAiMax (Invitrogen) according to manufacturer's instructions with 32 nM siRNA. siRNAs were obtained from Dharmacon: LRP6 On-Target Plus SmartPool siRNA (GCAGAUAUCAGACGAAUUUUU, CAGAUGAACUG GAUUGUUAUU, CCACAGAGCGAUCACAUUAUU, GCUCA ACCGUGAAGUUAUAUU) LRP5 On-Target Plus Smartpool

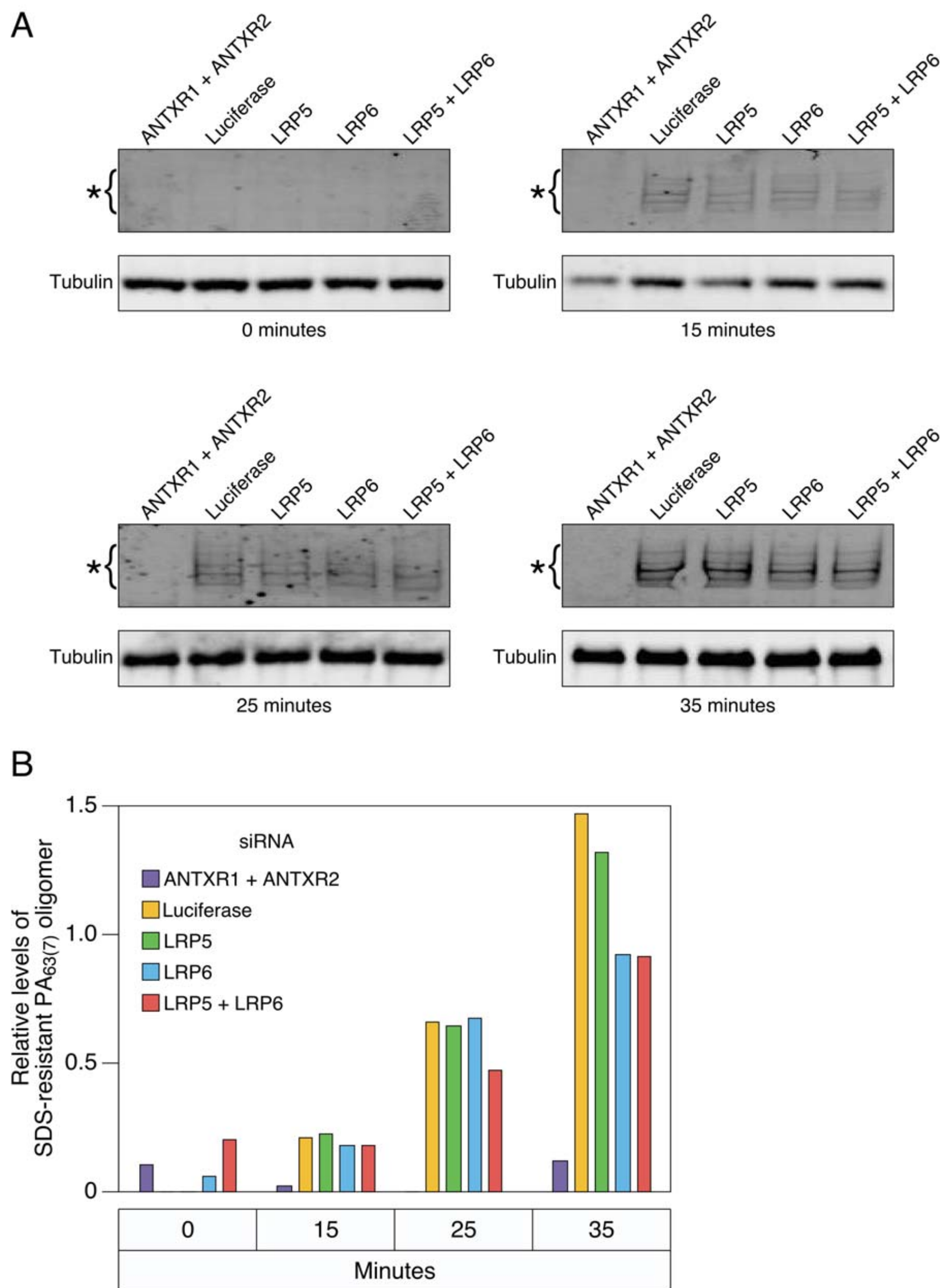

Figure 3. Reduced levels of LRP6 and/or LRP5 do not influence the kinetics of $PA_{63(7)}$ pore formation in HeLa cells. A) The kinetics of PA pore formation in siRNA-transfected HeLa cells was evaluated as described in materials and methods. The cells were lysed at different time points after initiating toxin entry (0, 15, 25, 35 mins) and the protein lysates subjected to SDS-PAGE and immunoblotting with anti-PA antibody to detect the SDS-resistant oligomeric $PA_{63(7)}$ pore species. (B) Quantitation of the levels of SDS-resistant pore species in the different time point samples shown in panel A. As described in materials and methods, the relative levels shown were determined by comparing the pore:tubulin ratio in each sample. The experiment shown is representative of three independent experiments.
doi:10.1371/journal.pone.0001817.g003

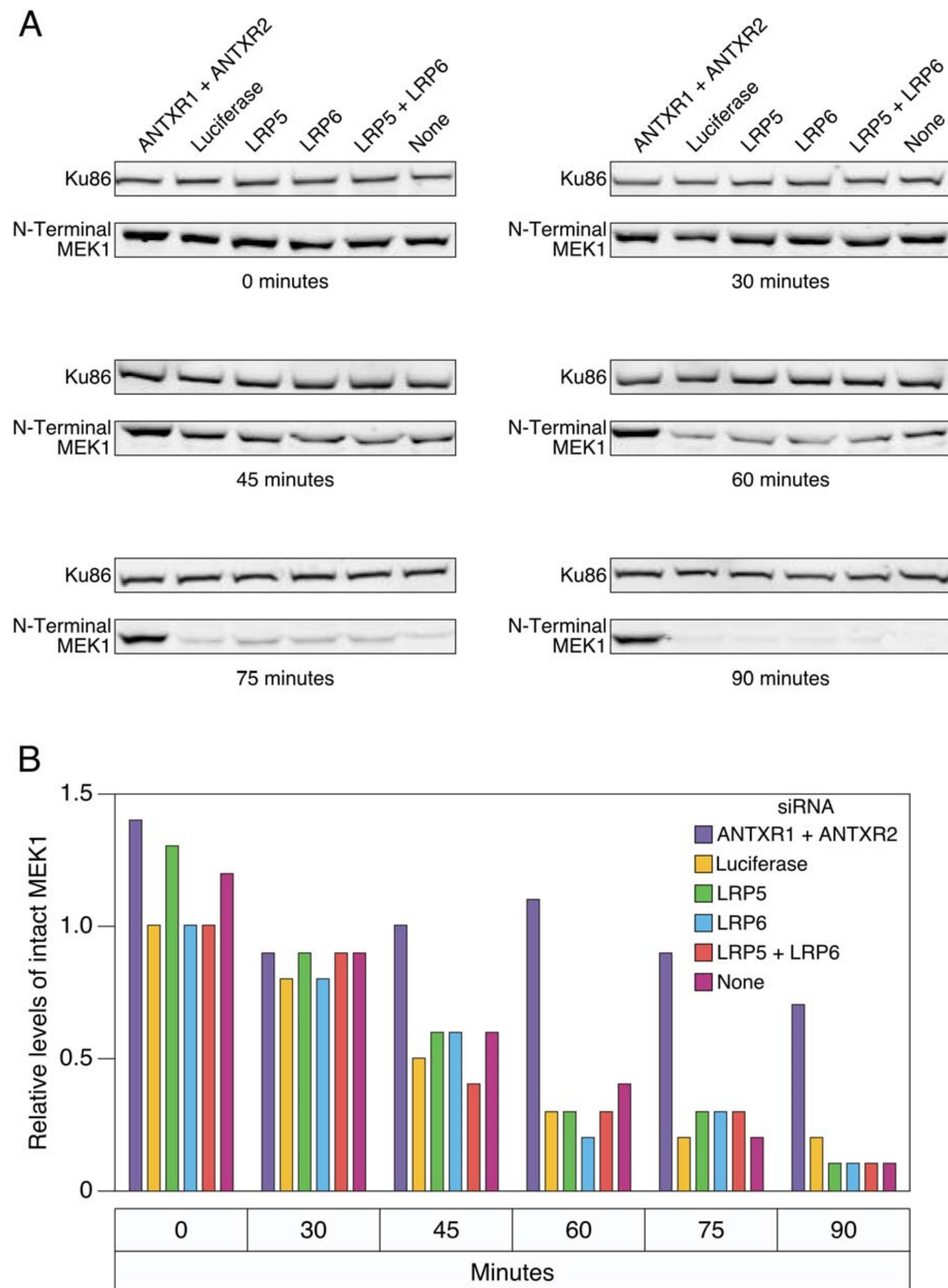

Figure 4. Reduced levels of LRP6 and/or LRP5 do not influence the kinetics of MEK1 cleavage in HeLa cells. A) HeLa cells transfected with different siRNAs were incubated with LeTx. Protein lysates were prepared at different time points and subjected to SDS-PAGE and immunoblotting with an anti-N-terminal MEK-1 antibody. Ku86, which is not an LF substrate, was also immunblotted as a loading control. B) The relative signal obtained with the N-terminal MEK1 antibody was compared with that associated with Ku86 in each sample shown in panel A. The experiment shown is representative of three independent experiments.
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siRNA (CGUCAAAGCCAUCGACUAUUU, CGUCAUGGGUG-GUGUCUAUUU, GGACGGACCUACGGAGGAUUU, GUAC AGGCCCUACAUCAUUUUU), ANTXR1 On Target SmartPool siRNA (CCAGUGAGCAGAUUUAUUAUU, GCUAAUAGGU-CUCGAGAUCUU, GAAGAAGUCCUGCAUCGAAUU, GGAA-CAACCUUAAUGAAACUU) ANTXR2 On Target SmartPool siRNA (Dharmacon GUAAAGGCUUGGAGGAUUUU, GCUA GCGAAUGAACAAAUUUU, GGGCUAGUGUUUAUUGUGU UU, UAUACUAGCUCAGUCAUGUUU) and GL2 firefly luciferase siRNA (target DNA sequence: CGTACGCGGAATACT TCGA)

LRP5 and LRP6 protein expression analysis

Approximately 5×10^4 HeLa cells plated in individual wells of a 12-well plate were reverse-transfected with different siRNA pools targeting LRP5, LRP6, both LRP5 and LRP6, or firefly luciferase (negative control). After 48 hours, cells were lysed with reducing gel sample buffer containing 2% SDS and 100 mM DTT [24]. Lysate samples were separated by denaturing SDS-PAGE and transferred to PVDF membranes (Millipore). These membranes were blocked and incubated with antibodies in TBS-T containing 5% milk: The same conditions were used for all immunoblotting experiments described in this report. The antibodies used were the LRP5-specific (Rabbit anti-LRP5, Zymed) diluted 1:200 and the LRP6-specific (LRP6 C5C7), Cell Signaling Technologies) diluted 1:1000. Tubulin was detected as a loading control (α/β-Tubulin antibody, Cell Signaling Technologies) diluted 1:1000. The Alexa Fluor 680-conjugated secondary antibody used in each case was the goat anti-rabbit IgG (Alexa Flour 680 goat anti-rabbit IgG, Invitrogen) diluted 1:20,000. The samples were then scanned and analyzed with the fluorescence-scanning Odyssey system and its associated software (Li-Cor).

RT-PCR analysis of LRP5 and LRP6 mRNA expression

Approximately 1×10^5 HeLa cells plated in individual wells of a 6-well plate were reverse-transfected with the different siRNA pools described above. RNA was harvested using the Qiagen RNeasy kit at 48 hours post siRNA transfection for RT-PCR analysis: The cDNA synthesis was performed with the SuperScript III reverse transcriptase system (Invitrogen) using 2 μg of total RNA from each sample and random hexamers as primers. PCR amplification was subsequently performed for 40 cycles (95°C for 15 seconds, 60°C for 1 minute) with an ABI Prism 7900HT instrument and with primers obtained from the validated library of Qiagen Quantitect primers along with Sybr green PCR master mix (Applied Biosystems). The relative levels of LRP5 or LRP6 mRNA transcripts were then determined using the Comparative Ct ($\Delta\Delta$Ct) method using GAPDH (CCTCTGACTTCAACAGC-GACAC, TTCCTCTTGTGCTCTTGCTGG) as an endogenous mRNA control.

Cell intoxication assays

Triplicate samples of approximately 5×10^3 HeLa cells seeded in individual wells of a 96-well plate were reverse-transfected with the different siRNAs. After 48 hours, cells were incubated with 5×10^{-10}M LF_N-DTA and with varying concentrations of PA for 48 hours [18]. Cell viability was then assayed with the Cell Titer-Glo assay (Promega) as previously described [24].

SDS-resistant pore formation

Formation of the SDS-resistant $PA_{63(7)}$ pore species was detected by immunoblotting as previously described [24], except cells were incubated with 2.5×10^{-8}M PA and 2×10^{-9}M LF for 2 hours at 4°C, washed and shifted to 37°C for the indicated times. Cells were lysed with reducing gel sample buffer, protein lysates were subjected to SDS-PAGE and SDS-resistant pore was detected with anti-PA (Goat anti-PA, List Labs) diluted 1:2,000. The secondary antibody used in this case was the Alexa Fluor-680 conjugated rabbit anti-goat (Invitrogen) diluted 1:20,000. The immunoblotting conditions were the same as described above and western blot scanning and analysis was conducted using the Odyssey system and its associated software (LiCor).

MEK1 Cleavage assays

MEK1 cleavage was detected by immunoblotting as previously described [24], except that samples were collected directly with reducing gel sample buffer. Membrane blocking and antibody dilutions were performed with TBS-T containing 5% milk as described above. The N-terminal MEK1 antibody (Anti-MEK1 N-terminal) was obtained from Calbiochem (used at 1:1,000) and the Ku86 antibody (Ku-86 B-1) was obtained from Santa Cruz Biotechnology (used at 1:500 dilution). Alexa Fluor-680 conjugated anti-Rabbit IgG and Alexa Fluor-680 conjugated anti-mouse IgG (Invitrogen) were used as secondary antibodies (both diluted 1:20,000). Western blot scanning and analysis was conducted using the Odyssey system and its associated software (LiCor).

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Author Contributions

Conceived and designed the experiments: JY PR. Performed the experiments: PR. Analyzed the data: PR. Wrote the paper: JY PR.

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3.3 Conclusions and subsequent LRP6 studies

siRNA-mediated knock-down of LRP6 had no effect on the kinetics of toxin pore formation or Mek1 cleavage. Based on these data, I concluded that LRP6 is not absolutely required for anthrax toxin entry into cells.

After our study was published, a fourth group led by Gisou van der Goot also found that that LRP6 is not absolutely required for toxin entry and plays no role in toxin binding to cells. In contrast to our results, they showed that LRP6 does appear to enhance internalization of the toxin/receptor complex (Abrami et al., 2008). In cells where LRP6 was overexpressed, ~15% more MEK1 cleavage was observed after 40 minutes compared to wild-type cells. In the absence of LRP6 expression, internalization kinetics are reduced. This group confirmed that LRP6 associates with ANTXR1 and ANTXR2 and made the surprising observation that LRP6 expression depends on that of the receptors, as downregulating receptor expression also led to downregulation of LRP6 expression. Possible reasons for the discrepancies between our results are discussed below.

3.4 Follow-up studies and models of LRP6 action

Since my results did not demonstrate a role for LRP6 in anthrax toxin internalization in HeLa cells, I attempted to resolve the conflicting LRP6 studies with various models of LRP6 action outlined below. Preliminary results pertaining to these models are presented here. After the van der Goot study

was published, our labs exchanged HeLa cells. Preliminary results concerning the differences between our HeLa cell populations are also presented here.

3.4.1 HeLa receptor expression.

Contrary to our findings, Abrami et al. found LRP6 has an effect on the kinetics of PA pore formation in HeLa cells. This may be due to differences between our HeLa cells, designated here as $HeLa^{JY}$, and the van der Goot Hela cells, designated here as $HeLa^{vdG}$, as the kinetics of toxin entry occurred at a faster rate in the $HeLa^{vdG}$ cells. Specifically, in $HeLa^{vdG}$ cells treated with toxin for 1hr at 4° and shifted to 37°, Mek1 cleavage begins within 10 minutes of the shift to 37° and is complete within 20-40 minutes (Abrami et al., 2008). In $HeLa^{JY}$ cells treated with toxin for 2hrs at 4° and shifted to 37°, MEK1 cleavage is not completed until somewhere between 75 and 90 minutes (Ryan and Young, 2008). Additionally, siRNA-mediated depletion studies indicate that $HeLa^{JY}$ cells require ANTXR1 for intoxication, while $HeLa^{vdG}$ cell use either ANTXR1 or ANTXR2 (Fig 3.1). These differences in receptor expression and toxin internalization kinetics may affect the role of LRP6 in intoxication.

3.4.2 Model 1: LRP6 plays a receptor-specific role in anthrax intoxication.

Thus far, human M2182 cells are the only cell-type to exhibit a profound reduction in anthrax intoxication in the absence of LRP6 expression. M2182 cells are thought to predominantly express ANTXR1, though the specific receptor isoform(s) has not been determined (Young et al., 2007). As mentioned previously,

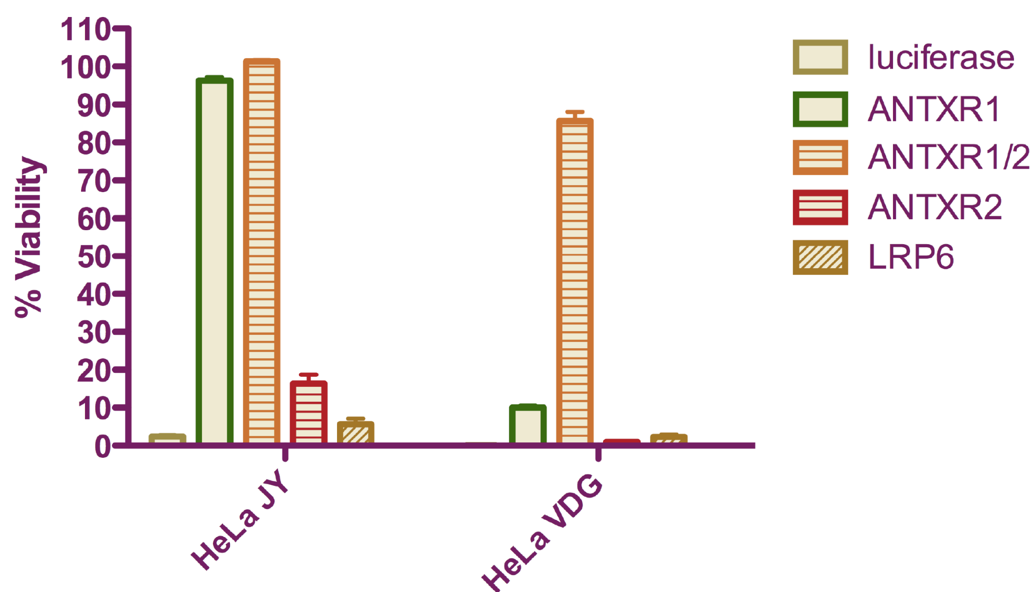


Figure 3.1.Different HeLa cell populations express different levels of receptor. $HeLa^{VDG}$ and $HeLa^{JY}$ cells were reverse transfected with siRNAs as indicated and challenged with $2.5x10^{-9}$ M PA and $5x10^{-10}$ M LF_NDTA. Viability was assessed 48 hours after toxin addition and normalized to a no toxin control.

the tail of ANTXR1 sv2 is truncated compared to the tails of ANTXR1 sv1 and ANTXR2. Given this lack of full-length receptor tail, it is possible that LRP6 overcomes an ANTXR1 sv2-mediated toxin internalization defect. Additionally, given the weaker PA binding affinity exhibited by ANTXR1, it is possible that LRP6 may play an important role in PA binding to this type of toxin receptor. Though no direct binding between LRP6 and PA has been observed, LRP6 enhances PA binding in M2182 cells (Wei et al., 2006). In receptor-negative CHO cells engineered to express high levels of either human ANTXR1 (splice variant not specified) or human ANTXR2 (splice variant not specified), LRP6 does not affect toxin internalization. This indicates that LRP6 does not affect ANTXR1-mediated intoxication, but this could be due to the artificially high levels of ANTXR1 or species-specific barriers blocking interactions between hamster LRP6 and the human receptor.

In order to rule out a receptor-specific role for LRP6, HeLa cells were engineered to express siRNA-resistant forms of ANTXR1 (sv1 or sv2) or $ANTXR2^{488}$. These cell lines allow us to knock down endogenous receptor in human cells and study the effect on LRP6 on each receptor type. Intoxication assays with these cells show that the absence of endogenous LRP6 expression has no effect on intoxication (Fig. 3.2A). However, the caveat in these studies is that the receptors are overexpressed (Fig 3.2B). This does argue that LRP6 is not absolutely required for toxin entry mediated by any receptor type in human cells,

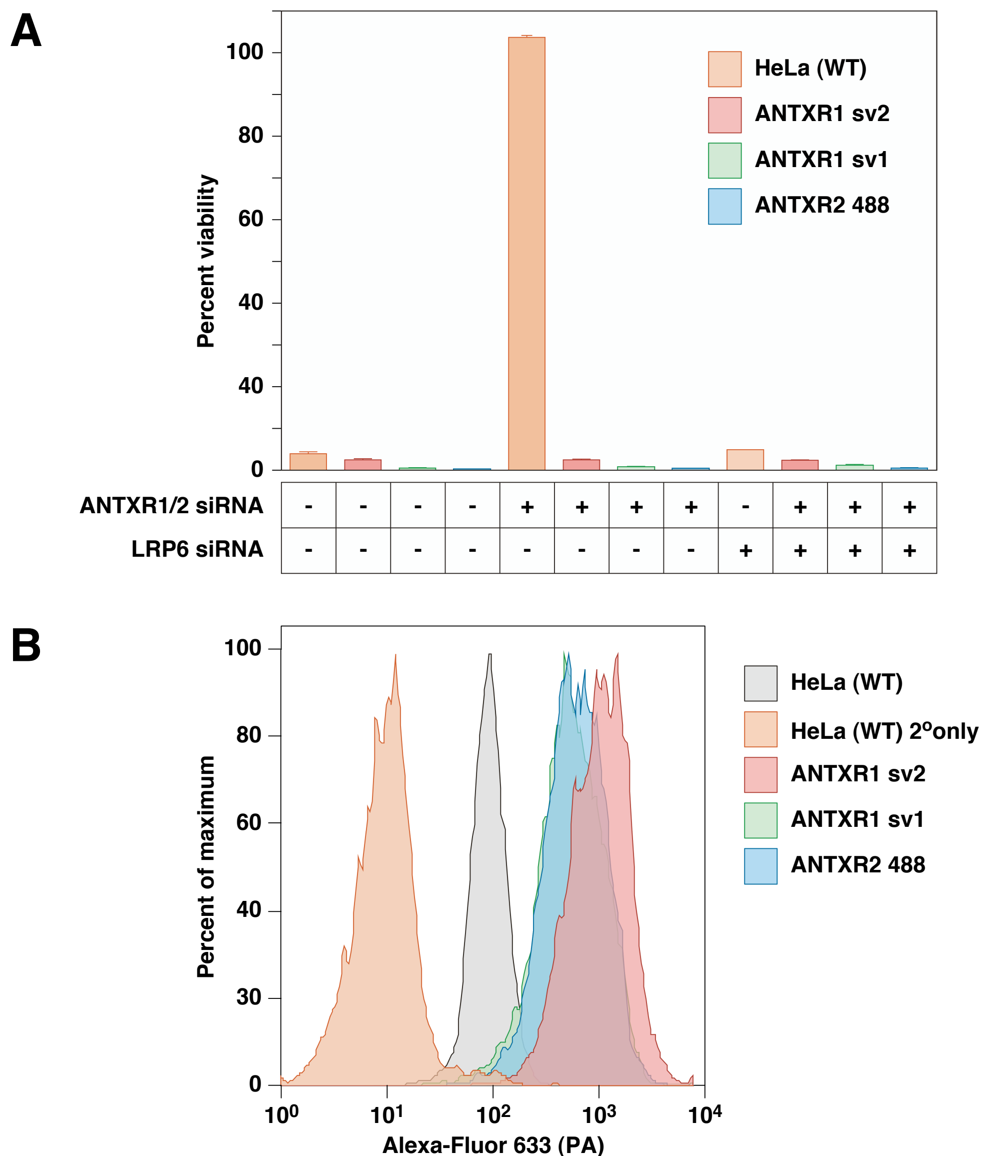


Figure 3.2. HeLa cells engineered to express siRNA resistant receptors do not require LRP6 for anthrax toxin internalization. A) HeLa cells were engineered to express different types of siRNA resistant anthrax toxin receptor as indicated. Cells were reverse transfected or not with siRNAs targeting LRP6 or endogenous receptor and after 48 hours challenged with 1.25×10^{-9} M PA and 3×10^{-10} M LF_NDTA. Viability was assayed 48 hours after toxin addition. B) PA binding was measured in cells engineered to express different siRNA resistant anthrax toxin receptors. Cells were incubated with 1×10^{-7} M PA, rabbit anti-PA antibody and goat anti-Rabbit IgG conjugated to Alexa-fluor 633. Fluorescence was measured by flow cytometry.

but in order to address more modest effects of LRP6 these cells must first be sorted for endogenous levels of receptor expression.

3.4.3 Model 2: The ratio of LRP6 to receptor expression determines efficiency of anthrax toxin entry.

A recent report showed that LRP6 plays a role in efficiently internalizing toxin in HeLa cells. It is possible that LRP6 expression levels vary between the two HeLa cell lines cultured in our respective labs and could account for differences seen in our results. If LRP6 works to recruit endocytic machinery to the toxin/receptor complex, cells that express a relatively low amount of LRP6 compared to receptors might not exhibit a discernable effect on toxin entry when LRP6 expression is reduced.

3.4.4 Model 3: ANTXR1 and LRP6 interaction is required for LRP6 modulation of pore formation.

Abrami et al. hypothesized that LRP6 acts as an efficiency factor by recruiting endocytic machinery to the toxin/receptor complex. Two groups have shown that ANTXR1 and ANTXR2 co-IP with LRP6. It is possible that the association of LRP6 with receptor is disrupted in our cells and under these conditions the absence of LRP6 expression does not alter the kinetics of toxin entry. Testing receptor/LRP6 association via co-IP in both cell lines would answer this question.

3.5 Summary and Discussion

Table 3.1 summarizes the results from various labs concerning the role of LRP6. All studies subsequent to the initial Wei et al. paper identifying LRP6 as an essential coreceptor are in agreement that it is not an essential factor for anthrax toxin binding and internalization, although differences remain over whether LRP6 plays a modest role in the kinetics of anthrax toxin internalization.

To date, most studies of LRP6 have been performed in immortalized cell lines. Given the disparate results concerning the effects of LRP6 on anthrax toxin entry among cell lines tested, it will be most instructive to determine whether LRP6 plays a role in anthrax toxin entry into cells that are relevant to anthrax intoxication, such as human endothelial cells and primary macrophages.

Chapter 3 contains a reprint of a paper as it appears in PLoSOne. Ryan, P.L., Young, J.A. *Evidence against a human cell specific role for LRP6 in anthrax toxin entry.* PLoS One, 2008. 3(3): p. 1817

Table 3.1. Effects of reducing LRP6 expression on intoxication. Results from LRP6 studies are shown. NA = Not addressed.

Author	Cell Type	Toxin Binding	Pore formation	Mek1 cleavage	Intoxication
Wei et al., 2006	M2182	Strongly Reduced	Strongly Reduced	NA	Strongly Reduced
Wei et al., 2006	Raw	NA	NA	NA	Modestly Reduced
Young et al., 2007	CHO	NA	NA	No Effect	No Effect
Young et al., 2007	MEFs (LRP6 KO)	NA	NA	No Effect	No Effect
Ryan & Young, 2008	HeLa	NA	No Effect	No Effect	No Effect
Abrami et al., 2008	HeLa	No Effect	Reduced	Reduced	NA

Chapter 4.

Identifying other host factors involved in anthrax toxin entry.

4.1 Background.

The work in this chapter will contribute to a manuscript in preparation. This chapter contains the results of a targeted siRNA screen for factors involved in anthrax toxin entry into HeLa cells and subsequent studies on these factors.

4.2 Introduction

Bacillus anthracis is a gram positive, spore-forming bacterium and causes the disease anthrax. *B. anthracis* produces a harmful exotoxin, termed anthrax toxin. Anthrax toxin is an AB-type toxin and consists of three proteins, protective antigen (PA), lethal factor (LF) and edema factor (EF). PA acts as the B-moiety and is responsible for receptor binding and translocation of the A moieties, LF and EF. LF is a zinc-metalloprotease that cleaves mitogen-activated protein kinase kinases (MEKs) and EF is an adenylate cyclase that raises cAMP levels (Leppla, 1982; Vitale et al., 1998). EF and LF combine with PA to form lethal toxin (LT) and edema toxin (ET), respectively.

Both 83 kD and 63 kD forms of PA can bind to either of two identified cellular receptors, ANTXR1 or ANTXR2 (Bradley et al., 2001; Scobie et al., 2003). Receptor bound PA_{83} is processed by a furin-like protease into a 63 kD form. PA_{63} oligomerizes into either a heptamer or an octamer, producing 3 or 4 binding sites, respectively, for EF and LF. The toxin receptor complex is internalized primarily via clathrin-mediated endocytosis and acidic pH

encountered within the endosome promotes PA pore formation and toxin translocation (Milne and Collier, 1993). It has been suggested that PA preferentially forms pores in the membrane of the intraluminal vesicles, resulting in toxin translocation into the lumen of these vesicles. Intraluminal vesicles then back-fuse with the limiting endosomal membrane allowing for toxin release into the cytosol (Abrami et al., 2004).

Several cellular factors play a role in anthrax toxin internalization. ARAP3 promotes toxin/receptor complex endocytosis through an as yet undefined mechanism (Lu et al., 2004). COPI and Alix are involved in intraluminal vesicle generation and facilitate toxin trafficking (Abrami et al., 2004; Abrami et al., 2003). It is likely that the anthrax toxin internalization involves even more cellular components that remain to be discovered.

Vesicular stomatitis virus (VSV) enters cells via a low-pH dependent pathway that is proposed to be similar to that used by anthrax toxin (Le Blanc et al., 2005). VSV glycoprotein (VSV-G) binds an unidentified receptor and the virus/receptor complex is taken up by clathrin-mediated endocytosis. In addition to being responsible for receptor binding, VSV-G undergoes a pH-dependent conformational change that is required for fusion of the viral envelope with the host membrane (Blumenthal et al., 1987). The acidic endosomal environment promotes VSV-G mediated fusion of the viral envelope with the membrane of intraluminal vesicles, resulting in capsid

release into the vesicle. Backfusion of the intraluminal vesicle with the limiting endosomal membrane results in capsid release into the cytosol.

A genome-wide siRNA library targeting approximately 20,000 human genes with six distinct siRNAs per gene was screened for host factors involved in VSV-G pseudotyped HIV and MLV retrovirus infection (Konig et al., 2008). Hundreds of candidate factors were identified, and preliminary staging experiments were performed by screening a subset of the hits for their involvement in 10A1 pseudotyped retrovirus infection. The 10A1 envelope mediates entry into cells in a pH-independent manner, likely by mediating fusion directly at the plasma membrane, thus candidate genes that have an effect on both 10A1 mediated retrovirus infection and VSV-G mediated retrovirus infection likely act at a stage post entry. Those factors that affect VSV-G mediated retrovirus infection but have an insignificant effect on 10A1-mediated retrovirus infection likely play a role in VSV-G mediated retrovirus entry.

Since anthrax toxin and VSV are proposed to use the same entry pathway to gain access to the cell, we hypothesized that they might share similar host-factor requirements for entry. In order to test whether cellular factors involved in VSV-G mediated retroviral entry and anthrax toxin entry overlap, 39 candidate genes from the VSV-G pseudotyped retrovirus screen described above were selected and screened for their involvement in anthrax

toxin internalization. This report identifies and characterizes those factors that were found to play a role in anthrax toxin intoxication.

4.3 Results

Thirty-nine candidate genes from the VSV-G pseudotyped retrovirus study were selected for further analysis based on several criteria. One subset of candidates was chosen if at least two of the siRNAs targeting the gene reduced infection of VSV-G pseduotyped retrovirus infection by at least 50%, and did not have a significant effect on 10A1 pseudotyped retrovirus infection (Table 4.1). Another subset of candidate genes was chosen based on their relative ranking as measured by a Redundant siRNA Activity (RSA) algorithm (Table 4.2) This algorithm identifies multiple and distinct siRNAs targeting the same gene that have a negative effect on VSV-G mediated infection and ranks genes based on these criteria (Konig et al., 2007). Candidate genes in this subset were included based on an RSA ranking of 5000 or less, which means they were among the top 5,000 genes whose cognate siRNAs had a negative effect on infection. Some candidates fulfilled both types of selection criteria. In addition to the criteria outlined above candidates were selected if they appeared to have a previously noted role in endocytosis, trafficking or membrane fusion. Candidates were excluded if they were required for cell viability. One additional factor, EHD1, was chosen based on its ability to reduce VSV-G psuedotyped HIV infection in post-screen follow-up

Table 4.1. One subset of candidates was chosen if at least two of the siRNAs targeting the gene reduced infection of VSV-G pseudotyped retrovirus infection by at least 50% and did not have a significant effect on 10A1 pseudotyped retrovirus infection. NA = Not applicable.

Gene Name	MLV infection ≤50%	HIV infection ≤50%	10A1infection ≥65%
AP1G2	√	√	√
B4GALNT4	√	√	√
CLTA	√	√	√
EXOSC10	√	√	√
FBLIM1	√	√	√
FER1L3	√	√	√
KCNJ11	√	√	√
MPDZ	√	√	√
NPHP3	√	√	√
NUMBL	√	√	√
PPM1K	√	√	√
STXBP1	√	√	√
TPPP	√	√	√
UBE2H	√	√	√
UBE2L3	√	√	√
CDC42EP3	√	√	NA
DMXL1	√	√	NA
DOCK10	√	√	NA
GANAB	√	√	NA
MAP4	√	√	NA
MDS032	√	√	NA
MID1IP1	√	√	NA
PTPRN2	√	√	NA
EHD1		√	NA

Table 4.2. One subset of candidate genes was chosen based on their Redundant siRNA Activity (RSA) algorithm ranking. This algorithm identifies multiple siRNAs targeting a specific gene that have a negative effect on infection and ranks genes based on these criteria.

Gene Name	RSA HIV ≤5000	RSA MLV ≤5000
CLTC	√	√
TUBA1B	√	√
REPS1	√	√
Rab11B	√	√
ACTC1	√	√
PTK2	√	√
CDKL3	√	√
SEPT2	√	√
ANKRD7	√	√
MAP1LC3C	√	√
NCKAP1	√	√
Rab17	√	√
ARL1	√	√
RABEP1	√	√
TSNAX	√	√
DGKB	√	√

experiments and its noted involvement in trafficking. For each candidate gene, 2 to 4 independent siRNAs were tested in the screen (Appendix Tables 1&2). In order to test each candidate for a role in anthrax toxin entry, HeLa cells were reverse-transfected with siRNAs and after 48 hours challenged separately with a PA + LF_NDTA intoxication screen and a PA+LF MEK1 cleavage screen (see Chapters 2 and 3). Nine of the 37 candidate genes were implicated in intoxication because depletion by at least two independent cognate siRNAs yielded a 2.5-fold increase in viability in the LF_NDTA intoxication screen (Fig. 4.1) (Appendix Tables 1&2). All but one of these nine hits from the intoxication screen were corroborated in the MEK1 cleavage screen (see below and Fig. 4.3 and Fig 4.4). siRNAs targeting Tubulin alpha 1B (TUBA1B) protected cells from intoxication but not MEK1 cleavage. It is possible TUBA1B siRNAs protect cells from intoxication at a stage post entry or are LF_NDTA specific, and this candidate was excluded from further study. Additionally, two siRNAs targeting ankyrin repeat domain 7 (ANKRD7) protected cells from LF-induced MEK1 cleavage, though this gene didn't qualify as a hit in the intoxication screen. This could be explained if ANKRD7 depletion slows or partially inhibits toxin entry, but does not block it completely. Thus, ANKRD7 was included as a candidate factor for further study. Many of the siRNAs targeting these candidates have been validated. Preliminary results indicate siRNAs depleted target mRNA, as expected (Figure 4.2)

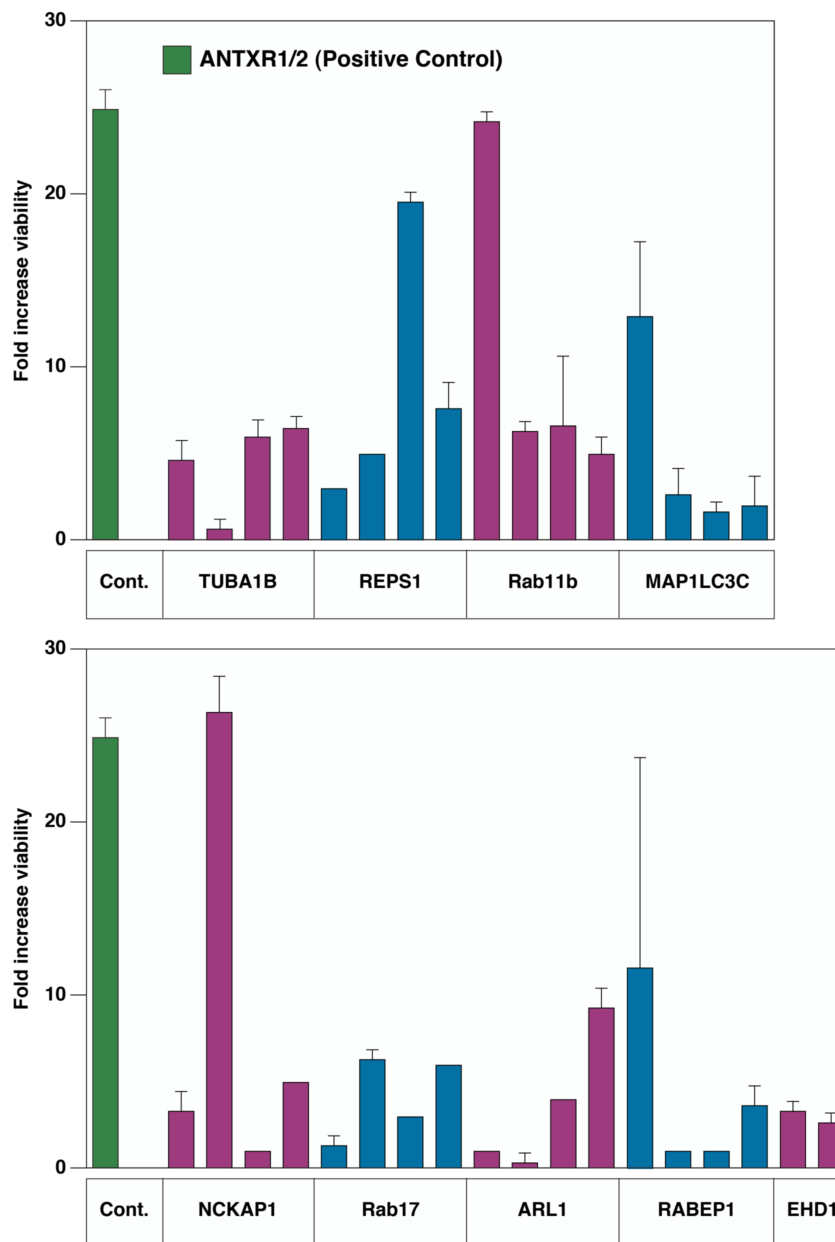


Figure 4.1. Multiple siRNAs targeting specific genes block intoxication. Multiple siRNAs targeting the genes indicated were reverse transfected into HeLa cells. After 48 hours cells were challenged with 1.25 $x10^{-10}$M PA and 2.5 $x10^{-10}$M LF_NDTA. Cell viability was measured 48 hours after toxin addition and was normalized to a no toxin control. If at least 2 siRNAs targeting a gene conferred a 2.5-fold increase in viability over the negative control siRNA, that gene was defined as positive.

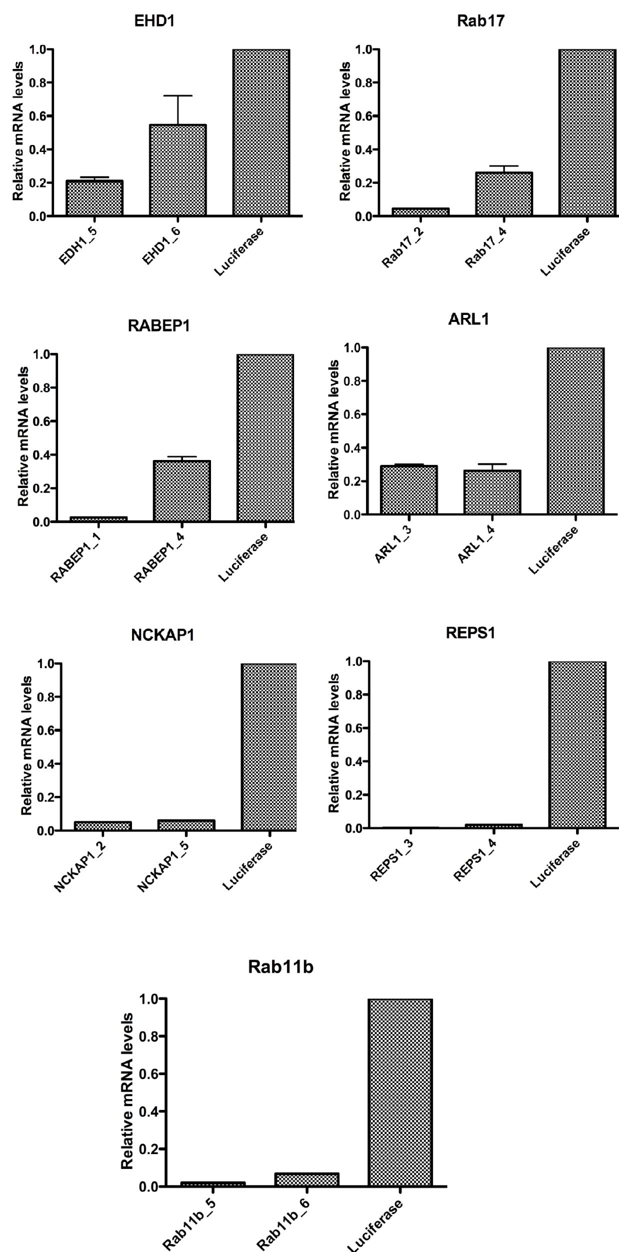


Figure 4.2. RT-PCR analysis of mRNA expression. siRNAs were reverse transfected into HeLa cells as indicated along with negative control siRNA luciferase. After 48 hours, RNA was harvested and RT-PCR analysis was performed. Relative mRNA levels were calculated with either the ΔΔCt method or standard curve analysis.

(Table 4.3)

To test each of these factors for involvement in PA binding to cellular receptors, siRNAs targeting the factors were reverse transfected into HeLa cells. After 48 hours, cells were harvested and incubated with PA on ice. Cells were stained using a primary PA antibody and a secondary antibody conjugated to AlexaFluor-633 and fluorescence was quantified with flow cytometry. A subset of siRNAs targeting candidate genes were found to play a role in PA binding, these candidate are: Eps homology domain containing 1 (EHD1), Rab11b, Rab GTPase-binding effector protein 1 (RABEP1), and RalBP1-associated Eps domain-containing protein 1 (REPS1) (Fig 4.3) (Table 4.1). All of these factors are involved in the endosomal recycling pathway. Of the two siRNAs targeting NCKAP1, one siRNA had little effect on PA binding and one siRNA reduced PA binding by 65%, despite similar levels of mRNA knockdown. The remaining candidate genes ARF-like GTPase (ARL1), Rab 17, Microtubule associated protein 1 light chain 3 gamma (MAP1LC3C) and ANKRD7 had no measurable effect on PA binding to cells, and presumably act at a later stage of the toxin internalization process (Fig. 4.4).

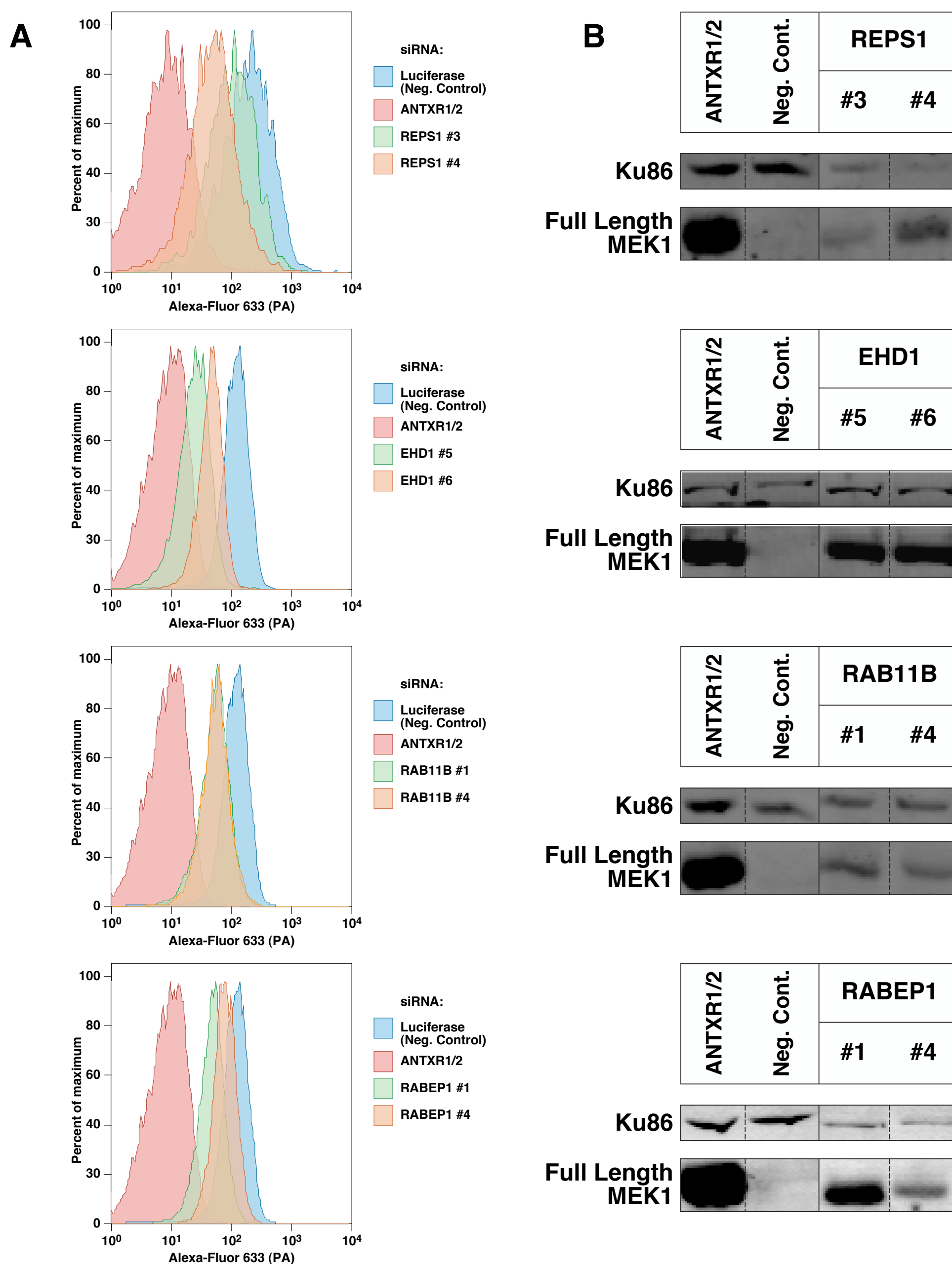


Figure 4.3. One subset of candidate siRNAs reduces PA binding to the cell surface. (A) siRNAs were reverse transfected into HeLa cells. After 48 hours cells were harvested with 1mM EDTA. Cells were incubated with $1x10^{-7}$M PA on ice then stained with Rabbit anit-PA antibody and goat anti-Rabbit IgG-AlexFluor 633 on ice. Fluorescence was assayed by flow cytometry. (B) siRNAs were reverse transfected into HeLa cells. After 48 hrs cells were challenged with $1.25x10^{-8}$M PA + $2.5x10^{-9}$M LF for 90 minutes. Cells were lysed and MEK1 cleavage was assessed with N-terminal MEK1 antibody immunoblotting.

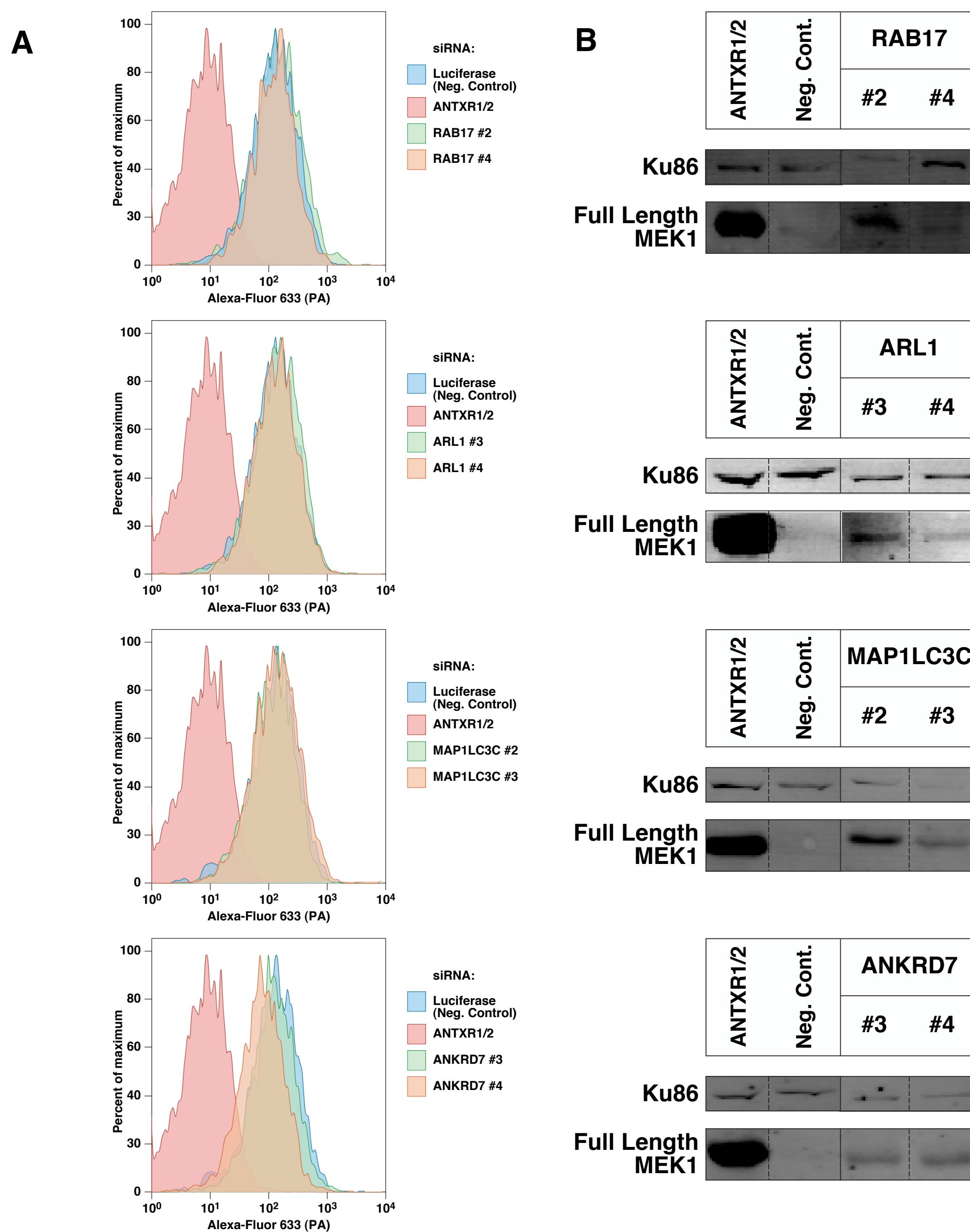


Figure 4.4. One subset of candidate siRNAs act at a stage post PA binding. (A) siRNAs were reverse transfected into HeLa cells. After 48 hours cells were harvested with 1mM EDTA and incubated with $1x10^{-7}$M PA on ice then stained with Rabbit anit-PA antibody and goat anti-Rabbit IgG-AlexFluor 633. Fluorescence was assayed by flow cytometry. (B) siRNAs were reverse transfected into HeLa cells. After 48 hrs cells were challenged with $1.25x10^{-8}$M PA + $2.5x10^{-9}$M LF for 90 minutes. Cells were lysed and MEK1 cleavage was assessed with N-terminal MEK1 antibody immunoblotting.

Table 4.3. Summary of results concerning candidate factors and cognate siRNAs.

Factor	mRNA (fold Knockdown)	Fold-increase viability	% reduction PA binding	Fold increase Full-length MEK1	Conclusions
ANTXR1/2 (+ control)	**20**	**24**	**90**	**20-30**	**N/A (+ control)**
Rab11b	10-50	5-24	50	5-11	Receptor cell surface expression
RABEP1	3-33	4-12	30-60	5-11	Receptor cell surface expression
EHD1	2-5	3-4	61-77	15-20	Receptor cell surface expression
REPS1	15-20	3-20	30-60	2-5	Receptor cell surface expression
Rab17	4-25	3-6	0	2	Internalization/trafficking
ANKRD7	Not tested	0	0-5	3-4	Internalization/trafficking
MAP1LC3C	Not tested	3-5	0	>2	Internalization/trafficking
ARL1	3-4	4-9	0	2-5	Internalization/trafficking
NCKAP1	10	3-26	5-65	2-4	Undetermined
TUBA1B	Not tested	4-7	Not tested	0	LF_NDTA specific

4.4 Discussion

This screen identified several factors involved in both VSV-G mediated retrovirus infection and anthrax toxin entry. Interestingly, preliminary results indicate that those siRNAs having the strongest effect on intoxication also reduce PA binding at the cell surface. These siRNAs target candidate factors involved in endocytic recycling: EHD1, Rab11b, RABEP1, REPS1. EHD1 is localized to membrane tubules that form part of the endocytic recycling compartment (ERC). EHD1 has been implicated in the recycling of several proteins, including transferrin receptor, beta-integrins, Major Histocompatability Class I (MHC-I) (Caplan et al., 2002; Jovic et al., 2007; Rapaport et al., 2006) and in the internalization of insulin growth factor receptor 1 (IGF-1) (Rotem-Yehudar et al., 2001). Rab11b is a small GTPase implicated in transferrin receptor recycling (Schlierf et al., 2000). REPS1 localizes to recycling endosomes and partners Rab11-Fip2, a Rab11 binding protein involved in endocytosis and recycling (Cullis et al., 2002). RABEP1 has been reported to localize to recycling endosomes and plays a role in transferrin receptor recycling (Deneka et al., 2003). It has been suggested that RABEP1 might be involved in sorting cargo into recycling and degradative pathways (Popa et al., 2005).

There are three destinations for proteins once they have been internalized and reach the early/sorting endosome. These are the plasma

membrane, the lysosome and the endocytic recycling compartment (ERC) (Grant and Donaldson, 2009). Proteins that return directly to the plasma membrane from the sorting endosome are said to pass through the "fast" recycling pathway. Those that pass through the ERC before returning to the plasma membrane via recycling endosomes are said to undergo "slow" recycling. Rab11b and EHD1 regulate the ERC, and thus play a role in the slow recycling pathway. REPS1 is found in recycling endosomes, which are also part of the slow recycling pathway. RABEP1 localizes to early/sorting endosomes and recycling vesicles and may play a role in both the fast and the slow recycling pathways.

Endocytic recycling is the major pathway by which internalized proteins are returned to the plasma membrane. Our data are consistent with a model in which the anthrax toxin receptors are internalized and sorted back to the plasma membrane via the recycling pathway. In the absence of a functioning recycling pathway due to siRNA-mediated depletion of critical recycling pathway components, the receptors are internalized but not returned to the plasma membrane, resulting in a reduced level of cell surface expression. To date, studies concerning anthrax toxin receptor internalization and trafficking have addressed this phenomenon in the context of anthrax toxin ligand binding. ANTXR1 binds to collagen type I and type VI and ANTXR2 binds to collagen IV and laminin (Bell et al., 2001; Nanda et al., 2004; Werner et al.,

2006). These molecules can be synthesized and secreted by HeLa cells (Furth et al., 1991), and it is possible receptor internalization occurs in response to natural ligand binding. It is also possible the receptors are undergoing ligand independent turnover.

In chapter 2 we put forth a model in which toxin bound to the ANTXR2 remains attached to the receptor until it reaches the late endosome. It is possible that in the absence of ligand or when bound to natural ligands, anthrax toxin receptor is internalized and shunted to the recycling pathway. Several other receptors display differential ligand-dependent sorting, including the Fc receptor and the epidermal growth factor receptor (Mellman et al., 1984; Roepstorff et al., 2009).

Four remaining factors do not have an effect on PA binding to cells and siRNAs targeting these factors presumably act at a step post-PA binding. These four factors are ARL1, Rab 17, MAP1LC3C and ANKRD7. ARL1 is widely conserved and localizes to the Golgi apparatus with some evidence pointing toward an involvement in trafficking from the endosome to the Golgi (Munro, 2005). Rab17 is expressed primarily in epithelial cells and is involved in transcytosis (Zacchi et al., 1998). ANKRD7 is not well characterized, though ankyrin repeat domains are involved in protein-protein interactions. MAP1LC3C is localized to autophagosomal membranes and plays a role in autophagy (Tanida et al., 2004). Interestingly, autophagy was recently

identified as playing a role in toxin release from late endosomal compartments (Ha et al., 2009).

4.6 Materials and Methods

Cell culture

HeLa cells were maintained in DMEM and 10% fetal bovine serum in the presence of penicillin, streptomycin and ciproflaxacin.

siRNA transfection

HeLa cells were reverse-transfected with 32 nM siRNA using RNAiMax (Life Technologies). siRNAs were obtained from Qiagen, except for ANTXR1 and ANTXR2 pooled siRNAs which were obtained from Dharmacon. ANTXR1 On Target SmartPool siRNA (CCAGUGAGCAGAUUUAUUAUU, GCUAAUAGGUCUCGAGAUCUU, GAAGAAGUCCUGCAUCGAAUU, GGAACAACCUUAAUGAAACUU) ANTXR2 On Target SmartPool siRNA (Dharmacon GUAAAGGCUUGGAGGAUUUU, GCUAGCGAAUGAACAAAUUUU, GGGCUAGUGUUUAUUGUGUUU, UAUACUAGCUCAGUCAUGUUU) and GL2 firefly luciferase siRNA (target DNA sequence: CGTACGCGGAATACT TCGA). Individual target sequences for remaining candidate genes are listed in Appendix Table 1.

Cell intoxication assay

Triplicate samples of approximately 15,000 HeLa cells were seeded into individual wells of a 96-well and reverse transfected with different siRNAs. After 48 hours, cells were incubated with 1.25×10^{-9} M PA and 2.5×10^{-10} M LF_NDTA for 48 hrs. Cell Viability was measured with Cell Titer-Glo (Promega) by removing incubation media and replacing with 50 μL per well Cell-Titer Glo

diluted 1:2 in PBS. Wells were assayed with a TopCount microplate scintillation counter. Measurements were normalized to a no toxin control.

MEK1 cleavage assay

Approximately 50,000 HeLa cells were seeded into 12-well plates and reverse-transfected with different siRNAs. After 48 hours cells were incubated with $1.25x10^{-8}$ M PA and $2.5x10^{-9}$ M LF in 500μL media. Cells were washed two times with PBS and collected with 200μL SDS reducing gel sample buffer. Samples were resolved on 10% Tris-Glycine SDS/PAGE and transferred to a polyvinylidene difluoride membrane. Membrane was blocked in PBS + 5% milk and detected with anti-MEK1 N-terminal antibody (Anti-MEK1 N-terminal, Calbiochem) diluted 1:1,000 in PBS + 5% milk and incubated overnight at 4°. Ku86 antibody (Ku-86 B-1, Santa Cruz Biotechnology) was used at 1:500 dilution and incubated overnight as described above. Alexa-Fluor-680 conjugated antibodies anti-Rabbit IgG and anti-mouse IgG were used as secondary antibodies (Life Technologies) and diluted 1:20,000. Western blot scanning and analysis was conducted using the Odyssey system and its associated software (LiCor).

PA binding assay

HeLa cells were seeded and reverse transfected into 12-well plates as described above. After 48 hrs cells were harvested with 1mM EDTA in PBS, washed with media cold media and incubated with $1x10^{-7}$M PA on ice for one

hour. Cells were washed once with media, and incubated for one hour on ice with rabbit anti-PA antibody (Ken Bradley) diluted 1:1000 in media. Cells were washed once and incubated with Alexa-Fluor-680 conjugated anti-Rabbit IgG (Life Technologies) for 45 minutes diluted 1:500. Cells were washed in PBS three times and fixed with 1% formaldehyde. Fluorescence was assayed with an LSR I flow cytometer (Becton Dickinson).

RT-PCR analysis of mRNA expression

Approximately $1X10^5$ cells were seeded into individual wells of a 6-well plate and reverse transfected with different siRNAs. RNA was harvested using Qiagen RNeasy kit and QiaShredder 48 hrs after transfection. cDNA synthesis was performed with the SuperScript III reverse transcriptase system (Life Technologies) using 2μg of total RNA from each sample and random hexamers as primers. PCR amplicfication was subsequently performed for 40 cycles (95°C for 15 seconds, 60° C for 1 minute) with an ABI Prism 7900 HT instrument. The relative levels of mRNA transcripts were then determined using either comparative Ct or standard curve method using GAPDH as an endogenous mRNA control.

Chapter 5.

Discussion and Future Directions

Experiments described in this dissertation contain several critically important contributions to our understanding of how anthrax toxin enters cells. The data presented in Chapter 2 represent my work in collaboration with a Young lab postdoctoral fellow, Jonah Rainey, which revealed receptor-specific pH requirements for anthrax toxin entry. This challenged the assumption that the characteristics of toxin entry mediated by ANTXR1 and ANTXR2 were the same. These findings carried implications for the accepted model of anthrax toxin entry, which was previously generated by combining data from several studies in which receptor type was often undefined. Additionally, we contributed the first line of biochemical evidence in favor of the hypothesis that the toxin-receptor interaction is weakened upon pore formation. Finally, in contrast to the hypothesis that acidic pH was required for toxin translocation, we reported the unexpected finding that toxin can translocate under near neutral pH conditions, albeit with slower kinetics.

Experiments in Chapter 3 dealt with the role of the putative anthrax toxin co-receptor, LRP6, in anthrax toxin internalization. The role of LRP6, if any, continues to be controversial. By utilizing siRNAs that ablated LRP6 expression in Hela cells, my findings contributed to a growing consensus that LRP6 is not absolutely required for toxin entry into cells.

Experiments outlined in Chapter 4 identified several cellular factors involved in anthrax toxin entry. Among these were several components of the

recycling pathway, which presumably control receptor expression at the cell surface. Additionally, I identified several other factors that play a role in intoxication at an as yet unidentified step downstream of PA binding.

5.1 Receptor specific pH requirements for anthrax toxin entry

Our findings outlined in Chapter 2 demonstrated that when bound to ANTXR1, PA oligomers form pores efficiently between pH 6.0-6.4, with some pore formation detectable at pH 6.8. In contrast, when PA binds ANTXR2, a much lower pH of 5.2 is required for pore formation. Consistent with this, toxin bound to ANTXR1 was capable of intoxicating cells in the presence of NH_4Cl, but toxin bound to ANTXR2 was not. This finding was unexpected since previous reports demonstrated a low-pH requirement for anthrax intoxication by showing lysosomotropic agents such as NH_4Cl block intoxication (Friedlander, 1986; Menard et al., 1996). However, studies demonstrating this low-pH requirement for entry were primarily done in mouse macrophage cell lines, which we now know primarily express ANTXR2 (Young et al., 2007).

These studies were subsequently corroborated using a patch-clamp based assay to measure pore formation induced on the plasma membrane in cells engineered to express ANTXR1 or ANTXR2 (Wolfe et al., 2005). The patch-clamp assay measures ionic currents through pores, such as those formed by anthrax toxin. When pore formation was measured in cells engineered to express ANTXR1, efficient pore formation occurred at pH ~6.2.

When measured in ANTXR2 expressing cells, pore formation occurred at pH ~5.3.

The structure of the heptameric prepore revealed that the pore lumen is too narrow to translocate EF and LF in their folded state. Thus it is thought that EF and LF must unfold, at least partially, in order to pass through the pore. Prior to our work, it was thought a low pH environment (~pH 5.0) was absolutely required for LF and EF unfolding and translocation, based on studies that used artificial lipid membranes or artificially induced translocation across the plasma membrane (Kochi et al., 1994; Krantz et al., 2004; Wesche et al., 1998). Contrary to this hypothesis, we demonstrated that at near-neutral pH conditions, PA is still capable of translocating LF when it is associated with ANTXR1. The kinetics of MEK1 cleavage occur at a slower rate, and this may be due to slower kinetics of toxin unfolding and translocation in the presence of near-neutral pH conditions. This evidence is consistent with a model in which acidic pH facilitates unfolding, but is not required. Subsequent studies have demonstrated a transmembrane proton gradient is required for toxin translocation (Krantz et al., 2006). This is consistent with my results, as NH_4Cl should not completely disrupt the pH gradient - cytosolic pH (which is thought to be equivalent to the lumen of intraluminal vesicles where LF and EF are delivered) is thought to be ~ 7.3, and NH_4Cl is estimated to raise

intraendosomal pH to >6.5 (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981).

It has been suggested that the receptors act as a molecular clamp to inhibit pore formation by inhibiting the conformational changes that take place during prepore-to-pore conversion. Thus, it has been hypothesized that receptor contacts must be weakened or released, at least temporarily, in order for PA oligomers to form pores. Conversely, it has also been suggested the receptors might remain attached to the pore in order to provide structural support. The patch clamp studies outlined above demonstrated that receptor expression is required for pore formation, so understanding the receptor/PA interaction during the prepore-to-pore conversion is of great interest. Using co-immunoprecipitation experiments, Rainey showed that cell-surface PA oligomers interact with receptor, but once exposed to low pH, this interaction is no longer detectable. This supports the idea that receptor/PA interaction is weakened coincident with pore formation. NMR experiments published after this work have demonstrated that the ANTXR2 vWA-domain dissociates from oligomeric PA when exposed to low pH (Rajapaksha et al., 2009). Other immunoprecipitation experiments have detected receptor/PA interaction when pore formation occurred within endosomes (Abrami et al., 2006). Ongoing structural studies of oligomeric PA/receptor interactions at varying pH levels

will allow us to address the nature of receptor/PA interaction during the prepore-to-pore conversion.

5.2 LRP6 and anthrax toxin internalization

An initial study showed that LRP6 was important for efficient intoxication of human M2182 prostate carcinoma cells and played a modest role in intoxication of RAW 264.7 macrophages. LRP6 was required for proper PA binding and pore formation in these cells. However, other studies performed with cells from knockout mice demonstrated no role for either LRP6 or the related LRP5 protein in anthrax toxin entry. I hypothesized that LRP6 might play a human cell-specific role in anthrax toxin internalization or might modulate the kinetics of toxin entry. I directly tested this in studies presented in Chapter 3 and showed that the absence of LRP6 did not affect the kinetics of anthrax toxin entry into HeLa cells. In contrast to our report, a fourth group, led by Gisou Van Der Goot, showed that though LRP6 is not required for entry, it modestly enhances toxin/receptor complex internalization. The results of LRP6 studies published to date are summarized in Table 3.1.

Several possible models were outlined in chapter 3 concerning the role of LRP6 in toxin internalization that might resolve the conflicting LRP6 data summarized in Table 3.1. These include the possibility that LRP6 modulates entry in a receptor-specific manner, that the ratio of LRP6 to receptor determines its ability to modulate toxin internalization, and that the

LRP6/receptor interaction is required for LRP6 to modulate internalization and this interaction is disrupted in some cell lines. To date, most studies of LRP6 have been performed in immortalized cell lines. Given the disparate results concerning the effects of LRP6 on anthrax toxin entry among cell lines tested, it will be most instructive to determine whether LRP6 plays a role in anthrax toxin entry into cells that are relevant to anthrax intoxication, such as human endothelial cells and primary macrophages.

5.3 Other host cell factors involved in anthrax toxin intoxication.

Chapter 4 detailed a targeted siRNA screen for factors involved in anthrax toxin entry that identified nine cellular genes. siRNA-mediated depletion of four of these factors results in reduced PA binding at the cell surface. The remaining siRNAs target factors that play a role at an as yet undetermined step in the intoxication process.

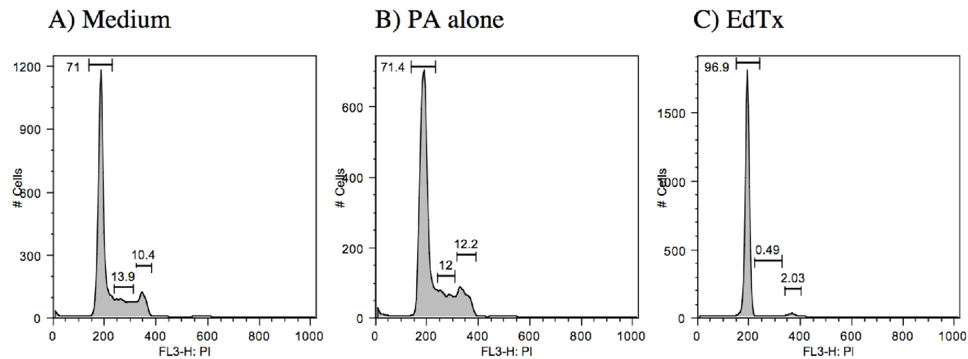
Four factors that play a role in PA binding are also part of the endocytic recycling pathway: EHD1, REPS1, RABEP1 and Rab11b. Endocytic recycling is the major pathway by which internalized proteins are returned to the plasma membrane. Presumably, the reduction in PA binding at the cell surface is caused by deficient recycling of the anthrax toxin receptors, resulting in a decreased level of receptor cell surface expression. Our data are consistent with a model in which the anthrax toxin receptors are internalized and recycled to the plasma membrane via the recycling pathway. It is also possible that

these factors affect the cell surface expression of other proteins required for PA binding. In order to rule out this possibility, it is imperative to detect anthrax toxin receptors at the cell surface directly. Given the lack of effective antibodies for detection of endogenous protein, receptor recycling will have to be measured using an epitope tagged protein. Early efforts in this regard proved challenging, as overexpression of the receptor ablated the effect of siRNAs targeting the recycling pathway (Appendix Figure 2). Using cells that express endogenous levels of tagged receptor will hopefully circumvent this problem. Alternatively, some success has been found with detecting overexpressed ANTXR2 with an antibody raised in chickens (Rainey, unpublished data). If this extends to endogenous levels of receptor this will be a useful tool in assessing ANTXR2 recycling.

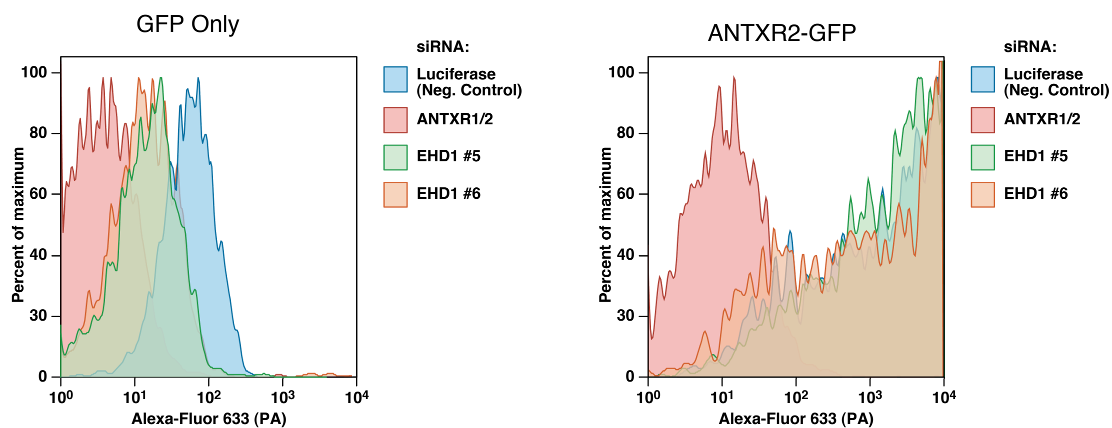
Studies done to date have assessed receptor trafficking in the context of anthrax toxin binding. ANTXR1 also binds to collagen type I and type VI and ANTXR2 binds to collagen IV and laminin (Bell et al., 2001; Nanda et al., 2004; Werner et al., 2006). These molecules can be synthesized and secreted by HeLa cells (Furth et al., 1991). It is possible that in the absence of ligand or when bound to natural ligands, anthrax toxin receptor is internalized and shunted to the recycling pathway. Several other receptors display differential ligand-dependent sorting, including the Fc receptor and the epidermal growth factor receptor (EGFR) (Mellman et al., 1984; Roepstorff et al., 2009).

Four remaining factors do not have an effect on PA binding to cells and presumably act at a step post-PA binding. These four factors are ARL1, Rab17, MAP1LC3C and ANKRD7. Further staging experiments should be performed in order to determine how each of these factors plays a role in anthrax toxin internalization. Assays that measure toxin/receptor complex lipid raft localization, receptor ubiquitination, toxin trafficking and pore formation would provide insight into the functions of these factors in intoxication. Interestingly, MAP1LC3C is thought to play a role in autophagy, a process recently identified as playing a role in toxin release from late endosomal compartments (Ha et al., 2009). One remaining factor, NCKAP1 appears to play a role in intoxication, but siRNAs targeting this factor have different effects on PA binding to cells. One siRNA has an insignificant effect on PA binding and one siRNA prevents PA binding to cells. Further studies, perhaps with additional siRNAs, will help resolve these inconsistencies and determine the role, if any, of NCKAP1 in anthrax intoxication.

APPENDIX I.



Appendix Figure 1. EdTx induces cell cycle arrest at G1 in RAW 264.7 macrophages. Cells were treated with A) medium B) PA or C) EdTx and collected after 24 hours. Cells were permeabilized and fixed with EtOH, treated with RNase, and DNA was stained with propidium iodide. Cells were analyzed by FACS and gated on the single ce population. DNA content for single cells is shown and percentage of cells in each phase is indicated.



Appendix Figure 2. HeLa cells engineered to express tagged receptor are not sensitive to disruption of the recycling pathway. HeLa cells engineered to express ANTXR2-GFP or GFP were reverse transfected with siRNAs targeting EHD1. 48 hours later, cells were harvested and incubated on ice with $1x10^{-7}$M PA, rabbit anti-PA antibody and Anti-Rabbit IgG-AlexaFluor633. Fluorescence was measured by flow cytometry.

Appendix Table 1. One subset (RSA) of candidate siRNAs sequences and corresponding viability results from one intoxication screen.

NCBI gene symbol	Entrez Gene Id	siRNA Target Sequence	Product Id	Product Name	well1	well2	well3	% viability(Avg)	St. Dev.
CLTC_11	1213	ACAGTTCTACTTAGAATTCAA	SI02651733	Hs_CLTC_11	11	7	7	8	2.3
CLTC_12	1213	CCGGAGAGATTTCTTCGTGAA	SI02651740	Hs_CLTC_12	3	4	4	4	0.6
TUBA1B	10376	TCCATCATATCTCAAAGTAAA	SI00084308	Hs_K-ALPHA-1_1	14	15	22	17	4.4
TUBA1B	10376	AAGGAGAGGAATACTAATTAT	SI00084329	Hs_K-ALPHA-1_4	1	2	2	2	0.6
TUBA1B	10376	CCCGCCCTAGTGCGTTACTTA	SI02636627	Hs_K-ALPHA-1_5	18	26	25	23	4.4
TUBA1B	10376	CAGCTTAACTGACAGACGTTA	SI02636634	Hs_K-ALPHA-1_6		25	29	27	2.8
REPS1	85021	TAGAAAGAGTTTCCCATTTAA	SI00701267	Hs_REPS1_1	12	13	11	12	1.0
REPS1	85021	ATGCAGGAAATGGAACTTAAA	SI00701274	Hs_REPS1_2	20	21	18	20	1.5
REPS1	85021	AACAAAGACAGTATTATGTAA	SI00701281	Hs_REPS1_3	80	78	77	78	1.5
REPS1	85021	ATGGCTATGATTTACCAGAAA	SI00701288	Hs_REPS1_4	36	33	25	31	5.7
RAB11B	9230	CACGGACGGACAGAAGCCCAA	SI00061145	Hs_RAB11B_1	101	94	94	96	4.0
RAB11B	9230	CGAGTTCAACCTGGAGAGCAA	SI00061166	Hs_RAB11B_4	26	23	24	24	1.5
RAB11B	9230	CCGCATCACCTCCGCGTACTA	SI02655296	Hs_RAB11B_5	35	36	7	26	16.5
RAB11B	9230	CCGCATCGTGTCACAGAAACA	SI02662695	Hs_RAB11B_6	23	20	17	20	3.0
ACTC1	70	TCCTAGCACCATGAAGATTAA	SI00291382	Hs_ACTC_2	4	4	3	4	0.6
ACTC1	70	TCCTAGCACCATGAAGATTAA	SI00291382	Hs_ACTC_1	5	5	4	5	0.6
ACTC1	70	CTGATCGTATGCAGAAGGAAA	SI00291389	Hs_ACTC_3	8	8	7	8	0.6
ACTC1	70	GACCAGGACTTGCAACCTAAA	SI00291396	Hs_ACTC_4	13	13	9	12	2.3
PTK2	5747	CCGGTCGAATGATAAGGTGTA	SI02622130	Hs_PTK2_10	1	1	1	1	0.0
PTK3	5747	CCCAGGTTTACTGAACTTAAA	SI02635584	Hs_PTK2_12	15	15	13	14	1.2
CDKL3	51265	TGGGCAGATAGTGGCCATTAA	SI02224782	Hs_CDKL3_5	1	1	18	7	9.8
CDKL4	51265	CAACATTATTGTCATGGACTA	SI02642311	Hs_CDKL3_6	8	9	7	8	1.0
SEPT2	4735	ACCCAGGACCTTCATTATGAA	SI00715029	Hs_SEPT2_3	47	39		43	5.7
SEPT2	4735	AACAAGTTTGCTAGTGACTAA	SI00715036	Hs_SEPT2_4	1	1	1	1	0.0
ANKRD7	56311	AAGGATGGGTATACTCCACTA	SI00296646	Hs_ANKRD7_2	7	6	5	6	1.0
ANKRD7	56311	AAGGATGGGTATACTCCACTA	SI00296646	Hs_ANKRD7_2	6	4	2	4	2.0
ANKRD7	56311	CACCTTATTCTTGGCACTACA	SI00296653	Hs_ANKRD7_3	2	2	1	2	0.6
ANKRD7	56311	CTGAGGGATATTCGTTATAAT	SI00296660	Hs_ANKRD7_4	83	71		77	8.5
MAP1LC3C	440738	AACCGTATTCTAAATAAGAAA	SI00580916	Hs_LOC440738_1	33	63	58	51	16.1
MAP1LC3C	440738	CTGCTCTTGAAAGTTATATAA	SI00580923	Hs_LOC440738_2	12	4	17	11	6.6
MAP1LC3C	440738	CCCGGTGGTAGTGGAGCGCTA	SI00580930	Hs_LOC440738_3	4	8	7	6	2.1
MAP1LC3C	440738	CGCAACCATGGCAGAGATCTA	SI00580937	Hs_LOC440738_4	4	2	14	7	6.4
NCKAP1	10787	CAGGCATATACTAGTGTCTCA	SI00102088	Hs_NCKAP1_1	8	14	16	13	4.2
NCKAP1	10787	TTCACTGAGATTATTCCTATA	SI00102095	Hs_NCKAP1_2		99	111	105	8.5
NCKAP1	10787	ACGCATGAACTATGTCCAGAA	SI00102102	Hs_NCKAP1_3	3	4	4	4	0.6
NCKAP1	10787	GAGGCTATAATAAACGTATTA	SI02640008	Hs_NCKAP1_5	20	18	18	19	1.2
RAB17	64284	TCGCCTGAGATATAAGTTGTA	SI00133756	Hs_RAB17_1	4	6	5	5	1.0
RAB17	64284	CAGGAAGGATTCCTTCCTCAA	SI00133763	Hs_RAB17_2	23	27	23	24	2.3
RAB17	64284	CAGCCTCTGGACAGAGAGGAA	SI00133770	Hs_RAB17_3	11	11	12	11	0.6
RAB17	64284	AAGTGAGATCCTGGAAGTGAA	SI00133777	Hs_RAB17_4	23	23	24	23	0.6
ARL1	400	CAGCGTTTGGATATTGATATA	SI00303205	Hs_ARL1_1	2	2	2	2	0.0
ARL1	400	CACAGTCTAACTCTTAAATAA	SI00303212	Hs_ARL1_2	1	2	1	1	0.6
ARL1	400	AAGGTATATATTCTATTTGTA	SI00303219	Hs_ARL1_3	14	16	15	15	1.0
ARL1	400	CAGAATATGTTCATAGTTGAA	SI00303226	Hs_ARL1_4	38	38	31	36	4.0
RABEP1	9135	CTGGAAGACTTCATAAAGCAA	SI00697571	Hs_RABEP1_1	4	99	36	46	48.3
RABEP1	9135	TAGGCTATAACTACAAAGCAA	SI00697578	Hs_RABEP1_2	4	4	4	4	0.0
RABEP1	9135	AAGCTGAATTAGAAAGAATAA	SI00697585	Hs_RABEP1_3	3	3	3	3	0.0
RABEP1	9135	CAGGATAAAGCCGAACTGGTA	SI00697592	Hs_RABEP1_4	18	13	13	15	2.9
TSNAX	7257	CTGGTTAACCTTTATCATTTA	SI00751975	Hs_TSNAX_1	3	3	3	3	0.0
TSNAX	7257	ACCCGTGATGTTGGCCTTTAA	SI00751982	Hs_TSNAX_2	19	22	20	20	1.5
TSNAX	7257	CAGCAATAAGACAATTGTCAA	SI00751989	Hs_TSNAX_3	8	5		7	2.1
TSNAX	7257	AGGGCATTTCTTAGAATCTAA	SI00751996	Hs_TSNAX_4	4	3	3	3	0.6
DGKB	1607	CTGCATTAAAGTGCTAATGAT	SI00059486	Hs_DGKB_3	4	3	4	4	0.6
DGKB	1607	AGCAAGTGAGTTGCCCATTAA	SI00059493	Hs_DGKB_4	0	0	0	0	0.0
DGKB	1607	CCCGAACTACTTCTCCTGCAA	SI02225265	Hs_DGKB_9	33	27	19	26	7.0
DGKB	1607	CTCATTCTAGTCCAATGGTAA	SI02225272	Hs_DGKB_10	51	47	54	51	3.5
				(-) control siRNA	4	5	4	4	0.6

Appendix Table 2. One subset of candidate siRNAs sequences and corresponding viability results from one intoxication screen.

NCBI gene symbol	Entrez Gene Id	siRNA Target Sequence	Product Id	Product Name	well1	well2	well3	% viability(Avg)	St. Dev.
AP1G2	8906	CCGCCAGGTGGTGTCCATCTA	SI00297290	Hs_AP1G2_2	11	9	4	8	3.8
AP1G2	8906	TGCGATGGCTTCAAATCCAAA	SI00297297	Hs_AP1G2_3	32	26	20	26	6.4
B4GALNT4	338707	TGGGTTCTATAAATACATGAA	SI00311668	Hs_Beta4GalNAc-T4_2	10	9	4	8	3.2
B4GALNT4	338707	CTGGAGAATTCACCAAGTTCA	SI00311682	Hs_Beta4GalNAc-T4_4	18	14	9	14	4.9
CDC42EP3	10602	AAGGAACTTCACGGAACTTTA	SI00341124	Hs_CDC42EP3_2	21	14	10	15	5.2
CDC42EP3	10602	CACGATGTCTTTGGAGATATT	SI00341131	Hs_CDC42EP3_3	59	45	48	51	7.7
CLTA	1211	CCGGATGCTGTTGATGGAGTA	SI02650683	Hs_CLTA_4	33	27	21	27	6.4
CLTA	1211	GTGCATTATTCTGAGAATAAA	SI02650697	Hs_CLTA_6	19	14	9	14	5.1
DMXL1	1657	CTGGGACTATTTCATAGCTAA	SI00370419	Hs_DMXL1_3	24	18	13	18	5.8
DMXL1	1657	CAGCGTCTTATGGAAATGTTA	SI00370426	Hs_DMXL1_4	34	22	17	24	8.7
DOCK10	55619	CTCGAGTAATTAGCAAAGCAA	SI00372547	Hs_DOCK10_3	36	26	23	28	6.5
DOCK10	55619	CTGGTTCTTCTTTGCAATTAT	SI00372554	Hs_DOCK10_4	28	23	17	23	5.3
EXOSC10	5394	CTGGAAGACAAGTTTGATTTA	SI00381864	Hs_EXOSC10_2	13	15	10	12	2.3
EXOSC10	5394	ACGGATGAGTCCTACCTTGAA	SI00381871	Hs_EXOSC10_3	11	9	6	8	2.6
FBLIM1	54751	CTCCACAATTGTTATAACCAA	SI00385147	Hs_FBLP-1_3	27	20	16	21	5.6
FBLIM1	54751	AAGATTCTTCTTCCCTTTGAA	SI00385154	Hs_FBLP-1_4	15	12	9	12	2.9
FER1L3	26509	AAGCTGATAGATGAAGTTATA	SI00386435	Hs_FER1L3_3	24	22	15	20	5.1
FER1L3	26509	TTGCATCATGTTATCTCTTAA	SI00386442	Hs_FER1L3_4	9	8	5	7	1.7
GANAB	23193	CCGGGATGTGCATAACATCTA	SI00107240	Hs_GANAB_1	16	14	10	13	3.1
GANAB	23193	TACCATCTCAGCACAATGATA	SI00107261	Hs_GANAB_4	22	17	8	16	6.8
KCNJ11	3767	CCGGCCTGAGGCTGGTATTAA	SI00011144	Hs_KCNJ11_2	33	27	24	28	4.7
KCNJ11	3767	AGGCTGGTATTAAGAAGTGAA	SI00011151	Hs_KCNJ11_3	25	22	18	22	3.2
MAP4	4134	AACCGGGAACTCAGAGTCAAA	SI00627802	Hs_MAP4_2	26	25	20	24	3.3
MAP4	4134	CCCAGATTTCCAAATCATTAA	SI00627809	Hs_MAP4_3	20	15	15	17	2.8
USE1	55850	ACCGGCCTCTGAGGTGATCAA	SI00629622	Hs_MDS032_2	11	7	6	8	2.7
USE1	55850	CTCAGAGAAAGCACTGGCCAA	SI00629636	Hs_MDS032_4	34	30	28	31	2.9
MID1IP1	58526	CAGCCACTACGTGCTTCTCAA	SI00645673	Hs_MID1IP1_3	45	38	39	41	3.9
MID1IP1	58526	CTCGCTCTTTAACGCCATGAA	SI00645680	Hs_MID1IP1_4	47	38	31	38	8.0
MPDZ	8777	AAGGACTATTAATATAGCAAA	SI00647633	Hs_MPDZ_3	27	16	15	19	6.8
MPDZ	8777	AAGGATTATGGGAATTAACTA	SI00647640	Hs_MPDZ_4	6	3	4	4	2.0
NPHP3	27031	AAGCTTTGCCATTATATGAAA	SI00660926	Hs_NPHP3_2	33	22	15	23	9.1
NPHP3	27031	AAGGATGTAGACACATTAGAA	SI00660940	Hs_NPHP3_4	31	25	23	26	4.1
NUMBL	9253	AACTTGAAGATTTGTATTATA	SI00662886	Hs_NUMBL_2	24	20	16	20	4.1
NUMBL	9253	GCCAATAAAGGAAGAATATAA	SI00662893	Hs_NUMBL_3	37	34	32	34	2.5
PPM1K	152926	CCAGACTTCCCTAATCCTAAA	SI00691341	Hs_PPM1K_3	44	32	27	34	8.4
PPM1K	152926	TACCAAGGAATAAGTATGAAA	SI00691348	Hs_PPM1K_4	20	16	12	16	4.4
PTPRN2	5799	ACGGATGTTGTCAGGAATCAT	SI00044373	Hs_PTPRN2_2	24	22	19	21	2.4
PTPRN2	5799	AGCGGACAGAATGATGCCAAA	SI00044387	Hs_PTPRN2_4	20	12	16	16	4.0
STXBP1	6812	ACGGTGGACTCCGATTATCAA	SI00735693	Hs_STXBP1_3	22	17	13	18	4.5
STXBP1	6812	GAGGACAAACTTGACACCAAA	SI00735700	Hs_STXBP1_4	10	5	3	6	3.5
TPPP	11076	AAGATATATATTTATACTGGA	SI00750197	Hs_TPPP_3	30	21	13	21	8.4
TPPP	11076	CCCGATAACAGCCCATCACAA	SI00750204	Hs_TPPP_4	57	46	45	50	6.7
UBE2H	7328	AACACAGGTTCTAAATTTAAA	SI02651271	Hs_UBE2H_8	15	12	10	12	2.9
UBE2H	7328	ATGAATAATAGCATTGATATA	SI02651278	Hs_UBE2H_9	18	15	14	16	2.5
UBE2L3	7332	ACCACCGAAGATCACATTTAA	SI00754642	Hs_UBE2L3_2	4	1	2	2	1.3
UBE2L3	7332	CACACTCCAGTTTGTAATAAA	SI00754649	Hs_UBE2L3_3	20	17	23	20	3.5
EHD1	10938	TTGAGCAATAAGAAACCAGAA	SI02638069	Hs_EHD1_5	70	63	94	76	15.9
EHD1	10938	CCGTCACTCCATACAGTATTA	SI02638076	Hs_EHD1_6	70	50	57	59	10.2
MARK1	4139	CCGGCCCAGTAGTGACTTAAA	SI00122507	Hs_MARK1_1	17	9	8	11	4.7
MARK1	4139	CCCGGTGTAGAAACTCCATTA	SI00122514	Hs_MARK1_2	8	3	4	5	2.4
				(-) control siRNA	18	19	25	21	3.4

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